Gap junctions in nematodes

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Summary. Gap junctions (GJ) are one of the most common forms of intercellular communication. They are considered to serve a similar function in all multicellular animals (Metazoa) and provide a general route for intercellular interactions in various tissues and cell types. GJ are composed of proteins that form a channel connecting the cytoplasm of adjacent cells. Two unrelated protein families are involved in this function: connexins, which are found only in chordates, and pannexins/innexins, which are present in both chordate and invertebrate genomes. Nematodes are recognised as extremely useful model organisms for various areas of biological studies with complete genomes sequenced in many species and the free-living nematode, *Caenorhabditis elegans*, is one of the best studied model organisms. Genomes of different nematode species contain 12-25 pannexins/innexins. We compared nematode GJ proteins with GJ proteins of other species, reviewed their expression patterns and their role in several biological functions.

Key words: *Caenorhabditis elegans,* connexin, innexin, nematode, pannexin.

Gap junctions **(**GJ) are fundamentally important and intensively studied. GJ are composed of proteins. Two unrelated protein families are involved: connexins, found only in chordates, and pannexins/innexins, found in both chordate and invertebrate genomes. In the past 45 years 16350 publications have been recorded in PUBMED referring to GJ, connexins, innexins or pannexins. GJ provide one of the most common forms of intercellular communication. They are composed of membrane proteins that form a channel that is permeable to ions and small molecules, connecting the cytoplasms of adjacent cells. They are considered to be a universal feature of all multicellular animals (Metazoa) and play important role in different biological functions. Nematodes are recognised as extremely useful model organisms in biological studies, since the groundbreaking works of Sydney Brenner on *Caenorhabditis elegans* (Brenner, 1974). Complete genomes are now sequenced for 79 nematode species, providing the basis for genomic studies. Some nematodes serve as satellite model organisms to *C. elegans*. The gene number in nematode genomes varies significantly from about 20000 (similar to the human gene count) to only 6712 genes in a plant parasite *Pratylenchus coffeae* (the smallest completely sequenced bilaterian genome until now) (Schaff *et al.*, 2015). GJ in *C. elegans* and other nematodes are reliably identified by transmission electron microscopy (White *et al.*, 1986). All of the electrical synapses in *C. elegans* (which are actually GJ) were identified in impressive research to achieve the complete reconstruction of the nematode nervous system. These were pioneering connectomic studies that started way before this new 'omic' term appeared. Nematode studies contributed significantly to different aspects of GJ studies and comparative approaches may further affect future research.

There are also several considerations in favour of using GJ as potential targets for anthelmintics. GJ in nematodes are made of pannexins, while in mammals this function is mostly occupied by connexins. Only three pannexins are preserved in mammalian genomes. It appears that their functions are not very critical to mammalian fitness. Pannexin knockouts in mammals have no apparent phenotypes, although some biological effects may be found in targeted experiments (Romanov *et al.*, 2012; Gaynullina *et al.*, 2014, 2015). By contrast, some pannexin mutations in nematodes lead to apparent morphological defects before lethal phenotype (Li *et al.*, 2003; Liu *et al.*, 2013; Simonsen *et al.*, 2014). Thus, strong blockers of pannexins could be expected to have much stronger effects on nematodes and other invertebrates than on vertebrates. Ivermectin is one of the most effective anthelmintic and it is used to treat numerous

parasitic infections of humans, pets, and livestock. Although its prime targets are probably glutamate-gated chloride channels, it was demonstrated that mutations of GJ genes may contribute to ivermectin sensitivity (Boswell *et al.*, 1990, Dent *et al.*, 2000).

INTERCELLULAR CHANNELS AND GAP JUNCTIONS

Intercellular communication is a basis for coordinated function of multicellular organisms. Numerous types of molecules were shown to transfer biological signals from one cell to another. Typically, such molecules are released by special mechanism from one cell and diffuse in the extracellular space to act on neighbouring or distant cells of the same multicellular organisms. Another route of communication requires direct cellular contacts that may use signal molecules and their receptors that are bound to membranes of the adjacent cells. Yet the most intimate intercellular contacts occur by direct linkage of cytoplasm of two cells. In some cases complete cell fusion may occur as in multinucleated syncytia. Otherwise, distinct specialised channels may link adjoining cells. Plants and fungi have intercellular channels. Their cytoplasmic bridges are formed by the cell membrane and such channels are called plasmodesmata and septal pores. In animals (Metazoa) intercellular channels are assembled from specialised proteins known as GJ. In excitable tissues (neurons, muscles) GJ are also referred to as electrical synapses. Two cells contribute to GJ channels by providing half-channels (or hemichannels). Two such hemichannels from adjacent cells may dock to form functional coaxial cell-cell junctional channels (Fig. 1). All studied GJ (including those found in vertebrate and invertebrate species) were considered to share similar hexameric structures, calculated pore size, voltage-gating properties and sensitivity to the same classes of pharmacological agents (Bruzzone *et al.*, 1996; Phelan & Starich, 2001; Levin, 2002). These seemingly very similar structures are assemblages of proteins belonging to two different unrelated protein families.

GJ MOLECULES

Connexins were identified as the molecular components of vertebrate GJ about 30 years ago (Paul, 1986). For long time it was assumed that, connexins were the only family of GJ proteins. Yet numerous attempts to clone connexins from invertebrates failed, and finally it was suggested that invertebrate GJ are assembled from proteins unrelated to connexins (Barnes, 1994). The *C. elegans* genome published in 1998 was the first sequenced animal genome that enabled searches for the presence of connexin homologues in invertebrates (The *C. elegans* Sequencing Consortium, 1998). The absence in *C. elegans* of the only known (at that time) connexin GJ protein family provided evidence that more than one protein family may be responsible for GJ function. This protein family was found in studies that analysed *Drosophila* and *C. elegans* mutants, and was originally designated OPUS for four founder protein family members: **o**gre, **p**assover, **u**nc-7 and **s**hakingB (Barnes, 1994; Phelan *et al.*, 1996; Starich *et al.*, 1996). Heterologous expression experiments with *C. elegans* and *Drosophila melanogaster* putative GJ proteins confirmed OPUS GJ function (Phelan *et al.*, 1998a; Landesman *et al.*, 1999; Stebbings *et al.*, 2000). Later they were renamed innexins, as it was suggested that they were specific invertebrate GJ proteins (Phelan *et al.*, 1998b).

Fifteen years ago, we found the presence of innexin homologues in humans and other vertebrates, and proposed to reclassify innexins and their vertebrate homologues into a bigger family named, pannexins (Panchin *et al.*, 2000). In this paper, we will use the terms innexins, pannexins or innexins/pannexins as synonymous. Despite their independent phylogenetic origin, both connexins and pannexins share a similar four transmembrane (4-TM) domains structure (Fig. 1A). Moreover, connexins and pannexins assemble GJ channels that presumably fit a similar subunit structure (Fig. 1B). According to existing models, a GJ hemichannel is formed by assembly of corresponding GJ protein subunits making a doughnut shaped structure. The proposed intercellular channel was named connexon or pannexon, respectively. A full operative channel is formed of a pair of opposing ‘doughnuts’ residing in membranes of neighbouring cells (Fig. 1D). Fully assembled GJ channels may differ depending on monomeric protein expression in the same or adjacent cells (Fig. 1D, E).

Until now, it was supposed that the channels assembled from proteins of both families show hexametric organisation with six subunits in one hemichannel. The high-resolution crystal structure of the GJ channel formed by human connexin 26 confirms the connexins hexametric structure (Maeda *et al.*, 2009) that was deduced previously by other methods. Similarly, the innexin hemichannels were believed to be hexameric. *Caenorhabditis elegans* INX-6 overexpression in insect cell culture demonstrated that it forms hexameric channels similar

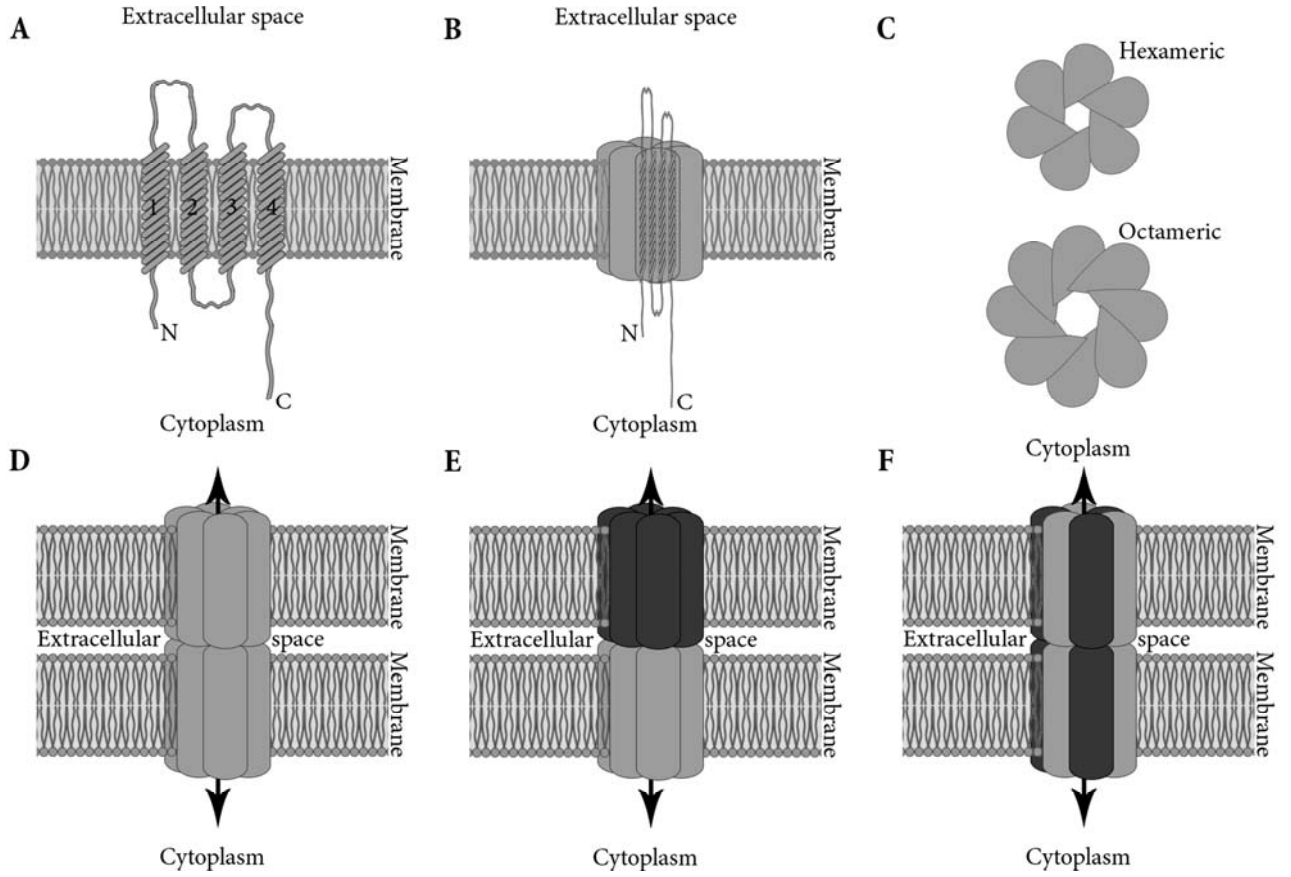

Fig. 1. Pannexin channel structure. A: Pannexin monomers display a similar transmembrane topology with four transmembrane domains, two extracellular loops, and a central cytoplasmic loop; both amino (N) and carboxyl (C) termini have an intracellular location. B: Assembled channel on a cell membrane, named pannexon/innexon, consists of six or eight monomers. They comprise the channel connecting a cytoplasm with extracellular space. This channel, so called 'hemichannel', may be in open or in closed condition. C: Top view of hexameric and octameric pannexon assemblies. D-F: The intercellular channel consists of two pannexons, one from each neighbouring cell. The arrow shows the aqueous pore that directly connects cytoplasms of apposed cells. D: Two hemichannels of similar composition form homotypic channels. E: Two hemichannels of different composition form heterotypic channels. F: Different pannexin monomers may combine to form a heteromeric channel.

to connexins (Oshima *et al.*, 2013). However, in a new paper from the same team it was reported that the actual geometry of the INX-6 channel is octameric and the whole channel of two hemichannels is hexadecameric (Fig. 1C) (Oshima *et al.*, 2016). Interestingly, in cross-linking and native gels studies mammalian Pannexin 2 also showed a banding pattern more consistent with an octamer structure (Ambrosi *et al.*, 2010). Thus, the paradigm of universal hexameric GJ structure may be wrong.

NON-JUNCTIONAL FUNCTIONS OF GAP JUNCTION PROTEINS

In vertebrate species, it was found that a single unpaired hemichannels might exist in the plasma membrane of a single cell as a regular ion channel. These properties of GJ proteins are called non-junctional and are found in both connexin and pannexin protein families (Dahl & Locovei, 2006). For mammalian Pannexins 1 and 3 it was also shown that they may form functional hemichannels in the endoplasmic reticulum membrane that serve as calcium leak channels (Vanden Abeele *et al.*, 2006; Ishikawa *et al.*, 2011).

Non-junctional functions of pannexins in nematodes and other invertebrates are poorly understood in general and require serious consideration. One accepted function of mammalian hemichannels is the transport of ATP into extracellular space. Therefore, pannexins may play a crucial role in purinergic signalling. Lately this research field was extended to invertebrate studies.

Expressed in frog oocytes, three leech pannexins were shown to form ATP permeable hemichannels (Bao *et al.*, 2008). Recently a putative pannexin hemichannel expressed in *C. elegans* touch-sensing neurons was characterised as a large mechanosensitive channel *in situ* and in culture (Sangaletti *et al.*, 2014).

An additional study on nematodes suggests that innexins are involved in some important cellular mechanisms that have no obvious connections to their GJ function. *unc-7* and *unc-9* mutants cause similar defects in the differentiation of electron dense presynaptic plasma membrane region (Yeh *et al.*, 2009). These regions are termed as active zones (Zhai & Bellen, 2004). It was proposed that in this case innexins work as plasma membrane hemichannels or as the hemichannels in some intracellular membrane structures regulating ATP and calcium concentrations (Bouhours *et al.*, 2011).

PHYLOGENETIC TREE OF GAP JUNCTION PROTEINS

We have hypothesised that pannexins are universal GJ proteins of multicellular animals, distinct from connexins that are characteristic only of chordates (Panchin, 2011). Pannexins are found in Chordates, Ctenophores, Cnidarians, and in most major groups of bilateral protostomes (flatworms, nematodes, arthropods, annelids and molluscs). Pannexins were detected in all sequenced nematodes genomes. The number of pannexin genes in different nematode species and in some representatives of other metazoan phyla is shown in Table 1.

Table 1. Predicted number of pannexin genes in various nematode species and in some representatives of other phyla.

Phylum	Name	Number of pannexins
Nematoda	*Caenorhabditis elegans*	25
Nematoda	*Trichinella spiralis*	22
Nematoda	*Pristionchus pacificus*	19
Nematoda	*Ascaris suum*	19
Nematoda	*Trichuris suis*	14
Nematoda	*Romanomermis culicivorax*	14
Nematoda	*Meloidogyne incognita*	13
Nematoda	*Pratylenchus coffeae*	13
Nematoda	*Brugia malayi*	12
Ctenophora	*Pleurobrachia bachei*	6
Cnidaria	*Hydra vulgaris*	11
Arthropoda	*Drosophila melanogaster*	13
Chordata	*Homo sapiens*	3

Phylogenetic trees for pannexin proteins of nematodes and several other animals are shown in Fig. 2A. To reconstruct the evolution of nematode pannexins, we used pannexins from different species belonging to Ctenophora, Cnidaria, Chordata, Arthropoda and pannexins from seven diverse nematode species. To compare the sequence relatedness of pannexin proteins from different organisms a global alignment of all available sequences was generated using Muscle (Edgar, 2004), and a phylogenetic tree was constructed, using PhyML (Guindon *et al.*, 2010).

Proteins from different phyla are not intermixed in tree nodes and the generated pannexin tree satisfies commonly accepted metazoan taxonomy (Fig. 2). All nematode sequences form one common cluster (Fig. 2). At the same time, smaller clusters inside the joint nematode cluster are comprised of proteins of different species (orthologous groups). This is also true for other big phyla if more than one species is included in the alignment (not shown; see Abascal & Zardoya (2013) and Hasegawa & Turnbull (2014). This observation indicates that only one precursor pannexin gene was acquired vertically from their common ancestor and was then diversified independently in each animal phylum.

Recently a new protein family has been suggested to be homologous to pannexins/innexins (Abascal & Zardoya, 2012). In chordates these proteins have four transmembrane domains similar to pannexins (Pannexin-like TM region of LRRC8 PF12534) and a number of leucine-rich repeats (LRR, PF00560). Outside chordates, PF12534 was detected only in *Nematostella* (Anthozoa), and it was even suggested that LRRC8 proteins evolved as the pannexin domain and LRR domain joined together

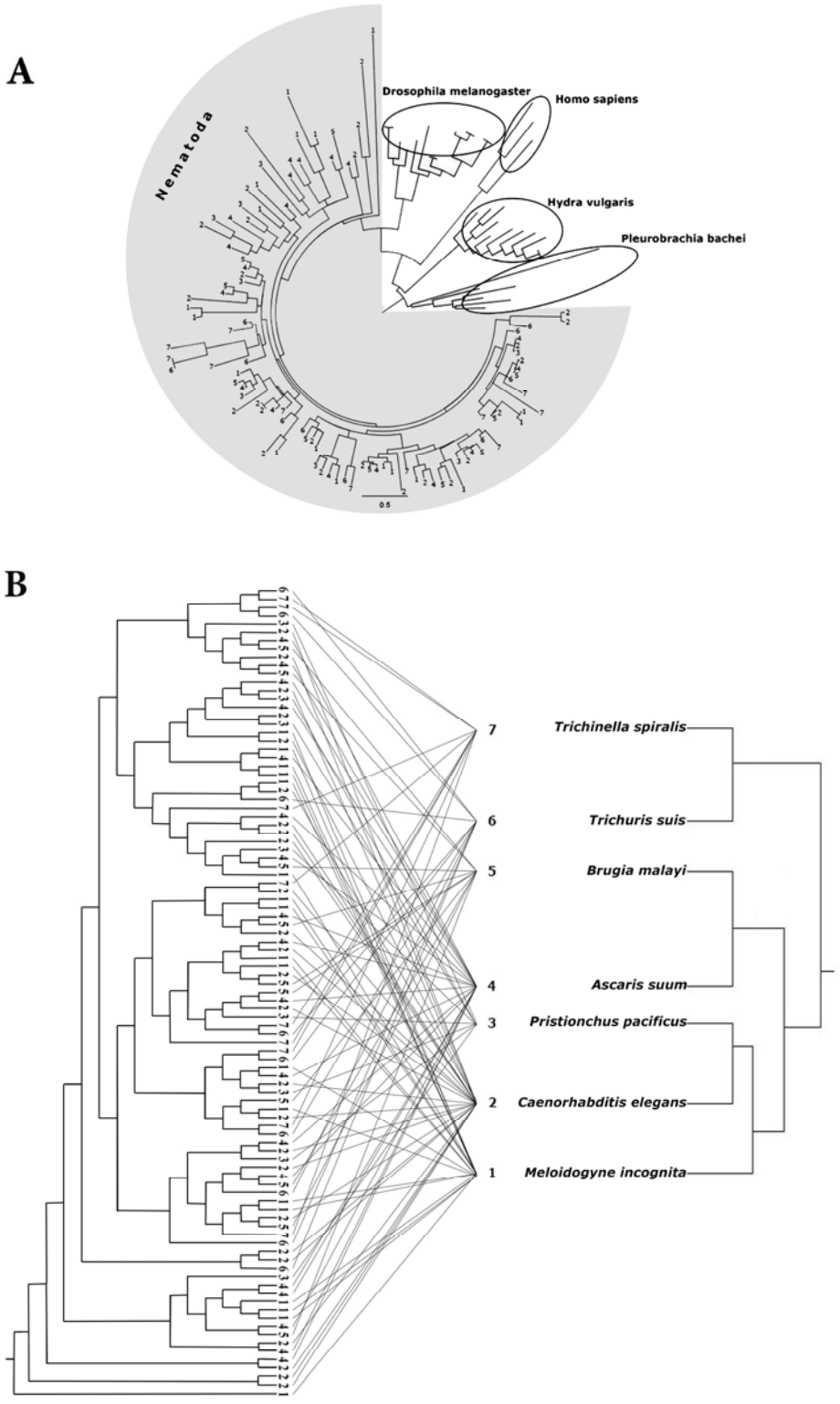

Fig. 2. Phylogeny of the pannexin/innexin protein family. A: Phylogenetic tree of pannexin family in different animal phyla (Ctenophora, Cnidaria, Chordata, Arthropoda, Nematode). Proteins from groups other than nematodes form individual clusters grouped by species (black oval outlines). Genes from diverse nematode species form one big cluster (grey area). B: Pannexins phylogeny in nematodes. A generally accepted nematodes evolutionary tree is presented on the right panel. Each species is assigned a number (1-7) to show the correspondence of different innexins proteins in the tree in left panel.

and this combined gene was later acquired by *Nematostella* by horizontal gene transfer (Abascal & Zardoya, 2013). Although LRRC8 proteins are homologous to pannexins no evidence exist that they participate in GJ function. They were reported to form volume-regulated anion channel VRAC (Voss *et al.*, 2014).

EXPERIMENTAL APPROACHES FOR GJ STUDIES

Several experimental approaches significantly contributed to GJ studies in different model animals. These approaches were successfully applied in nematode research and are reviewed below.

Dye injection experiments. Dye injection experiments have shown that small fluorescent dye molecules injected into one cell can actually diffuse into adjacent cells (Chailakhyan, 1990; Dermietzel *et al.*, 1990; Levin, 2002). Intracellular injections of fluorescent dyes allow the detection GJ and the estimation of channel size and properties (Fig. 3A).

Fluorescent dyes were injected into living developing embryo cells in experiments with marine nematodes *Enoplus brevis* and *Pontonema vulgare* (Voronov *et al.*, 1986; Voronov & Panchin, 1998). It was shown that GJ between blastomers are permeable for Lucifer Yellow CH and fluorescein but not permeable for carboxyfluorescein (Bossinger & Schierenberg, 1996). Similarly, Lucifer Yellow was used to study GJ in soil nematodes *Cephalobus* sp. and *C. elegans*. Using fluorescein dye injection techniques it was revealed that the pattern and timing of GJ development in these two species differs (Bossinger & Schierenberg, 1996).

In adult *C. elegans* pharyngeal muscles are dye-coupled and carboxyfluorescein injection was used to detect GJ. Normally carboxyfluorescein spread throughout all parts of the pharynx. In *inx-6* mutants, dye injected into the terminal bulb spread to the isthmus and the metacorpus but did not leak into the procorpus. The finding suggests that in *inx-6* mutant dye-coupling is disturbed and this confirms that the *inx-6* gene codes a GJ protein (Li *et al.*, 2003).

Electrophysiology and optogenetic tools. Ions may pass GJ channel, so the establishment of the electrical coupling between adjacent cells is one of the basic techniques in GJ studies. Actually this approach first demonstrated the very existence of electrical synapses/GJ (Furshpan & Potter, 1958).

The application of electrophysiological techniques to GJ studies is illustrated in Figs 3B and C. Two electrodes designated as I1 and V1 are located within one cell, whereas the third electrode, called V2 is impaled in the adjacent cell. Negative current pulses are applied through the I1 electrode resulting in the subsequent changes in voltage on the membrane that are recorded using both V1 and V2 electrodes. A fraction of I1 current from the first cell is delivered to the second cell through GJ, and leads to the voltage shift on the second cell membrane. The magnitude of trans-junctional current depends on GJ conductance. The $\Delta V2/\Delta V1$ ratio, which represented the coupling coefficient, is used as a measure of electrical coupling.

One obstacle in intracellular microelectrodes usage in *C. elegans* is the small cell size of this animal. Nevertheless, state of the art experiments in *C. elegans* made some electrophysiology possible. Liu *et al.* (2006) used *in situ* dual whole cell voltage clamp techniques to study electrical coupling in *C. elegans* body-wall muscle cells. They have directly measured biophysical properties of *C. elegans* GJ and demonstrated that body-wall muscle cells were electrically coupled in a highly organised and specific pattern.

Alternatively, other nematode species with bigger cell size could be used as models for GJ studies. Electrical coupling between *Ascaris* muscle cells was shown as far back as 1985 (Walrond & Stretton, 1985).

Another possibility is to take advantage of big cells of the early embryo. Blastomers are much bigger than developed cells and this allow intercellular electrical coupling to be measured by conventional electrophysiological methods (Voronov *et al.*, 1986; Voronov & Panchin, 1998).

Optogenetic tools may greatly contribute to GJ studies, especially when microelectrode techniques are not easily applicable. Optogenetics uses light-sensitive ion channels like channel rhodopsin to control membrane potentials in genetically modified living cells. Recordings could be made with the help of sensors for calcium or membrane voltage (Nguyen *et al.*, 2015; Venkatachalam *et al.*, 2016. In nematode GJ research calcium sensors were used to study GJ mediated coupling in the gut (Peters *et al.*, 2007) and muscles (Liu *et al.*, 2011).

Heterologous expression. Heterologous expression of GJ proteins in big cells, such as *Xenopus* oocytes, is another way to overcome small cell size restrictions that limit usage of microelectrode techniques. This method allows the production of target protein in cells that do not naturally produce it. Oocytes of the South African clawed frog, *Xenopus laevis*, have some obvious advantages.

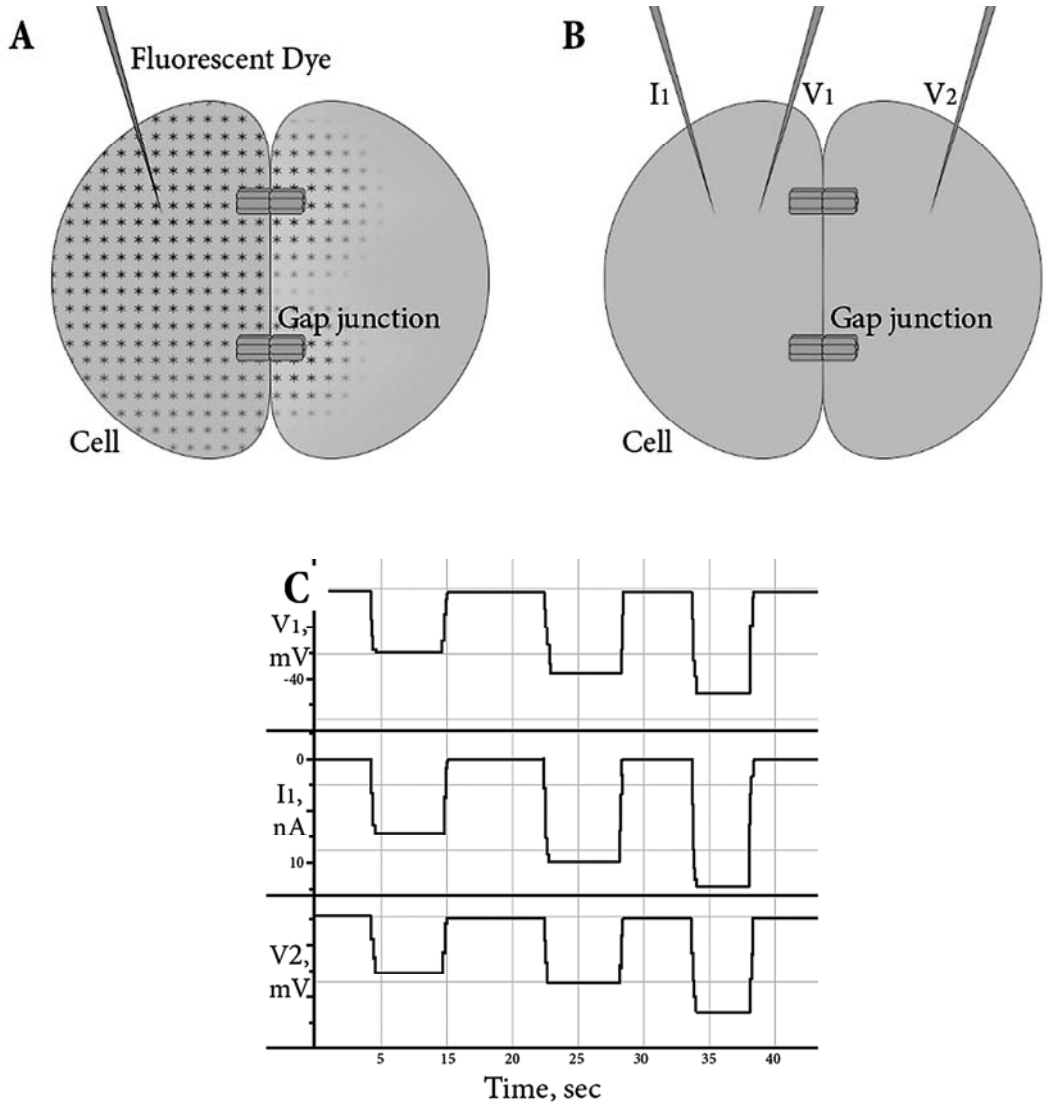

Fig. 3. Dye injection experiments and electrophysiological measurements of cell coupling are two methods that mostly contributed to GJ research. A: Small fluorescent dye molecules injected into one cell can spread directly into adjacent cells through the GJ. B & C: Intracellular microelectrodes are used for measuring the GJ conductance and calculating the coupling coefficient between cells. See text for details.

- They are large and easily manipulated, which is especially convenient for electrophysiological experiments.
- Oocytes can be cultured in a simple saline buffer without addition of nutrients during several days.
- In mature oocytes most of endogenous protein syntheses are arrested until fertilisation.
- *Xenopus* oocytes could be injected with constructs in which foreign mRNA is flanked by 5' and 3' untranslated sequences of *Xenopus*' own globin or other proteins. This procedure facilitates the first initiation phase of ribosomal translation and provides a robust synthesis of heterologous protein.

Usually from 5-20 ng of cRNA is pressure-injected to each oocyte through a glass pipette (Fig. 4). *Xenopus* oocyte expression system resulted in generation of functional pannexin channels, which enabled investigation of their properties such as conductance, ion and voltage sensitivity and response to acidification (Phelan *et al.*, 1998a; Stebbings *et al.*, 2000; Bao *et al.*, 2004; Bruzzone *et al.*, 2003, 2005). Voltage clamp is the most common method for functional characterisation of GJ channels expressed in *Xenopus* oocyte membranes. These data are important because *in situ* each cell has many different types of channels and their properties overlap. Five innexins/pannexins from *C. elegans* were investigated in *Xenopus* oocytes: INX-3, EAT-5, UNC-7, UNC-9 and INX-19 (NSY-5) (Landesman *et al.*, 1999; Chuang *et al.*, 2007; Starich

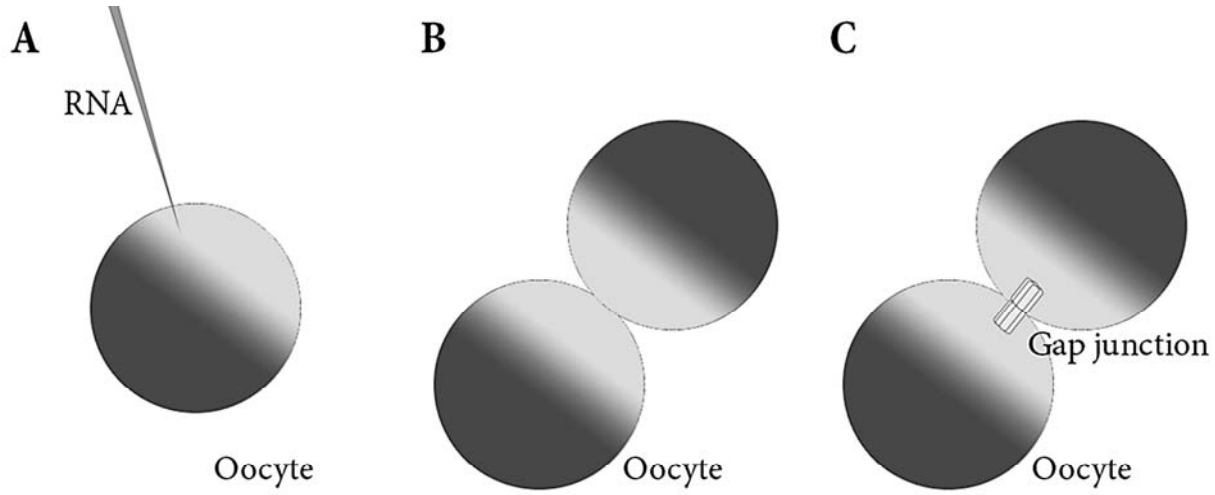

Fig. 4. The heterologous expression method. A: oocytes are injected with target RNA. B: Oocytes are manually paired. The vitelline membranes of oocytes have been previously removed by mechanical dissection. C: In 24-48 hours, GJ are formed.

et al., 2009). INX-3 and UNC-7 GJ were shown to be voltage dependent (Landesman *et al.*, 1999; Starich *et al.*, 2009). The function of UNC-9 and INX-19 GJ did not depend on transjunctional potential (Chuang *et al.*, 2007; Starich *et al.*, 2009). INX-3 GJ were closed in response to cytoplasm acidification (Landesman *et al.*, 1999). INX-19 GJ were sensitive to calcium concentration (Chuang *et al.*, 2007). EAT-5 protein was not able to form intercellular channels in *Xenopus* oocytes (Landesman *et al.*, 1999). The functional properties of INX-6 were not studied in *Xenopus* oocytes; however, INX-6 was expressed in the insect cell line Sf9 (Oshima *et al.*, 2013). Curiously, INX-6 did not form GJ in mammalian HeLa cells. Therefore, the method of heterologous expression has some obvious restrictions, including requirement of protein modifications.

GAP JUNCTION IN DIFFERENT NEMATODE ORGANS AND TISSUES

Pharynx. The pharynx is one of the most complex parts of the nematode body. It is a neuromuscular machine, somewhat similar to the vertebrate heart (Mango, 2007). Through the nematode's life, the pharynx is constantly contracting, resulting in food pumping. The pharynx consists of muscle cells, neurons, epithelial cells, marginal cells and gland cells. According to Altun *et al.* (2009) seventeen of twenty-five *C. elegans* innexins are expressed in the pharynx and ten of them are strongly expressed in muscular cells (Table 2). The pharynx contains twenty pharyngeal muscle cells with three-fold symmetry that are connected *via* GJ. Eight muscle segments (pm1-8) form four parts of the pharynx (Fig. 5A). The first and the last segments contain just one cell each, while the other six segments include three muscle cells (Altun *et al.*, 2009). The first three segments assemble to the procorpus, pm4 is the main part of the metacorpus, pm 5 composes the isthmus and lastly the terminal bulb is formed by pm6-8. Three-fold symmetry spreads also on nine marginal cells. Three mc1 are located in the corpus, three mc2 are located in the isthmus and three mc3 are located on the terminal bulb. Motor neurons are required for a frequent and rapid pumping of pharynx (Raizen & Avery, 1994; Raizen *et al.*, 1995). Such neuronal control directs the metacorpus muscles.

It is considered that pharyngeal innexins ensure the pumping synchrony and lead to consistent food delivery. Each muscle segment strongly expresses three to five different innexins and some innexins are expressed in more than one segment. Despite the difference between innexin expression patterns in different pharyngeal cells, there is always at least one innexin that is presented in adjacent muscle segment cells (Fig. 5B). Li *et al.* (2003) showed that the *inx-6 (rr5)* mutant has abnormalities in GJ coupling. Using a fluorescent dye it was shown that the procorpus was uncoupled from the other parts of

Table 2. Localisation of pharynx muscle segments and innexin expression.

Part of the pharynx	Segment	Expressed innexins
Procorpus	Pm1	INX-1, INX-6, INX-8, INX-10, INX-20
	Pm2	INX-6, INX-10, INX-20, EAT-5
	Pm3	INX-6, INX-8, INX-10
Metacorpus	Pm4	INX-6, INX-11, EAT-5
Isthmus	Pm5	INX-3, INX-7, EAT-5
Terminal Bulb	Pm6	INX-2, INX-3, INX-10
	Pm7	INX-2, INX-3, INX-10
	Pm8	INX-2, INX-3, INX-10, INX-11, INX-20

the pharynx in *inx-6 (rr5)* mutant nematodes and the dye injected in the terminal bulb region did not diffuse to the procorpus muscles. Video recording and electropharyngeograms revealed the asynchrony of pro- and metacorpus activity in *inx-6 (rr5)*. Synchrony of the metacorpus and the terminal bulb depends on EAT-5 innexin (Starich *et al.*, 1996). In *eat-5 (ad464)* mutants, the metacorpus muscles (pm4) and the terminal bulb muscles (pm6-8) pump independently and are not synchronised. The *inx6 (rr5)* mutant phenotype could be rescued by EAT-5 expression under the control of the *inx-6* promoter (Li *et al.*, 2003). In such animals, the entire pharynx works synchronously. These experimental data could be viewed as shown on Fig. 5A. It could be suggested that for the normal activity all muscle cell segments along the pharynx have to be connected by GJ. Let us consider that two cells form GJ if they produce identical hemichannels assembled by the same protein. Then electrical coupling of the entire pharynx could be established by cooperated expression of three proteins: INX-6 (shared by procorpus and metacorpus muscles), EAT-5 (shared by metacorpus and isthmus muscles) and INX-3 (shared by isthmus and terminal bulb muscles) (Altun *et al.*, 2009). If *eat-5 (ad464)* and *inx-6 (rr5)* mutations are deleterious, the lack of INX-6 in *inx6 (rr5)* mutant uncouples pro- and metacorpus (Fig. 5C), while the lack of EAT-5 innexin in *eat-5 (ad464)* mutant uncouples anterior and posterior parts of the pharynx (Fig. 5D).The EAT-5 expression under the control of *inx-6* promoter in *inx-6 (rr5)* mutants will provide EAT-5 expression in the procorpus and in the metacorpus. Restoration of their coupling is in agreement with experimental data (Fig. 5E).

Intestine. The intestine is one of the major organs of *C. elegans*, comprising roughly one third of the total somatic mass. While eating *C. elegans* is rhythmically defecating with the stable period of 45-50 seconds (Croll & Smith, 1978). In the nematode intestine cells calcium waves are regularly generated and spread from cell to cell. Each calcium wave is followed by contractions of the posterior body muscles, anterior body muscles and enteric muscles. This sequence of events comprises the defecation motor program (Teramoto & Iwasaki, 2006). One of the important component of the timekeeping mechanism of this rhythm is the inositol-1, 4, 5-trisphosphate (IP3) receptor (Espelt *et al.*, 2005; Branicky & Hekimi, 2006). In wild-type animals, calcium spikes in the posterior intestine start the initiation of the motor program, and these spikes are absent in the IP3 receptor mutant (Dal Santo *et al.*, 1999). The contraction of body muscles in the defecation cycle is regulated by pH changes in the pseudocoelomic space caused by Na^+/H^+ transporter activity in intestinal cells.

It was shown that four types of pannexins are expressed in the *C. elegans* intestine: INX-2, INX-11, INX-15, INX-16 (Altun *et al.*, 2009). INX-2 is also present in the pharynx, and hypothetically may participate in connections between the pharynx and intestine. INX-16 is expressed only in intestine cells. It is well known, that calcium wave generation is endogenous to intestine cells and is not controlled by the nervous system (Dal Santo *et al.*, 1999). Peters *et al.* (2007) studied the role of pannexins in calcium wave propagation along the intestine. They investigated *inx-16 (ox144)* mutants that had constipated phenotype. In most of the mutant animals, the propagation of the calcium wave was eliminated. It was shown that in mutant animal's intestine cell calcium spikes were generated asynchronously and appeared to be independent from neighbouring cells. It could be suggested that INX-16 plays general role in electrical synchronisation between intestine cells similar to innexins that are common in excitable cells such as muscles or neurons. Peters *et al.* (2007) also suppose that IP3 may diffuse between cells through

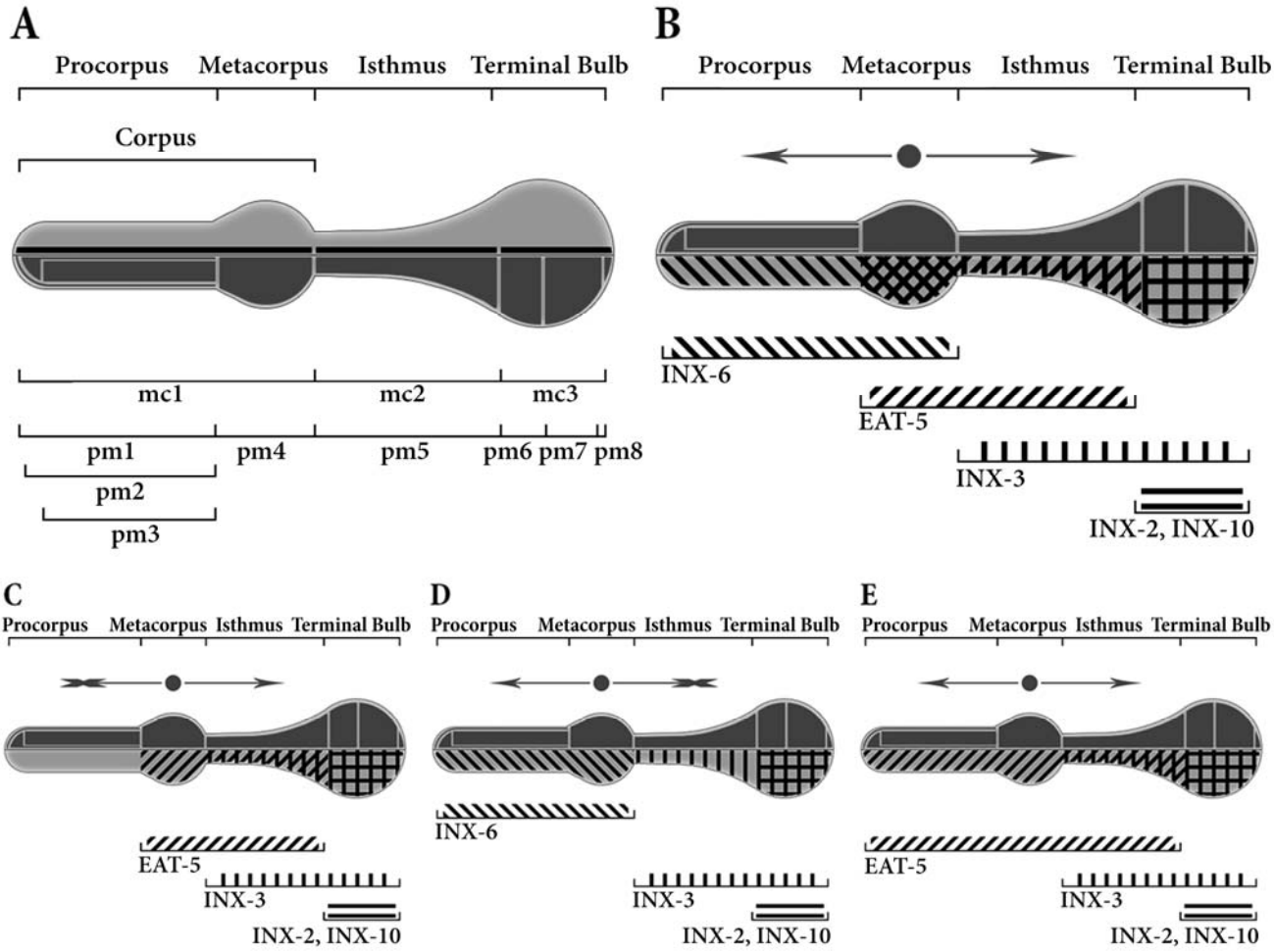

Fig. 5. Pharynx structure and innexin formation in the wild type and mutant *Caenorhabditis elegans*. Expression space of the most influenced in pharynx cell coupling innexins is hatched. The suggested synchrony centre is marked by the dot and arrows show the direction of connected and coordinated cells. A: One side of the three-fold symmetrical pharynx. Eight muscle segments (pm1-8), three marginal cell segments (mc1-3) and four physiological parts of the pharynx. B: Coupled by innexins all four parts work in synchrony. C: Presumable schematic representation of *inx-6 (rr5)* mutation, where the lack of INX-6 uncouples pro- and metacorpus. D: Presumable schematic representation of *eat-5 (ad464)* mutation, where the lack of EAT-5 uncouples posterior and anterior pharynx. E: Presumable schematic representation of *inx-6 (rr5)* mutation rescued by EAT-5 expressed under the control of *inx-6* promoter that have restored connection between pro- and metacorpus.

GJ channels composed of INX-16. IP3 activates the IP3 receptor, and it is conceivable that IP3 passes through the junctions to coordinate the calcium waves in adjacent cells.

Body wall muscle. *Caenorhabditis elegans* locomotion is produced by synchronised sinusoidal waves of the body wall muscles. Liu *et al.* (2011) studied the effects of GJ protein mutations in the neighbouring muscle cells. Null mutations of some GJ genes led to significant decrease of cell coupling coefficients, junctional conductance or synchrony of action potentials and decrease of the Ca^{2+} spread area (Liu *et al.*, 2013). UNC-7, UNC-9, INX-1, INX-10, INX-11 and INX-18 expression was detected in the body wall muscle cells (Altun *et al.*, 2009; Simonsen *et al.*, 2014). Later, INX-16 was also demonstrated to be expressed in the muscle cells. Effect of knockout of this and other innexins on body wall muscle cells coupling was studied by electrophysiological methods and with optical calcium sensors. *inx-3*, *inx-12*, *inx-13* mutants are lethal or sterile, so they were excluded from the analyses. UNC-9, INX-1, INX-10, INX-11, INX-16, and INX-18 contributed the most to the cell electrical connections (Liu *et al.*, 2013). The list of body wall muscle innexins and the list of innexins

that are most important for electrical coupling are mostly similar (Table 3). UNC-7 is the remarkable exclusion. It is interesting that UNC-7 similarly with UNC-9 expresses in muscle cells and its mutant phenotype is similar to *unc-9*, but knockout of gene had no impact on junctional conductance of muscle cells (Liu *et al.*, 2006). Starich *et al.* (2009) demonstrated that in heterologous oocyte system UNC-7 expression was sufficient to produce GJ. Thus, it was not clear why its knockout was not affecting junctional conductance *in situ*. The authors concluded that apparently similar *unc-7* and *unc-9* phenotypes resulted from different mechanisms. UNC-7 was shown to be involved predominantly in neuronal GJ; therefore, its absence could result in uncoordinated locomotion.

Liu *et al.* (2011) showed that knockout of *unc-9* decreased the junctional conductance by 67%, so it could be predicted that other innexins would not exceed 33% in overall junctional conductance share. However, Liu *et al.* (2013) found that any of six previously described innexins had an impact greater than 50%. Thus, impact of individual innexins is not additive. The authors explained this as follows. It is well known that innexon hemichannel consists of six subunits. If subunits are identical, the innexon is called homomeric and if subunits are different, such hemichannel is called heteromeric. If innexon is heteromeric, the lack of any subunit may dramatically decrease its efficiency (Liu *et al.*, 2013). Understanding the model of real subunit association is complicated due the large number of possible variants.

Neurons. Unexpectedly an important role of GJ was revealed in coordination of balance between the forward and backward locomotion in *C. elegans* (Kawano *et al.*, 2011). *Caenorhabditis elegans* surprisingly violates the classical "principle of final common path". Due to this principle formulated by the famous physiologist, Charles Scott Sherrington, motoneurons integrate a large number of inputs and send their final output to muscles. Thus, the same motoneurons may participate in a great variety of different motor activities, *i.e.*, swimming and dancing recruit the same motoneurons (Sherrington, 1906). In nematodes, forward and backward locomotion is controlled by specialised motoneurons (Chalfie *et al.*, 1985; Wicks *et al.*, 1996). There are five pairs of locomotor interneurons and four classes of body muscle motoneurons. This network controls a locomotor behaviour including forward and backward movement. AVA and AVB interneurons work reciprocally and are connected with A and B motoneurons, respectively. AVA interneurons and A motoneurons promote backward movement and are connected both by GJ and chemical synapses. AVB interneurons and B motoneurons promote forward locomotion and are connected only by GJ. *unc-7* and *unc-9* mutants are uncoordinated and are impaired in forward movement, but exhibit backward locomotion. *unc-7* and *unc-9* encode GJ proteins that form electrical connections between inter- and motor- neurons. In mutant animals, B motor neurons are uncoupled from the locomotor network because their only connection to AVB interneurons is dysfunctional. By contrast, A motor neurons retain chemical connection from AVA interneurons. Thus, the backward movement phenotype dominates in *unc-7* and *unc-9* mutants (Kawano *et al.*, 2011).

NON-GAP JUNCTION PROTEIN CONTROLS GAP JUNCTION EFFICIENCY

It was generally suggested that innexons were standalone channels; they did not have regulatory or ancillary proteins. However, the discovery of a GJ regulation by stomatin-like protein UNC-1 challenged this opinion. Analysis of different *unc-1* and *unc-9* mutants in body wall muscle showed that there was no significant difference between single or double mutants (Chen *et al.*, 2007). In addition, all single mutants decreased junctional conductance by the same value. It was suggested that UNC-1 and UNC-9 had similar function in the same

Table 3. *Caenorhabditis elegans* muscle expressed innexins (first row), marked by plus (Simonsen *et al.*, 2014). INX-16 muscle expression was revealed later (Liu *et al.*, 2013), marked by asterisk. On the second row UNC-7 is shown to have no contribution to the body wall muscle cell electrical connections (Liu *et al.*, 2006), marked by minus. Other innexins have significant impact and are marked by plus.

	UNC-7	UNC-9	INX-1	INX-10	INX-11	INX-18	INX-16
Body wall muscle expression	+	+	+	+	+	+	+
Contribution to the electrical connections	–	+	+	+	+	+	+

pathway. By using fusion proteins, it was shown that UNC-1 and UNC-9 were co-expressed in muscle cells and neurons. Furthermore, by using bimolecular fluorescence complementation it was revealed that they appeared to be physically very close at intercellular junctions. Interestingly, UNC-9 localisation in *unc-1* mutants and UNC-1 localisation in *unc-9* mutants appeared to stay normal. Therefore, it was proposed that UNC-1 had no impact on synthesis, membrane transport, or localisation of UNC-9 protein, but had a direct effect on the GJ gating properties. Surprisingly, it appeared that UNC-9::GFP fusion protein produced gain-of-function GJ channels that were independent from UNC-1. Chen *et al.* (2007) revealed that UNC-1 promoted UNC-9 channel opening, while UNC-9::GFP fusion protein permanently maintained an open state. UNC-1 is one of ten stomatin-like proteins in *C. elegans*. Another stomatin-like UNC-24 protein mutant revealed a similar phenotype with *unc-9* mutants. It was concluded, that UNC-24 played a stabilisation role for UNC-1, because *unc-24* mutants have greatly reduced amount of UNC-1 protein (Morgan *et al.*, 2007). It is possible that other stomatin-like proteins also have regulatory effects on GJ function.

GJ IN NEMATODE DEVELOPMENT

GJ are present in embryonic cells from early stages of the developing organism. For instance, the presence of GJ was experimentally shown by electrophysiological methods and by dye coupling in *Enoplus brevis* embryos from the two blastomers stage (Voronov & Panchin, 1998). It is known that several innexin mutations in *C. elegans* result in severe developmental defects (Li *et al.*, 2003; Simonsen *et al.*, 2014).

The role of innexins in embryogenesis has been well studied for INX-19. This innexin determines the fate of AWC olfactory neuron pair in *C. elegans*. During embryogenesis and at the first-stage larva (L1) it is implicated in GJ formation between AWC and some other sensory neurons (Chuang *et al.*, 2007). Left and right AWC are not coupled with GJ directly (White *et al.*, 1986). Left and right AWC are morphologically identical but they differ in their reaction to olfactory stimulus. The AWC^{ON} neuron is activated by odour stimulus onset and the AWC^{OFF} neuron is activated by odour stimulus cessation. ON or OFF fates of AWC neurons is established randomly in animal development, so 50% of worms have this neuron on the left side of body, and another 50% have it on the right side (Wes & Bargmann, 2001). The AWC^{ON} neuron function is to detect odours and it expresses G-protein coupled receptor STR-2 (Bauer Huang *et al.*, 2007; Troemel *et al.*, 1999). It was shown that INX-19 is important for AWC functional asymmetry. *inx-19* mutants generate two AWC^{OFF} neurons, whereas overexpression of INX-19 produces two AWC^{ON} neurons (Troemel *et al.*, 1999; VanHoven *et al.*, 2006; Chuang *et al.*, 2007; Schumacher *et al.*, 2012). A complex chain of interactions that leads to specific asymmetrical gene expressions and determinates AWC functional asymmetry was recently studied (Hsieh *et al.*, 2012; Schumacher *et al.*, 2012; Alqadah *et al.*, 2016).

GJ PROTEIN DIVERSITY AND ADDITIONAL FAMILIES HYPOTHESIS

Earlier we pointed out that two distinct protein families are presently shown to be implicated in GJ formation and pannexins/innexins were suggested to be the universal protein family for metazoan species. It was also anticipated that more GJ proteins types may exist (Litvin *et al.*, 2006; Shestopalov & Panchin, 2008; Panchin, 2011; Popova *et al.*, 2012). This speculation was based on the observation that GJ appear to be a very important and universal feature of Metazoa that contradicted the growing evidence that some multicellular animals lack both types of these proteins. No pannexins or connexins were found in Porifera, Placozoa, Echinoderms, Hemichordates and Xenoturbella. Recent genome sequencing of Tardigrada and Onychophora species suggests that these animals have no pannexins or connexins. Thus, some Ecdysozoans may have lost pannexins from their genomes. Nematodes provide additional perspective to this comparative view. Advanced and reliable methods for *C. elegans* expression study have been developed, including the green fluorescent protein under the targeted protein promoter essay. In the remarkable investigation that used this method for all 25 *C. elegans* innexin it was shown that in some cells with documented GJ no innexin expression could be detected (Li *et al.*, 2003; Altun *et al.*, 2009; Hobert, 2010). These include twenty non-pharyngeal neuron types (Hobert, 2010). Similar result for four pharyngeal neurons also could be deduced (Li *et al.*, 2003; Altun *et al.*, 2009; Hobert, 2010). One explanation for this observation implies that some unknown protein family is involved in the GJ formation. Therefore, nematodes that already greatly contributed to GJ studies could further help in resolving the general problem of GJ protein diversity and function.

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В.П. Кузнецов, Г.А. Сливко-Кольчик, Л.Б. Попова и Ю.В. Панчин. Щелевые контакты у нематод.

Резюме. Щелевые контакты (GJ – *gap junctions*) представляют собой одну из наиболее распространенных форм межклеточных взаимодействий. Считается, что они выполняют сходную функцию у всех многоклеточных организмов (Metazoa) и обеспечивают межклеточные взаимодействия в самых разных тканях и клетках. Щелевой контакт представляет собой белковый канал, который объединяет цитоплазму двух соседних клеток. Два семейства неродственных белков вовлечены в формирование GJ: коннексины, обнаруживаемые только у хордовых, и паннексины/иннексины, присутствующие в геноме как хордовых, так и беспозвоночных. Нематоды являются исключительно удобной моделью для исследований в различных областях биологических исследований, поскольку для многих видов нематод известны полные геномы, а свободноживущая нематода *Caenorhabditis elegans* представляет собой наиболее изученный к настоящему времени модельный организм. В геномах различных нематод имеется от 12 до 25 паннексинов/иннексинов. В обзоре проведено сравнение GJ белков нематод с таковыми других животных. Проанализированы особенности их экспрессии и роль в различных биологических процессах.