Ion channels of the intestinal hyperpolarising action potential in nematodes

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Accepted for publication 6 July 2019

Summary. The defaecation motor program in nematodes is controlled by the intestinal central pattern generator, coupled with propagating calcium waves and electrical membrane potential oscillations. Recent electrophysiological experiments in *Heterorhabditis megidis* have indicated that the intestinal cells of nematodes produce unusual all-or-none hyperpolarisation 'action potential' (HAP). HAP resembles the action potential generation in regular excitable tissues, neurons and myocytes, having the reversed polarity of current and voltage. It can be considered that rhythmic electrical potential generation in the intestinal cells is based on the cooperation of voltage-gated ion channels. Here we combined our electrophysiological study with molecular-genetics of the nematode intestine and recent single-cell RNA-seq open-access data of *Caenorhabditis elegans*. Single-cell RNA-seq data confirm the unique spectrum of the ion channel expression in the gut cells, which is highly distinct from other nematode tissues. We have compared electrophysiological data with the channel expression data from nematode tissues implementing regular action potential and HAP to find ion channel candidates that can fit HAP model.

Key words: *Caenorhabditis elegans*, CPG, defaecation, ion channels, scRNA-seq data.

The transmission of electrical signals in living tissues has been known about since the time of the work of Luigi Galvani in the 18th century. In 1963, Alan Hodgkin and Andrew Huxley received the Nobel Prize for elucidating the biophysical principles of the depolarisation action potential (AP) in neurons and muscles (Hodgkin & Huxley, 1952). In 1964, a work published in Nature described the hyperpolarising action potential (HAP) in *Ascaris lumbricoides* pharynx, opposite in sign to the usual AP. That research became the basis for studying the HAP phenomenon (Del Castillo *et al.*, 1964). It was shown that similarly to AP, pharynx muscle cells can generate HAP in response to small hyperpolarisation impulses, and the role of K^+ channels in this process was also described (Del Castillo & Morales, 1967; Byerly & Masuda, 1979).

As it was later discovered, the negative spike in nematode pharynx turned out to be a repolarising part of depolarising AP; the interest in this phenomenon declined. Recent works on intestine cells in nematodes show that 'pure' HAP exists in that tissue (Kuznetsov *et al.*, 2017; Slivko-Koltchik *et al.*, 2018a). Thus nematode gut cells' electrical activity and expression data require a comprehensive molecular model and call for a reevaluation of the excitable cells definition.

What is AP? Electrical AP is reflected in transient all-or-none depolarisation from the initial negative level of the membrane potential (MP). With this definition, electrically excitable cells include neurons, muscle cells and some secretory cells. AP is generated by the activation of specific voltage-gated ion channels. The prominent feature of AP is the presence of positive feedback mechanisms of cell membrane ion channels. The voltage shift activates ion channels that produce current, which leads to cell membrane voltage changes in the same direction and further causes more channels to open, producing a higher electric current across the cell membrane. HAP that resembles the action potential generation in normal excitable tissues, neurons and myocytes, but with the reversed polarity of the voltage and current, calls for the molecular model that can explain its mechanism. Most voltage-gated ion channels are open upon depolarisation and are not suitable for HAP. In this paper, we compare electrophysiology

data with channel expression data from nematode tissues that have regular AP and HAP and search for ion channel candidates that fit HAP activity model.

HAP as a critical element of the nematode defaecation motor program. The defaecation motor program (DMP) controlled by the central pattern generator (CPG) was initially discovered in the intestine cells of *Caenorhabditis elegans* nematode (Thomas, 1990), and CPG activity was associated with rhythmically propagated calcium waves (Teramoto & Iwasaki, 2006). Later in our works on other nematodes (*Heterorhabditis megidis* and *Enoplus brevis*), it was shown that intestinal CPG activity is also reflected in electrical MP oscillations (Kuznetsov *et al.*, 2017; Slivko-Koltchik *et al.*, 2018a, b). In those studies, it was established that nematode intestinal cells produce unusual all-or-none hyperpolarisation 'action potential' HAP with the fixed duration, period, and amplitude, specific for different species (Fig. 1A).

HAP could be triggered by a short hyperpolarisation current pulse, and the new cycle of MP changes starts. Thus, the CPG phase could be shifted by a short hyperpolarisation current pulse, assuming the involvement of plasma membrane voltage-gated mechanisms and highlighting the similarity of HAP and action potential generation in classic excitable tissues, neurons, or myocytes. In excitable tissues, a common spike is also triggered by the shift of the MP beyond a certain threshold. The ongoing chain of spike rate depends on the MP and could be influenced by artificial current pulses. This description is also accurate for the nematode gut cells' electrical activity but with the reversed polarity of voltage and current. In line with those characteristics, it may be considered that the intestinal cell rhythmic electrical potential generation is based on the cooperation of the voltage-gated ion channels. However, CPG cycling also remained in experiments where the MP of intestine cells was continuously clamped at steady voltage levels, indicating that intracellular voltage-independent mechanisms are also implicated in the process of rhythm generation (Kuznetsov *et al.*, 2017).

We have also shown that neighbouring gut cells are strongly linked through gap junctions (GJ), and endogenous pacemakers of individual cells can be synchronised by electrical coupling (Kuznetsov *et al.*, 2017; Slivko-Koltchik *et al.*, 2018b).

The findings of two different molecular oscillators that act together in gut cells came up with the idea of searching for genes involved in intestinal pacemaker activity.

Intestinal pacemaker activity is reflected in plasma membrane electrical activity and in Ca^{2+} oscillations that also engage endoplasmic reticulum (ER) Ca^{2+} store (Dal Santo *et al.*, 1999; Teramoto & Iwasaki, 2006). Fig. 1B depicts three primary model situations that can produce Ca^{2+} oscillations and show their link to the MP.

HAP is a new type of cell excitability that is distinct from the depolarising spike (Kuznetsov *et al.*, 2017). It is predicted to be generated by particular types of voltage-gated ion channels embedded in a cell's plasma membrane. The simplest model for HAP generation includes only three plasma membrane ion channels with g_{a1}, g_{a2}, and g_b conductance. g_{a1} can be attributed to Na^+ channels. Hypothetical g_b is caused by K^+ channel. g_{a2} can represent a putative membrane Ca^{2+} channel. Fig. 1C summarises the role of three different ion currents in the plasma membrane for the gut DMP activity.

Differential expression of HAP and DMP intestinal genes. For decades of *C. elegans* studies, many genes potentially implicated in DMP were identified and characterised (Thomas, 1990; Dal Santo *et al.*, 1999). At the same time, detailed data of gene expression patterns in individual cells and tissues, including intestine, became recently available from nematode single-cell RNA-seq studies (Cao *et al.*, 2017; Packer *et al.*, 2019). Several genes are expressed in specific tissues at much higher rates than in others. We assumed that this differential expression reflects tissue-specific functions. Taking into account the unique type of intestine cells' electrical properties, we expected them to employ a unique set of genes that control MP oscillations in comparison to other cell types.

We selected a set of *C. elegans* ion channels (IC) and analysed their expression rate in different excitable and non-excitable nematode tissues. As the biophysical properties of nematode enterocytes appear to be unique, we suggested this selection as an independent and unbiased approach to filter potential candidates for HAP and DMP activity.

Caenorhabditis elegans IC were selected according to the wormbase (https://wormbase.org/) annotation and excellent wormbook reviews (Salkoff *et al.*, 2005; McGhee, 2007; Nehrke, 2014).

Dendrogram clustermap and UMAP plot built from the full dataset of 20271 and 222 IC set genes using open-access single-cell RNA-seq data (Cao *et al.*, 2017) show that *C. elegans* IC genes have functional clustering to different tissue types, and most IC are tissue-specific (Fig. 2). In particular, this confirms the unique spectrum of ion channel

expression in gut cells that are highly distinct from other nematode tissues.

Ca^{2+} dynamics in intestinal oscillations. The discovery of rhythmically propagated calcium waves in the intestinal cells was a significant step in DMP studies (Dal Santo *et al.*, 1999; Espelt *et al.*, 2005; Teramoto & Iwasaki, 2006). Calcium dynamics is one of the essential ways of cellular regulation. There are two distinct ways to regulate plasma Ca^{2+} concentrations. One controls Ca^{2+} exchange between extracellular medium and cytoplasm, while another mechanism utilises internal Ca^{2+} stores in membrane-bound organelles.

It was already known that Ca^{2+} oscillations go along with DMP cycles even before intracellular recordings in the nematode gut were performed (Dal Santo *et al.*, 1999; Teramoto & Iwasaki, 2006). So it seemed possible that Ca^{2+} is entering enterocyte *via* voltage-gated calcium channels (VGCC) similar to vertebrate heart, nematode pharynx, or some neurons and myocytes (Fig. 2B, the second row) (Verkhratsky & Parpura, 2014). Single-cell RNA-seq data show that the expression of VGCC in the intestine is much lower than in normal excitable tissues.

Most animals utilise voltage-gated sodium channels (VGSC) as the primary channels responsible for AP generation. However, nematodes are the exception, and their genomes encode no VGSC (Goldin, 2002).

The role of ER calcium release mediated through inositol 1,4,5-trisphosphate (IP_3) and its single receptor ITR-1 is thought to be the most crucial element in the intestinal cell Ca^{2+} oscillator in *C. elegans* (Dal Santo *et al.*, 1999; Walker *et al.*, 2004; Xing & Strange, 2010; Baylis & Vázquez-Manrique, 2012). Indeed, *itr-1* mutants demonstrate long defaecation cycle periods or no defaecation behaviour at all, while overexpression of *itr-1* gene results in short defaecation cycle periods (Dal Santo *et al.*, 1999). The persistence of the DMP rhythmicity, even under the voltage-clamp conditions in *H. megidis*, is consistent with the idea of the crucial role of the IP_3 receptor in nematode DMP pacemaker. Potentially rhythmical changes of Ca^{2+} concentrations in intestinal cells may proceed entirely on intracellular machinery based on the exchange with ER Ca^{2+} storage. In this case, rhythmical Ca^{2+} intake from ER and its reabsorption does not affect the MP (Fig. 2B, the first row). The latest single-cell RNA-seq expression data demonstrate (Packer *et al.*, 2019) that *itr-1* is expressed almost exclusively in the nematode intestine cells (Fig. 3).

By contrast, according to single cell RNA-seq data (Cao *et al.*, 2017) another calcium channel located in the endoplasmic reticulum – ryanodine receptor (*unc-68*) (Maryon *et al.*, 1996) is predominantly expressed in muscles and moderately in neurons. Interactions between ITR-1 and IP_3 are prone to produce calcium oscillations *via* calcium-induced calcium release (CICR) mechanism. CICR and IP_3 receptor channel activity depends on the interplay of two intracellular signals: Ca^{2+} and IP_3. Consistently the role of another player of these interactions – phospholipase C β (*egl-8*) – in intestinal oscillations was also demonstrated (Baylis & Vázquez-Manrique, 2012). PLCβ is an essential part of the outlined mechanism, and single-cell RNA-seq data show elevated expression of this gene in neurons and intestine cells. CICR loop operation often requires Ca^{2+} influx from the extracellular medium, usually *via* voltage-gated Ca^{2+} channels. These channels are not expressed in the intestine. Strange and colleges (Yan *et al.*, 2006; Xing & Strange, 2010) suggested transient receptor potential (TRP) channel family take part in this process. The potential role of Na^+ and Ca^{2+} channels in DMP cycling would be considered later.

HAP and voltage-gated potassium channels. As mentioned earlier (Fig. 1A), the self-sustained plasma membrane hyperpolarisation induced by hyperpolarising current is the most prominent and unusual feature of intestinal electrical oscillations. It was proposed to be caused by a hypothetical K^+ channel (g_b conductivity).

We have analysed different types of K^+ channels as candidates for this function. The negative spike in nematode pharynx (that is also the rapid repolarisation at the end of the action potential plateau phase) resembles intestinal HAP. In *A. lumbricoides* pharynx, it was discovered that the negative spike is controlled by K^+ channels (Del Castillo *et al.*, 1964; Del Castillo & Morales, 1967; Byerly & Masuda, 1979), and it is proposed that similar activity in *C. elegans* is controlled in the same way with involvement of EXP-2 protein.

Unusual EXP-2 channel properties can explain the positive feedback upon hyperpolarising potential shift. *exp-2* encodes a potassium channel subunit related to the voltage-gated Shab (Kv2)-like potassium family (Davis *et al.*, 1999; Fleischhauer *et al.*, 2000; Shtonda & Avery, 2005). *exp-2* null animals exhibit abnormally prolonged pharyngeal contractions. EXP-2 provides rapid repolarisation at the end of the electrical potential plateau in the pharyngeal muscle. It was also demonstrated that the initiation of the negative-going action potential could be independently triggered. EXP-2 channels open upon hyperpolarisation, but only being preceded by a prior depolarisation (Davis *et al.*, 1999;

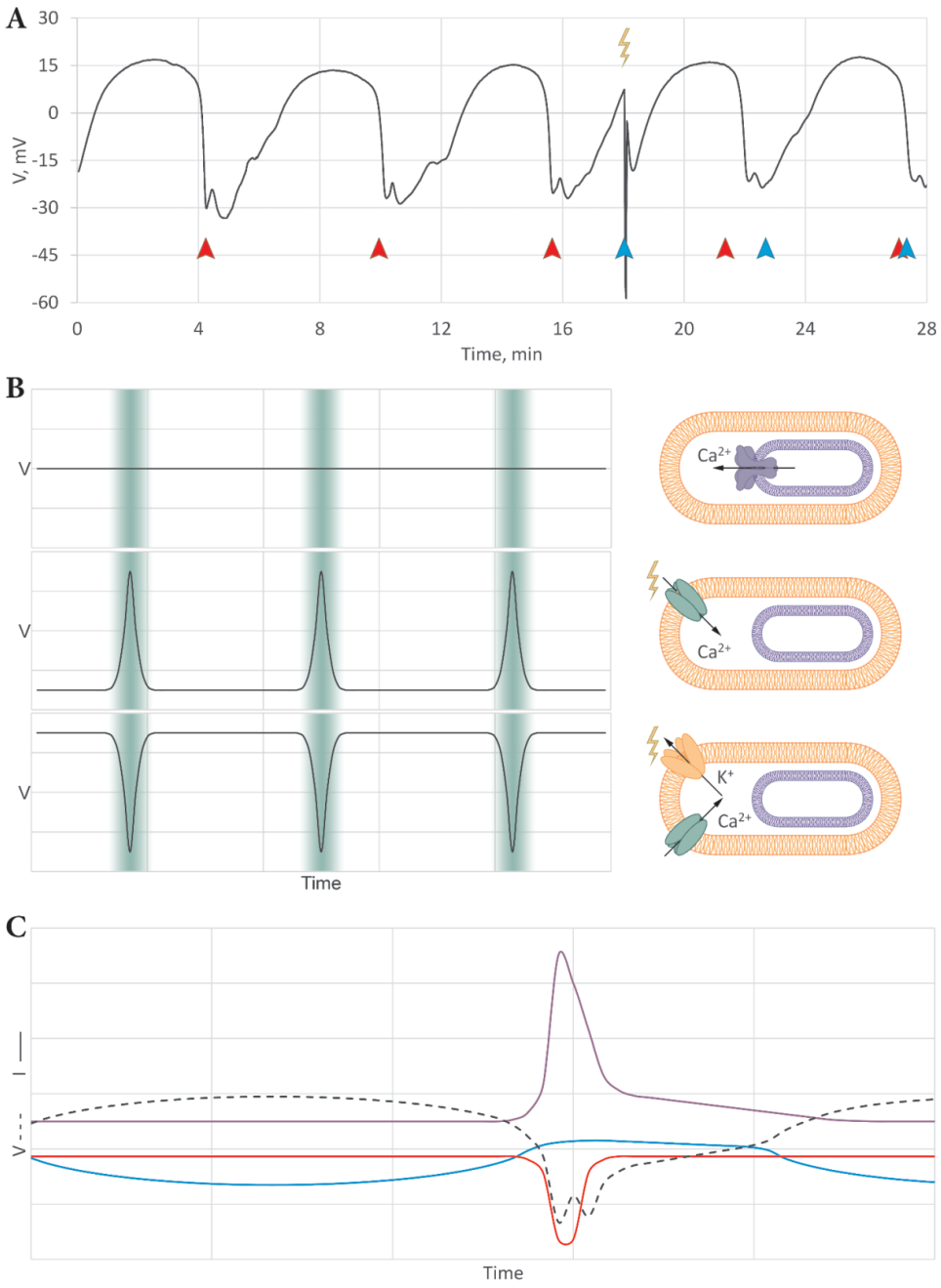

Fig. 1. HAP properties. A. Intracellular microelectrode recording of membrane potential oscillations in *Heterorhabditis megidis* nematode intestine (5 cycles). One cycle period is approximately 3 min with an amplitude of 50 mV; the first three HAP are indicated by red arrows. Lightning represents the moment of the artificial hyperpolarising current input (3 s; -80 nA). It leads to the premature HAP and the phase shift. Red arrowheads indicate the initial DMP period. Blue arrowheads indicate phase after reset. B. Theoretical mechanism of intracellular Ca^{2+} concentration oscillations (green shade) and their coupling with MP. The first row: Ca^{2+} influx from intracellular storage (endoplasmic reticulum) *via* the IP_3-dependent mechanism. MP is constant during Ca^{2+} oscillations. The second row: voltage-gated Ca^{2+} spike. When voltage-gated calcium channels are open, intracellular Ca^{2+} concentration grows, and the membrane depolarises. The third row: voltage-gated K^+ HAP pulls Ca^{2+} in the cell by resulting voltage gradient *via* leak channels. Ca^{2+} concentration increases while the membrane hyperpolarises. C. The simplest model of the voltage changes V(t) during the complete HAP cycle (black dashed line) produced by currents I(t) through ion channels of three types: sodium (blue; g_{a1} conductance), calcium (red; g_{a2} conductance), and potassium (purple; g_b conductance).

Fleischhauer *et al.*, 2000; Shtonda & Avery, 2005). Unusual properties (slow activation and very rapid inactivation in response to depolarisation and rapid recovery from inactivation on repolarisation) make the Kv-type EXP-2 channel an inward rectifier suitable for HAP generation.

Single-cell RNA-seq data confirm strong differential *exp-2* expression in *C. elegans* pharynx. These data also shows no substantial expression of this protein in intestinal cells. Besides, there are no *exp-2* orthologs in *C. elegans*, and they cannot contribute to intestinal HAP.

According to single-cell RNA-seq studies, two other potassium voltage-gated ion channels that belong to KQT K^+ channels family (*kqt-2* and *kqt-3*) are strongly overexpressed in the gut cells. Mammalian KCNQ voltage-gated K^+ channels, implicated in long-QT cardiac syndrome are homologous to *kqt-2* and *kqt-3* open by depolarisation and participate in the usual spike repolarisation phase (Jan & Jan, 2012). The properties of nematode *kqt* genes (including pharynx specific *kqt-1*) were studied *via* heterologous expression in *Xenopus* oocytes (Wei *et al.*, 2005). In such conditions, *C. elegans* proteins form channels with no inward rectification properties expected for potassium g_b current. At the same time, KQT channels were previously implicated in intestinal cell DMP function (Kwan *et al.*, 2008).

The channels *kqt-2* and *kqt-3* that are highly expressed voltage-gated intestine channels might still determine HAP *in vivo* by the mechanism similar to EXP-2 channels activity in the pharynx (see above). EXP-2 K^+ channels open on depolarisation and close on hyperpolarisation, while the inactivation-reactivation mechanism radically shapes K^+ currents. K^+ channel inactivation occurs on depolarised voltage level while the channel remains open, but stops passing ions. Channel inactivation terminates on hyperpolarisation. The activity of KQT homologs in mammals is known to be strongly modulated by accessory proteins like KCNE1 beta-subunit. It is possible that *in situ kqt-2* and *kqt-3* channel properties are different from that in the heterological expression system in *Xenopus* oocytes, and they exhibit inward rectification.

Similarly, EXP-2 properties are different *in situ* and in heterological expression *in vitro* system in *Xenopus* oocytes (Davis *et al.*, 1999). Our data show that in the intestinal cell in nematodes, MP is maintained as unusual to other cell types depolarised level close to 0 mV. Therefore, we suggest that at that MP level K^+ channels are open, but inactivated. Natural or artificial hyperpolarisation can transiently reactivate some K^+ channels resulting in hyperpolarisation with further K^+ channel reactivation. Under-threshold hyperpolarisation and K^+ channels closure together with refractory mechanisms can cause HAP cycling. Unfortunately, crucial experiments of simultaneous inactivation of both *kqt-2* and *kqt-3* on DMP were not reported.

Other types of potassium channels. Inward rectification of EXP-2 channels can explain HAP generation in pharyngeal muscle cells while the presence of such properties in intestinal voltage-gated *kqt-2* and *kqt-3* channels was not shown. However, there is another well-studied family of inward rectifier potassium channels with two transmembrane domains per subunit (2TM) and a single P domain. Three *C. elegans* genes have this subunit structure, yet no mutants have so far been reported to be associated with these genes (Salkoff *et al.*, 2005), and they show no elevated expression in the intestine. Besides 2TM inward rectifiers open only at much more negative potentials, than we observe for HAT gb current. An alternative mechanism to produce hyperpolarising spike can utilise calcium or sodium-activated potassium channels. We can expect that Ca^{2+} or Na^+ influx to cytoplasm caused by natural or artificial hyperpolarisation can activate K^+ channel in positive feedback mode. Such channels exist and are present in the nematode genome (BK and SK) (Salkoff *et al.*, 2005), yet they are not highly expressed in the gut cells.

Two-pore-domain channel proteins (TWK) form the most prominent *C. elegans* potassium channel family with 46 genes. Four genes from this family are overexpressed in intestinal cells (*twk-26*, *twk-33*, *twk-37* and *twk-45*) (Nehrke, 2014). There is no evidence that these genes are directly linked to the intestinal CPG activity (Salkoff *et al.*, 2005). The channels composed of proteins from this family are not voltage-gated, although their functions are regulated by various mechanisms that may participate in DMP function (Czirják *et al.*, 2004). First, some two-pore-domain potassium channels form 'leak channels' shaping plasma membrane resting potential. Intestinal cells have unusually depolarised MP, and TWK channels may help to control this cellular feature. Several mechanisms capable of regulating these channels are also known, including Ca^{2+}, pH, signalling lipids, oxygen tension, mechanical stretch, and G-proteins. Ca^{2+} (Dal Santo *et al.*, 1999; Teramoto & Iwasaki, 2006) and pH cycling are known (Pfeiffer *et al.*, 2008; Allman *et al.*, 2009) to accompany intestinal oscillations, so Ca^{2+} and acidic sensing channels may participate in the coupling between ion oscillations and potassium currents and membrane voltage.

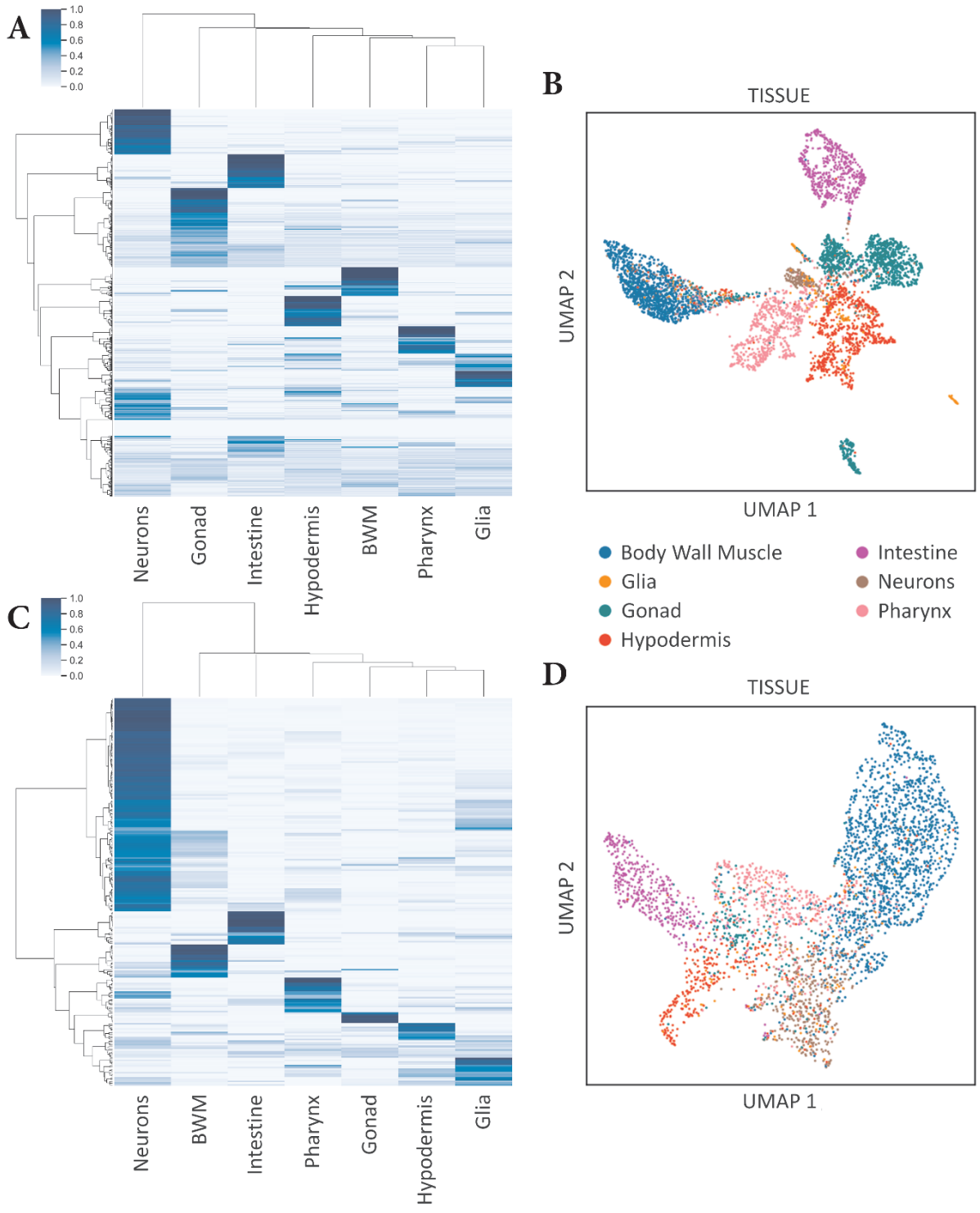
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Fig. 2. Differential expression of intestinal genes. Clustermap dendrogram (A, C) and UMAP (B, D) for scRNA-seq expression data of all genes in *Caenorhabditis elegans* (A, B) and ion channels in *C. elegans* (C, D). A, C. Clustermap dendrogram. To compare gene expression between different tissue types (neurons, gonad, hypodermis, pharynx, body wall muscles, glia, and intestine, (Cao *et al.*, 2017; Table S3)), for each gene the tissue/total ratio of gene expression was calculated. For this, the expression value for each gene, and each tissue was divided by the sum of the gene expression values for each tissue. These values vary between 0 and 1, and the sum for each gene is equal to 1. Data were clustered with Ward's method (Müllner, 2011). B. Result of dimensional reduction (UMAP (McInnes *et al.*, 2018)) using all expressed genes. Graph generated from the data of all gene expression forms apparent clusters. D. Similar graph generated from the expression data of 222 ion channels. The cells of intestine tissue demonstrate similar to all gene expression graph results. The collection of the ion channel genes is tissue-specific. In both cases we have used the first six principal components of the analysed space; the number of neighbours for each point in UMAP calculations was set to 15. UMI per cell tables and gene expression per tissue have been taken from (Cao *et al.*, 2017). Analysis has been conducted *via* SCANPY and SEABORN (Wolf *et al.*, 2018; https://zenodo.org/record/883859#.Xc1b-1czaHs) for Python 3. Cell-to-tissue annotation has been derived from the initial dataset. The collection consists of 222 genes: *cca-1*, *ccb-1*, *ccb-2*, *egl-19*, *itr-1*, *nca-2*, *orai-1*, *stim-1*, *tag-180*, *unc-2*, *unc-36*, *unc-68*, *unc-77*, *che-6*, *cng-1*, *cng-2*, *cng-3*, *tax-2*, *tax-4*, *acc-1*, *acc-2*, *acc-3*, *acc-4*, *avr-14*, *avr-15*, *exp-1*, *gab-1*, *ggr-1*, *ggr-2*, *ggr-3*, *glc-1*, *glc-2*, *glc-3*, *glc-4*, *lgc-32*, *lgc-33*, *lgc-34*, *lgc-35*, *lgc-36*, *lgc-37*, *lgc-38*, *lgc-39*, *lgc-40*, *lgc-41*, *lgc-42*, *lgc-43*, *lgc-44*, *lgc-45*, *lgc-46*, *lgc-47*, *lgc-48*, *lgc-49*, *lgc-50*, *lgc-51*, *lgc-52*, *lgc-53*, *lgc-54*, *lgc-55*, *mod-1*, *unc-49*, *C08B6.5*, *F59E12.8*, *glr-1*, *glr-2*, *glr-3*, *glr-4*, *glr-5*, *glr-6*, *glr-7*, *glr-8*, *nmr-1*, *T25E4.2*, *W02A2.5*, *ZK867.2*, *kqt-2*, *twk-26*, *twk-37*, *kqt-3*, *twk-33*, *twk-45*, *twk-28*, *twk-46*, *irk-2*, *twk-20*, *slo-2*, *kcnl-3*, *twk-47*, *twk-3*, *twk-31*, *twk-40*, *twk-7*, *twk-34*, *kcnl-2*, *egl-2*, *twk-9*, *twk-24*, *twk-14*, *twk-29*, *unc-58*, *twk-8*, *twk-42*, *twk-12*, *kqt-1*, *twk-48*, *kvs-4*, *twk-11*, *twk-13*, *irk-3*, *twk-30*, *egl-36*, *twk-35*, *twk-18*, *twk-16*, *twk-25*, *twk-21*, *twk-2*, *kcnl-1*, *kvs-5*, *unc-103*, *kvs-1*, *twk-43*, *egl-23*, *twk-32*, *twk-1*, *twk-10*, *shk-1*, *shw-1*, *slo-1*, *twk-49*, *exp-2*, *irk-1*, *kcnl-4*, *kvs-3*, *shl-1*, *shw-3*, *sup-9*, *twk-17*, *twk-22*, *twk-23*, *twk-36*, *twk-39*, *twk-4*, *twk-44*, *twk-5*, *twk-6*, *acd-1*, *acd-2*, *acd-3*, *acd-4*, *acd-5*, *asic-1*, *asic-2*, *deg-1*, *degt-1*, *del-1*, *del-10*, *del-2*, *del-3*, *del-4*, *del-5*, *del-6*, *del-7*, *del-8*, *del-9*, *delm-1*, *delm-2*, *egas-1*, *egas-2*, *egas-3*, *egas-4*, *flr-1*, *mec-10*, *mec-4*, *unc-105*, *unc-8*, *pezo-1*, *cup-5*, *ced-11*, *gon-2*, *gtl-1*, *gtl-2*, *lov-1*, *ocr-1*, *ocr-2*, *ocr-3*, *ocr-4*, *osm-9*, *pkd-2*, *spe-41*, *trp-1*, *trp-2*, *trp-4*, *trpa-1*, *trpa-2*, *trpl-2*, *trpl-3*, *trpl-4*, *trpl-5*, *eat-5*, *inx-1*, *inx-10*, *inx-11*, *inx-12*, *inx-13*, *inx-14*, *inx-15*, *inx-16*, *inx-17*, *inx-18*, *inx-19*, *inx-2*, *inx-20*, *inx-21*, *inx-22*, *inx-3*, *inx-5*, *inx-6*, *inx-7*, *inx-8*, *inx-9*, *unc-7* and *unc-9*.

DEG/ENaC/ASIC channels. In *C. elegans*, the DEGenerin/Epithelial Na^+ Channels/Acid-sensing ion channels (DEG/ENaC/ASIC) super-family contains 30 genes (Nehrke, 2014). They are trimeric sodium channels with subunits that consist of two transmembrane helices and an extracellular loop. The amino- and carboxy- termini are located in the cytosol. Single-cell RNA-seq data analysis reveals that five genes from this family are expressed in intestinal cells much higher than in other tissues (*acd-5*, *flr-1*, *del-5*, *acd-1* and *acd-2*) (Fig. 3). According to WormBase (https://www.wormbase.org), the observed mutant phenotypes of *acd-5*, *del-5*, *acd-1* and *acd-2* do not affect the function of the intestine, but the *flr-1* loss-of-function mutants show very short defaecation cycle periods and at the same time low efficiency of defaecation, which will lead to the formation of a constipated phenotype (Iwasaki *et al.*, 1995; Take-Uchi *et al.*, 1998). Transgenic worms with deleted C-terminal intracellular region of FLR-1 protein demonstrated prolonged defaecation cycle periods. As *flr-1* loss-of-function mutants exhibit short defaecation cycle periods, the deletion of the regulatory C-terminal intracellular region might have a gain-of-function effect on the FLR-1 ion channel (Kobayashi *et al.*, 2011). The electro-physiological causes of the described effects of mutations have not yet been investigated.

In the simplest model for *H. megidis* HAP generation, g_{a1} conductance was attributed to plasma membrane Na^+ channels (Kuznetsov *et al.*, 2017). DEG/ENaC/ASIC channels are predicted to have sodium channel activity, but they are not voltage-gated. How could DEG/ENaC/ASIC channels participate in HAP/DMP generation? Acid-sensing sodium channels from this family (ASIC) are activated by extracellular protons. pH cycling in pseudocoelom fluid is known to accompany intestinal oscillations (Beg *et al.*, 2008), so such acidic sensing channels may participate in the coupling between protons concentration oscillations and sodium currents and membrane voltage.

Transient receptor potential channels (TRP channels) are a group of ion channels located mainly on the plasma membrane of various animal cells. Generally, they are not voltage-gated but activates by various ligands or sensory inputs. Usually, TRP channels have a relatively non-selective permeability to cations, including sodium, calcium, and magnesium (Kadowaki, 2015). The *C. elegans* genome contains 23 TRP genes. Single-cell RNA-seq

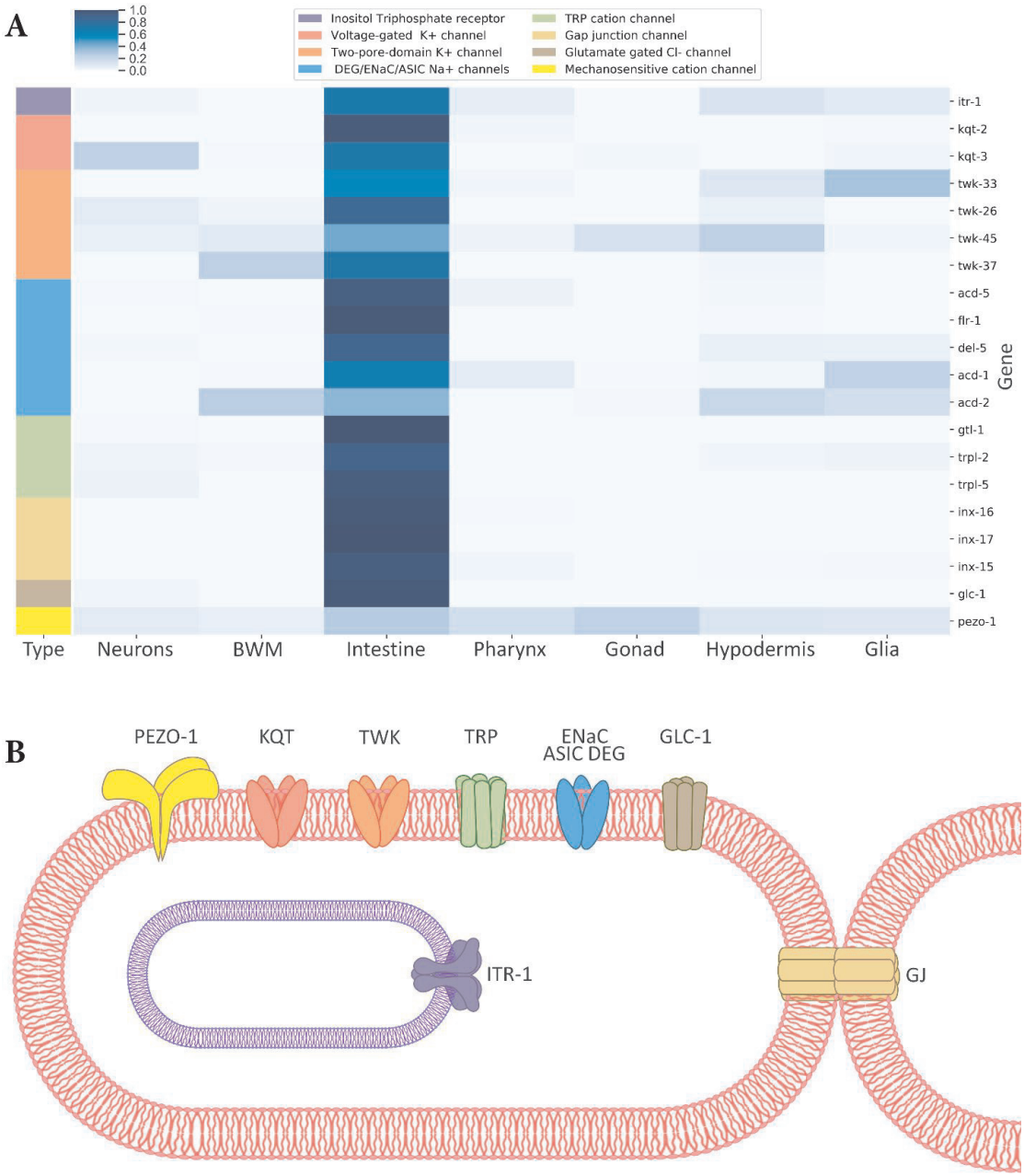

Fig. 3. Predominantly expressed in intestine genes. A. Heatmap of 20 genes with maximum relative expression in nematode intestine. For each gene, the tissue/total ratio of gene expression was calculated. For this, the expression value for each gene and each tissue was divided by the sum of the gene expression values for each tissue. The higher gene expression in a particular tissue has a higher heatmap unit saturation and the data value closer to 1. The leftmost column color-based represents the gene types described in the legend and B. All 20 genes are labeled on the right. B. All types of ion channels, present in scRNA-seq data in *C. elegans*. Ion channel colors correspond with Type coloring in panel A. Two cells are connected *via* GJ (INX-15, INX-16, INX-17). Intracellular Ca^{2+} storage (ER) has ITR-1 receptor on the membrane (purple). The cell membrane is permeated by six types of ion channels. Two K^{+} channel types: KQT-family (KQT-2, KQT-3) and TWK channels (TWK-26, TWK-33, TWK-37, TWK-45). DEG/ENaC/ASIC group (ACD-5, FLR-1, DEL-5, ACD-1, ACD-2). TRP channels (GTL-1, TRPL-2, TRPL-5). Cys-loop receptor GLC-1 and mechanosensory receptor PEZO-1.

data analysis reveals that three genes from this family are expressed in intestinal cells much higher than in other tissues: *gtl-1*, *trpl-2* and *trpl-5* (Fig. 3). According to WormBase, within these channels, a mutant phenotype associated with impaired defaecation was found for *gtl-1* only. *gtl-1* is an ortholog of human TRPM7 (transient receptor potential cation channel subfamily M member 7). In *gon-2* and *gtl-1* mutants Ca^{2+} oscillation rhythmicity is abnormal as *gon-2* and *gtl-1* interact with components of the calcium signalling machinery (Xing *et al.*, 2008). GON-2 and GTL-1 might participate in the generation of g_{a2} conductance in HAP model as they are outwardly rectifying Ca^{2+} current channels.

In the intestine *gtl-1* gene is reported to be co-expressed with *gon-2*, and they probably form heteromeric channels (Xing *et al.*, 2008). These channels are important in Mg^{2+} and Ca^{2+} intracellular concentration regulation: they mediate selective Ca^{2+} influx and function to regulate IP_3 receptor activity and, possibly, to refill endoplasmic reticulum Ca^{2+} stores (Teramoto *et al.*, 2005; Kwan *et al.*, 2008; Xing *et al.*, 2008).

Gap junctions. Gap junctions are composed of membrane protein molecules and form intercellular channels that connect the cytoplasm of adjacent cells and are permeable to ions and small molecules (Slivko-Koltchik *et al.*, 2019). In nematodes, as in most invertebrates, GJ channels are formed by innexin/pannexins family proteins that are encoded by 25 genes in the *C. elegans* genome (Kuznetsov *et al.*, 2016). According to single-cell RNA-seq data, three genes from this family are overexpressed in *C. elegans* intestinal cells (*inx-16*, *inx-17* and *inx-15*). This observation is in agreement with high-resolution expression map generated with the green fluorescent protein under the control of innexin/pannexins promoter regions (Altun *et al.*, 2009). In addition to intestinal *inx-16*, *inx-17* and *inx-15*, some expression of *inx-2* was reported in anterior intestine as well as in the pharynx.

The role of innexin/pannexins was studied in *C. elegans* intestinal calcium wave propagation (Peters *et al.*, 2007), and the presence of GJ between the gut cells and their role in electrical synchronisation in *H. megidis* was confirmed by direct physiological studies with both dye injection and electrical coupling experiments (Kuznetsov *et al.*, 2017). Peters *et al.* (2007) investigated the role of pannexins in calcium wave propagation along the intestine. They studied *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 the mutant animal's intestine, cell calcium spikes were generated asynchronously and appeared to be independent of neighbouring cells. It appeared that INX-16 plays a critical role in the synchronisation between intestine cells. It is not clear why two other highly expressed *inx-17* and *inx-15* genes are not compensating for INX-16 loss of function. It is known that the intercellular channel consists of two pannexons, one from each neighbouring cell, and different pannexin monomers may combine to form a heteromeric channel. All three intestinal GJ proteins may contribute to such heteromeric channel with an indispensable INX-16 subunit. Alternatively, *inx-17* and *inx-15* may not participate in GJ function at all. It is known that innexin/pannexin proteins are not only capable of forming intercellular gap junctions but also functioning in the plasma and ER membranes as hemichannels (Dahl & Locovei, 2006; Vanden Abeele *et al.*, 2006; Sangaletti *et al.*, 2014).

Transporters and exchangers. Transporters are much slower than channels and are less critical in fast voltage transitions in neurons and muscles. However, ultradian oscillations in nematode intestine are much slower, and transporter/exchanger activity could be important for intestinal oscillations. The activity of the majority of transporters is not electrically neutral and can directly contribute to electrical potential oscillations, which occur on intestinal cell membrane, while changes of pH or Ca^{2+} concentrations can regulate ion channel properties. Single-cell RNA-seq data also show strong differential expression of transporter/exchanger genes in intestinal cells. The comprehensive DMP model will require the inclusion of transporters/exchangers and some regulatory genes in combination with described ion channels. Due to space limitations of this publication this task is retained for the future.

External signalling and sensory reception. The nematode gut contains no neuronal or muscle cells, and the CPG resides in the intestine itself. Rhythmical cycling is autonomous being generated by intestinal cells' intrinsic properties. Some external stimuli were found to modulate defaecation. Food availability (leaving the bacterial lawn) and moulting suppress the DMP (Liu & Thomas, 1994). It was also shown that light-touch mechanosensation could reset the defaecation phase (Thomas, 1990; Liu & Thomas, 1994). At the same time, the participation of the nervous system in DMP control appears to be minimal, which was revealed by laser microbeam neuron ablation. Only one neuron elimination affects DMP (Wang *et al.*,

2013). It is known that killing AVL neuron causes defects of the anterior body wall muscle contraction (aBoc) step. Although AVL is GABAergic neuron, GABA dysfunction mutants have a normal aBoc step. Overall, the mechanisms of DMP modulation by the external stimuli are poorly understood. Intestinal ion channel expression revealed by single-cell RNA-seq shed some light on this issue. Expression data shows high and differential expression of the unusual glutamate-gated chloride-permeable ionotropic ligand-gated Cys-loop receptors (*glc-1*).

C. elegans glc-1 messenger RNA expressed in *Xenopus* oocytes encodes an avermectin-sensitive glutamate-gated chloride channel (Cully *et al.*, 1994). Thus, glutamate could be a signal molecule that can modulate intestinal function. This observation is essential for the electrophysiological study of intestinal cells giving a tool to control enterocyte activity by glutamate application. No direct synaptic projections from glutamatergic neurons to the intestine are known, and the source of this signalling molecule is obscure. *glc-1* receptors might sense nutritional glutamate in the intestinal lumen.

The *pezo-1* gene is another overexpressed one in the intestine channel. Conservative Piezo proteins form mechanosensitive ion channels in many species (Bagriantsev *et al.*, 2014). Its presence in intestinal cells can explain how gentle touch can reset the defaecation phase without nervous system participation.

CONCLUSIONS

Here we have compared single-cell RNA-seq data with the available models of DMP generation, proposed in *C. elegans* and *H. megidis* studies. The comparison made it possible to confirm or dispute the role of specific proteins in the work of DMP and to propose new candidates for participation in this molecular network. In many cases, single-cell RNA-seq data confirm earlier observations of specific intestinal gene expression and function, yet it also indicates the new candidates for the model. Comparative analysis of the expression data from the selected gene set (ion channels) confirms that single-cell RNA-seq is a reliable and powerful technique.

Starting with Hodgkin and Huxley studies, it appeared that depolarisation AP could be almost fully described and modelled in terms of plasma membrane voltage-dependent ion channels. In this paper, we have restricted our analysis to ion channels expressed in nematode intestine. Although it helps to clarify some properties of DMP cycling, it seems essential for the comprehensive DMP model to include transporters/exchangers and regulatory genes in combination with the ion channels.

ACKNOWLEDGEMENTS

This work was supported by the RFBR [grant number 18-04-00705].

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В.П. Кузнецов, Г.А. Сливко-Кольчик, С.В. Исаев, Д.А. Воронов и Ю.В. Панчин. Ионные каналы кишечного гиперполяризационного потенциала действия у нематод.

Резюме. Моторная программа дефекации нематод включает распространяющиеся кальциевые волны и колебания электрического потенциала мембраны клеток кишечника и контролируется центральным генератором. Недавние электрофизиологические эксперименты на *Heterorhabditis megidis* показали, что кишечные клетки нематод генерируют необычный гиперполяризационный «потенциал действия» (ГАП). ГАП похож на потенциалы действия в обычных возбудимых тканях, нейронах и миоцитах, но с обратным знаком тока и напряжения. Можно предположить, что генерация ритмического электрического потенциала в клетках кишечника тоже основана на взаимодействии потенциал-зависимых ионных каналов. Здесь мы объединили наши электрофизиологические исследования и молекулярно-генетический анализ механизмов работы кишечных клеток нематод с недавними данными по секвенированию РНК одиночных клеток *Caenorhabditis elegans*, находящимися в открытом доступе. Данные по секвенированию РНК одиночных клеток подтверждают уникальный, сильно отличающийся от других тканей нематод, спектр экспрессии ионных каналов в клетках кишечника. Сравнение электрофизиологических данных с данными по экспрессии ионных каналов в клетках, генерирующих обычные потенциалы действия и ГПД, позволяет решить, какие из этих каналов могут соответствовать модели ГПД.