

Innexins in the lobster stomatogastric nervous system: cloning, phylogenetic analysis, developmental changes and expression within adult identified dye and electrically coupled neurons

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Abstract

Gap junctions play a key role in the operation of neuronal networks by enabling direct electrical and metabolic communication between neurons. Suitable models to investigate their role in network operation and plasticity are invertebrate motor networks, which are built of comparatively few identified neurons, and can be examined throughout development; an excellent example is the lobster stomatogastric nervous system. In invertebrates, gap junctions are formed by proteins that belong to the innexin family. Here, we report the first molecular characterization of two crustacean innexins: the lobster *Homarus gammarus* innexin 1 (Hg-inx1) and 2 (Hg-inx2). Phylogenetic analysis reveals that innexin gene duplication occurred within the arthropod clade before the separation of insect and crustacean lineages. Using *in situ* hybridization, we find that each innexin is expressed within the adult and developing lobster stomatogastric nervous system and undergoes a marked down-regulation throughout development within the stomatogastric ganglion (STG). The number of innexin expressing neurons is significantly higher in the embryo than in the adult. By combining *in situ* hybridization, dye and electrical coupling experiments on identified neurons, we demonstrate that adult neurons that express at least one innexin are dye and electrically coupled with at least one other STG neuron. Finally, two STG neurons display no detectable amount of either innexin mRNAs but may express weak electrical coupling with other STG neurons, suggesting the existence of other forms of innexins. Altogether, we provide evidence that innexins are expressed within small neuronal networks built of dye and electrically coupled neurons and may be developmentally regulated.

Introduction

Gap junctions mediate cell-to-cell communication by directly linking the cytoplasm of adjacent cells. In the central nervous system, gap junctions enable both direct electrical communication between neurons and diffusion of small molecules such as ions, amino-acids and second messengers. They are widespread early in development and play a crucial role in the early elaboration of neural networks (Sutor, 2002; Montoro & Yuste, 2004). In the adult, modelling studies have suggested that electrical synapses play a major role in the genesis and coordination of neuronal oscillations (Sherman & Rinzel, 1992; Traub, 1995; Manor *et al.*, 1997; Draguhn *et al.*, 1998; Skinner *et al.*, 1999; Bem *et al.*, 2002). Moreover, electrical synapses often operate in parallel with inhibitory chemical synapses in neural networks. As the coexistence of these two types of coupling may promote the expression of a large variety of activity patterns (Nomura *et al.*, 2003; Bem & Rinzel, 2004; Bem *et al.*, 2005; Merriam *et al.*, 2005;

Pfeuty *et al.*, 2005), the analysis of the dynamics and roles of electrical coupling is very challenging.

Therefore, in order to study the roles of gap junctions in the development and operation of neural networks, an obvious approach is to use a simple system such as invertebrate motor networks, which are built of few well-characterized identified neurons. Among these model systems, the stomatogastric nervous system (STNS) of the lobster *Homarus gammarus*, is particularly suitable. In this system, the approximately 30 neurons of the stomatogastric ganglion (STG) are present early in development (Fénelon *et al.*, 1998) and in the adult are linked through electrical and chemical synapses. These STG neurons are organized in a single motor network in embryos and larvae (LI–LIII) and then subdivide into two motor networks, gastric and pyloric, after metamorphosis (Casasnovas & Meyrand, 1995). Modelling studies have suggested that this reconfiguration into two motor networks may result from a decrease in electrical coupling within the STG neuronal population (Bem *et al.*, 2002).

An important step in studying the role of gap junctions within this system is to determine the molecular basis of crustacean gap junctions. In invertebrates, gap junctions are formed by proteins that belong to the innexin family (Phelan *et al.*, 1998a, b). In the present study, we have cloned two lobster innexins, characterized the

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expression of their mRNA in the adult and developing STNS using *in situ* hybridization, and explored whether innexins are expressed within adult identified STG neurons that are dye or electrically coupled with network partners. Our present findings (i) extend the molecular characterization of the innexin family within the Ecdysozoans and suggest that the evolution of the multigene innexin family occurred before the divergence of crustacean and insect lineages; (ii) indicate that the expression of the two lobster innexin mRNAs undergoes a marked down-regulation after metamorphosis, suggesting a possible role for gap junctions in the ontogeny of neural networks, and (iii) provide evidence that adult STG neurons expressing at least one innexin are dye and electrically coupled to at least one other STG neuron.

Materials and methods

Molecular cloning and analysis of lobster neuronal innexins

Adult European lobsters, *Homarus gammarus*, were cold anaesthetized. Brain, STNS and thoracic ganglia were dissected out and immediately frozen on dry ice. Total RNA was then extracted using the RNeasy kit (Qiagen, Crawley, UK) and single-strand cDNA synthesis was performed using random hexamers as primers and SuperscriptII enzyme (Promega, Southampton, UK), according to the manufacturer's protocol. Consensus-degenerate (CODEHOP) primers (Rose *et al.*, 1998) were used. These primers are degenerate at their 3' end and this 'core' region corresponds to four or five amino-acids. However, the 5' end of the primer, the 'clamp', is a consensus, nondegenerate sequence. This method is suitable for amplifying novel sequences even when minimal homology is present. Three CODEHOP primers were designed against the sequence alignment of the transmembrane regions of five selected innexins (*Hirudo medicinalis* Inx1, *Cliona limacina* Inx1, *Drosophila melanogaster* Inx2, *Caenorhabditis elegans* Inx3, *Giardia tigrina* Inx1). The TM1-CODE forward primer was designed against the first putative transmembrane domain (CAG TAC GTC GGA GAC CCA ATC CAC TGY TGG KKY CC), the TM2-CODE forward primer was designed against the second putative transmembrane domain (AG TAC GTC GGA GAC CCA ATC CAC TAY TAY CAR TGG) and the TM4-CODE reverse primer was designed against the fourth putative transmembrane domain (GAA CCA GAA CCA GAG GAA GAT GWA NAY YTT YTC). For all sequences, the nomenclature for incompletely specified nucleic acid sequences that we used can be found at <http://www.chem.qmul.ac.uk/iubmb/misc/naseq.html>. PCR products were run on a 1% agarose gel. Products of the expected size (PCR-prod1 and PCR-prod2; 550–600 bp each) were then extracted using a gel extraction kit (QIAquick gel extraction kit from Qiagen) and subcloned into the pCRII-TOPO vector (Invitrogen, Paisley, UK). Subcloned DNA fragments were sent for sequencing to MWG Biotech (London, UK), both from Sp6 and T7 vector primers.

In order to get the full sequence of both cloned fragments, 5'- and 3'-RACE (rapid amplification of cDNA ends) PCR was used (SMARTTM method and kits from Clontech, Mountain View, CA, USA). Gene specific primers were as follow for PCR-prod1 (5' RACE GGC CTG CCA GGA TGG TGG TGA ACA GCC CGC CC; 3' RACE CTC GCA TCT TCC CCC GCG TCA CCA AGT GCA CC) and for PCR-prod2 (5' RACE GGC CTT GCG TTC CCG TTT GAC ATC GTC ATC C; 3' RACE CCC GTC ACG ACC CAA TGG ATG AAG TAT TCC CC). All PCR products were subcloned into the pCRII-TOPO vector (Invitrogen) and sent for sequencing to MWG Biotech. Overlapping cDNA sequences were used to reconstruct the full cDNA sequence of each innexin.

Phylogenetic analysis

Phylogenetic trees were constructed using the Neighbour-joining algorithm, a distance matrix operating in conjunction with the CLUSTAL W alignment interface (Thompson *et al.*, 1994). Protein distance matrix and trees were computed using PHYLIP software (Felsenstein, 1989). The data were bootstrapped 1000 times to assess the robustness of the phylogeny. Branch lengths between the nodes and genes were drawn in proportion to the calculated evolutionary distance.

mRNA *in situ* hybridization

Digoxigenin-labelled RNA sense and antisense probes were generated using the first cloned product of each innexin (PCR-prod1 and PCR-prod2) as templates using DIG RNA labelling mix from Roche (Meylan, France). In some experiments probes were obtained by PCR amplification using a PCR DIG probe synthesis kit (Roche) with a set of appropriate primers (CCC CGC TAC CTG TGG AAG and CAG CCA GAA CCA GAT GAA GAT for innexin 1; ATT CCC CGG TAT CTA TGG AAA and GAA CCA GAA CCA GAG GAA AAT for innexin 2). However the overall staining was identical whichever probe was used. Determination of embryonic and larval stages and dissections were performed as previously described (Le Feuvre *et al.*, 2001). The STNS was dissected out in *Panulirus* saline (NaCl 479.12 mM, KCl 12.74 mM, CaCl₂ 13.2 mM, MgSO₄ 10 mM, Na₂SO₄ 3.2 mM, and HEPES 5 mM, pH 7.45), pinned down into small Sylgard coated Petri dishes and desheathed before paraformaldehyde fixation (4% PFA from Sigma in NaCl 115.5 mM, KCl 4 mM, Na₂HPO₄ 81 mM, NaH₂PO₄ 19 mM, pH 7.5) for 12–24 h at 4 °C. Preparations were rinsed at least three times in PBS and then incubated in proteinase K (Sigma, 20 µg/mL) in PBT (0.1% Tween 20, Sigma, in PBS) for 1 min. Preparations were rinsed five times (5 min each) in PBT and then postfixed in 4% PFA for 15 min. After five rinses in PBT, preparations were incubated in hybridization solution (50% deionized formamide, 5× saline sodium citrate (SSC, NaCl 750 mM, sodium citrate 2H₂O 75 mM, pH 7), 100 µg/mL tRNA, 50 µg/mL heparin, 0.1% Tween 20) at 55 °C for 1 h. Labelled RNA probe was then added to hybridization solution and incubation proceeded at 55 °C for 24 h. Successive rinses in hybridization solution (15 min 55 °C) then SSC-5× (2 × 15 min 55 °C), SSC-3× (2 × 15 min 55 °C) and SSC-1× (2 × 15 min room temperature) were performed. Unbound probes were removed by incubating the preparations in RNase (20 µg/mL RNaseA in 500 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8.2) for 20 min at 37 °C. To detect hybridized labelled RNA, an alkaline-phosphatase conjugated digoxigenin antibody was used (Roche, polyclonal sheep 1 : 2000 in PBT 5% normal goat serum, 3 h at room temperature) and the colour reaction was carried out using 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (Roche) for 12 h at 4 °C following the manufacturer's instructions.

Intracellular injections

In order to assess whether innexin expression within identified neurons is correlated with dye coupling between neurons, neurons identified with conventional electrophysiological methods were injected with two different kinds of neuronal tracers. Firstly, identified neurons were injected with high molecular weight dyes (Dextran Tetramethyl Rhodamine or Dextran Lucifer Yellow, 3000 MW, Molecular Probes, Cergy Pontoise, France, diluted 10% in 2 M potassium acetate or Lucifer Yellow, 457 MW, Sigma–Aldrich, diluted

10% in 2 M LiCl) using pressure injection (Picospitzer II, Parker Hannifin, Cleveland, OH, USA, 20–40 p.s.i.). These dyes do not cross gap junctions and therefore stain only the injected cell. These preparations were later processed for *in situ* hybridization in order to determine which innexins are expressed by these identified labelled neurons. Secondly, using different preparations, identified neurons were injected with Neurobiotin™ (*N*-2-aminoethyl biotinamide hydrochloride; 323 MW, Vector Laboratories, Peterborough, UK, diluted 10% in 2 M Potassium acetate) using current injection (1 Hz, 1–3 nA, 500 ms, 30–60 min) in order to determine if they displayed dye coupling. Neurobiotin™ is known to diffuse through gap junctions (Vaney, 1991). Neurobiotin™ was allowed to diffuse overnight before fixation.

Visualization of Neurobiotin™

Neurobiotin™ injected preparations were fixed in 4% PFA for 10–12 h at 4 °C. They were then rinsed five times over a 2-h period with phosphate buffer saline. Preparations were incubated at 4 °C for 12 h in Avidin DCS-fluorescein (Vector Laboratories, 1 : 200 in 0.3% Triton X-100). Preparations were rinsed five times in PBS and then mounted in 90% glycerol-10% NaHCO₃ before confocal microscope observation. Two control preparations in which no Neurobiotin™ was injected were treated as detailed above; these displayed no staining.

Acquisition of images

Preparations processed for *in situ* hybridization were observed either directly in the Petri dish using a Nikon Coolpix digital camera (Nikon, Champigny sur Marne, France) coupled to a binocular microscope or after mounting in 90% glycerol-10% NaHCO₃ using a Nikon digital camera DXM 1200 on a Nikon Eclipse E600 or a SPOT RT digital camera on a Zeiss Axioskop microscope (Zeiss, Welwyn Garden City, UK). All preparations combining fluorescence identification of neurons and *in situ* labelling were observed using a Nikon digital camera DXM 1200 on a Nikon Eclipse E600 with a Y-FL epifluorescence attachment. Images showing double labelling were reconstructed with Corel Draw software (Corel, Neuilly-sur-Seine, France) using additive combinations of the two images (*in situ* hybridization and fluorescence). In embryos, larvae and adults, cells that were labelled after *in situ* hybridization treatment were carefully counted within the STG and the oesophageal ganglion (OG) using several micrographs of the preparation taken at different levels of focus. Neurobiotin™ injected preparations were observed with an Olympus (Rungis, France) confocal microscope (Fluoview). Typically, 20–60 optical sections of thickness 1–5 µm each were acquired. Individual stacks were then loaded into Scion Image software (Scion corporation, Frederick, MD, USA) and converted to grey scale. For each neuron the mean grey level was measured on the slice estimated to capture the largest diameter of the neuron. Background was measured in three zones of equal areas in the middle of the STG in a given stack and the mean value of the noise was then subtracted from the values measured for the cell bodies. This allowed the unambiguous determination of neurobiotin stained cells.

Electrophysiology and electrical coupling characterization

Adult stomatogastric nervous systems pinned onto sylgard coated Petri dishes were constantly perfused with chilled *Panulirus* saline. Intracellular recordings were performed using conventional sharp electrodes filled with 2 M K-acetate (15–40 MΩ) in the soma of STG

neurons. Intracellular signals were amplified using an Axoclamp 2B (Axon Instruments, Union City, CA, USA) and digitized using a CED device (Cambridge Electronic Design, Cambridge, UK). Data acquisition and analysis were performed using Spike 2 software.

STG neurons were isolated from modulatory inputs and network partners by superfusing a mixture of picrotoxin (PTX, 10^{−6} M) and tetrodotoxin (TTX, 10^{−7} M), respectively. To demonstrate the presence of electrical coupling between a pair of identified neurons, a brief current pulse (500 ms, −20 nA) was injected into one cell (the presynaptic neuron) and the variation of voltage was measured in the cell of interest (the postsynaptic neuron). For coupling coefficient measurements, two electrodes, one for current injection, the other for voltage measurement, were inserted into the presynaptic neuron. Brief (500 ms) current pulses (range −20 nA to +20 nA, 4 nA steps, ten times each) were injected into the presynaptic neuron and the voltage deflection measured both in the presynaptic (injected) and postsynaptic neurons. The mean of ten voltage deflections for each value of injected current was used to construct input–output curves. The coupling coefficient is defined as the slope of the curve (in its linear part) representing postsynaptic voltage variation as a function of presynaptic voltage variation. In the case of rectifying electrical synapses, different slopes for negative and positive current pulses are obtained and the higher value of the slope is used to determine the coupling coefficient. For all experiments, the coupling coefficient between the electrodes (all electrodes in the bath) as well as between the bath and the postsynaptic cell was measured (only electrode injecting current in the bath). No coupling could be detected.

Results

Molecular characterization of two lobster neural innexins: *Hg-inx1* and *Hg-inx2*

We used RT-PCR in RNA extracts from lobster nervous tissue to isolate innexin related products (see Materials and methods). Two distinct PCR products were obtained with the same set of primers (TM2-CODE and TM4-CODE), which were elongated to full-size cDNA using RACE PCR. Full-length cDNA sequences were reconstructed by aligning overlapping regions of the middle fragment (PCR-Prod1 and PCR-Prod2) and RACE extensions. Using a BLAST search, these two sequences showed statistically significant similarity to other innexins (*E*-values < e^{−90}), and were therefore identified as members of the innexin family and named *Homarus gammarus*-innexin 1 (*Hg-inx1*; accession number AM182905) and *Homarus gammarus*-innexin 2 (*Hg-inx2*; AM182906). The deduced amino-acid sequence for *Hg-inx1* is 367 amino-acids long (calculated molecular mass of 42.3 kDa) and shows most homology to the *Drosophila* and grasshopper innexin 2 (45 and 47% identity, respectively). Similarly, *Hg-inx2* is 362 amino-acids long (molecular mass of 42.1 kDa) and also shows most homology to grasshopper and *Drosophila* innexin 2 (64 and 62%, respectively). Figure 1A shows alignment of *Hg-inx1* and *Hg-inx2* with most closely related innexins in *Schistocerca americana* (Sa-inx1, Sa-inx2) and *Drosophila melanogaster* (Dm-inx1 and Dm-inx2). In common with other innexins known in other species, both lobster innexins showed two conserved cysteine amino-acids in each of their extracellular loops (see boxes in Fig. 1A at positions 54, 68, 242 and 260). Hydropathy profiles of the two lobster innexins show four highly hydrophobic regions in each protein as shown in Fig. 1B (calculation using Kyte-Doolittle algorithm; bioinformatics.weizmann.ac.il/hydroph/plot_hydroph.html). The four hydrophobic regions are likely to constitute the four transmembrane domains, which are highly conserved among the innexin family. This finding

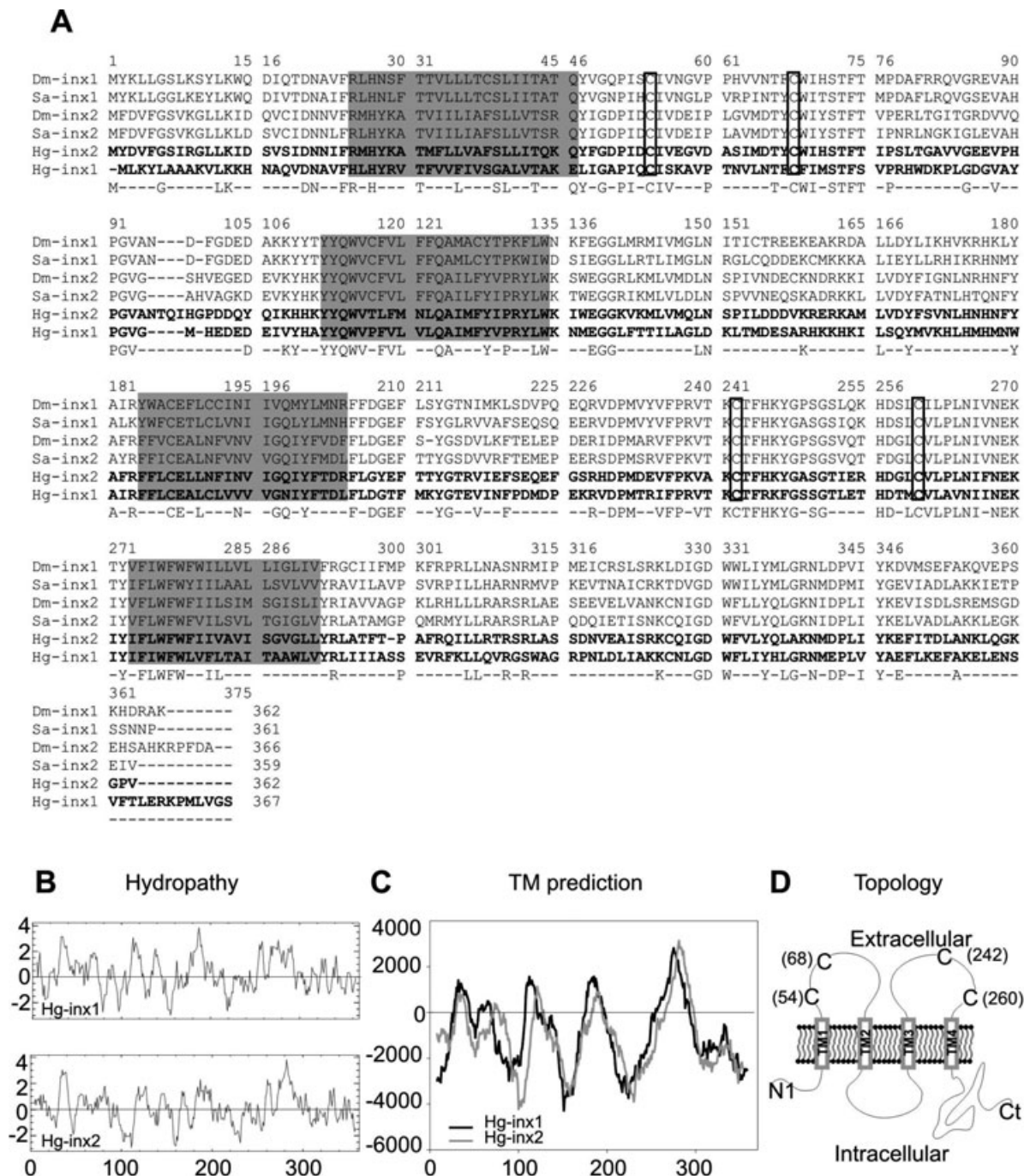


FIG. 1. Deduced amino-acid sequence, sequence analysis and predicted topology of lobster innexin 1 (Hg-inx1) and lobster innexin 2 (Hg-inx2). (A) Alignments of Hg-inx1 and Hg-inx2 proteins with most closely related proteins (*Drosophila melanogaster* innexin 1, Dm-inx1; *Drosophila melanogaster* innexin 2, Dm-inx2; *Schistocerca americana* innexin 1, Sa-inx1; *Schistocerca americana* innexin 2, Sa-inx2). Grey boxes highlight the putative hydrophobic transmembrane domains (see B and C) while conserved cysteines are surrounded by black rectangles. The last line of the alignment represents amino-acids conserved in at least five of the presented sequences. (B) Hydropathy plots for Hg-inx1 (top panel) and Hg-inx2 (bottom panel) proteins determined by Kyte and Doolittle's algorithm. (C) Prediction of transmembrane domains for Hg-inx1 (black curve) and Hg-inx2 (grey curve) as computed by the TMPred program (http://www.ch.embnet.org/software/TMPRED_form.html). High values indicate a high probability that an amino-acid belongs to a transmembrane domain. (D) Schematic representation of the predicted topology for Hg-inx1 and Hg-inx2 with four transmembrane domains (TM1–TM4) and the two conserved cysteines located in each of the extracellular loops (adapted from Phelan *et al.*, 1998a).

was supported using a transmembrane domain prediction program (Fig. 1C; TMPred program, http://www.ch.embnet.org/software/TMPRED_form.html) that also predicted a four transmembrane domain structure for each lobster innexin as shown on the schematic diagram in Fig. 1D. The amino-acids corresponding to transmembrane domains are highlighted on Fig. 1A (grey areas).

Phylogenetic analysis of the innexin protein family

We then studied the phylogenetic relationships of the two novel lobster innexins with other known innexins; after CLUSTAL W alignment, a phylogenetic tree (Fig. 2) was constructed using the Neighbour-joining algorithm. This analysis shows three major clades

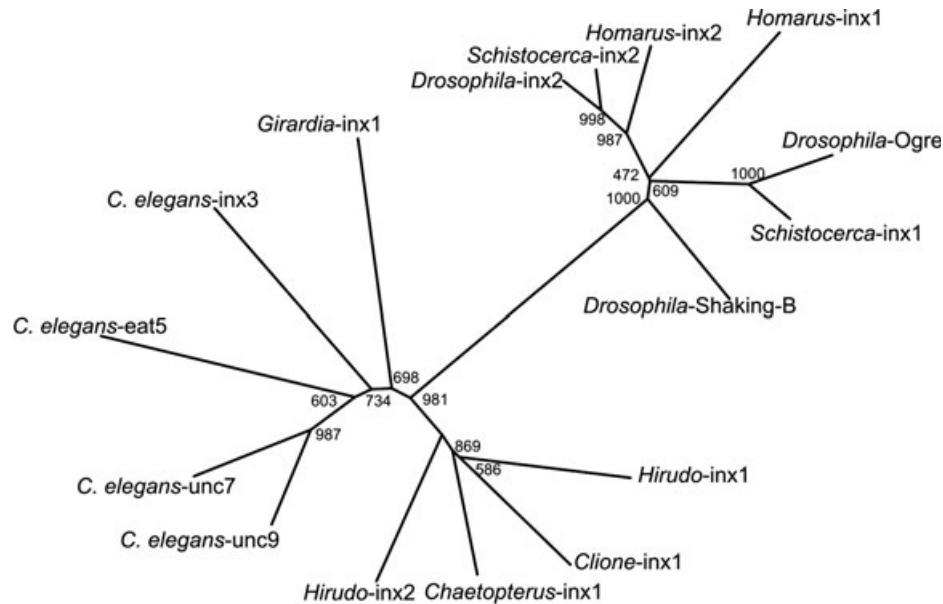


FIG. 2. Phylogenetic analysis of the relationship of Hg-inx1 and Hg-inx2 proteins to some other known innexins. The tree is unrooted. The numbers indicate bootstrap values per 1000 replicates. Note that the two lobster innexins together with the *Schistocerca* and *Drosophila* innexins form a robust arthropod clade, well separated from the *C. elegans* and the Lophotrochozoan (*Hirudo*, *Chaetopterus*, *Clione*) clades.

within the innexin family. The first clade includes all Lophotrochozoans (molluscs and annelids), the second clade includes all *C. elegans* innexins and the third includes all arthropod innexins. The *Girardia* innexin lies outside of these clades. Interestingly, within the arthropod clade, Hg-inx2 is more related to *Drosophila* and grasshopper innexin-2 than to Hg-inx1, suggesting that the establishment of the innexin multigene family occurred before the divergence of the crustacean and insect lineages. It is also worth noting that, although *C. elegans* and arthropods are all Ecdysozoans, the *C. elegans* innexins form a separate well defined clade that is distinct from the arthropod clade; this suggests that innexin gene duplication within Ecdysozoans occurred independently within each of these two groups.

mRNA expression of Hg-inx1 and Hg-inx2 within the adult STNS

We then addressed whether *Hg-inx1* and *Hg-inx2* are expressed within the STNS. This neural system consists of four interconnected ganglia: the stomatogastric ganglion (STG), which contains the motor networks, and the oesophageal ganglion (OG) and the two paired commissural ganglia (CoG), which contain the modulatory input neurons to the STG as well as the oesophageal motor neurons (Fig. 3A).

We first focused our attention on the STG because most neurons constituting this ganglion are interconnected through electrical synapses. *In situ* hybridization with *Hg-inx1* RNA antisense probe or *Hg-inx2* RNA antisense probe revealed some cells with a dense deep blue/purple granular staining surrounding the nucleus (Fig. 3B and C). This staining consists in most cells in a ring of dark granular and perinuclear staining (arrowheads; Fig. 3, B1, B2, C1 and C2). In some cells with a low cytoplasm/nucleus volume, almost all the cytoplasm displays this staining (see cell marked by an arrowhead in Fig. 7A and see also embryonic and larval cells on Fig. 5). Therefore, these cells clearly express *Hg-inx1/2* mRNA. It is striking that *Hg-inx1* and *Hg-inx2* mRNA only appears to be expressed within a subset of STG neurons (Fig. 3, B1 and C1). Whereas physiological findings obtained in *H. gammarus* as well as other crustacean decapod species

suggest that most of the 34 STG neurons are electrically coupled to one or several STG neurons (Johnson *et al.*, 1993; Combes *et al.*, 1999), we typically only found three to seven stained soma for *Hg-inx1* mRNA (range 0–11 on 19 STG; see Fig. 3, B3) and four to seven cells for *Hg-inx2* mRNA (range 0–12 on 18 STG; see Fig. 3, C3). However, in the absence of unambiguous cell identification, it was impossible to conclude from these preparations whether the cells stained for *Hg-inx1* were also stained for *Hg-inx2*.

In contrast, in preparations treated with sense probe for *Hg-inx1* (Fig. 3, D1, D3 and D4; $n = 4$) or for *Hg-inx2* (Fig. 3, D2; $n = 3$) and in STNS treated without any probe (Fig. 3, D5; $n = 2$), the dense ring of granular staining surrounding the nucleus was never observed. In four out of these nine preparations, no colouration was observed (Fig. 3, D1 and D2). In the remaining preparations (five out of nine controls), a faint homogenous colouration of the entire cytoplasm of a few cells was observed (Fig. 3, D3 and D5, asterisks in Fig. 3, D4). This colouration in control preparations must be regarded as a background artefact of the histological procedure and therefore any similar level of colouration in the experimental STG was considered background (Jezzini *et al.*, 2005; asterisks in Fig. 3, B2 and C2). In all subsequent experiments, the presence of the dense granular staining surrounding the nucleus (restricted to a ring or invading almost all the cytoplasm) was used as the criterion of whether a cell was regarded to express detectable levels of innexin mRNA.

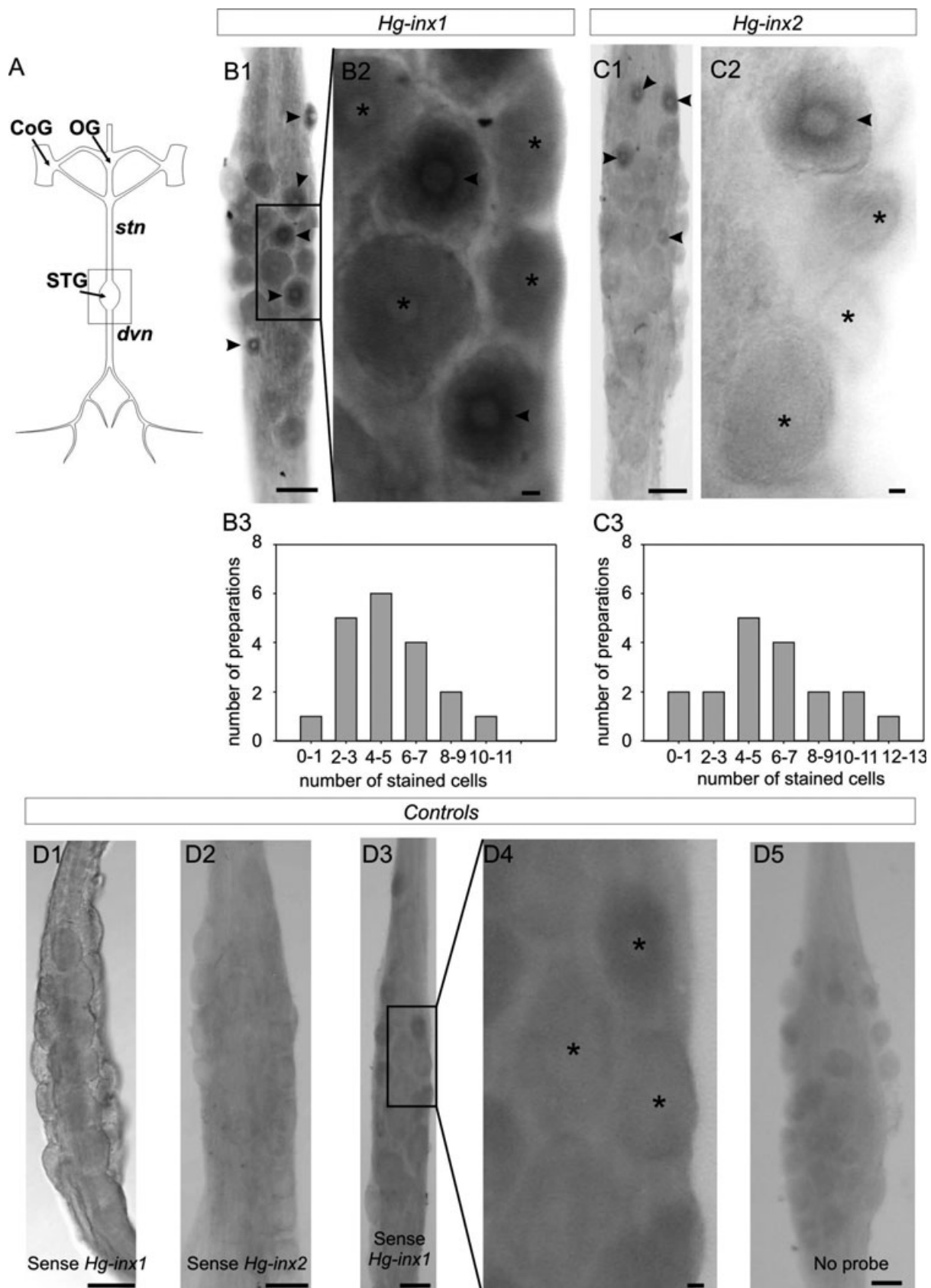
In other ganglia of the STNS, we observed numerous stained somata in the CoG with both *Hg-inx1* (Fig. 4, B1; $n = 7$) and *Hg-inx2* (Fig. 4, C1; $n = 11$) probes. In contrast, no staining was observed in the OG with either *Hg-inx1* (Fig. 4, B2; $n = 10$) or *Hg-inx2* (Fig. 4, C2; $n = 8$) probe.

mRNA expression of Hg-inx1 and Hg-inx2 within the developing STNS

We performed *in situ* hybridization using *Hg-inx1* and *Hg-inx2* probes on embryos (at least 60% of embryonic development) and the three larval stages. When sense probes (control) were used, preparations

were totally devoid of staining (insert in Fig. 5, B1). By contrast, antisense probes revealed that both innexin mRNAs are expressed within the STG, OG (Fig. 5) and CoG (data not shown). Indeed, a

dense granular staining surrounding a large nucleus (embryonic and larval STG neurons have a higher nucleus/cytoplasm volume ratio than adult neurons) appeared intensely stained in most cells within



embryonic and larval STG (Fig. 5, A1, A2, B1 and B2) and OG (Fig. 5, A3, B3). Statistical analysis showed that the number of stained soma for *Hg-inx1* or *Hg-inx2* was significantly higher in embryonic and larval STG ($n = 11$ preparations for *Hg-inx1*, 7–27 stained somata; $n = 9$ for *Hg-inx2*, 15–26 stained somata) than in the adult STG (Fig. 5A4 and B4; $n = 19$ for *Hg-inx1*, 2–11 stained neurons; $n = 18$ for *Hg-inx2*, 2–10 stained cell bodies; Mann–Whitney rank sum test, $P < 0.001$) while no significant difference was found between the number of stained soma in embryos, LI, LII and LIII STG for both innexins (one-way ANOVA). In contrast to adult OG, which show no innexin stained cells (see Fig. 4), embryonic and larval OG present several cells expressing *Hg-inx1/2* mRNA (Fig. 5, A3 and B3). Not surprisingly, the number of stained cells for *Hg-inx1* or *Hg-inx2* was significantly higher in larval OG ($n = 8$ and $n = 6$ preparations, respectively) than in the adult OG ($n = 10$ and $n = 8$, respectively) (Fig. 5, A4 and B4; Mann–Whitney rank sum test, $P < 0.001$ and $P < 0.01$, respectively). In summary, *Hg-inx1* and *Hg-inx2* mRNA are expressed in a large subset of STG neurons in premetamorphic stages (embryo and larva) whereas a limited fraction of STG neurons express these mRNA in the postmetamorphic stage (adult). Therefore, innexin expression undergoes a marked down-regulation after metamorphosis.

Hg-inx1 and *Hg-inx2* mRNA expression within identified adult STG neurons

To determine which cells within the STG consistently express *Hg-inx1* and/or *Hg-inx2*, some adult STG neurons (one to four per preparation) identified on the basis of their electrical properties and nerve projections were intracellularly injected with high molecular weight fluorescent dyes (see Materials and methods). These preparations were then processed for *in situ* hybridization using either *Hg-inx1* or *Hg-inx2* antisense probe. This procedure allowed unequivocal identification of neurons after the *in situ* hybridization procedures. For example, injection of Dextran Tetramethyl Rhodamine or Dextran Lucifer Yellow into the soma of the electrically coupled pyloric dilator (PD) neurons (Fig. 6, A2 and B2) followed by *in situ* hybridization with *Hg-inx1* (Fig. 6A1) or *Hg-inx2* (Fig. 6, B1) probe revealed that the two PDs express both *Hg-inx1* and *Hg-inx2* (see the superposition of fluorescent labelling and *in situ* hybridization images in Fig. 6, A3, $n = 4$ and Fig. 6, B3, $n = 3$). In contrast, using the same procedure, we found that LP neuron, visualized here after Lucifer Yellow injection (Fig. 7, A2 and B2) does not express either *Hg-inx1* (Fig. 7, A1 and A3, $n = 3$) or *Hg-inx2* (Fig. 7, B1 and B3, $n = 3$), although some other cells in these preparations were clearly stained with either *Hg-inx1* (see asterisk in Fig. 7, A1 and A3) or *Hg-inx2* probe (asterisk in Fig. 7, B1 and B3).

To extend the characterization of innexin expression in identified cells, the soma location of many other STG neurons (up to 22 in a single preparation), identified on the basis of their electrical properties

and nerve projections, were recorded on a micrograph of each STG ($n = 15$) prior to the *in situ* hybridization protocol. Comparison of the position of innexin expressing somata with the position of identified neurons recorded on the STG micrograph allowed identification of some of the innexin expressing neurons. The results of all these experiments are summarized in Table 1. First, a few neurons appeared to be consistently labelled for both *Hg-inx1* and *Hg-inx2* expression; PD, anterior burster (AB), ventral dilator (VD) and inhibitory postsynaptic potential (IPSP) cells were found to express an innexin in more than 80% of the cases. Although the dorsal gastric (DG) neuron was identified in fewer preparations than these neurons (only five preparations), there was evidence of innexin expression in 60% of the cases. Second, some cells [LP, $n = 9$ and inferior cardiac (IC), $n = 7$] showed no evidence of innexin expression in any of the preparations. Third, pyloric motor (PY), lateral pyloric (LPG) and gastric motor (GM) neurons were found to express only *Hg-inx2*. Fourth, in some cases, only a subset of electrically coupled neurons innervating a given stomach muscle was found to express an innexin. For example, in some preparations in which both PDs were identified, only one PD was labelled ($n = 2$ in the ten preparations in which this could be assessed). There are eight PYs and ten GMs, but only a maximum of two neurons for each type was labelled for *Hg-inx2* expression in any preparation. Finally, lateral gastric (LG) was found to express *Hg-inx1* in one out of three preparations.

Innexin expressing neurons display both dye and electrical coupling

To determine if neurons expressing innexin mRNA (summarized in Table 1) display gap-junctional coupling, we performed both dye and electrical coupling experiments. For dye-coupling experiments, each type of identified STG neuron expressing at least one innexin mRNA was intracellularly injected with Neurobiotin™ (Fig. 8 right, white arrows), a low-weight molecular tracer that crosses gap junctions (see Materials and methods). This procedure allowed us to label the population of STG neurons dye-coupled (see micrographs on Fig. 8, white asterisks) to the injected neuron. We found that all STG neurons expressing at least one innexin mRNA are dye coupled to at least one other STG neuron (PD, $n = 12$; AB, $n = 2$; VD, $n = 4$; PY, $n = 5$; IPSP, $n = 3$; DG, $n = 3$; LPG, $n = 1$; GM, $n = 2$; LG, $n = 1$). For electrical coupling experiments, a -20 nA current pulse was injected into potential presynaptic neurons and a voltage variation was recorded in the postsynaptic (innexin expressing) neuron (see examples of recordings on Fig. 8). These experiments demonstrate that all STG neurons expressing at least one innexin mRNA are electrically coupled to at least one other STG neuron. In conclusion, all STG neurons that were found to express at least one innexin mRNA were also found to be dye and electrically coupled to other STG neurons.

Fig. 3. *In situ* hybridization expression pattern of *Hg-inx1* and *Hg-inx2* mRNA within the stomatogastric ganglion of *Homarus gammarus*. (A) Schematic drawing of the lobster stomatogastric nervous system showing the four ganglia (stomatogastric, STG; oesophageal, OG; and two commissural CoG). The main nerves are indicated in italicized characters. The box highlights the region observed in the micrographs B1, C1, D1, D2, D3 and D5. (B and C) Images of *in situ* hybridization staining of the STG using antisense (labelling; B1, B2, C1, C2) or sense (control; B3, C3) probe for *Hg-inx1* (B) and *Hg-inx2* (C). B2 shows a higher magnification of preparation in B1 whereas C2 is not the same preparation as C1. These preparations treated with antisense probes display labelled cells with a clear ring of dense granular staining surrounding the nucleus (black arrowheads) that is not visible in unstained cells (asterisks in B2 and C2). A quantification of the number of stained cells is shown in B3 for *Hg-inx1* ($n = 19$) and in C3 for *Hg-inx2* ($n = 18$). B1 is a photomontage from six photographs. In all pictures *stm* is at the top and *dvn* at the bottom. (D) Control STG preparations treated with sense *Hg-inx1* probe (D1, D3, D4), sense *Hg-inx2* probe (D2) or no probe (D5) show no cell with dense perinuclear staining. However in some of these preparations a few cells with homogenous colouration (marked with asterisks in D4) can be seen; this is regarded as a background artefact of the methodology. Scale bars, 100 μ m (B1, B3, C1 and C3) and 10 μ m (B2 and C2). CoG, commissural ganglion; *dvn*, dorsal ventricular nerve; OG, oesophageal ganglion; STG, stomatogastric ganglion; *stm*, stomatogastric nerve.

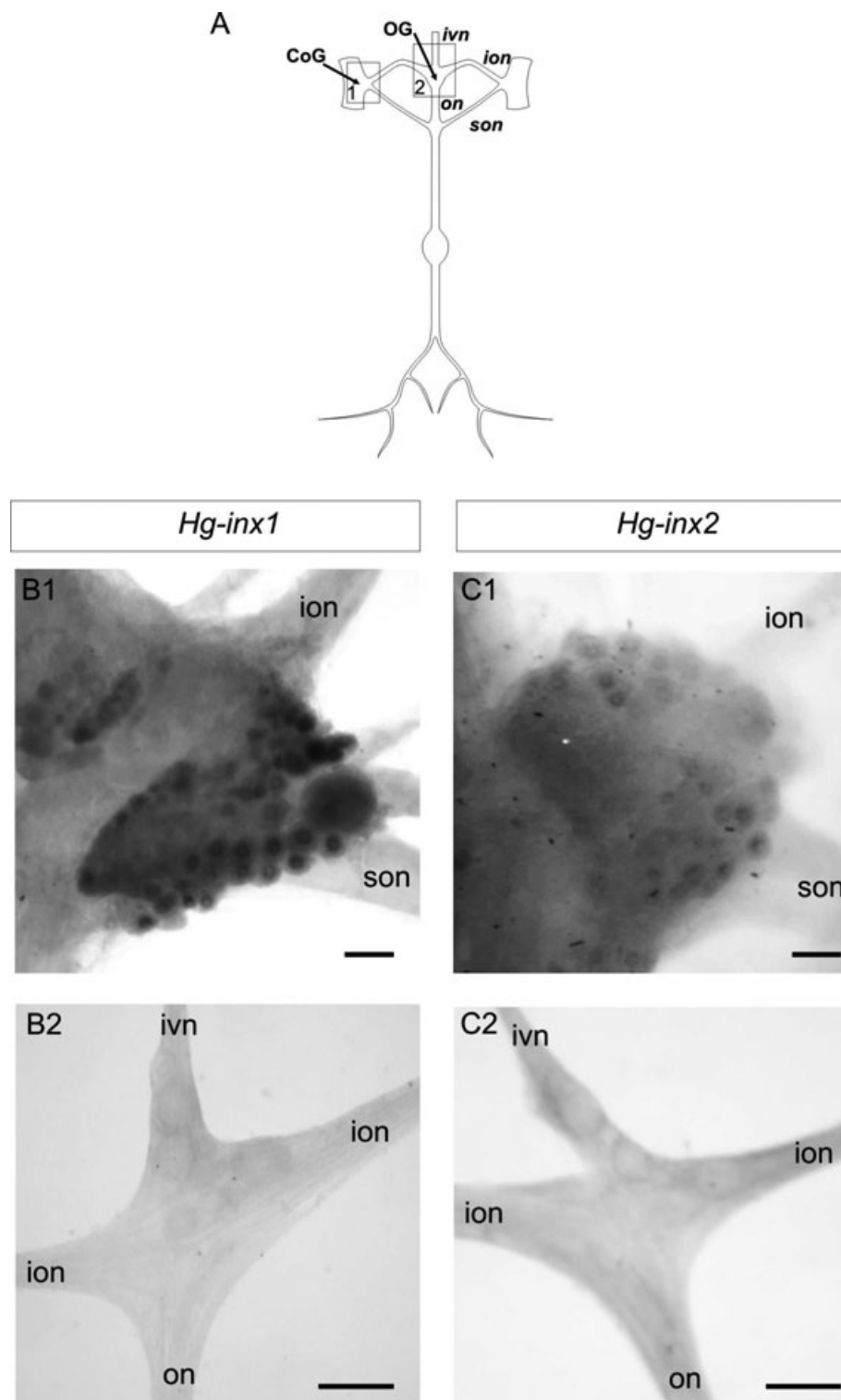


FIG. 4. *In situ* hybridization expression pattern of *Hg-inx1* (B) and *Hg-inx2* (C) mRNA in the CoG (B1 and C1) and OG (B2 and C2). (A) Schematic drawing of the preparation showing the location of the CoG (box 1) and OG (box 2) modulatory ganglia. (B and C) *In situ* hybridization reveals numerous stained soma within the CoG (B1 and C1) using antisense probe for *Hg-inx1* (B) and *Hg-inx2* (C), while no staining is apparent in the OG (B2 and C2). Scale bars, 100 μ m. Abbreviations as in Fig. 3 and *ion*, inferior oesophageal nerve; *ivn*, inferior ventricular nerve; *on*, oesophageal nerve; *son*, superior oesophageal nerve.

Neurons lacking innexin expression display weak electrical coupling and in some rare cases dye coupling

Among STG neurons, we found that LP and IC show no evidence of *Hg-inx1* or *Hg-inx2* expression. We therefore addressed whether

these neurons are dye and electrically isolated from other STG neurons. Neurobiotin injected into the soma of LP ($n = 4$) or IC ($n = 2$) remained within these cells (micrographs in Fig. 9, A1 and A2) and did not pass into other STG neurons; we conclude that LP and IC are dye isolated. However, using electrophysiological

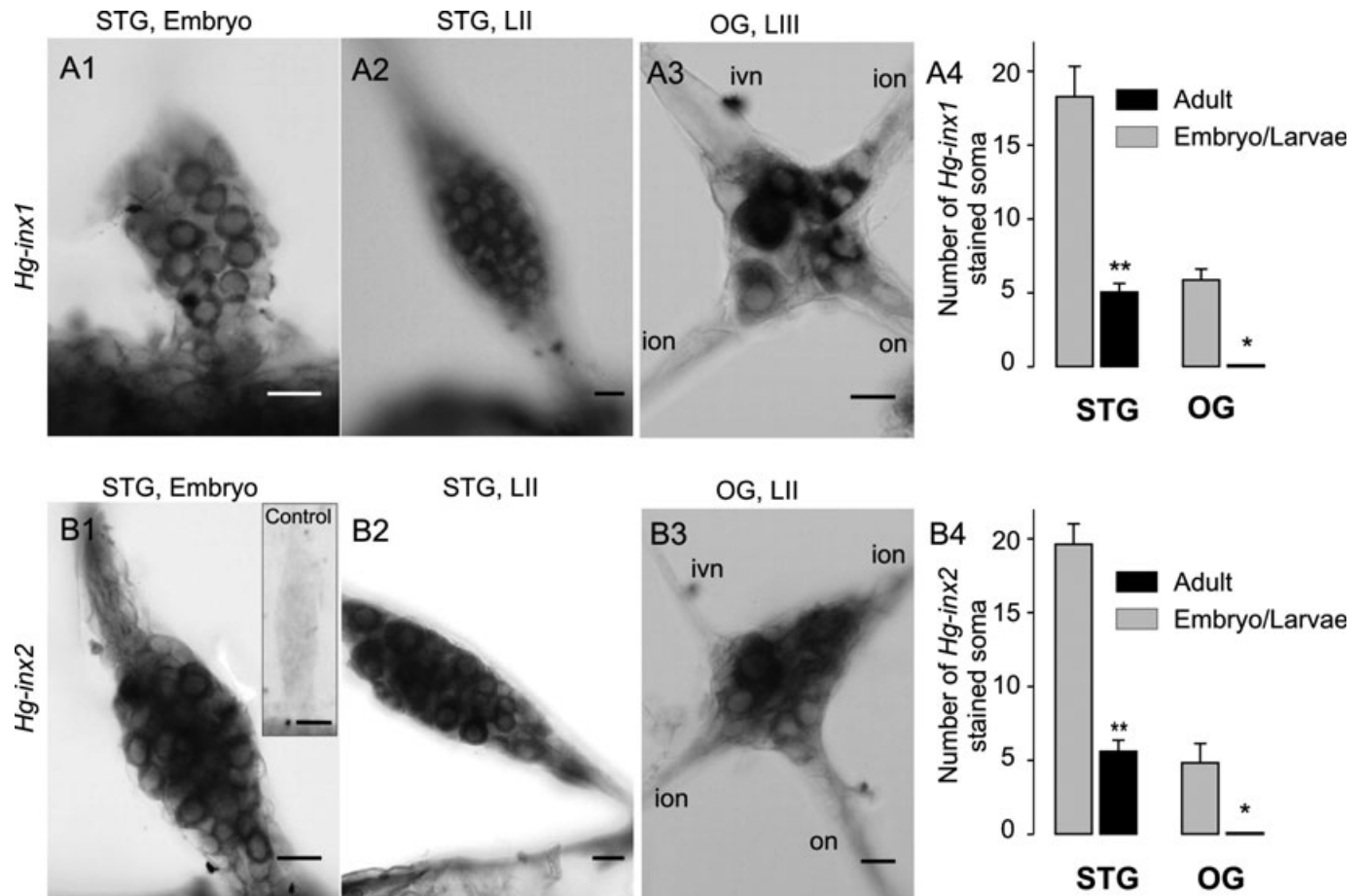


FIG. 5. Innexin expression within the STNS throughout embryonic and larval development. (A) *Hg-inx1* mRNA is expressed in the embryonic (A1) and larval (LII, A2) STG as well as in the larval OG (LIII, A3). The number of *Hg-inx1* stained cells in the embryonic or larval ($n = 11$) STG is significantly higher than in the adult ($n = 19$, A4). The number of *Hg-inx1* stained cells in the embryonic or larval ($n = 8$) OG is also significantly higher than in the adult OG ($n = 10$, A4). (B) Similarly, *Hg-inx2* mRNA is expressed in the embryonic (B1) and larval (LII, B2) STG as well as in the larval OG (LII, B3). The insert in B1 is an embryonic STG treated with sense *Hg-inx2* probe; note the absence of staining. The number of *Hg-inx2* stained cells in the embryonic/larval ($n = 8$) STG is significantly higher than in the adult ($n = 18$, B4). The number of *Hg-inx2* stained cells in the embryonic or larval ($n = 6$) OG is also significantly higher than in the adult OG ($n = 8$, B4). All comparisons were performed using the Mann–Whitney rank sum test; ** $P < 0.001$; * $P < 0.01$. In all micrographs of the STG, the *stm* is at the top. Scale bars, 20 μ m.

experiments, we found that both LP and IC display weak but significant electrical coupling with, respectively, PY and PD (mean coupling coefficient was 0.008 ± 0.001 for PY to LP synapse and 0.003 ± 0.001 for PD to IC compared for example to a coupling coefficient of 0.18 ± 0.03 for the PD to AB synapse). The discrepancy between the absence of dye coupling and the presence of electrical coupling led us to address whether the absence of neurobiotin staining within neurons electrically coupled to LP or IC could result from specific properties of these electrical synapses. Coupling coefficient measurements revealed that both synapses display rectification (curves in Fig. 9, A1 and A2). Because NeurobiotinTM may not diffuse through rectifying electrical synapses equally in both directions, we attempted to dye fill LP and IC from their known electrically coupled partners (respectively PY and PD). NeurobiotinTM injected into the soma of either one PY or one PD neuron resulted in the staining of several other neurons, but LP or, respectively, IC identified by Dextran Tetramethyl Rhodamin injection did not appear to be stained by NeurobiotinTM in three out four preparations for each (micrograph on the left in Fig. 9, B1 and B2). However, in the remaining preparation, we found that LP and IC were dye coupled to, respectively, PY and PD ($n = 1$ each,

micrograph on the right in Fig. 9, B1 and B2). Altogether these results show that, despite no detectable levels of *Hg-inx1* or *Hg-inx2* mRNA, LP and IC do display weak electrical coupling, and in some rare cases dye coupling, with some other STG neurons.

Discussion

In the present study, we have cloned two lobster innexins *Hg-inx1* and *Hg-inx2*. These constitute the two first crustacean members of the innexin family. Our combination of anatomical, molecular and physiological approaches provide characterization of gap-junctional coupling within small pattern-generating neural networks. Indeed, these two innexins are expressed within the developing and adult lobster STNS. A marked decrease is observed in the number of innexin expressing neurons in the adult compared to the embryo, which may suggest a possible role for innexin down-regulation in network ontogeny. All adult neurons expressing at least one innexin also display dye and electrical coupling with at least another network partner. However, neurons that express neither *Hg-inx1* nor *Hg-inx2* do display electrical coupling but are rarely dye coupled to other network partners.

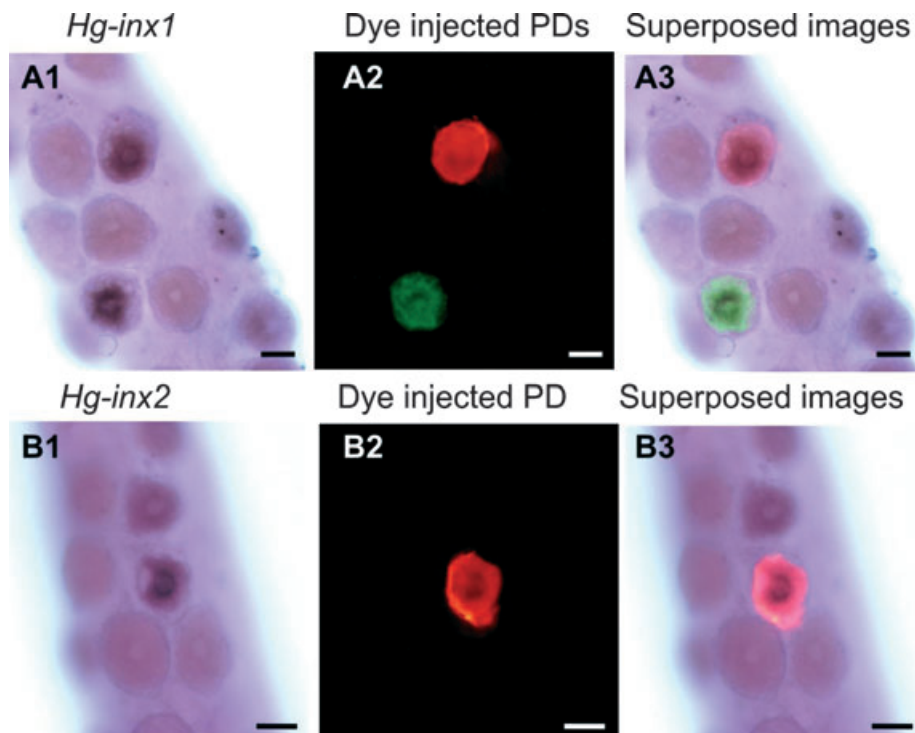


FIG. 6. Expression of *Hg-inx1* and *Hg-inx2* in pyloric dilator (PD) neurons. (A) *Hg-inx1* *in situ* hybridization of the STG (A1) after injection of high molecular weight dyes (Dextran Tetramethyl Rhodamine, red staining; Dextran Lucifer Yellow, green staining) in the two identified PD neurons (A2). Superposition of the images A1 and A2 clearly shows that both PD neurons express *Hg-inx1* mRNA (A3). (B) Similarly, superposition (B3) of *Hg-inx2* *in situ* hybridization (B1) and Dextran Tetramethyl Rhodamine injected PD neuron (B2) clearly demonstrates that this PD neuron expresses *Hg-inx2* mRNA. Scale bars, 50 μ m.

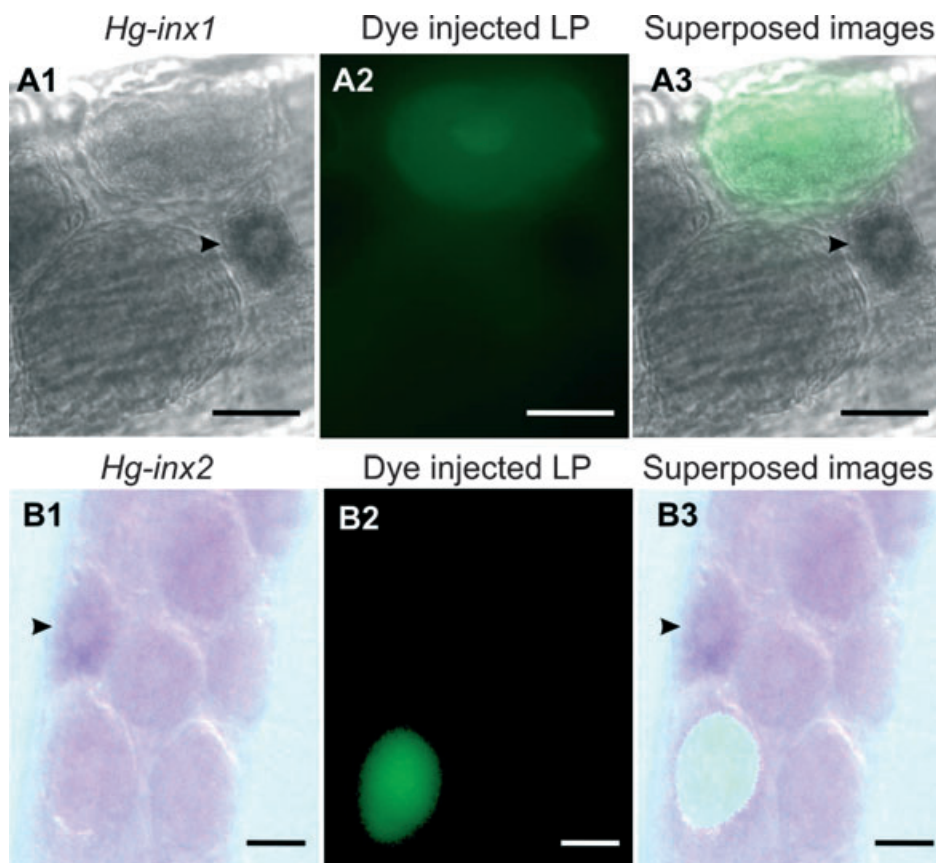


FIG. 7. Lack of expression of *Hg-inx1* and *Hg-inx2* in lateral pyloric (LP) neuron. (A) *Hg-inx1* *in situ* hybridization of the STG (A1) after injection of Lucifer Yellow in an identified LP neuron (A2). Superposition of the two previous pictures (A3) clearly shows that LP does not express *Hg-inx1* mRNA. Note that although LP is not stained with the *in situ* probe, one clearly stained cell is visible (arrowhead). (B) Similarly, superposition (B3) of *Hg-inx2* *in situ* hybridization (B1) and Lucifer Yellow injected LP neuron (B2) clearly demonstrates that LP does not express *Hg-inx2* mRNA. However an adjacent neuron (arrowhead) is clearly expressing *Hg-inx2*. Scale bars, 50 μ m.

The evolution of gap-junction proteins

Vertebrate gap junctions are comprised of the connexin family of proteins; humans have more than 20 connexins. The completion of

the *Drosophila* and *C. elegans* genome projects showed unequivocally that invertebrates have no connexins (Adams *et al.*, 2000; *C. elegans* Sequencing Consortium 1998), but instead use the innexin protein family to build gap junctions (Phelan *et al.*, 1998a, b).

TABLE 1. Occurrence of *Hg-inx1* and *Hg-inx2* expression in the identified STG neuronal population

Neuron	PD	AB	LP	IC	VD	PY	DG	LPG	GM	LG	IPSP
<i>Hg-inx1</i>	77% (<i>n</i> = 13)	67% (<i>n</i> = 3)	0% (<i>n</i> = 4)	0% (<i>n</i> = 3)	83% (<i>n</i> = 6)	0% (<i>n</i> = 11)	67% (<i>n</i> = 3)	0% (<i>n</i> = 9)	0% (<i>n</i> = 21)	33% (<i>n</i> = 3)	72% (<i>n</i> = 11)
<i>Hg-inx2</i>	83% (<i>n</i> = 12)	100% (<i>n</i> = 3)	0% (<i>n</i> = 5)	0% (<i>n</i> = 4)	80% (<i>n</i> = 5)	30% (<i>n</i> = 10)	50% (<i>n</i> = 2)	45% (<i>n</i> = 11)	20% (<i>n</i> = 25)	–	100% (<i>n</i> = 4)

The table summarizes *Hg-inx1* and *Hg-inx2* expression (in percentage of the observed cells) in each type of STG neuron of the pyloric (PD, AB, LP, IC, VD, PY), gastric (DG, LPG, GM, LG) and oesophageal (IPSP) networks, identified in 15 preparations. Each number in brackets represents the total number of neuron of a given type identified in the eight preparations processed with *Hg-inx1* probe or in the seven preparations processed with *Hg-inx2* probe. Note that only LP and IC express neither *Hg-inx1* nor *Hg-inx2*. LG was identified only once in preparations treated with *Hg-inx2* probes (no staining), therefore no percentage is provided.

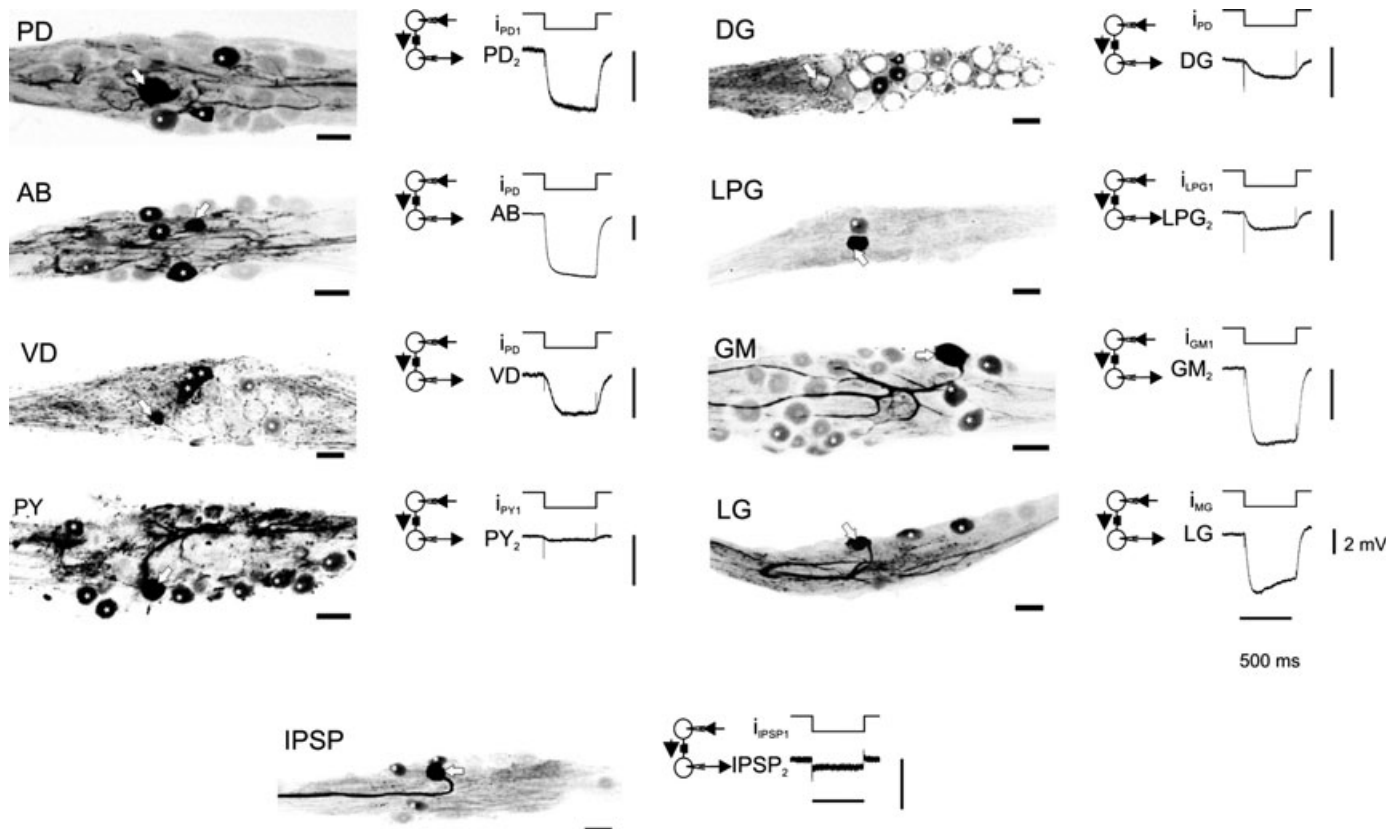


FIG. 8. Neurons expressing *Hg-inx1* and/or *Hg-inx2* are dye and electrically coupled with other STG neurons. Micrographs illustrate dye coupling after Neurobiotin™ injection into each type of STG neuron expressing at least one innexin (injected neuron is indicated by a white arrow). Note that all these neurons are dye coupled to one or several cells within the STG (white asterisks). All images are projections of a full confocal stack except for DG, which is a single 2.5-μm thick focal plane from a confocal stack. It is noteworthy that in several preparations in which DG was injected, little staining remained in the soma of DG whereas dye-coupled neurons were more intensely stained. Recording on the right of each micrograph shows the voltage variation (mean of ten traces) recorded in the identified neuron following intrasomatic injection of a square current pulse (−20 nA) into an electrically coupled neuron, indicated in small characters. Numbers following PD, PY, IPSP, LPG and GM are provided to indicate that current injection and voltage recordings are made in two different neurons of the same group. Scale bars, 100 μm.

The innexins have the same four transmembrane topology but no sequence homology to connexins. Recent work has shown that the simple diploblastic organism hydra, representing an evolutionary grade before the divergence of the protostome and deuterostome lineages, appears to possess at least five innexin but no connexin genes (Alexopoulos *et al.*, 2004). This key finding, plus the observation that the protocordate *Ciona intestinalis* has connexin and innexin homologues (Dehal *et al.*, 2002; Sasakura *et al.*, 2003), and that presumptive innexin homologues (the pannexins), data-

mined from the human and mouse genome (Panchin *et al.*, 2000), form functional gap junctions when expressed in *Xenopus oocytes* (Bruzzone *et al.*, 2003), suggest a parsimonious scenario for gap-junction evolution. Diploblasts, such as hydra, evolved innexins to mediate gap-junction communication that were inherited by the protostome and deuterostome lineages. In the deuterostome lineage the connexins evolved *de novo*, perhaps as a result of gene duplication, allowing the innexin to form the pannexin subfamily.

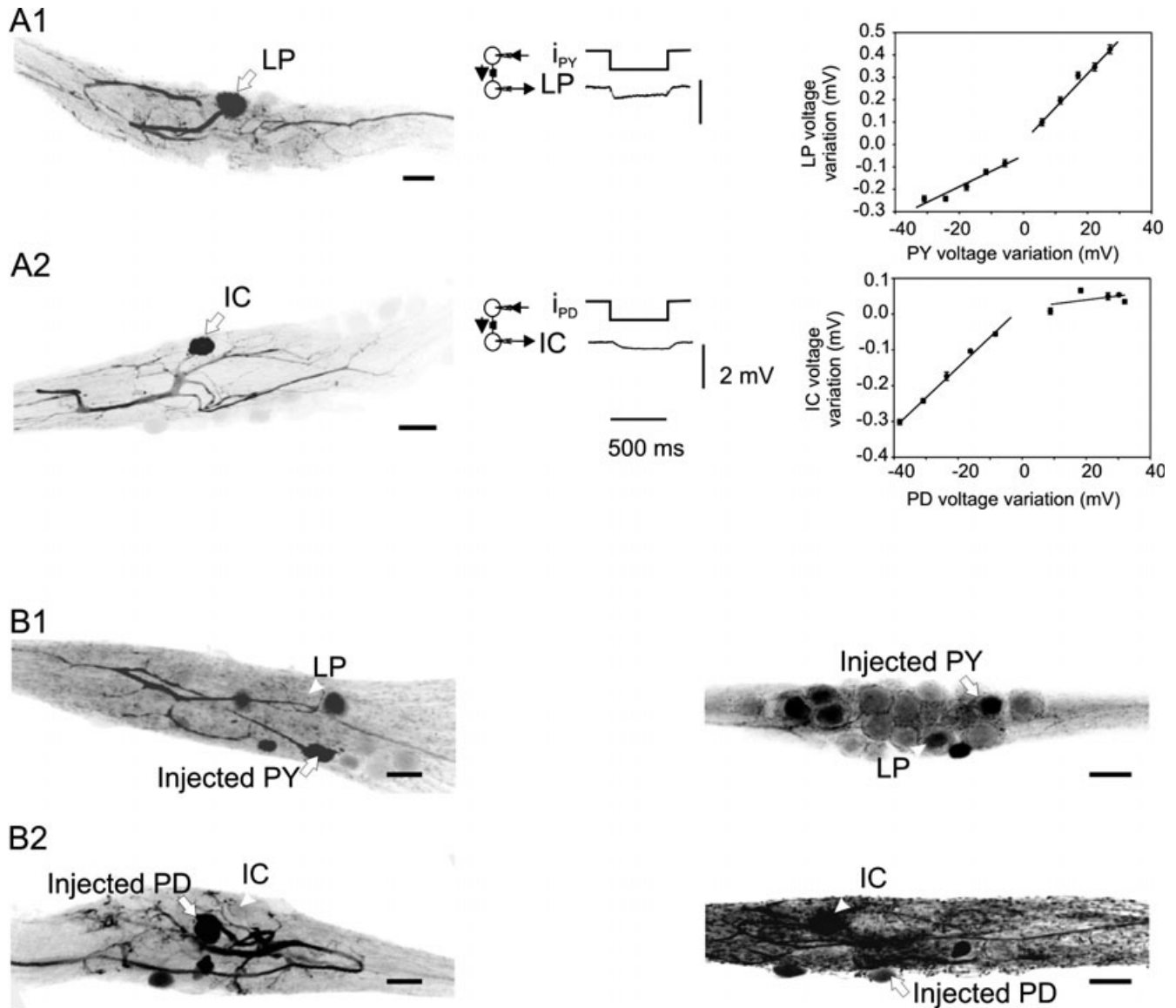


FIG. 9. Neurons lacking *Hg-inx1* and *Hg-inx2* mRNA expression display weak electrical and rare dye coupling with other STG neurons. (A1) NeurobiotinTM injected into the soma of LP (white arrow) does not stain any other STG neurons, suggesting that LP is not dye coupled to other STG neurons (micrograph on the left). However, current injection into a PY neuron induces voltage deflection into LP (recording in the middle panel, mean of ten traces) demonstrating that LP is electrically coupled to some of the PYs. The two different slopes of the curve showing the variation of LP voltage as a function of PY voltage (right panel) shows this electrical coupling to have rectifying properties. (A2) Similarly, NeurobiotinTM injected into the soma of IC (white arrow) does not pass into other STG neuron (micrograph on the left), but current injection into a PD neuron induces voltage deflection in IC (recording in the middle panel, mean of ten traces) demonstrating that IC is electrically coupled to PD. This electrical coupling is also clearly rectifying (right panel). (B1) NeurobiotinTM injection into the soma of PY (white arrow) that is electrically coupled to LP (see A1) results in no staining in LP in three out of four preparations (one example shown on left). However, in one preparation out of four, LP was among the stained neurons (left). (B2) NeurobiotinTM injected into the soma of PD that is electrically coupled to IC (see A2) does not pass in IC in three out of four preparations (one example shown on left). However, in one preparation out of four, IC was among the stained neurons (left). All images are projections of a full confocal stack except for right panel of B1, which is a single 2.5- μ m thick focal plane from a confocal stack. Scale bars, 100 μ m.

The present study, which is the first characterization of crustacean innexins, provides vital additional data to understanding the evolution of the innexin protein family within protostomes. It was known that *Drosophila* has at least ten (Stebbins *et al.*, 2002), and *C. elegans* has 25 innexin proteins (Starich *et al.*, 2001). In addition to this, innexin genes have been cloned in Lophotrochozoans such as the leech *Hirudo medicinalis* (Dykes *et al.*, 2004), the molluscs *Lymnaea stagnalis* (Alexopoulos *et al.*, 2000) and *Clione limacina* (Panchin *et al.*, 2000) and the polychaete annelid *Chaetopterus variopedatus* (Potenza *et al.*, 2002). A recent phylogenetic analysis

of innexin genes supports the existence of the molluscan and annelid Lophotrochozoan clade, but that the Ecdysozoan *C. elegans* innexin clade is more closely related to the Lophotrochozoans than it is to the arthropod Ecdysozoan clade. This suggests rapid evolution of the innexins in the arthropod clade (Dykes *et al.*, 2004). The findings in the current paper suggest that arthropod innexins arose as an intraclade duplication of an ancestral Ecdysozoan innexin. Within arthropods this ancestral innexin then underwent gene duplication and sequence divergence leading to different innexins before the separation of crustaceans and insects.

Correlating dye coupling, electrical synapses and innexin expression within identified neurons

Among the adult STG neurons, we show that all identified neurons investigated, with the exception of LP and IC, express *Hg-inx1* and/or *Hg-inx2* in at least one preparation. For all those neurons expressing at least one innexin, NeurobiotinTM injection shows that they are dye coupled to at least one other STG neuron. Furthermore, all neurons displaying innexin expression and dye coupling also display electrical coupling with at least another STG neuron.

Only two of the identified STG neurons investigated in this study, LP and IC, do not express either *Hg-inx1* or 2. We found that LP and IC are rarely and unidirectionally dye coupled to PY and PD, respectively, and that LP and IC display electrical coupling with the neuron to which they are dye coupled as already reported in a related species, *Panulirus interruptus* (Johnson & Harris-Warrick, 1990; Johnson *et al.*, 1993).

There are two obvious explanations for this apparent discrepancy between lack of innexin expression and the presence of dye (albeit rare) and electrical coupling. The first is that LP and IC may express other innexins. Confirmation of this requires the molecular cloning of the complete set of innexins within the *Homarus gammarus* STNS. Additional innexin genes may well be expressed in the lobster STNS because six innexin genes are expressed in *C. elegans* neurons (Starich *et al.*, 2001) and at least seven in *Drosophila* neurons (Stebbins *et al.*, 2002). The second, perhaps most parsimonious, explanation is that LP and IC may express low levels of *Hg-inx1* and *Hg-inx2*, subthreshold for our *in situ* techniques; such very low levels of innexin expression may underlie the rare dye coupling and weak electrical coupling displayed by LP and IC.

In the STG, we found variability in mRNA expression in PD neurons (only 80% of the identified PDs were found to express at least one innexin and in 20% of the preparations where this could be assessed, only one PD was stained) although they always display strong electrical coupling. Variability of innexin expression appeared even more pronounced in the two large groups of PY and GM neurons, in which less than 30% of the identified neurons were stained for *Hg-inx2*. For example, in preparations on which four PY and/or eight GM neurons were identified, only one PY and one GM were labelled for *Hg-inx2*. Such apparent variability in gene expression from one animal to the other and within groups of neurons innervating a given muscle has already been reported within the STG for the *shab* gene, which encodes delayed rectifier-like channels (Baro *et al.*, 1994), as well as for BK-KCa mRNAs that encode large conductance calcium activated potassium channels (Schulz *et al.*, 2006).

This variability in innexin mRNA staining is consistent with there being a relative high threshold of detection of *Hg-inx1* and 2, and so, all neurons expressing weak levels of innexin mRNAs would remain undetected. The use of enzymatic techniques for *in situ* hybridization, although quite sensitive, is not as sensitive as other techniques such as single cell RT-PCR and may be responsible for this variable staining. Alternatively, the apparent variability in innexin expression may result from a long half life of innexin proteins, and perhaps innexin mRNAs are transcribed only transiently when required. Preliminary experiments reported in Curtin *et al.* (2002) show that innexins have a half-life of approximately 6 h in *Drosophila* and it seems reasonable to assume that innexin half-life is significantly longer in the cold-water living lobster. Apparent variability of innexin expression may also result from a difference in the lobster's state (sex, moulting episode, social status, etc.). Despite this variability, it is the case that in the pyloric network, neurons that are most frequently labelled (PD, AB, VD) are those that exhibit strong coupling coefficients with their

partners, whereas cells that are rarely or never labelled such as PY, LP and IC have weak coupling coefficients (data not shown). Establishing a correlation between the level of innexin mRNA expression and the strength of electrical coupling could not be achieved in this paper in the absence of quantitative RT-PCR measurements. However, it should be noted that establishing a correlation between mRNA expression and the phenotype expressed by individual cells may be hard to achieve. Indeed, several studies have emphasized that mRNA levels alone are unreliable indicators of the corresponding protein levels and therefore unreliable indicators of individual cell phenotype (reviewed in Pradet-Balade *et al.*, 2001).

Innexin expression is down-regulated in the STNS during development

Modelling studies have suggested that the switch, after metamorphosis, from a single embryonic network to multiple adult networks within the STNS could be mediated entirely by a large decrease in electrical coupling between neurons (Bem *et al.*, 2002). We found a clear decrease in the mean number of adult STNS neurons expressing *Hg-inx1* and/or *Hg-inx2* compared to embryonic and larval STNS. Such decrease could result from a down-regulation of innexin gene expression, which in turn suggests that electrical coupling may be stronger and more widespread in the embryonic and larval STNS than in the adult and provide strong support for hypothesis that emerged from the modelling study of Bem *et al.* (2002). Alternatively, this decrease could result from a difference in innexin mRNA half-life between the embryo and the adult.

The decrease in the number of innexin expressing neurons is seen in both the STG and the OG. Indeed, no innexin expressing cell could be found in the adult OG, whereas several stained neurons are present in the embryos and larvae. The OG is known to contain some oesophageal motoneurons (Selverston & Moulins, 1987) which are components of the unique embryonic motor network (Casasnovas & Meyrand, 1995). Therefore, a decrease in electrical coupling within these oesophageal neurons may well contribute to the reconfiguration of the single embryonic network to the multiple adult networks (oesophageal, gastric and pyloric).

A decrease in electrical coupling throughout development has been reported in vertebrate CNS using dye-coupling techniques (Connors *et al.*, 1983; Peinado *et al.*, 1993a, b), electrophysiology (Chang *et al.*, 1999; Vandecasteele *et al.*, 2005) and connexin-expression studies (Dermietzel *et al.*, 1989; Nadarajah *et al.*, 1997; Sohl *et al.*, 1998). For neocortical development, it has been proposed that early widespread gap-junctional coupling is vital for neuronal migration, cortical lamination and the development of functional architecture (reviewed in Montoro & Yuste, 2004). In spinal cord, it has been suggested that the decrease in coupling between motoneurons may play a role in the developmental transition from multiple to single innervation of skeletal muscle fibers (Chang *et al.*, 1999; Personius & Balice-Gordon, 2001). Our present results suggest that a decrease in electrical coupling is also present in small developing invertebrate neural networks. Consequently, our model will provide a simple and more accessible preparation to decipher the role of diminishing electrical coupling in network maturation.

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Abbreviations

AB, anterior burster; CoG, commissural ganglia; DG, dorsal gastric; GM, gastric motor; Hg-inx1, *Homarus gammarus*-innexin 1; Hg-inx2, *Homarus gammarus*-innexin 2; IC, inferior cardiac; IPSP, inhibitory postsynaptic potential; LG, lateral gastric; LP, lateral pyloric; LPG, lateral posterior gastric; OG, oesophageal ganglion; PBT, 0.1% Tween 20 in PBS; PD, pyloric dilator; PY, pyloric motor; RACE, rapid amplification of cDNA ends; SSC, saline sodium citrate; STG, stomatogastric ganglion; STNS, stomatogastric nervous system; VD, ventral dilator.

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