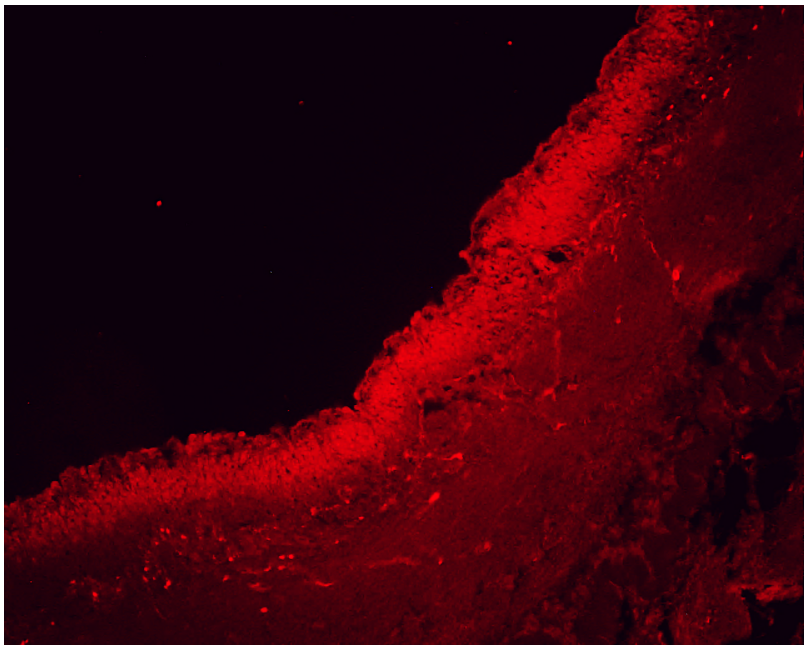
UNIVERSITY OF GOTHENBURG

DEPARTMENT OF BIOLOGICAL AND ENVIRONMENTAL SCIENCES

LOCALISATION OF THE INTERSTITIAL CELLS OF CAJAL IN RAINBOW TROUT



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Degree project for Bachelor of Science with a major in Biology
BIO602, bachelor's degree project 15 hec
First cycle
Semester/year: Spring 2022
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Photograph by Ellen Nilsson (2022)

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Abstract

The interstitial cells of Cajal (ICC) are primarily placed in the gastrointestinal tract, where they play a crucial role in the initiation and control of motility by operating as pacemaker cells. They also have a role in neurotransmission. ICC's functions have been investigated, especially in mammals, but less is known about the cells in fish. This study explored the presence and distribution of ICC in rainbow trout (*Oncorhynchus mykiss*) using immunohistochemistry on tissue preparation from six different regions of the gastrointestinal tract. This was done to investigate the difference in the distribution of ICC between the regions. The primary antibodies were directed against anoctamin 1 (Ano-1), targeting the ICC, and the neuronal marker acetylated tubulin (AcT), some preparations were simultaneously incubated with Ano-1 and AcT. Ano-1 immunoreactive structures were found in the *proximal-, mid-, and distal intestine,* but no Ano-1 immunoreactive cells were found in the *cardiac stomach, pyloric stomach,* or *rectum.* Wholemount preparations of the myenteric plexus, situated between the two muscle layers, showed singular fibres with branched extensions. These cells were located above AcT immunoreactive nerve fibres closer to the circular muscle layer. Nerves and potential ICC could be distinguished situated in the same area, but there was no overlap between them. The immunohistochemistry performed on the paraffin sectioned preparations resulted in Ano-1 immunoreactivities found in the myenteric plexus and the circular muscle layer. Occasional cells were located deeper into the circular muscle layer. The distribution and location of the Ano-1 immunoreactive structures suggest that they are ICC. The result also shows that even though most of the ICC are located adjacent to the myenteric plexus, some cells are also found in the circular muscle layer. Further studies are required to examine if there are anatomically and functionally differences between the cells.

Keywords
Interstitial cells of Cajal; Immunohistochemistry; Paraffin section; Gastrointestinal; Slow waves

Sammanfattning

De interstitiella Cajal-cellerna är primärt distribuerade i mag-tarmkanalen där de spelar en roll i initieringen och kontrollen av motiliteten genom att agera pacemakerceller men de har även en roll i neurotransmission. Forskning kring de interstitiella Cajal-cellernas (ICC) funktion har gjorts främst i däggdjur medan det i fisk finns mycket kvar att ta reda på. Denna studie undersökte distributionen av ICC i regnbåge (*Oncorhynchus mykiss)* med immunhistokemi på vävnadspreparat från sex regioner från mag-tarmkanalen för att undersöka hur förekomsten och distributionen av ICC skiljde sig åt i de olika delarna. De primära antikropparna var riktade mot anoctamin 1 (Ano-1) för lokalisering av ICC och nervmarkörerna acetyl tubulin (AcT). Ett antal vävnadspreparat var simultant dubbelmärkta med Ano-1 och AcT. Ano-1 reaktiva celler återfanns i *proximal-, mid-* och *distal intestine* men inga spår av immunreaktiva celler återfanns i *cardiac stomach, pyloric stomach* eller *rectum.* Utbredningspreparat av de myenteriska plexat belägen mellan de två muskellagerna, uppvisade singulära fibrer med förgrenade utskott utan distinkta cellkroppar. Dessa celler var placerade ovanför AcT immunreaktiva nervfibrer, närmare det cirkulära muskellagret. Nerver och potentiella ICC belägna i samma område var möjliga att särskilja och överlappade ej varandra. Immunohistokemin utförd på den paraffinsnittade vävnaden visade en majoritet av immunreaktiviteten i det myenteriska plexat och ytligt i det cirkulära muskellagret. Enstaka celler återfanns även djupare i det cirkulära muskellagret. Distributionen av de Ano-1 immunreaktiva strukturerna tyder på att de är ICC. Resultatet visar även att trots att majoriteten av cellerna är placerade intill de myenteriska plexat, återfinns även celler i det cirkulära muskellagret. Vidare forskning är erforderlig för att avgöra huruvida det finns någon anatomisk och funktionell skillnad mellan cellerna.

Nyckelord
Interstitiella Cajal celler; Immunhistokemi; Paraffinsnitt; Mag-tarmkanalen; Slow waves

Introduction

The gastrointestinal tract extends from mouth to anus and enables organisms to assimilate nutrition, making the organs essential for survival (Olsson & Holmgren, 2001). The motility of the gastrointestinal tract has several vital features; peristalsis transports the food through the gastrointestinal tract mainly in an anterograde direction, standing contraction mixes the food, other contractions create compartmentalisation and migrating motor complexes probably transport waste products (Olsson & Holmgren, 2001).

The motility is achieved by contractions and relaxations of the smooth muscle cells. These contractions are coordinated between the inner circular and outer longitudinal layers (Fig. 1). The smooth muscle cells are connected through gap junctions, enabling electrical stimuli to spread between them. This makes it possible for the smooth muscle cells to interact, creating unitary motions of larger muscle sections by promoting a coordinated contraction of the muscle cells (Olsson & Holmgren, 2001).

The gastrointestinal movement is initiated and controlled in several ways with cooperation between the smooth muscle cells, hormones, the autonomic nervous system, especially the enteric nervous system, and the interstitial cells of Cajal (ICC) (Olsson & Holmgren, 2001).

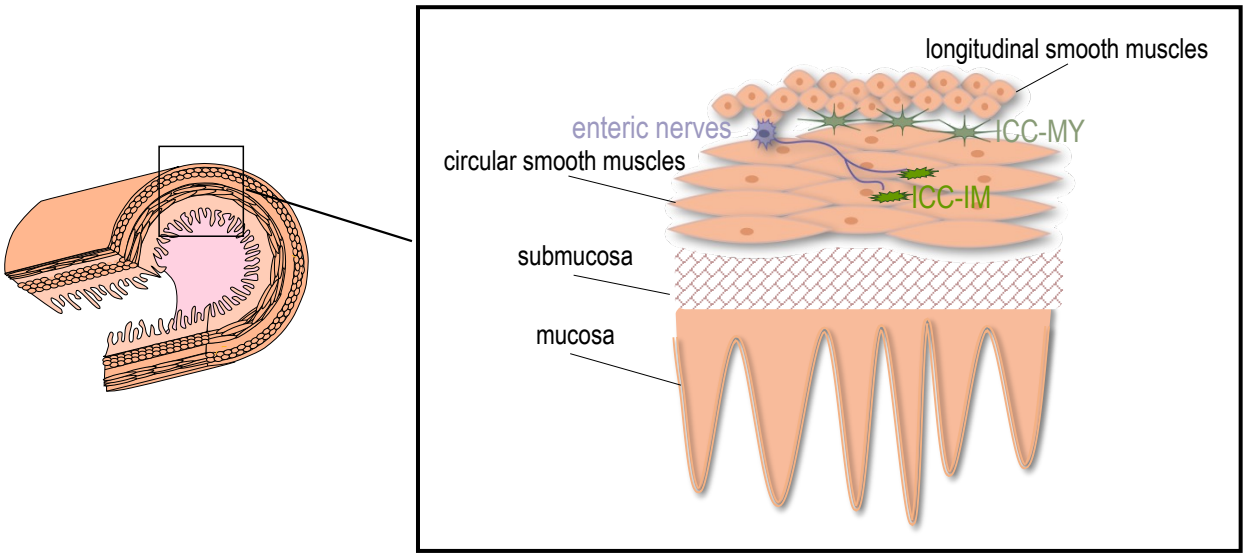

Figure 1. Schematic figure of the different layers of the gastrointestinal tract. Schematic figure showing the different layers of the gastrointestinal tract containing two subtypes of ICC relative to a nerve cell. Modified from J. Brijs, 2017 and C. Olsson, unpublished.

Interstitial cells of Cajal

Santiago Ramón y Cajal first identified the Interstitial cells of Cajal (ICC) in the early 20th century (Cajal, 1911). The ICC are a type of mesenchymal cell found in the gastrointestinal wall (Ward et al., 2004). They have several vital functions in the gastrointestinal tract. For instance, they operate both as a pacemaker cell that controls the frequency of muscle contractions and have parts in neurotransmission. Cajal himself first described the cells as primitive neurons. Taxi later distinguished them from other cell types by using electron microscopy, while Langton was the first to point out its electrical properties (Al-Shboul, 2013; Cajal, 1911; Langton et al., 1989; Taxi, 1961, 1964).

In mammals, ICC are primarily found close by the myenteric plexus but also in the muscle layers. There are different subtypes of ICC that differ in both form and function. ICC placed in the myenteric plexus is called ICC-MP or ICC-MY (Fig. 1). This ICC subtype contains branched structures on the cell surface. These cells are often distributed in networks and are placed mainly between the longitudinal and circular muscle layers. Another type is the ICC found in the circular muscle layer called ICC-CM; these cells are spindle-shaped and are not distributed in networks to the same extent as the ICC-MY. Instead, they are more independent. ICC-LM are much like ICC-CM but often found in lower quantities. Therefore, there is a collective term for ICC-CM and ICC-LM, called ICC-IM (Fig. 1). ICC-DMP are connected to nerve

cells in the deep muscular plexus. The deep muscular plexus is a neural plexus in the small intestine placed between the smooth muscle cells in the circular muscle layer (Henry et al., 1998). ICC-SM and ICC-SMP are loosely connected cells in the submucosal area (Al-Shboul, 2013).

The ICC in the gastrointestinal tract are connected through gap junctions; this connection makes it possible for electric currents to propagate between the ICC and from ICC to the syncytium of smooth muscle cells (Kito, 2011). ICC act by generating spontaneous so-called slow wave currents (Ward et al., 2004). The intercellular channels probably enable the slow waves generated by the ICC to propagate to the smooth muscle cells. The slow waves facilitate for the smooth muscle cells to reach the limit of the threshold, which can lead to an action potential and contraction of the muscles. These responses increase the likelihood for the smooth muscle cells to contract. Hormonal and neural signalling can promote or inhibit action potential by altering the electrical conductivity between the smooth muscle cells and the ICC and affecting the degree of depolarization (Ward et al., 2004). ICC also has a role in neurotransmission. Receptors on the ICC surface react to neurotransmitters released from the neuron's axon terminal. The stimuli are further propagated to the smooth muscle cells.

Localisation techniques of the ICC

The first observations of ICC were made using traditional histochemical staining techniques. The most used method for localising the ICC is immunohistochemistry (Komuro, 2012).

Immunohistochemistry is a method that detects specific antigens in tissue using a compatible antibody. The areas where the antigen is distributed can then be visualised with a fluorescent dye, enzymes, radioactivity or colloidal gold. This can be directly linked to the primary antibody or to a secondary antibody that binds to the primary by being directed against the species of the primary antibody. The main benefit of using both a primary and secondary antibody is that the fluorescent is amplified because several secondary antibodies can target the same primary antibody (Jackson Immunoresearch, n.d.). The advantage of immunohistochemistry over traditionally staining techniques is that it can be more specific in targeting proteins, tissue structures, or enzymes.

The first specific marker of ICC that could be used for immunohistochemistry in mammals was a receptor tyrosine kinase called Kit (CD117). Studies utilising a blockade of c-Kit showed abnormal ICC properties indicating that the receptor tyrosine kinase was central for the cells (Maeda et al., 1992; Torihashi et al., 1995). Some subtypes of ICC, for example, the ICC placed in the deep muscular plexus in the small intestine in humans, do not express c-Kit and some other cells than ICC express c-Kit. But most cells expressing c-Kit are the ICCs, and it is still a sufficiently specific marker for localising ICC (Al-Shboul, 2013). A more recent marker for the ICC is the ion channel anoctamin-1 (Ano-1), which targets several subtypes of ICC in humans and mice (Al-Shboul, 2013).

ICC in the gastrointestinal tract of fish

The first study showing the ICC in the gastrointestinal tract of fish was in Common snakehead (*Ophiocephalus striatus*) and a species of *Scylliorhinus* using Methylene Blue. (Kirtisinghe, 1940). Several attempts were made to use the antibody C-kit for the immunohistochemistry in fish of several different species but failed, which questioned the efficiency of the antibody (C.Olsson, personal communication). Since then, no reports on the occurrence of ICC in fish were published until 2007, when the first localisation of ICC using immunohistochemistry was established using zebrafish. The antibody used for this study was c-Kit, targeting a receptor tyrosine kinase (Rich et al., 2007). A couple of years later, the analysis was successfully repeated using the same antibody (Ball et al., 2012).

In 2013 the antibody targeting anoctamin-1, an ion channel occurring in ICC, was tried in zebrafish and successfully located some cells in the myenteric region of the tract, which most likely were the ICC (Uyttebroek et al., 2013). A similar study was followed in shorthorn sculpin, where Ano-1 immunoreactive cells appeared close to the myenteric plexus. This study only investigated the distribution in the proximal intestine. Still, unpublished data shows the presence of immunoreactive cells in the myenteric plexus for all parts of the gastrointestinal tract of high density in shorthorn sculpin. This study argues that the antibody Ano-1 works as a more reliable marker for ICC (Brijs et al., 2017b). The Ano-1 positive cells found in shorthorn sculpins are linked in network-like structures (Fig. 4).

There are indications from previous studies that ICC may have similar functions as in mammals, like generating slow waves. Brijs et al. (2017b) study suggests that they found ICC in the myenteric region like the ICC in mammals. The contractile movement of the gastrointestinal tract was inhibited after blocking Ano-1 with benzbromarone. However, the movement still occurred when adding a neural blocker (TTX), indicating that ICC has an even more comprehensive role in gut motility than ICC in mammals. (Brijs et al., 2017b). In rainbow trout, an attempt to stain the cells using immunohistochemistry and blockading of Ano-1 using benzbromarone have been made. The blockade of Ano-1 partially inhibited the contractile movement of the gut, an indication that Ano-1 occurred in the ICC. However, some movement still occurred, indicating that not all of the ICC with pacemaker activity were targeted by the Ano-1 blockade. The study suggests Ano-1 immunoreactive cells in the myenteric plexus (Julien, 2020). Despite this, there is a lot left to find out about the ICC in the fish.

No studies have examined the distribution of the ICC in the different layers of the gastrointestinal tract in fish. Nor if there are different subtypes of ICC in fish that differs anatomically or in function from one another. Major studies showing successful ICC labelling in rainbow trout do not exist either.

Aim of the study

This study investigated the ICC distribution in different parts and layers of the gastrointestinal tract in rainbow trout (*Oncorhynchus mykiss).* This was implemented by using paraffin sectioned tissue preparation to be able to observe the different layers. Immunohistochemistry with antibodies against anoctamin-1 was utilised for the targeting of ICC. In addition, the relation between ICC and nerves has been described using simultaneous double labelling against neuronal markers.

Material and Methods

Experimental organism

The experimental animal used for this study was rainbow trout (*Oncorhynchus mykiss),* obtained from Vänneåns Fiskodling AB. The rainbow trout was kept in freshwater tanks at the University of Gothenburg and fed three times a week. The photoperiod was 12 hours light and 12 hours darkness, and the water temperature was approximately 10 °C. The animals were held under the ethical guidelines with the permit number: 5-8-18-06591-2019, approved by the Ethical Committee on Animal Research in Gothenburg.

Tissue preparation

The rainbow trout was put to death by a blow to the head and cutting the gills. The gastrointestinal tract was then removed from the interior and divided into six segments: *cardiac stomach, pyloric stomach, proximal intestine, mid intestine, distal intestine* and *rectum*. Each section was then stretched out and pinned to dental wax, fixated in 4 % formaldehyde (0.1 M phosphate buffer 0.9 % NaCl, pH 7), and kept in the fridge for four hours. After that, the tissues were rinsed using 0.1 M phosphate buffer saline containing 0.9 % NaCl, pH 7.2 (PBS). The tissue was stored in the fridge in 0.1 M PBS until further processing.

Two types of preparations were made. For the wholemount tissue preparations, the mucosa, submucosa, and most of the circular muscle layer were gently peeled off under a dissection microscope. Approximately 5x5 mm sections were cut out to be used for the immunohistochemistry treatment.

For the paraffin sectioning, the tissue was placed in individual chambers and then dehydrated. For dehydration, the tissue chamber was first soaked in 50 % ethanol for 1-2 hours, then in 70 % ethanol for 1-2 hours, 90 % ethanol for further 1-2 hours, and 100 % ethanol for 1-2 hours two times. Then, the ethanol was removed from the tissue by replacement with toluene because the ethanol and paraffin cannot mix (but the toluene and paraffin can). The tissue was transferred from 100 % ethanol to a mixture of one part toluene and three parts 100 % ethanol for an hour. It was then changed to one part toluene and one part 100 % ethanol for one hour, then three parts toluene with one part 100 % ethanol for an additional one hour and then 100 % toluene for 3-4 hours, two times. The tissue samples were, after that, ready for the paraffin infiltration.

The tissue was transferred to one part paraffin and two parts toluene in a 45° C oven for one hour, then to a mixture of two parts paraffin and one part toluene and incubation in a 45° C oven for one hour; the tissue chamber was then transferred to 100 % paraffin just above the melting point (46-68 ° C) for 2-4 hours, two times. Lastly, the tissue was embedded in paraffin and cooled down to enable the paraffin block to harden.

The block of paraffin-embedded tissue samples was then sectioned at 5 µm thickness using a Shandon Finesse ME. The sections were placed in a tempered water bath, captured on microscope slides and then left at room temperature to air dry. Before the immunohistochemistry treatment could be done, the slides with tissue section had to be deparaffinised by being transferred to a xylene bath for 2 x 10 minutes. The samples were after that rehydrated by being put in 100 % ethanol for 2 x 10 minutes, followed by 5 minutes in 90 % ethanol, 5 minutes in 80 % ethanol, 5 minutes in 70 % ethanol, 2 x 5 minutes in deionised water and 2 x 5 minutes in PBS.

Immunohistochemistry

Both wholemount preparations and the paraffin sections were incubated overnight with primary antibodies against the ICC marker anoctamin-1 (Ano-1), both alone and together with one neural marker targeting the antigen acetylate tubulin (AcT). After the incubation period, the tissue samples were rinsed with PBS for 3x10 minutes and incubated additional 2 hours with the fluorescent secondary antibodies with the property to target the primary antibody. For simultaneous double labelling, secondary antibodies with different fluorophores were used (Table 1). Next, the tissue was again rinsed with PBS for 3x10 minutes.

The tissue was then mounted on microscope slides using carbonate-buffered glycerol as the mounting medium and a coverslip on top of the mounted tissue. The tissue samples were then analysed with an epifluorescence microscope (Nikon Eclipse E800) fitted with a Nikon Digital Camera DXM 1200 combined with the software *ACT-1*. The epifluorescence microscope works by an excitation filter that sorts out a particular wavelength of the white light that is directed towards the tissue. Which wavelength depends on which fluorophore is used. The fluorescent molecule absorbs this light and, in turn, emits a wavelength collected by an emission filter, making the fluorescent molecules observable to us. Different fluorophores absorb different wavelengths of white light and emit different wavelengths creating different colours (Termo Fisher Scientific, n.d.). The cells were identified, and from that, it was determined which layers the cells were located and where most of them were located. Pictures of the tissue containing the labelled cells were taken, and the images' exposure, contrast, and sharpness were edited in *preview version 11.0, Apple Inc.*

Table 1. The antibodies used for the immunohistochemistry.

PRIMARY ANTIBODIES					
Antigen	*abbreviation*	*code*	*host*	*dilution*	*supplier*
anoctamin-1 (TMEM16A)	Ano-1	ab53212	rabbit	1:200	Abcam
tubuline acetylerat	AcT	T-6793	mouse	1:1000	SIGMA
SECONDARY ANTIBODIES					
Fluorophore	*abbreviation*	*code*	*host*	*dilution*	*supplier*
Fluorescein isothiocyanate	DaM-FITC	715-095-150	donkey	1:100	Jackson
Fluorescein isothiocyanate	DaR-FITC	711-095-152	donkey	1:100	Jackson
Cyanine-3	DaR-CY3	711-165-152	donkey	1:800	Jackson

Results

Presence and distribution of Ano-1 positive cells

The immunohistochemistry performed showed Ano-1 immunoreactive cells in the wholemount tissue preparation, and the paraffin sectioned tissue. The wholemount preparations showed densely packed Ano-

1 positive cell-like structures containing long branches with lacking network structures. Also nerve bundles were successfully stained. The Ano-1 positive structures were found in the *proximal intestine, mid intestine,* and *distal intestine* of the gastrointestinal tract of the rainbow trout but not in the *cardiac stomach* or *rectum*. The paraffin sectioned tissue also showed Ano-1 immunoreactive cells only in the intestine. No Ano-1 immunoreactivity was found in the cardiac stomach, pyloric stomach, or rectum (Fig. 3 B). In the paraffin sectioned tissue, the cells were mainly located in between the muscle layers in the myenteric plexus and the periphery of the circular muscle layer (Fig. 3 C, D, E, F). Traces of Ano-1 reactive cells in a lower quantity were discovered even deeper into the circular muscle layer (Fig. 3 D, F). Nerve cells were successfully stained in all six regions, with most nerve bundles placed in the myenteric plexus.

The simultaneous double labelling using the primary antibodies Ano-1 and AcT of the wholemount tissue preparation worked without problems. The result showed that Ano-1positive cells were located above the AcT positive cells closer to the circular muscle layer, but there was no overlap between the two structures (Fig. 2 B). In the simultaneous double labelling of paraffin sections, however, there was a high degree of overlap in the red filter where also nerve cells were visible. To understand this outcome, various new immunohistochemistry staining was implemented. The primary antibodies were incubated separately on separate tissue samples and successfully labelled different structures. Different fluorophores of the secondary antibody were used but did not label anything. AcT was incubated alone with both secondary antibodies, and there was still overlap. AcT was incubated only with the secondary antibody of Ano-1, and there were no structures stained. The tissue was also preincubated in 5 % normal donkey serum for 30 minutes before the antibodies were added to prevent nonspecific binding of the secondary antibody. No improved result was established from the preincubation. Ano-1 positive cells were only successfully labelled when single labelled.

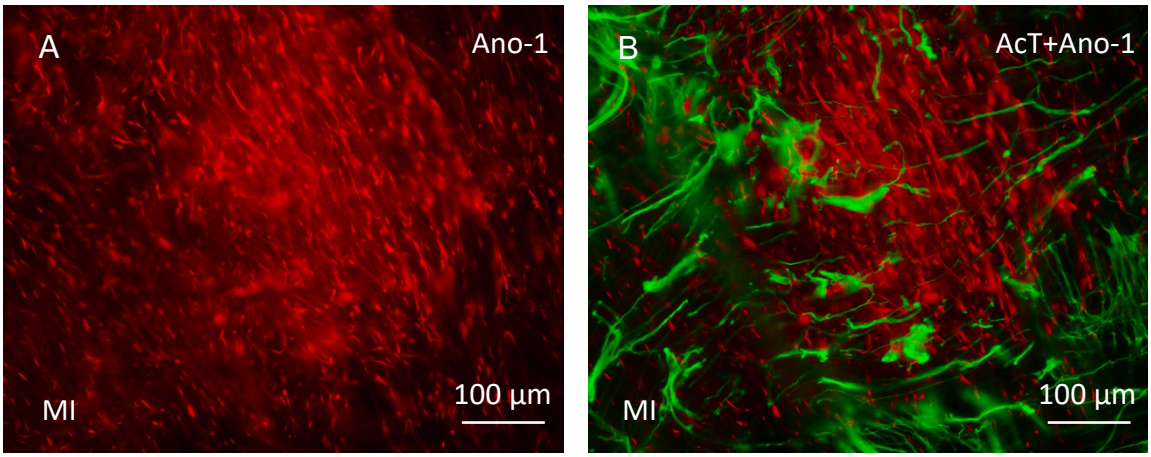

Figure 2. Immunohistochemistry labelling of wholemount tissue in the gastrointestinal tract of rainbow trout (*Oncorhynchus mykiss)*. Scale bars are placed in the lower right corner of every sub picture. MI stands for the middle intestine. (A) Ano-1 positive cells in the middle intestine of shorthorn sculpin. (B) The combination of the AcT positive cells and Ano-1 positive cells in the mid intestine of shorthorn sculpin.

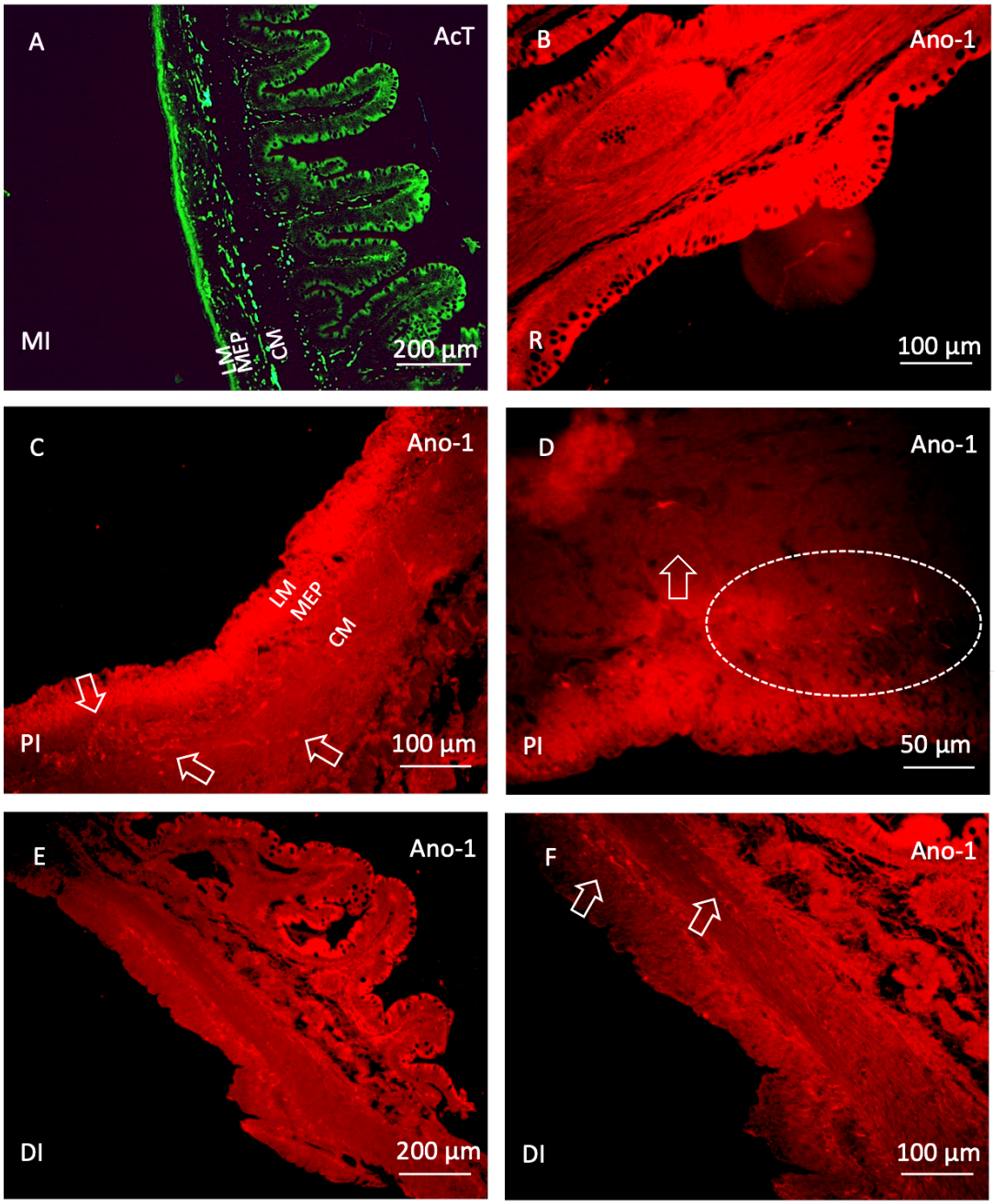

Figure 3. Immunohistochemistry labelling of paraffin sectioned tissue in rainbow trout (*Oncorhynchus mykiss)*. Longitudinal muscle layer (LM), Myenteric plexus (MEP), and the circular muscle layer (CM). Mid intestine (MI), rectum (R), proximal intestine (PI) and distal intestine (DI). (A) Nerve cells are marked with the primary antibody Act in the middle intestine. The three layers of the tissue are marked. (B) Tissue treated with the primary antibody Ano-1 in the rectum, no seen cells are labelled. (C) Ano-1 positive cell-like structures are labelled with Ano-1 in the proximal intestine. Arrowheads point at the cell-like structures marked with immunohistochemistry. The three tissue layers are marked with abbreviations. (D) Ano-1 positive cell in the proximal intestine of rainbow trout. Most of the Ano-1 positive cells are within the dashed line, and single Ano-1 positive cells deeper in the circular muscle layer are highlighted with the arrowhead. (E) Ano-1 immunoreactive cells in the *distal intestine*. (F) Arrowheads refer to Ano-1 positive cells in the distal intestine.

Discussion

This study reveals a deeper understanding of ICC in rainbow trout, including the cells' location, distribution, and structure. Established through immunohistochemistry, using paraffin sectioning and wholemount preparations, Ano-1 positive cells were labelled in the three gastrointestinal parts: *proximal intestine*, *mid intestine* and *distal intestine.* Most of the cells were distributed in the myenteric region and a short distance

into the area of circular muscles. Single cells were also located deeper into the circular muscle layer. The formations were labelled using the antibody anoctamin-1, and the structure of these formations corresponds with how the cells could look. The location where they were distributed fits with where the ICC would most probably be located, indicating that what was found in the experiments most likely was the ICC.

The ICC subtypes

There is still a question about whether the ICC in fish consists of different subtypes, and if they are comparable to mammalian ICC subtypes. This study could not fully establish this because it did not determine whether there were any morphological and functional differences between the cells found. But regarding their location, the Ano-1 positive structures found in this study could be ICC-DMP, ICC-IM and ICC-MY.

Most of the Ano-1 positive cells found in the experiment were placed in the myenteric region. It is most possible that the cells would be comparable to the ICC-MY found in the same area in mammals. The Ano-1 positive cells found deeper in the circular muscle layer would most probably be ICC-DMP based on their location. The ICC-DMP are only found in the small intestine of mammals, where they mediate neurotransmission. The role of ICC-MY in mammals is mainly generating slow waves and conducting electrical stimuli to the smooth muscle cells through the gap junction. The ICC-MY of mammals is distributed in the small intestine, large intestine, and the stomach but only in the antrum of the stomach (Zhou et al., 2017). In terms of the shape seen from the wholemount tissue, they are more like the group ICC-IM in mammals that are spindle-shaped and not distributed in an extensive network like ICC-MY (Al-Shboul, 2013). This structure seen from the wholemount tissue preparations is very different from the structures found in shorthorn sculpin and zebrafish, where they form clear networks (Fig. 4). Worth mentioning is that it is hard to see precisely how the morphology of the cells looks from the wholemount preparation. Hence, it is not sure that the species have morphological differences of the ICC. ICC-IM is found in mammals' intestines and stomachs, where they generate slow waves, producing spontaneous depolarisations and mediating neural transmission (Zhou et al., 2017).

What could be the reason for only finding ICC in the intestine of the study? It could be that the part of the stomach where the ICC was distributed was not examined. For example, the ICC-MY in mammals are only restricted to the antrum of the stomach (Zhou et al., 2017), the same restrictions could be in fish, and therefore ICC in the stomach was missed because the parts used for analysing did not contain the antrum. On the contrary, the antrum in mammals is equivalent to the position of the pyloric stomach in fish. But paraffined sectioned tissue did not show any Ano-1 immunoreactive structures in the pyloric stomach. It could also be that the antibody anoctamin-1 does not target every ICC subtype in fish because they do not hold the ion channel that the antibody targets or simply that rainbow trout do not have ICC in their stomach or rectum.

The disparity of the Ano-1 positive structures

The Ano-1 immunoreactive cells labelled in the experiment differed significantly from the ICC marked in shorthorn sculpin and zebrafish. The Ano-1 positive cells in previous studies from shorthorn sculpin showed a more branched network of cells (Fig. 4), while in this study, the cells are more solitary with branched structures extending from the cell's surface. The differences could be due to that it is different subtypes that look anatomically different. Previous studies have shown that the motility pattern differs depending on whether the fish have been acclimated to freshwater or saltwater (Brijs et al., 2017a). When rainbow trout were exposed to salt water, there was an increase in contractile activity, probably to increase the intestinal water and ion absorption to maintain the osmotic homeostasis which therefore suggests that saltwater have a direct effect on ICC (Brijs et al., 2017a). If the motility pattern differs in fish depending on the surrounding circumstances, the processes behind it will also work and look anatomically different. This could explain why the ICC differed in the other species' gastrointestinal tracts. One hypothesis is that there are a higher proportion of the ICC-DMP in the gastrointestinal tract of rainbow trout. ICC-DMP was the cells involved in neurotransmission restricted in the intestine of mammals. That could explain both the morphology we seemed to see in the mounted tissue preparations and the reason for only finding Ano-1 immunoreactive cells in the three intestinal parts.

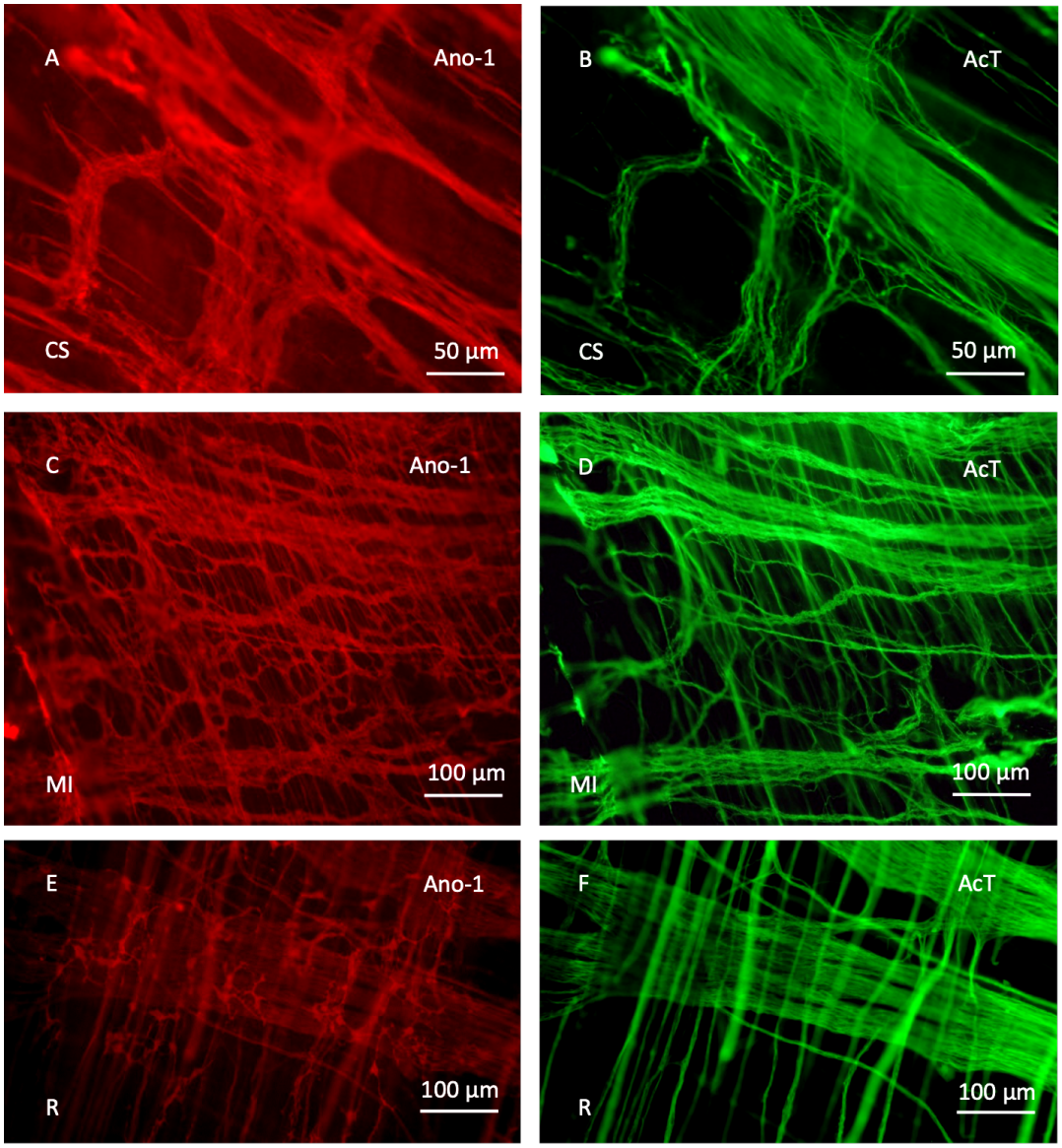

Figure 4. Ano-1 immunoreactivity in shorthorn sculpin (*Myoxocephalus scorpius*). Cardiac stomach (CS), middle intestine (MI) and rectum (R). (A) Ano-1 positive cells in the cardiac stomach arrange in a network. (B) Nerve cells of the cardiac stomach are labelled with the primary antibody AcT. (C) Ano-1 positive cells in the mid intestine of shorthorn sculpin. (D) Network of nerves labelled with the primary antibody AcT in the mid intestine. (E) Ano-1 positive cells in the rectum of shorthorn sculpin. (F) Broad nerve bundle labelled with AcT in the rectum of shorthorn sculpin—image: Catharina Olsson, unpublished.

Difficulties in simultaneous double labelling of immunohistochemistry

The difficulties of the simultaneous double labelling using Ano-1 together with AcT could have several explanations. The problem arose because nerve cells were labelled with the secondary antibody DaR-CY3 even though their target was Ano-1, the primary antibody supposed to target ICC explicitly. One suspicion was that the primary antibody Ano-1 attached to the ion channel found in ICC but also something present in nerve cells. By doing the immunohistochemistry using only Ano-1 and its secondary antibody, that explanation could be rejected because no nerve cells were stained. The problem only occurred when simultaneous double labelling. The question was then if the secondary antibody for Ano-1 also binds to AcT, if the background colouring viewed in the microscope bled over to nerves or if it was DaR-CY3 that attached to the secondary antibody DaM-FITC? When using AcT together with only the secondary antibody for Ano-1 (DaR-CY3), no nerve cells were stained, rejecting the hypothesis that DaR-CY3 was bound to AcT. When doing the immunohistochemistry only with AcT with its suitable secondary antibody, only nerve cells were stained, and no structures were detected under the filter for CY3, which excluded that it was due to bleed-through of the microscopic background colouring. When using AcT and both secondary antibodies, nerve cells were stained in CY3, indicating that DaR-CY3 binds to the secondary antibody

DaM-FITC. That could explain why nerve cells were overlapped in red filter when simultaneous double labelling using the Ano-1 and AcT and their secondary antibodies.

Further research

It has been shown that ICC can be affected by temperature changes in several species of mammals (Kito & Suzuki, 2007). How the ICC in the gastrointestinal tract in fish is affected by possible changes in temperature remains to be investigated. It may be of particular interest because the gastrointestinal tract is in direct contact with the surrounding water when consuming food and could therefore be strongly affected by temperature fluctuations linked to today's climate changes (Brijs et al., 2014). There is thus a great value in studying ICC in fish to increase knowledge and understanding of its functions and interactions in the gastrointestinal tract of the fish and examine the occurrence of subtypes and all its functions and interactions with the rest of the body.

Conclusions

Experiments executed during my study indicate that ICC is distributed in rainbow trout in different areas of the intestine. Immunohistochemistry on paraffin sectioning of the tissue partially worked and revealed that most of the Ano-1 positive cells were located in the myenteric plexus and shallow into the circular muscle layer. Single cells were also found deeper into the circular muscle layer. The findings suggest that there could be different subtypes of ICC in rainbow trout with distinct functional and anatomically differences, but further investigation is required to understand what functions the ICC have in fish and if there are different subtypes comparable to the ICC found in mammals. This study opens new scientific questions on how the ICC works and how environmental factors could affect it.

Acknowledgement

First, I especially want to thank my supervisor Catharina Olsson for her great support in my writing and guidance during laboratory work. Thanks to everyone in the ECG group who has helped me during my work and to improve my finished result. I would also like to thank Henrik Sundh for his help with the paraffin sectioning.

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Appendix

Table 1. The different combinations of secondary and primary antibodies used for the paraffin sectioned tissue.

	PRIMARY ANTIBODY:		SECONDARY ANTIBODY:			RESULT:
	Ano-1	AcT	DaR-CY3	DaM-FITC	DaR-FITC	
1	X	X	X	X		Overlap between the filters
2	X		X			Sucessfully labelled structures
3	X				X	No labelled structures
5		X		X		Sucessfully labelled structures
6		X	X	X		Overlap between the filters
7	X		X	X		Sucessfully labelled structures
8		X	X			No labelled structures