Localization of Receptors for Calcitonin Gene-Related Peptide to Intraganglionic Laminar Endings of the Mouse Esophagus: Peripheral Interaction between Vagal and Spinal Afferents?

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Abstract

The calcitonin gene-related peptide (CGRP)-receptor is a heterodimer of calcitonin receptor-like receptor (CLR) and receptor activity modifying protein 1 (RAMP1). Despite the importance of CGRP in regulating gastrointestinal functions, nothing is known about the distribution and function of CLR/RAMP1 in the esophagus, where up to 90% of spinal afferent neurons contain CGRP. We detected CLR/RAMP1 in the mouse esophagus using immunofluorescence and confocal laser scanning microscopy and examined their relationship with neuronal elements of the myenteric plexus. Immunoreactivity for CLR and RAMP1 colocalized with VGLUT2-positive IGLEs, which were contacted by CGRP-positive varicose axons presumably of spinal afferent origin, typically at sites of CRL/ RAMP1 immunoreactivity. This provides an anatomical basis for interaction between spinal afferent fibres and IGLEs. Immunoreactive CLR and RAMP1 also colocalized in myenteric neurons. Thus, CGRP-containing spinal afferents may interact with both vagal IGLEs and myenteric neurons in the mouse esophagus, possibly modulating motility reflexes and inflammatory hypersensitivity.

Keywords:

IGLEs

Myenteric ganglion

CLR

RAMP1

Enteric nervous system

Introduction

Intraganglionic laminar endings (IGLEs) derive from nodose ganglion neurons and enwrap myenteric ganglia (Rodrigo et al. 1975; Rodrigo et al. 1982; Berthoud and Powley 1992; Neuhuber 1987). IGLEs are the most prominent terminal structures of vagal afferent neurons in the gastrointestinal tract (Berthoud et al. 1997a; Phillips and Powley 2000; Wang and Powley 2000). On morphological grounds, IGLEs have been proposed to function as mechanosensors (Neuhuber 1987; Neuhuber and Clerc 1990; Phillips and Powley 2000), and subsequently were shown to represent low-threshold tension sensors (Zagorodnyuk and Brookes 2000; Zagorodnyuk et al. 2001). In addition, IGLEs establish synaptic contacts of yet undetermined function onto myenteric neurons, as revealed by electron microscopy (Neuhuber 1987; Neuhuber et al. 2006; Phillips and Powley 2007; Neuhuber and Clerc 1990). Several neurotransmitters and their respective transporters and receptors have been found on IGLEs and associated structures, including VGLUT1 (Ewald et al. 2006; Kraus et al. 2007; Zagorodnyuk et al. 2003), VGLUT2 (Raab and Neuhuber 2005, 2004, 2003; Zagorodnyuk et al. 2003), substance P (Kressel and Radespiel-Tröger 1999; Raab and Neuhuber 2004), $P2X_2$, $P2X_3$ and $P2X_{2/3}$ receptors (Kestler et al. 2009; Raab and Neuhuber 2005; Wang and Neuhuber 2003). VGLUT2-immunoreactivity is considered a specific marker for IGLEs of the mouse and rat esophagus (Raab and Neuhuber 2003, 2004, 2005).

The first report of heterogeneity among calcitonin gene-related peptide (CGRP) receptors described two receptor subtypes, CGRP1 and CGRP2 receptor (Dennis et al. 1989). The CGRP1-receptor, now referred to as the "CGRP-receptor" (Hay et al. 2008), is comprised of two proteins: calcitonin receptor-like receptor (CLR), a seven transmembrane G protein-coupled protein, and the accessory receptor activity modifying protein 1 (RAMP1), one of three single transmembrane-spanning proteins of the RAMP-family (Hay et al. 2008; McLatchie et al. 1998). Additionally, a receptor component protein (RCP) couples CLR/RAMP1 to the cellular signal transduction pathways (Evans et al. 2000). RAMPs

chaperone intracellular CLR to the cell surface where both proteins heterodimerize to form a functional CGRP receptor (McLatchie et al. 1998). Depending on the various RAMP isoforms (RAMP1-3), the preference of the heterodimeric receptor with CLR changes (McLatchie et al. 1998; Mallee et al. 2002). Thus, dimerization of CLR and RAMP1 forms a receptor which preferentially, but not exclusively binds CGRP (Bomberger et al. 2005; Cottrell et al. 2012; McLatchie et al. 1998), whereas adrenomedullin-1 binds preferentially receptors that are comprised of RAMP2 or RAMP3 coupled to CLR (Bomberger et al. 2005). Adrenomedullin-2/ intermedin can act through CRL coupled to all three RAMPs with its highest affinity to the CRL/RAMP3 (Adrenomedullin-2) -receptor (Hong et al. 2012). Besides the CLR all three RAMPs additionally interact with the vasoactive intestinal peptide (VIP)/pituitary adenylate cyclase-activating polypeptide (PACAP) 1 ($VPAC_1$) and $VPAC_2$-receptors (Kuwasako 2013). CGRP is a widely distributed peptide of the calcitonin family of regulatory peptides with different functions depending on its area of release (Wimalawansa 1997; Rosenfeld et al. 1983). Its two isoforms, α-CGRP (mainly localized in spinal afferents) and β-CGRP (main isoform in the enteric nervous system), regulate numerous physiological pathways including nociception, vasodilatation, motility, secretion, feeding, olfaction and audition (Cottrell et al. 2012; Brain et al. 1985). Within the mouse and rat gastrointestinal tract, CGRP has a major role as a neurotransmitter that regulates motility and secretion (Cottrell et al. 2012; Sternini 1992; Esfandyari et al. 2000; Cox et al. 1989).

CGRP and substance P are expressed by visceral afferent fibres originating from dorsal root ganglia (DRG; Rodrigo et al. 1985; Sternini 1992). Up to 90% of spinal afferents innervating the esophagus contain CGRP (Dütsch et al. 1998) and are evenly distributed throughout the whole organ (Neuhuber et al. 2006). However, in the rat cervical esophagus CGRP is not only contained in spinal afferents but is also found in vagal afferents innervating the mucosa and submucosa (Wank and Neuhuber 2001). Therefore, CGRP should be considered as a specific marker for spinal afferents only in the thoracic and abdominal esophagus. In the esophagus

CGRP-positive spinal afferent fibres are known to supply the mucosa and form close contacts to myenteric neurons (Furness 2006; Mazzia and Clerc 1997), suggesting a role in local effector functions (Clerc and Mazzia 1994; Mazzia and Clerc 1997). In rat and mouse esophagus, intimate contacts between CGRP-immunoreactive varicosities and IGLEs have been observed (Dütsch et al. 1998; Raab and Neuhuber 2003). This close relationship suggested possible peripheral interactions between IGLEs and spinal afferents. Interactions between vagal and spinal afferents have been identified within the spinal cord (Chandler et al. 1991; Randich and Gebhart 1992), prompting the question, if such interaction also existed in the periphery.

In contrast to previous studies of the localization of CLR, RAMP1 and CGRP in the gastrointestinal tract of rat and human (Cottrell et al. 2012; Cottrell et al. 2005), the distribution of these proteins has not been described in the esophagus. This dearth of data prompted us to focus on the two components of the CGRP-receptor, CLR and RAMP1, and their relationship to IGLEs, myenteric neurons and CGRP-containing varicosities in myenteric ganglia of the mouse esophagus.

Materials and methods

Animals

Adult mice (n=29; C57Bl/6, stock number 000664, inbred; The Jackson Laboratory, Bar Harbor, USA) were used. The federal animal welfare legislation implemented by the local government was followed for all procedures performed on the animals.

Tissue preparation and fixation

Animals were killed with carbon dioxide and thiopental (Trapanal, Byk Gulden, Konstanz Germany; 250 mg/kg i.p.). When they were unresponsive to nociceptive stimuli, the abdominal and thoracic cavities were opened and 20 ml Ringer solution containing 1,000 IE/ 100 ml heparin was perfused through the left ventricle, followed by 80 ml 3% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and 10 ml 15% sucrose phosphate buffer (pH 7.4). For tyramid signal amplification (TSA) tissue was post-fixed for 5 h. Spinal cord with spinal ganglia, brainstem and esophagi from the cricoid cartilage to the gastroesophageal junction were removed and transferred to 15% sucrose phosphate buffer (pH 7.4) at 4°C. The following day esophagi were divided into three segments of similar length, the cervical (= upper third), thoracic (= middle third) and abdominal (= lower third) parts, together with spinal cord and spinal ganglia mounted in Tissue-Tek (GSV; Slee Technik, Mainz, Germany), frozen in methylbutane at -70°C and stored at -20°C. For immunohistochemistry, 12-µm thick sections were cut in a cryostat and mounted on poly-L-lysine-coated slides.

Antibodies, detection systems and reagents

A rabbit antibody to CLR has been described (Cottrell et al. 2005). Rabbit antibody to RAMP1 was from Santa Cruz Biotechnology (Heidelberg, Germany). Due to the same host origin of CLR and RAMP1, in some cases a goat CLR antibody was used (Santa Cruz). The

rabbit RAMP1 polyclonal antibody was raised against amino acids 1-148 representing the full length peptide of human origin. The polyclonal CLR antibody raised in goat was directed against a peptide mapping near the C-terminus of human CLR, whereas the rabbit antibody was raised against a peptide corresponding to the penultimate 18 residues of rat CLR (Cottrell et al. 2005).

All other primary antibodies used in this study were either raised in guinea pig, goat or rabbit, detected by different kinds of secondary antibodies. All antibodies and detections systems used are listed in **Tables 1** and **2**.

Specificity of Primary Antibodies

To test the specificity of all three primary antibodies, we performed preadsorption and normal serum controls:

rCLR

The specificity of rabbit anti-CLR has previously been tested in rat by preadsorption and normal serum controls (Lennerz et al. 2008). Additionally, own preadsorption protocols in mouse esophagus were performed with the corresponding CLR-peptide. The rCLR antibody was blocked with 1- and 10-fold antigen excess overnight at 4°C. The mixtures were spun for 15 minutes at 14,000 rpm to sediment precipitating antigen-antibody complexes to avoid high background staining. Instead of a primary antibody, the supernatant diluted in TRIS-buffered saline (TBS) containing 1% bovine serum albumin (BSA; Roth, Germany) and 0.5% Triton X-100 (Merck, Darmstadt, Germany) was used. Double immunohistochemistry with antibodies against vesicular glutamate transporter 2 raised in guinea pig (gpVGLUT2; Chemicon, Temecula, CA) followed as described below.

gCLR

The specificity of the goat-CLR antibody was verified by preadsorbing the antibody with the corresponding peptide (V20, sc-18007p, Santa Cruz, Biotechnology, Heidelberg, Germany).

Preadsorption of the respective antibody with 1-, 2-, 5-, 10-, and 20-fold antigen excess was incubated overnight at 4°C and processed as described above. For counterstaining of IGLEs additional immunohistochemistry for gpVGLUT2 antibody was performed as described below.

rRAMP1

The specificity of rabbit-RAMP1 antibody could not be verified by preadsorption protocols due to the unavailability of the corresponding peptide. Western blots for testing specific recognition of the respective antigen were done by the supplier. Additionally, the specificity was tested performing dilution series: This antibody was diluted 1:200, 1:500, 1:1,000, 1:1,500, 1:2,000 and 1:5,000 in TBS. The resulting signal intensities were compared and decrease of staining intensity was taken as further indicator of specificity. As recommended by the supplier we also performed controls using 2% normal rabbit serum (Dako, Glostrup, Denmark; X0902) instead of the primary antibody. Furthermore, positive controls from mouse spinal cord and spinal ganglia were performed and compared with already published results (Cottrell et al. 2005).

Negative Controls

Specificity of primary and secondary antibody was tested by replacing the primary antibody with TBS. These controls were run with each immunohistochemical protocol.
Additionally, normal serum controls were performed. Tissues were first incubated with 5% normal donkey serum (Dianova, Hamburg, Germany) containing 0.5% Triton X-100 and 1% BSA in TBS for 1h. Then the primary antibody was replaced by normal serum from the host of the primary antibody in the same concentration as the replaced antibody.

Double-label and triple-label immunohistochemistry

Twelve μm thick longitudinal cryostat sections were processed after mounting on poly-L-lysine-coated slides and drying for 1h at room temperature. Sections were then preincubated with 5% normal donkey serum (Dianova) containing 0.5% Triton X-100 and 1% BSA in TBS for 1 h. After being rinsed in TBS the sections were incubated for double or triple immunolabeling with primary antibodies against VGLUT2, CLR, RAMP1, CGRP, choline acetyltransferase (ChAT) and neuronal nitric oxide synthase (nNOS). Due to the same species origin (rabbit) of RAMP1 and CLR antibodies, rabbit anti-CLR was replaced by goat anti-CLR for triple immunohistochemistry. All antibodies were diluted in TBS, containing 1% BSA and 0.5% Triton X-100 and incubated at room temperature overnight.

Followed by another rinse with TBS the next day, sections were incubated for 1 h with two or three of the following secondary antibodies: donkey anti-rabbit secondary antibodies coupled to Alexa Fluor® 488/ Alexa Fluor® 555, donkey anti-goat secondary antibodies coupled to Alexa Fluor® 488/ Alexa Fluor® 555/ Alexa Fluor® 647 or Cy™3/Cy™5-conjugated donkey anti-guinea pig secondary antibodies, all diluted in TBS containing 0.5% Triton X-100 and 1% BSA for 1h at room temperature. Sections were finally rinsed with TBS, coverslipped in TBS-glycerol (pH 8.6) and viewed under a Nikon fluorescence microscope (Nikon Eclipse E1000M, Tokyo, Japan). Controls with replacing the primary antibodies by normal serum were run with each staining protocol.

Triple immunohistochemistry of RAMP1, CLR and CGRP

A triple label protocol using RAMP1, CLR and CGRP to determine the close proximity of CGRP to the receptor could not be performed since all antibodies were derived either from goat or rabbit.

Tyramide signal amplification (TSA) followed by subsequent conventional immunostaining

In order to sidestep the problem of two antibodies raised in the same species (here rabbit) we followed the principle of TSA first published by Shindler and Roth (1996). In this commonly applied immunostaining protocol a detection system of different sensitivities was used in two steps. In the first step the primary antibody was diluted up to 200-fold (Adams 1992) and detected by the TSA method. The high dilution is necessary to prevent the secondary antibody of subsequent conventional immunostaining from detecting and binding the primary antibody. Our previous studies showed unwanted staining due to endogenous biotin which could be reduced significantly by using 5 h post-fixed tissue and a streptavidin-biotin blocking system (Horling et al. 2012). Therefore we used only 5 h post-fixed esophagi for TSA protocols. TSA-protocol was performed as described before (Horling et al. 2012). In brief, endogenous peroxidase activity of cryostat sections was blocked by H_2O_2 (0.3% in 40% methanol in TBS, 30 min). After rinsing, a preincubation step with 5% normal donkey serum (diluted with 0.5% Triton X-100 and 1% BSA in TBS) followed for 1h at room temperature. Endogenous biotin was blocked by a streptavidin-biotin blocking system (Linaris GmbH; 4 drops of streptavidin solution per 1 ml of 5 % normal donkey serum; diluted in TBS containing 0.5 % Triton X-100 and 1% BSA for 1h at room temperature). Sections including the negative control were then immersed in biotin solution for 15 minutes before adding the primary antibodies. In order to determine the suitable concentration of the rRAMP1antibody a titration series with dilutions 1:200, 1:500, 1:1,000, 1:1,500, 1:2,000 and 1:5,000 was performed .

For TSA the rRAMP1 antibody has to be diluted 1:5,000 in TBS (containing 1% BSA and 0.5% TritonX-100) and was exposed at room temperature overnight. The rabbit anti-RAMP1 was detected by donkey anti-rabbit antibody conjugated to biotin (1:500, for 1 h, room temperature; Dianova; Hamburg, Germany) followed by adding streptavidin coupled to horseradish peroxidase (HRP, 1:500; Dianova) for another 1 h at room temperature. Subsequently, biotin-conjugated tyramide diluted 1:250 in “amplification solution” was incubated for 20 minutes. Finally, streptavidin-Cy™3 (1:1,000 in TBS, 1 h at room

temperature; Dianova) was added to visualize incorporated biotin-tyramide. The last step contained the conventional incubation with the second primary antibodies rCLR and gpVGLUT2 or gCGRP, respectively, as described above. Finally, the slides were coverslipped in TBS-glycerol (pH 8.6) and viewed under a Nikon fluorescence microscope (Nikon Eclipse E1000M).

Confocal microscopy and image analysis

Confocal images were collected using a Nikon C1 laser scanning confocal unit (Nikon D-Eclipse C1, Nikon, Düsseldorf, Germany) attached to a fluorescence microscope (Nikon Eclipse E1000M) and a laser system composed of three different lasers (488 nm argon laser, 543 nm helium neon laser (both Melles Griot, Carlsbad, California, USA) and red diode laser emitting light at 635 nm (Coherent, Santa Clara, California, USA). To acquire images, the following objectives were used: A 20x dry lens (numerical aperture: 0.75), a 40x dry lens (numerical aperture: 0.95) or a 60x oil immersion lens (numerical aperture: 1.40) combined with electronic zoom factors between 1.0 and 3.0. Images were captured with Nikon EZ-C1 scan software (gold version 3.70). The absence of bleed-through between two fluorescence channels was confirmed. Z-series of up to 18 optical sections of 512x512 and 1024 x1024 pixels at z-increments of 0.5 µm were created.

For investigating colocalization, single sections were taken from the z-staples and the two different channels were merged. Further processing of images was done with EZ-C1 Freeviewer software (version 3.60, Nikon). In addition, Photoshop (Adobe Photoshop CS4; Adobe Systems, Unterschleißheim, Germany) and Coreldraw software (Coreldraw, version X6; Corel Corporation, Dublin, Ireland) were used to add text and scale bars, to adjust brightness and contrast and to organize the final layout.

Results

Controls

Staining in rCLR preadsorption protocols could completely be abolished with a 10-fold excess of antigen (**Fig. 1a**). Preadsorption controls for the gCLR showed a clear staining reduction with the 20-fold antigen excess, although staining was not completely abolished (**Fig. 1c**)**.** Dilution series of rRAMP1 antibody showed progressive staining reduction from the 1:200 to the 1:5,000 concentrations. Additionally, normal serum controls (using rabbit and donkey normal serum) did not yield any positive staining (not shown). No immunofluorescence was observed in any of the negative controls. Spinal ganglia and spinal cord were immunostained as positive controls. RAMP1-IR was found in perikarya of mostly large diameter spinal ganglion neurons and in fibers of the superficial dorsal horn (not shown).

CLR- and RAMP1-immunoreactivity (IR)

Both, rCLR- and RAMP1-antibodies, showed similar but not identical staining throughout the whole esophagus. rCLR-IR was observed as intense and punctate staining around, and diffuse homogeneous staining of lower intensity within neuronal cell bodies (**Fig. 1b**). The staining pattern and distribution of RAMP1 antibody was similar to rCLR staining results although both intensity and abundance of RAMP1 staining were lower.

CLR-IR using two different antibodies

Due to the same host species rabbit, CLR- and RAMP1 antibodies could not be used simultaneously. In order to avoid this antibody problem two possibilities were considered: replacing the antibody host of CLR or RAMP1 by goat. gRAMP1 did not show any staining in mouse esophagus, whereas gCLR did show a similar although less intense staining pattern compared to rCLR (**Fig. 1d**). Therefore TSA was tested as an alternative staining method.

However, spuriously positive stained negative controls in TSA protocols occurred as already described (Horling et al. 2012) and despite using blocking reagents, results were suboptimal: In TSA protocols the rCLR-IR, observed as intense and clear staining quality in non-TSA immunohistochemistry protocols, resulted in hardly visible homogeneous staining. Therefore TSA was not considered a possible staining alternative. gCLR antibody was double stained with rCLR to verify identic staining results. The two antibodies showed a high degree of colocalization (**Fig. 2**) and gCLR was chosen to be a suitable alternative. Thus, we used the polyclonal gCLR in combination with rabbit RAMP1 for triple label immunohistochemistry, although gCLR was not as brilliant as rCLR-staining. Both CLR antibodies derived from rabbit and goat, respectively, were chosen depending on the host of the other primary antibodies used.

VGLUT2 and rCLR

Analysis of 214 myenteric ganglia of all parts of the mouse esophagus (66 in cervical, 78 in thoracic and 70 in abdominal parts) in merged two-channel single confocal sections showed CLR-IR and VGLUT2-IR to be highly colocalized within the same profile, resulting in yellow mixed color in numerous spots (**Fig. 3c, f**). VGLUT2, a specific marker for IGLEs in mouse esophagus, showed the previously described "fine contiguous immunopositive dots in profusely arborizing laminar structures enveloping myenteric ganglia" (Raab and Neuhuber 2003, 2004, 2005). The unspecific gpVGLUT2 staining of myenteric cell bodies in mouse esophagus was found as described before (Raab and Neuhuber 2004). Immunostaining for rCLR antibody in myenteric neuronal cell bodies showed a homogeneous staining of lower intensity (**Fig. 3e**). No differences in intensity or distribution of CLR-IR in the upper, middle or lower parts of mouse esophagus were observed.

VGLUT2 and rRAMP1

Altogether, a sample of 136 myenteric ganglia of all parts of mouse esophagus (42 in the cervical, 37 in the thoracic and 57 in the abdominal part) was investigated. VGLUT2 staining, specifically labeling IGLEs, was similar as described above. RAMP1-IR and VGLUT2-IR also showed several spots of colocalization (arrows in **Fig. 4c**), although less frequently than CLR- and VGLUT2- colocalization.

Triple immunohistochemistry of VGLUT2, CLR and CGRP

Triple immunohistochemistry was performed on 44 myenteric ganglia of all parts of the esophagus using antibodies against VGLUT2, CLR and CGRP. This experiment as well as the following one was performed to investigate the relationship of the agonist CGRP and the receptor proteins. VGLUT2-IR (red) and CLR-IR (green) showed the yet described numerous yellow colored spots throughout the whole myenteric ganglion, indicating significant localization of the CGRP receptor on IGLEs. CGRP-IR was found as intense staining of numerous varicose passing fibres. Areas of close apposition of both CLR- (green) and CGRP- (blue) immunoreactivities resulting in the mixed color turquoise at the site of contact were frequently seen **(Fig. 5a-d).** However, these areas of close proximity were considerably fewer than those of colocalization of VGLUT2-IR and CLR-IR, shown as yellow mixed color in the merged image **(Fig. 5d).** VGLUT2-IR and CGRP-IR formed consistently some close relationships indicated by a few purple spots mixed from red (VGLUT2-IR) and blue (CGRP-IR). However, we found no colocalization of VGLUT2-IR and CGRP-IR within the same profiles (Raab and Neuhuber 2003). Combined detection of VGLUT2, CLR and CGRP demonstrated 1-2 CGRP-positive varicosities per section in close apposition to VGLUT2-/CLR-positive IGLEs-profiles appearing in the triple mixed color white (white arrow in **Fig. 5a-d**). We never found triple colocalization within the same profile. In conclusion, we observed close proximity of CGRP-positive varicosities to CLR positive sites on VGLUT2-positive IGLEs.

Triple immunohistochemistry of VGLUT2, RAMP1 and CGRP

Triple labeling for VGLUT2 (red), RAMP1 (green) and CGRP (blue) did not show as many spots of triple close proximity as seen in the VGLUT2, CLR and CGRP protocols. The staining pattern of VGLUT2-IR and RAMP1-IR was seen as described above with few spots of colocalization. Triple labeling of VGLUT2, RAMP1 and CGRP again showed close relationships of blue CGRP-IR and red VGLUT2-immunopositive IGLE-profiles resulting in the mixed color purple and blue CGRP-IR and green RAMP1-IR, resulting in the mixed color turquoise, respectively, in some spots, both indicating areas of close proximity beyond the resolution of the confocal technique. A triple colocalization in the same profile was not observed. However, colocalization of RAMP1-IR and VGLUT2-immunopositive IGLE-profiles and additional close proximity of CGRP-immunopositive varicosities resulting in the mixed color white were found in few sections (white arrow in **Fig. 6** and zoom-ins).

Triple immunohistochemistry of VGLUT2, RAMP1 and gCLR

RAMP1 and gCLR were triple labeled together with the IGLE-marker VGLUT2. Investigating the staining results in detailed confocal analysis of 40 representative randomly selected myenteric ganglia of all parts of the esophagus, we observed different types of colocalization.

In both, IGLEs and myenteric neurons spots of colocalization of RAMP1- (red) and gCLR (green) -IR resulted in the mixed color yellow, indicating expression of the functional CGRP-receptor parts (**Fig. 7**)Turquoise colored spots indicated spots of colocalization of VGLUT2-(blue)-immunopositive IGLE-profiles and CLR-(green)-IR which could be detected more frequently than spots of colocalization of VGLUT2- (blue)- and RAMP1-(red) -IR, resulting in purple mixed color. Spots of triple colocalization of VGLUT2, RAMP1- and CLR-IR resulted in the mixed color white, which could only be detected in some instances per section

studied (see white arrows in **Fig. 7**), indicating expression of both functional CGRP-receptor components CLR and RAMP1on IGLE-profiles.

CLR and RAMP1 on different myenteric neurons

CLR and nNOS or ChAT

Nitrergic (nNOS) and cholinergic (ChAT) markers were used to examine the relationship of the CLR and RAMP1 with myenteric neurons in the mouse esophagus. nNOS immunolabeling appeared as intense, homogenous cytoplasmic staining of cell bodies and proximal dendrites sparing the nuclei. CLR-IR resulted in intense staining in nitrergic neuronal perikarya (**Fig. 8b**). Analysis of 26 myenteric ganglia in merged two-channel single confocal sections revealed high colocalization of nNOS-IR and CLR-IR in the cytoplasm of over 90% of the nNOS-positive neurons, resulting in brilliant yellow mixed colors (**Fig. 8c)**. nNOS immunopositive fibres of the neuropil were unstained by CLR-IR. ChAT-IR appeared as diffuse homogeneous cytoplasmic and dendritic staining of myenteric neurons and as intense punctiforme staining of varicose fibres. Analysis of 19 myenteric ganglia and fibres in merged double-channel single optical sections showed colocalization of ChAT- and CLR-IR in 66% of myenteric perikarya (**Fig. 9**). Colocalization of CLR-IR and ChAT-IR was found in about 50% of ChAT-positive varicosities and ChAT-positive neurons, (**Fig. 9**). In conclusion, CLR-IR was found highly colocalized in both nitrergic and cholinergic myenteric neuronal perikarya as well as in ChAT-positive varicosities.

rRAMP1 and nNOS or ChAT

RAMP1-staining was additionally found weakly distributed in the myenteric neuropil around nitrergic cell bodies. Double labeling of 6 representative myenteric ganglia of all parts of the mouse esophagus resulted in a bright yellow mixed color indicating spots of high colocalization in nNOS immunopositive myenteric neurons. Analysis of 8 representative

myenteric ganglia of all parts of the esophagus revealed colocalization of RAMP1-IR and ChAT-IR within 40% of investigated myenteric neuronal perikarya, resulting in yellow mixed color (data not shown). Colocalization was similar compared to that of CLR and ChAT but not as numerous. Additionally, we saw spots of colocalization in numerous varicose fibres within the myenteric neuropil. In summary, RAMP1-IR was found in both nitrergic and cholinergic cell bodies in myenteric ganglia.

Discussion

This study describes location and distribution of the CGRP-receptor (CLR/RAMP1) in the mouse esophagus using immunohistochemistry. We found both receptor components located in myenteric ganglia throughout the esophagus. Counterstaining with markers for IGLEs and myenteric neurons revealed colocalization, indicating receptor occurrence on IGLEs as well as on myenteric neurons. CGRP-IR spinal afferents were found closely related to IGLEs, in particular to their CLR/RAMP1 bearing areas, but no colocalization of CGRP-IR and VGLUT2-IR, or with either receptor components was observed.

Methodological considerations

Due to the same host origin of CLR and RAMP1, we were unable to perform triple label immunohistochemistry using CLR, RAMP1 and CGRP. Some authors avoided this problem by using TSA (Marvizón et al. 2007). However, since our previous investigations revealed false negative controls in mouse gastrointestinal tract (Horling et al. 2012), we did not pursue this approach.

Throughout all staining procedures a noticeable difference in the staining intensity between RAMP1-IR and CLR-IR was obvious. This difference was described before (Cottrell et al. 2005; Lennerz et al. 2008; Marvizón et al. 2007) and might be due to the greater abundance of CLR which dimerizes not only with RAMP1 but also with other members of the RAMP family (Hay et al. 2008; Husmann et al. 2003; McLatchie et al. 1998). Thus, RAMP1 is suggested to be expressed at a lower level and more widely than CRL (Cottrell et al., 2005). Basically, it has to be considered, that CLR forms the ligand binding site for CGRP but also for adrenomedullin 1 (Conner et al. 2007). RAMP1 converts the CLR into a rather specific receptor for CGRP with little affinity for adrenomedullin 1 (Poyner et al. 2002). Intermedin or adrenomedullin 2 showed selectivity for the CLR/RAMP3 receptor, but also binds to CRL/RAMP1 and CLR/RAMP2 (Hong et al. 2012). Thus, CLR/RAMP1, the considered

CGRP-receptor, may also bind to a lower extend to adrenomedullin1 and intermedin, which was not examined in this study.

Another aspect has to be considered concerning the CGRP-receptor subunits: recently published data showed that also RAMP1 is able to combine with other G-protein coupled receptors than CLR, e.g. with the calcitonin and VIP-Receptors VPAC1 and VPAC2 (Kuwasako et al. 2013).As we also found close contacts of VGLUT2 immunopositive IGLEs and VIP-immunopositive varicosities within myenteric ganglia (Raab and Neuhuber 2004) the occurrence of RAMP1-IR on IGLEs might also be part of the VIP-receptor. This aspect underlines the intricate relationships of IGLEs within the myenteric ganglion, but was not focused in this study.

Contacts of spinal afferents to IGLEs

Spinal afferents are known to supply collateral branches on their way to the spinal cord (Fig. 10 for summary; Mazzia and Clerc 1997; Neuhuber et al. 2006; Furness 2006). Previously, our group described close relationship of CGRP-immunopositive spinal afferents to IGLEs, as identified by VGLUT2 and calretinin, specific markers for IGLEs in the mouse (Raab and Neuhuber 2003, 2004, 2005) and rat (Dütsch et al. 1998) esophagus, respectively. CGRP has been shown to be contained in more than 90% of DRG-neurons innervating the esophagus (Dütsch et al. 1998) and can be considered a marker for spinal primary afferent fibres, at least in the thoracic and abdominal esophagus of rat, mouse and guinea pig. In the cervical esophagus, CGRP has been additionally found in numerous fine vagal afferent fibers (Wank and Neuhuber 2001). In this study, we found the functional CGRP-receptor, CLR and RAMP1, on VGLUT2 positive IGLEs that are contacted by CGRP-positive varicosities within the esophageal wall. This occurrence on IGLEs is in line with results of the dorsal horn, where the CLR was described to colocalize with synaptophysin and VGLUT2 in axon terminals (Marvizón et al. 2007). Describing close contacts between spinal afferents and

myenteric neurons, a local effector function of spinal afferents has been hypothesized (Mazzia and Clerc 1997; Holzer 1988). This hypothesis might now be extended to the interaction between spinal afferents and IGLEs suggesting a peripheral interaction between vagal and spinal afferents in the thoracic and abdominal esophagus, and of both spinal and vagal CGRP afferents and vagal IGLEs in the cervical esophagus, due to their intimate relationship (Neuhuber et al. 2006). As central vagal-spinal afferent modulation is well-known at various levels of the neuraxis (Chandler et al. 1991; Randich and Gebhart 1992), this idea is not so far-fetched.

Functional considerations

Reflex circuits

Having in mind our new findings concerning the possible interaction between spinal afferents and IGLEs, we suppose an extension of the generally accepted vago-vagal reflex circuit (see Fig. 10 for summary; Jansson 1969; Miolan and Roman 1984; Travagli et al. 2003; Altschuler et al. 1989; Hermann et al. 2006). Originating in the wall of the esophagus, CGRP-releasing spinal primary afferents travelling through the myenteric ganglion may forward information to CLR/RAMP1 positive myenteric neurons but also to CLR/RAMP1 positive IGLEs. If and how IGLEs were affected by CGRP-release still remains unclear. Previous studies in other systems described receptor internalization, elevated cAMP and increased intracellular calcium levels as known CGRP effects (Bomberger et al. 2005; Husmann et al. 2003; Kuwasako et al. 2000; McLatchie et al. 1998; Parsons and Seybold 1997; Ryu et al. 1988; Segond von Banchet et al. 2002; Wimalawansa 1996). As proposed by Lennerz et al., elevated cAMP could lead to phosphorylation of ion channels that subsequently supports the conduction of action potentials (Lennerz et al. 2008). Transferred to IGLEs serving as low-threshold tension sensors (Zagorodnyuk and Brookes 2000; Zagorodnyuk et al. 2001), their excitability might be influenced through CGRP release and this in turn might affect vago-vagal reflex circuits

relayed through the nucleus of the solitary tract (Altschuler et al. 1989; Barrett et al. 1994; Shapiro and Miselis 1985) to esophageal motor neurons of the nucleus ambiguus terminating in the tunica muscularis of the esophagus (Barrett et al. 1994; Bieger and Hopkins 1987; Cunningham and Sawchenko 1989). This vago-vagal reflex circuit could be modulated peripherally by information picked up from the mucosa and connected via spinal afferent collaterals to IGLEs. Clearly, this has further to be determined.

Furthermore, involvement in a local reflex circuit is conceivable. As contacts between spinal afferents and myenteric neurons were already described (Mazzia and Clerc 1997; Furness 2006), a short circuit could be considered modulating myenteric neurons and probably also IGLEs. Synapse-like contacts between IGLEs and myenteric neurons have been described (Neuhuber 1987; Neuhuber and Clerc 1990; Phillips and Powley 2007). Thus, information coming from mucosa via spinal afferents might also be forwarded to IGLEs and, via the presumed transmitter release, may be passed onto myenteric neurons. Nevertheless, functional proof for this is still pending. From myenteric neurons information could be transmitted to motor endplates co-innervated by nitrergic enteric neurons. Functional studies corroborated this hypothesis: mucosal (spinal) primary afferents were found to play a key role in mediating the inhibitory effect of capsaicin on vagally induced striated muscle contractions in an in vitro vagus nerve - esophagus preparation (Boudaka et al. 2007; Izumi et al. 2003).

A third potential circuit concerning the pain pathway could be hypothesized as follows: CGRP-containing primary afferent fibers establish synaptic contacts with neurons of the spinothalamic tract (STT), the major ascending nociceptive tract (Carlton et al. 1990). Information from esophageal mucosa might be transmitted via spinal afferent collaterals to IGLEs. If we assume that an action potential is triggered by this influence, it might be forwarded along the vagus nerve to the central subnucleus of the NTS (Altschuler et al. 1989; Barrett et al. 1994; Shapiro and Miselis 1985) and probably beyond. The NTS functions as a relay between peripheral vagal afferent input and centrally located pain control mechanism

(Ren et al. 1990). Vagal afferents terminating in the NTS synapse, among others, on neurons projecting to the spinal cord (McNeill et al. 1991) which in turn could elicit an inhibitory effect on STT neuron activity (Ammons et al. 1983; Chandler et al. 1991) thus determining the intensity of painful sensations from the esophagus (Neuhuber et al. 2006).

CGRP, CGRP-receptor, IGLEs and myenteric neurons in inflammation

Previous studies have suggested a role for CGRP and SP co-released from spinal afferent nerve fibers in inflammation of the gastrointestinal tract (Holzer 2002; Foreman 1987; Sternini 1992). Furthermore, a bidirectional communication between immune cells, e.g. mast cells and CGRP/SP releasing spinal afferents has been shown to be involved in regulation of the inflammatory response (De Jonge et al. 2004). Myenteric neurons were found to be influenced through both SP and CGRP, since both NK1 receptors (Kuramoto et al. 2004) and CRL/RAMP1 (this study) were detected on myenteric neuronswhile IGLEs were equipped only with CRL /RAMP1.

In different species, including human, studies have documented that acute acid infusion into the esophagus leads to both a decrease in threshold for sensations to mechanical distension of the esophagus (Peghini et al. 1996) and an increase in the firing of vagal motorneurons, sensitive to esophageal distension (Partosoedarso and Blackshaw 1997). Medda et al. demonstrated that vagal afferent fibers, but not spinal neurons, exhibited an increase in action potential firing upon esophageal distension or acid-pepsin infusion (Medda et al. 2005) which has been shown to be inhibited by TRPV1 antagonists (Peles et al. 2009). Most IGLEs in the esophagus and stomach are resistant to capsaicin (Berthoud et al. 1997b), which corresponds to a lack of immunohistochemically detectable vanilloid receptor VR1/TRPV1 (Patterson et al. 2003). Recent functional data from TRPV1 knockout mice provided further substantial evidence that in addition to the spinal afferent pathway a subtype of vagal esophageal afferent fiber displays nociceptive characteristics, including responsiveness to low pH and

inflammatory mediators (Harrington et al. 2013). Thus, the hypothesized CGRP positive spinal afferent collaterals to IGLEs and their equipment with the CGRP receptor might probably be the anatomical correlation for this acid-induced enhanced mechano-hypersensitivity (Demir et al. 2013; Harrington et al. 2013).

In summary, we demonstrated the occurrence of CGRP receptors on IGLEs throughout the esophagus. CGRP-containing spinal afferents are found in close vicinity to IGLEs, in particular at their GCRP receptor bearing sites, and therefore raise the suspicion of possible interaction between primary spinal afferents and vagal IGLEs. This connection leads to some new speculations concerning reflex pathways and inflammatory processes, but further functional investigation is required.

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Figure legends

Fig. 1 Specificity controls

a Single optical section of an esophageal myenteric ganglion after a 1:10 block of rCLR with the corresponding control peptide. Specific staining is no longer detectable. The area of myenteric ganglion is outlined.

b All-in-focus projections of 5 single optical sections through an esophageal myenteric ganglion. The Image shows a positive control of rCLR-staining compared to the preadsorption (Fig. 1a).

c All-in-focus projections of 5 single optical sections of a myenteric ganglion in the mouse esophagus. Very low staining is retained after a 20-fold excessive block of gCLR with the blocking peptide. The area of myenteric ganglion is outlined.

d All-in-focus projections of 7 single images of an esophageal myenteric ganglion. Positive control of gCLR-staining.

Scale bars 20 µm

Fig. 2 Double immunostaining of rCLR and gCLR

a, b All-in-focus projection of 7 single optical sections through an esophageal myenteric ganglion immunostained with rCLR (red; **a**) and gCLR (green; **b**). gCLR immunoreactivity was found in the same location as rCLR-IR. *Arrows* exemplarily point to spots of colocalization within IGLEs, displayed in yellow spots in the merge (**c**).

Scale bar 20 µm

Fig. 3 Double immunostaining of gpVGLUT2 and rCLR

a-c Merged overview of an esophageal myenteric ganglion immunostained with gpVGLUT2 (red; **a**) and rCLR (green; **b**). Spots of colocalization appear as yellow spots in the merged image (*arrows*; **c**). However, rCLR was also localized in areas immunonegative for VGLUT2 as a spotted or fibre-like staining (*white lined arrows*; **b-c**). *Scale bar 20 µm*

d-f All-in-focus projection of fifteen single optical sections of an esophageal myenteric ganglion double-labeled for gpVGLUT2 (red; d) and rCLR (green; e). *Arrows* point to spots of colocalization

within one profile resulting in yellow mixed color (merge; **f**). In contrast to the unspecific staining of neuronal cell bodies of VGLUT2-IR, rCLR shows fine homogeneous staining of neuronal perikarya indicated by *asterisks*. *Scale bar 20 µm*

Fig. 4 Double immunostaining of gpVGLUT2 and rRAMP1

a, b Merged z-series of thirteen single images through an esophageal myenteric ganglion immunostained with gpVGLUT2 (red; **a**) and rRAMP1 (green; **b**). Spots of colocalization within one profile result in the mixed color yellow in the merge (**c**) and are indicated by *arrows*. Similar to rCLR staining, rRAMP-IR shows fine homogeneous staining of neuronal cell bodies, indicated by asterisks (**b, c**). *Scale bar 20 µm*

Fig. 5 Triple labeling of VGLUT2, rCLR and CGRP

a-c Single optical section through an esophageal ganglion immunostained for VGLUT2 (red; **a**), rCLR (green; **b**) and CGRP (blue; **c**).

d In the merged image different spots of colocalization (*yellow arrow)* or close proximity (*white, turquoise arrows*), respectively, can be found. Areas of close apposition of all three antibodies result in the mixed color white (*white arrows*), colocalization of VGLUT2 and rCLR only is shown in yellow spots indicated by the *yellow arrow*. Close proximity of CLR and CGRP appears turquoise in the merge (*turquoise arrow*) indicating close proximity beyond the resolution of the confocal technique. *Scale bar 20 µm*

Fig. 6 IGLEs triple labeled for VGLUT2, rRAMP1 and CGRP

a-c Single optical section through a myenteric esophageal ganglion incubated with VGLUT2 (red; **a**), rRAMP1 (green; **b**) and CGRP (blue; **c**). *Arrows* point to the spot of close apposition and overlay of all three antibodies (merge; **d**).

Zoom-ins Higher magnification of the *white dotted boxes* are seen below every image. *Arrows* indicate the mentioned spot of close proximity in the merged image.

Scale bars 20 µm for **a-d** and *5 µm* for *zoom-ins*

Fig. 7 esophageal myenteric ganglion triple labeled for gpVGLUT2, rRAMP1 and gCLR

a-c Single optical section immunostained for rRAMP1 (red; **a**), gCLR (green; **b**) and VGLUT2 (blue; **c**). *Arrows* show areas of close proximity and overlay in the merge (**d**). *Scale bar 15 µm.*

e-f All-in-focus projection of six single optical sections of a myenteric esophageal ganglion. *Asterisks* indicate the nuclei of a myenteric neuron immunopositive for CLR (green; **f**) and RAMP1 (red; **e**). Note, that gpVGLUT2 causes an unspecific labeling of myenteric neuronal perikarya. Arrows point to spots of colocalization of both, CLR and RAMP1, appearing yellow in the merge (**h**).*Scale bar 20µm.*

Fig. 8 Double immunostaining of rCLR and nNOS

a, b All-in-focus projection of five single optical sections of a myenteric esophageal ganglion. *Rings* indicate a neuronal cell body immunopositive for nNOS (red; **a**) and CLR (green; **b**), respectively, with omission of the nuclei (*asterisks*). In the merge (**c**) the overlay resulted in yellow mixed color within the myenteric perikaryon (*rings*). *Scale bar 15 µm*

Fig. 9 Double immunostaining of rCLR and ChAT

a, b Projected image of fourteen single optical sections through an esophageal myenteric ganglion immunostained with ChAT (red; **a**) and rCLR (green; b), respectively. *Asterisks* indicate ChAT immunopositive neuronal cell bodies, rCLR positive neuronal cell bodies are marked with *rings.*

c The merged image shows CLR-IR within cholinergic neuronal cell bodies indicated by *asterisks* and *rings* simultaneously. However, rCLR was also found in neuronal cell bodies immunonegative for ChAT (*single ring* in merge). *Arrows* point to spots of colocalization resulting in the mixed color yellow.

Scale bar 20 µm

Fig. 10 Simplifying diagram of esophageal innervation

Spinal afferents originating in the mucosa (M) supply collateral branches to myenteric neurons (black shaded white ellipse illustrated an exemplary myenteric ganglion), IGLEs (attached green semi-ellipse) and sympathetic ganglia (SYMP). Spinal afferents have their neuron in the dorsal root ganglion (DRG) and terminate mainly in the superficial dorsal horn of the spinal cord (SC). In the spinal cord they synapse with the classic pain pathway, the spinothalamic tract (STT). Information from IGLEs is send via the nodose ganglion (NOD) to the central subnucleus of the solitary complex (NTSce) and relayed to motor neurons in the nucleus ambiguus (AMB). From there, innervation of striated muscle via motor endplates (MEP) originates. Neurons from the dorsal motor nucleus (DMX) innervate excitatory cholinergic myenteric neurons (DMXro) as well as inhibitory nitrergic myenteric neurons (DMXcd). Furthermore, inhibitory (nNOS; black circle) and excitatory (ChAT; white circle) myenteric neurons are illustrated as an exemplary single neuron. Vagal afferents terminating in the nucleus of the solitary tract (NTS) additionally synapse on neurons projecting to the spinal cord leading to a general non-specific inhibitory effect on STT cells activity.
Reciprocal communication between myenteric neurons and IGLEs is suggested by double arrows.
LMM, Lamina muscularis mucosae; TM, tunica muscularis; THAL, Thalamus.

Diagram adapted from Neuhuber (Innervation of the mammalian esophagus) and modified

Table 1 List of primary antibodies and antisera used for immunohistochemistry

Antigen	**Host**	**Source**	**Working dilution**
Calcitonin gene-related peptide (CGRP)	Goat	Biotrend, Cologne, Germany; Code 1720-9007	1:250
Calcitonin receptor-like receptor (CLR)	Rabbit	(Cottrell et al. 2005), Code RK11	1:2,000
Calcitonin receptor-like receptor (CLR)	Goat	Santa Cruz, Biotechnology, Heidelberg, Germany; Code Sc-18007	1:50
Choline acetyltransferase (ChAT)	Goat	Chemicon, Temecula, CA; USA; Code AB 144P	1:40
Neuronal nitric oxide synthase (nNOS)	Guinea pig	Progen, Biotechnology, Heidelberg, Germany; Code 16059	1:1,000
Receptor activity modifying protein 1 (RAMP1)	Rabbit	Santa Cruz, Biotechnology, Heidelberg, Germany; FL-148, Code Sc-11379	1:50, 1:5,000
Vesicular glutamate transporter 2 (VGLUT2)	Guinea pig	Chemicon, Temecula, CA; Code AB5907	1:1,500

Table 2 List of secondary antibodies, antisera and detection systems used for immunohistochemistry

Secondary antibodies and antisera	**Conjugated to**	**Source**	**Working dilution**
Donkey anti-guinea pig	Cy™3	Dianova, Hamburg, Germany; Code 706-165-148	1:1,000
Donkey anti-guinea pig	Cy™5	Jackson ImmunoResearch; West Grove, PA (purchased by Dianova; Code 706-176-148)	1:1,000
Donkey anti-goat	Alexa Fluor® 488	Molecular Probes, Eugene, OR; Code A-11055	1:1,000
Donkey anti-goat	Alexa Fluor® 555	Molecular Probes; Code A-21432	1:1,000
Donkey anti-goat	Alexa Fluor® 647	Molecular Probes; Code A-21447	1:1,000
Donkey anti-rabbit	Alexa Fluor® 488	Molecular Probes; Code A-21206	1:1,000
Donkey anti-rabbit	Alexa Fluor® 555	Molecular Probes; Code A-31572	1:1,000
Donkey anti-rabbit	Biotin	Dianova, Hamburg, Germany; Code 711-065-152	1:500
Horseradish peroxidase (HRP)	Streptavidin	Dianova; Code 016-030-084	1:500
Streptavidin/Biotin blocking kit	-	Linaris GmbH, Sp-2002	4 drops per 1 ml of preincubation/antibody solution
Streptavidin	Cy™3	Dianova; Code 016-160-084	1:1,000
Tyramid	Biotin	Perkin-Elmer, Massachusetts, USA; NEL 700A001KT	In stock solution 1:250

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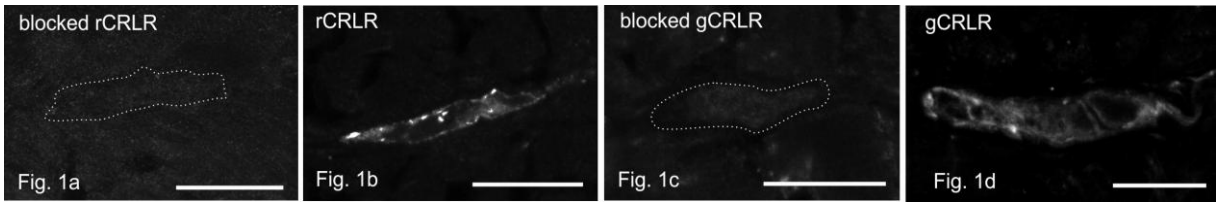

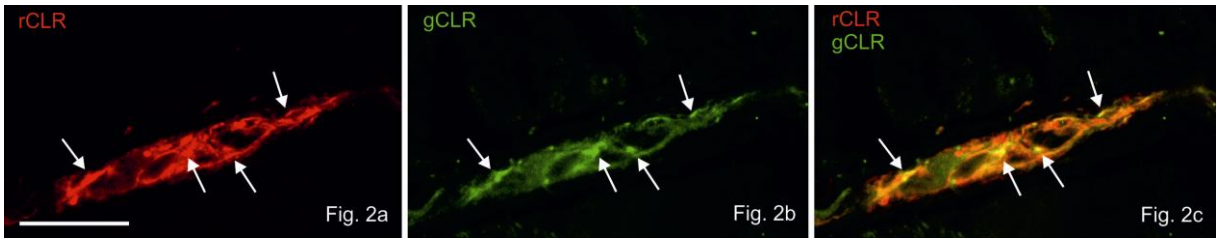
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Fig. 2a
gCLR
Fig. 2b
rCLR
gCLR
Fig. 2c

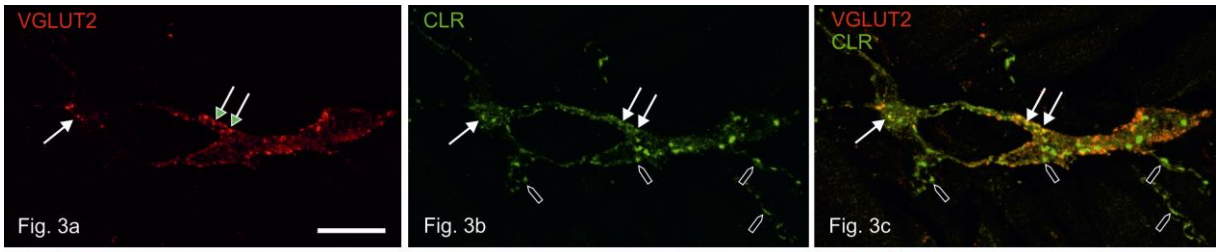
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Fig. 3a
CLR
Fig. 3b
VGLUT2
CLR
Fig. 3c

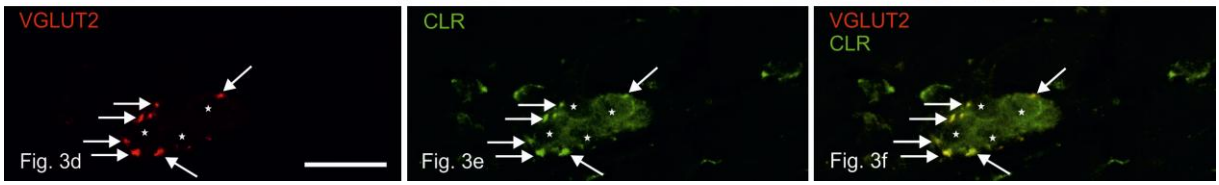
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Fig. 3d
CLR
Fig. 3e
VGLUT2
CLR
Fig. 3f

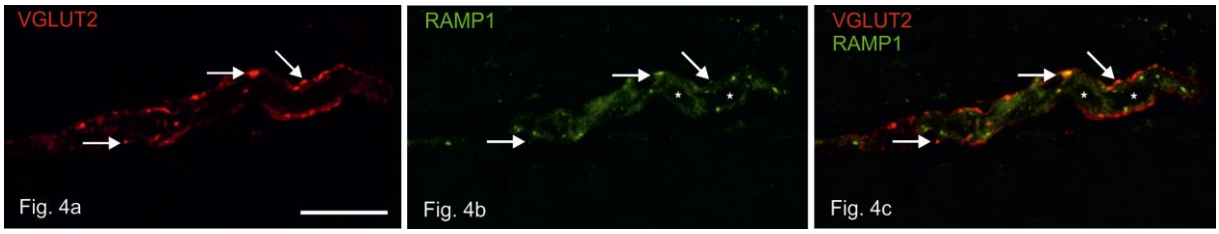
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Fig. 4a
RAMP1
Fig. 4b
VGLUT2
RAMP1
Fig. 4c

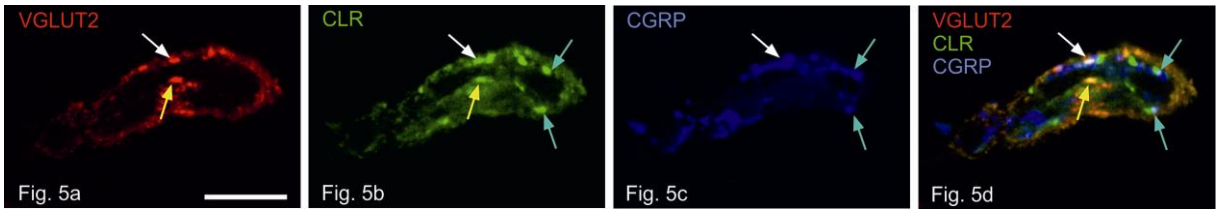
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Fig. 5d

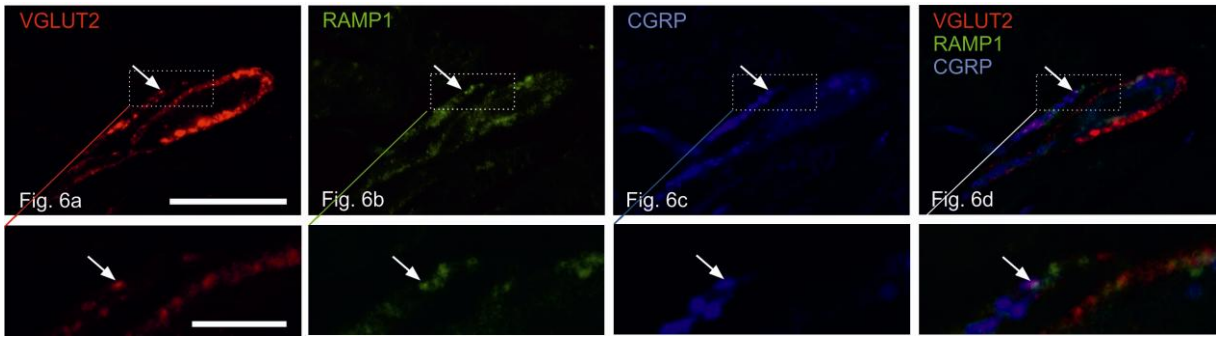
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Fig. 6a
RAMP1
Fig. 6b
CGRP
Fig. 6c
VGLUT2
RAMP1
CGRP
Fig. 6d

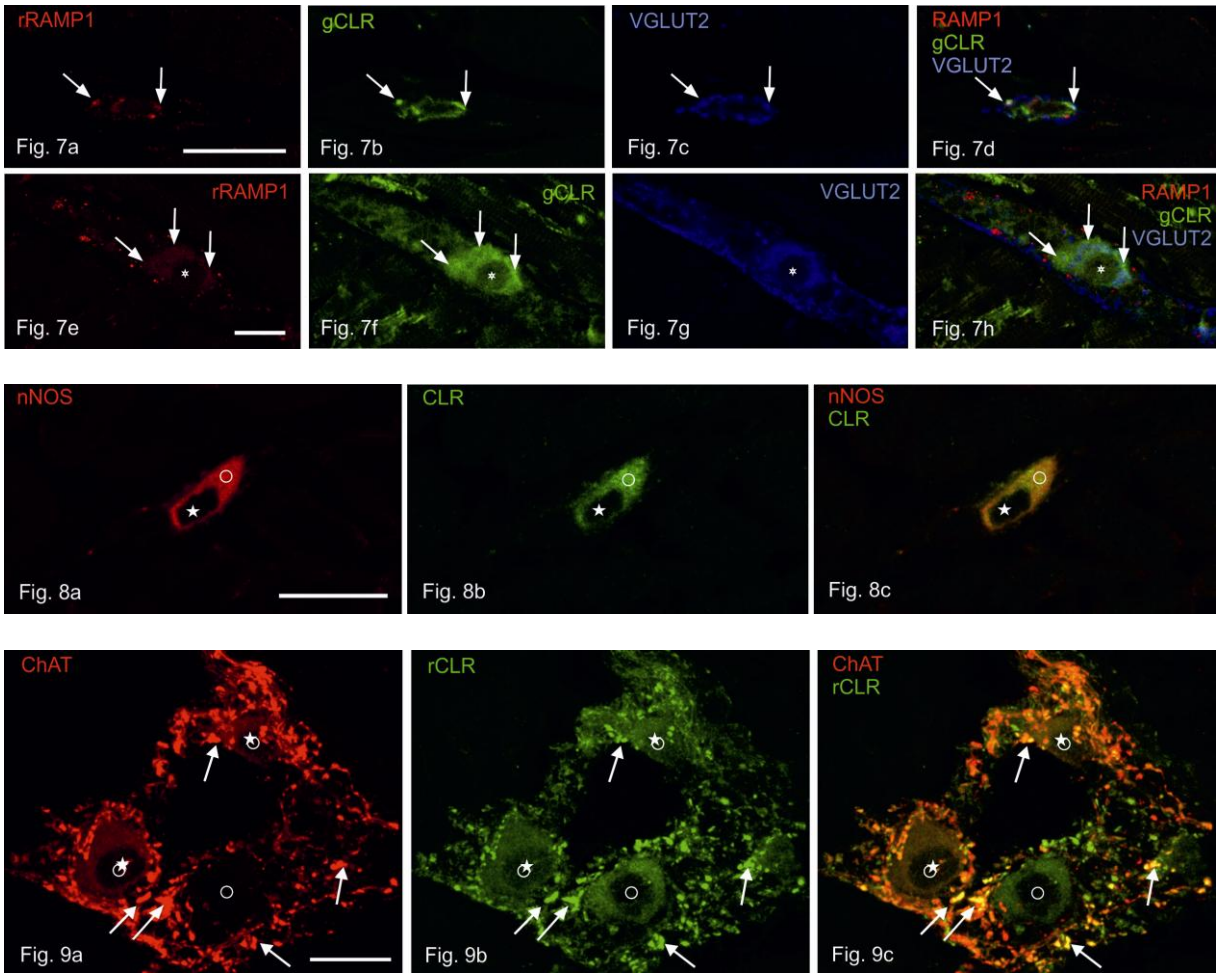
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Fig. 7a
gCLR
Fig. 7b
VGLUT2
Fig. 7c
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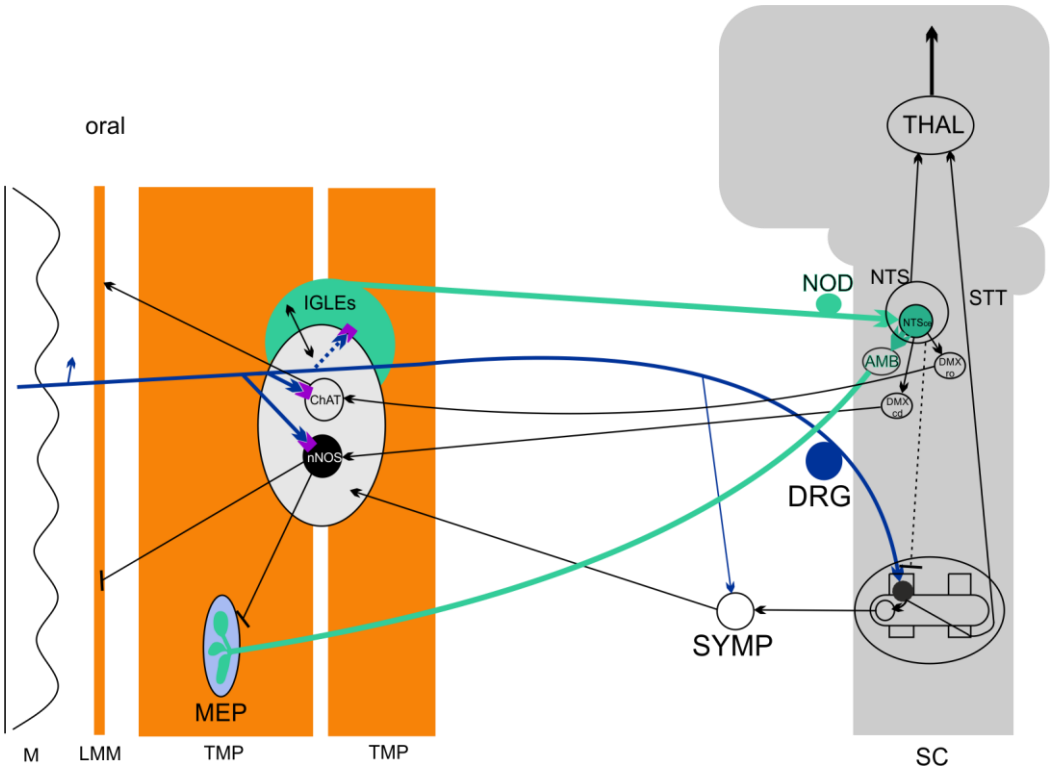
gCLR
VGLUT2
Fig. 7d
rRAMP1
Fig. 7e
gCLR
Fig. 7f
VGLUT2
Fig. 7g
RAMP1
gCLR
VGLUT2
Fig. 7h
nNOS
Fig. 8a
CLR
Fig. 8b
nNOS
CLR
Fig. 8c
ChAT
Fig. 9a
rCLR
Fig. 9b
ChAT
rCLR
Fig. 9c

Figure. 10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TMP
SC

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