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Identification of enteroendocrine cells that express TRPA1 channels in the mouse intestine

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Short Title: TRPA1 expression in EEC

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Abstract

TRPA1 is an ion channel that detects specific chemicals in food, and also transduces mechanical, cold and chemical stimulation. Its presence in sensory nerve endings is well known, and recent evidence indicates that it is expressed by some gastrointestinal enteroendocrine cells (EEC). The purpose of the present work was to identify and quantify EEC that express TRPA1 in the mouse gastrointestinal tract. Combined *in situ* hybridisation histochemistry for TRPA1 and immunofluorescence for EEC hormones was used. TRPA1 expressing EEC were common in the duodenum and jejunum, were rare in the distal small intestine and were absent from the stomach and large intestine. In the duodenum and jejunum, TRPA1 occurred in EEC that contained both cholecystokinin (CCK) and 5-hydroxytryptamine (5HT) and in a small number of cells expressing 5HT but not CCK. TRPA1 was absent from CCK cells that did not express 5HT and from EEC containing glucagon-like insulinotropic peptide. Thus TRPA1 is contained in very specific EEC populations. It is suggested that foods such as garlic and cinnamon that contain TRPA1 stimulants may aid digestion by facilitating the release of CCK.

Keywords: Intestine; enteroendocrine cells; cholecystokinin; serotonin; TRP receptors

Introduction

Transient receptor potential ankrin 1 (TRPA1) channels are non-selective cation channels that have been extensively studied in primary afferent neurons. TRPA1 channels transduce mechanical, cold and chemical stimulation at nociceptive nerve endings, more powerfully when the endings are sensitised by inflammatory cytokines (Kwan et al. 2006; Brierley et al. 2009). The channels can also be activated by chemicals that occur in various foods, including allyl isothiocyanate (AITC), a component of garlic and wasabi, and cinnamaldehyde from cinnamon (Holzer 2011). Components of other herbs, including mustard, horseradish, onion, ginger, oregano, wintergreen and clove, also stimulate TRPA1 channels (Holzer 2011). It is therefore significant that TRPA1 channels have been reported in enteroendocrine cells (EEC) of human and rat intestine (Nozawa et al. 2009), because these cells are directly exposed to the chemical constituents of foods.

However, it is not yet established which of the approximately 12 mammalian EEC subtypes (Rehfeld 2004; Furness et al. 2013) express TRPA1 channels, or whether the channels are expressed by EEC of the mouse, which is a species in which gene manipulation can be used to analyse roles of TRPA1 channels. By *in situ* hybridisation histochemistry, *Trpa1* expression has been localised to EEC in the rat and human small intestine (Nozawa et al. 2009). Most of the *Trpa1*-expressing cells were immunoreactive for 5HT. An investigation of mouse intestine found immunoreactivity for

TRPA1 in enteric neurons but not in EEC (Poole et al. 2011). Nevertheless, the transcript was detected in extracts of the duodenal, ileal and colonic mucosa, and by immunohistochemistry a low level of staining was detected in enterocytes of the small intestine and colon (Poole et al. 2011). In other RT-PCR studies, channel expression has been detected in mouse duodenal mucosa, STC-1 cells, an endocrine cell line derived from mouse small intestine, rat colonic mucosa and human duodenum and colon (Purhonen et al. 2008; Kaji et al. 2011; Kaji et al. 2012).

The present work was aimed at re-evaluating the expression of TRPA1 in EEC of the mouse gastrointestinal tract, and determining the relation of expression to specific populations of the EEC.

Materials and Methods

Tissue preparation

The localisation of *Trpa1* expression was investigated in the stomach, small and large intestines of C57Bl/6 mice and *Trpa1* deleted mice (Kwan et al. 2006). Male mice of 6 to 8 weeks of age (25 to 30g in weight) were used. The mice had free access to food and water and were taken for investigation in the morning. The experiments were approved by the University of Melbourne Animal Ethics Committee. Mice were killed by cervical dislocation before tissues were taken. The gastrointestinal tract from the stomach to the colon was carefully removed and the individual regions were dissected in ice cold diethylpyrocarbonate (DEPC) treated phosphate buffered saline (PBS: 0.15M NaCl in 0.01M sodium phosphate buffer, pH 7.2). The samples were placed in the fixative (4% paraformaldehyde) overnight at 4°C.

The next day the tissue samples were washed in DEPC-PBS at 4°C. The blocks were then placed in DEPC-PBS containing 0.1% sodium azide and 30% sucrose as a cryoprotectant and stored at 4^{o}C until the tissue was fully saturated and sank. Tissue was embedded in OCT compound (Tissue Tek, Elkhart, IN, USA) and frozen. Complete transverse sections of 12μm thickness were cut, air dried for 1hr, then washed with DEPC-PBS to remove residual OCT.

Combined in situ hybridisation with immunohistochemistry

Digoxigenin (DIG) labeled RNA probes were hybridised with sections overnight at 60°C. Both antisense and sense RNA probes were transcribed with T7 and SP6 primers, respectively, from the pCRII-TOPO vector (Invitrogen, Melbourne, Australia) containing 912bp of partial TRPA1 cDNA (responsible for bp 2432-3344 of TRPA1 mRNA, NM_177781.4). The hybridisation buffer contained 50% formamide, 1.3x standard salt concentration (SSC, pH 7.5, Sigma-Aldrich, Sydney, Australia), 10% dextran sulphate (Fisher Scientific, Victoria, Australia), 1x Denhardt's solution (Sigma-Aldrich), and 0.1 mg/mL yeast RNA (Roche Products, Dee Why, NSW, Australia). The next day the sections were washed twice with washing solution (50% formamide, 1x SSC, 0.1%

Tween-20), 5 x 30 min at 60°C and with MABT (150 mM NaCl containing 100 mM maleic acid and 0.1% Tween20, pH 7.5) for 10 min at room temperature. The sections were incubated with blocking solution, consisting of 2% Roche Blocking Reagent (Roche) and 20% sheep serum in MABT for 1.5 hr and anti-DIG conjugated with alkaline phosphatase (DIG-AP; Roche) 1:2000 overnight at 4°C. The following day sections were washed with MABT 4 times for 5 min at room temperature and incubated with AP buffer (100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris pH 9.5, 0.1% Tween-20) 2 times for 10 min before NBT/BCIP substrate (Nitro Blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate; Roche), 20 µl/mL in 5% polyvinyl alcohol, was applied. Staining was developed for 4~6 hr at room temperature.

In situ hybridisation was followed by incubation with 10% normal horse serum for 30min. Sections were then incubated with primary antibodies; goat anti-5HT (1:10000, #20079, Immunostar, Hudson, WI, USA), rabbit anti-CCK (1:2000, R183B, gift from Dr Robert Elde), rabbit anti- glucagon-like insulinotropic peptide (GIP, 1:2000, Ab22624, Abcam, Cambridge, UK) at 4°C, overnight. The anti-5HT was raised against 5HT coupled to bovine serum albumen. Staining is completely eliminated by pretreatment of the diluted antibody with 100 µg of serotonin/BSA conjugate (manufacturer's data). The anti-CCK was raised against CCK1-8, and its labeling in immunohistochemistry is eliminated by incubating the diluted antiserum with CCK1-8, 1 nM (R. Elde, personal communication). The anti-GIP was raised against the full length purified protein from pig, conjugated to keyhole limpet hemocyanin. The sections were then incubated with labeled secondary antibodies, donkey anti-sheep immunoglobulin conjugated with Alexa Fluor® 488, 1:500, donkey-anti rabbit immunoglobulin conjugated with Alexa Fluor® 594, 1:1000, for 1 hr at room temperature. Fluorescence and bright field images were taken either at 40x or 63x using an AxioImager microscope (Carl Zeiss, Sydney, Australia). For green fluorescence (Alexa Fluor® 488) we used filter set 38 HE, with excitation band pass 470 (40) nm, dichroic mirror 495 nm and emission band pass 525 (50) nm. For red fluorescence (Alexa Fluor® 594) we used filter set 45 HQ, with excitation band pass 560 (40) nm, dichroic mirror 585 nm and emission band pass, 630 (75) nm. There was no evidence of optical cross over in our studies. Positive EEC labeling was defined as labelling of a cell with EEC morphology that could be revealed as granular (located to putative storage vesicles, see Fig 2) and was strong on image capture settings at which no background staining of adjacent enterocytes could be observed (Fig. 2, panels e, f).

Detection of TRPA1 by reverse-transcription polymerase chain reaction

The tissue was opened longitudinally, stretched mucosal side up, washed with cold PBS and the mucosa of each region was scraped off using a scalpel blade. The mucosa was snap frozen. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Melbourne, Australia) and was reverse

transcribed using Superscript III reverse transcriptase (Invitrogen, Melbourne, Australia). Following quantification using a NanoDrop 1000 spectrophotometer, 100 ng of cDNA from each region was used to amplify mouse *Trpa1* (accession number: NM_177781) mRNA by polymerase chain reaction (PCR) using 5PRIME MasterMix (5PRIME GmbH, Hamburg, Germany) with intron spanning primers. TRPA1 (forward, 5' GTGTTGACTGAGCACAAG 3'; reverse, 5' CAATGTTCCAGGATGCAG 3', predicted product size 628 bp). cDNA from dorsal root ganglia was used as a positive control. Negative control reactions omitted reverse transcriptase. Products were separated by electrophoresis and sequenced.

Results

In situ hybridisation histochemistry for *Trpa1* expression revealed many cells with the morphology of EEC in the mucosa of the proximal small intestine. Few EEC were observed in the mucosa of the distal small intestine and none were present in the gastric or colonic mucosa. These regions were not further analysed. The number of TRPA1 positive cells in the duodenum and jejunum were 16 ± 2 and 23 ± 2 per complete transverse section (*n*=19 and 13 sections from 3 animals), respectively. No mucosal cells were revealed in tissue from the *Trpa1*-/- mice or when the sense probe was used in sections from wild type mice (Fig 1).

Combined immunofluorescence and *in situ* hybridisation histochemistry was used to identify the EEC that expressed *Trpa1*. There was no significant autofluorescence following *in situ* hybridisation. Combined staining revealed that sub-populations of *Trpa1* expressing EEC were immunoreactive for CCK and 5HT, but none were immunoreactive for GIP (Fig 2). Immunoreactivity for 5HT occurred in 96 ± 1 % of duodenal *Trpa1* positive cells and 97 ± 1 % of jejunal cells (*n*=300 *Trpa1* cells for each region from 3 animals). In the duodenum, 73 ± 4 %, and in the jejunum, 81 ± 8 %, of *Trpa1* positive cells were immunoreactive for CCK and all *Trpa1* /CCK positive cells were 5HT immunoreactive (Fig 3a). A small number of cells (~ 4% of *Trpa1* expressing EEC) had immunoreactivity for neither 5HT nor CCK

The total populations of EEC with immunoreactivity for 5HT and CCK were quantified from the same tissue. The numbers of 5HT positive EEC per section were 101 ± 7 and 91 ± 7 in duodenum and jejunum (*n*=9 sections from 3 animals), respectively. The CCK positive cell population was similar, being 106 ± 9 and 97 ± 6 in duodenum and jejunum, respectively. The numbers of EEC that contained both 5HT and CCK were slightly more in the duodenum compared to jejunum, but this was not statistically different (unpaired t-test, $P>0.05$; Fig 3b).

In summary, the duodenum and jejunum contained CCK/5HT cells, CCK/- cells and 5HT/- cells. About 27% of CCK/5HT cells, no CCK/- cells and about 10% of 5HT/- EEC expressed the *Trpa1* gene.

Trpa1 mRNA was detected by RT-PCR in extracts of mucosa from the duodenum and jejunum and corresponded in size to *Trpa1* mRNA in extracts of dorsal root ganglia (Fig 4). As previously reported (Poole et al. 2011), mRNA was detected in mucosal extracts from other regions of the gastrointestinal tract, from stomach to colon. Bands had the predicted molecular sizes (628 bp) and their identity was confirmed by sequencing.

Discussion

Expression of TRPA1 mRNA

Previously, *Trpa1* gene expression was reported in extracts of the whole wall of mouse stomach, jejunum, ileum, proximal colon and distal colon (Penuelas et al. 2007; Nozawa et al. 2009). However, myenteric neurons included in these extracts are reported to express TRPA1 (Poole et al. 2011) and thus these previous studies do not distinguish between myenteric neurons and EEC as repositories of *Trpa1*. In the present work, the presence of *Trpa1* mRNA in the duodenal and jejunal mucosa was confirmed by RT-PCR. A previous analysis of mucosal extracts from mouse revealed a strong signal for *Trpa1* in the mucosa of the ileum, and weak signals in extracts of duodenum and colon mucosa (Poole et al. 2011). It was not resolved whether the gene expression was in EEC or in other cells in or associated with the mucosa, which could include submucosal neurons.

In the present work, we have revealed *Trpa1* expression in murine EEC for the first time. Previous studies had revealed *Trpa1* expression in EEC of rat and human (Nozawa et al. 2009). Some of these rat and human cells also expressed 5HT. We have confirmed colocalisation with 5HT and have shown in addition that the cells are confined almost entirely to the proximal small intestine. No *Trpa1* expressing EEC were found in the stomach or large intestine. In the duodenum and jejunum, where almost all *Trpa1* expressing EEC occur, 96-97% also contained 5HT and 75-80% of these contained CCK. Thus the majority of *Trpa1* expressing EEC are CCK/5HT EEC. It was interesting that cells expressing CCK but not 5HT (CCK/- cells), which we showed were numerous in the mouse proximal intestine, and GIP expressing cells, which are also numerous, did not express *Trpa1*. Thus *Trpa1* is expressed by very specific subgroups of EEC. So far as we are aware, only one previous study reported that there is a population of EEC that express both 5HT and CCK in the mouse intestine (Aitken et al. 1994). However, cells expressing *Trpa1* are a minority, about 27% of the total population of CCK/5HT cells. It is possible that *Trpa1* is expressed at lower levels in other cells, including enterocytes, or in other regions of the gastrointestinal tract and was below the detection level of the *in situ* hybridisation histochemistry applied in the present study.

Possible roles of TRPA1 channels of duodenal and jejunal EEC

The largest group of cells that expressed *Trpa1* also expressed CCK and 5HT, but nevertheless this was a minority of CCK and 5HT cells. TRPA1 is a non-specific cation channel whose activation allows an inward flow of Ca^{2+} ions, which would be predicted to trigger the release of CCK and 5HT. In fact, there is good evidence that TRPA1 receptor agonists cause the release of 5HT from rat small intestinal EEC (Nozawa et al. 2009). The major role of CCK is to aid digestion of food components by causing the release of digestive enzymes from the pancreas and bile salts from the gallbladder. It also induces satiety, slows gastric emptying and reduces gastric acid secretion (Raybould 2007). Secretion of pancreatic enzymes is an indirect effect, mediated by activation of vagal endings in the connective tissue close to EEC by CCK (Owyang 1996). Components of food that stimulate TRPA1 channels may thus facilitate digestion by releasing CCK. It is also possible that CCK released by TRPA1 activation could contribute to satiety following the meal (Raybould 2007). However, experimental evidence indicates that CCK influences the pattern of food intake, but does not change overall food intake (Raybould 2007). Therefore foods that contain TRPA1 agonists probably will not reduce food intake if given regularly.

The significance of TRPA1 being expressed by a minority of CCK cells, and this minority also contains 5HT. 5HT has a number of effects in the digestive tract (Gershon 2013). In the context of the present study, its most pertinent role may be as a promoter of digestive enzyme release. The non-CCK mediated stimulation of pancreatic secretion by luminal nutrients is blocked by a $5HT_3$ receptor antagonist, which implies that 5HT and CCK act synergistically to promote digestive enzyme release (Li et al. 2000). TRPA1 agonists, given orally to rats, slow gastric emptying and the effect is reduced by the $5HT_3$ receptor antagonist, granisetron (Doihara et al. 2009). This may also aid digestion by extending the exposure time of the small intestine to nutrients. Because $5HT_3$ receptors are on vagal nerve endings that respond to luminal stimuli (Li et al. 2000), it is reasonable to assume that the slowing of gastric emptying is vagally mediated. Selective knockout of the synthetic enzyme for EEC 5HT (tryptophan hydroxylase 1) suggests that EEC derived 5HT also contributes to mucosal inflammatory responses (Gershon 2013). Although both CCK and 5HT activate vagal nerve endings, and there are interactions between some EEC hormones at the level of the endings (Raybould 2007; Dockray 2013), whether these hormones modify each other's effects at the endings is not known.

It is notable that herbs or herb-derived chemicals are used in very small amounts (parts per million, i.e., grams per tonne) in animal feed to improve the nutritional status of the animals. This includes cinnamon and garlic which are stimulants of TRPA1 channels (Rochfort et al. 2008; Windisch et al. 2008; de Lange et al. 2010; Michiels et al. 2010; Hashemi and Davoodi 2011).

There is evidence that herbs added to food can improve weight gain and food conversion rates, although the outcomes of existing trials are variable (Franz et al. 2010). Thus, the possibility that specific food additives aid digestion by activation of TRPA1 channels needs further investigation. The major stimulants of CCK release from EEC are fats, breakdown products of proteins and amino acids (Furness et al. 2013). It is possible that food additives that activate TRPA1 facilitate the release of CCK and 5HT by fats and other nutrients.

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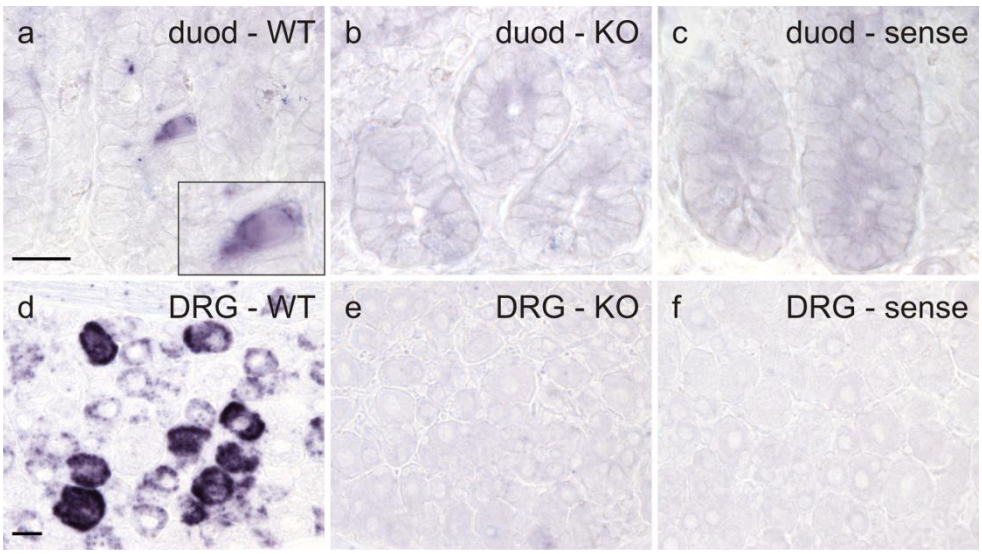
Figure Descriptions

Fig. 1. *In situ* hybridisation histochemistry showing EEC in the small intestine (**a**) and neurons in dorsal root ganglia (**d**) from wild-type mice (WT) that express *Trpa1*. The positive EEC in **a** is enlarged in the inset. No EEC (**b**) or dorsal root ganglion cells (**e**) were observed in tissues from *Trpa1*-/- mice (KO). Likewise, no EEC (**c**) or dorsal root ganglion cells (**f**) were observed in tissue sections from wild-type mice that were incubated with sense probes. The reaction conditions were the same for the small intestine and dorsal root ganglion sections. Scale bars: 20 µm.

Fig. 2. Simultaneous labeling for *Trpa1* gene expression using antisense probes, and for EEC hormones, using immunohistochemistry. **a**, **b**, **c**: An EEC that expresses both *Trpa1* and 5HT. To allow the merge, the TRP image has been converted to white on black in images **c**, **g** and **j**. **d** - **g**: An EEC that expresses *Trpa1*, 5HT and CCK. Most *Trpa1* expressing EEC had this pattern of colocalisation. **a**, **b**, **c**: An EEC that expresses *Trpa1* and an EEC that expresses GIP in the same field. There were no examples of *Trpa1* and GIP colocalisation. Scale bars: 20 µm.

Fig. 3. **a**: Quantitation of EEC populations that express *Trpa1*. The most numerous cells were those with immunoreactivity for both 5HT and CCK. Most of the remaining cells had only 5HT immunoreactivity, no cells with only CCK were encountered (indicated by arrows), and very few cells expressed neither 5HT nor CCK. **b**: Relative number of cells (with or without *Trpa1*) that expressed 5HT only, 5HT plus CCK or CCK only. The CCK only group (which did not express *Trpa1*) is about as numerous as the other groups.

Fig. 4. RT-PCR gel showing expression of *Trpa1* in extracts of dorsal root ganglia (DRG) and of the mucosa from the mouse duodenum and jejunum. Results with (+) and without (-) reverse transcriptase (RT) are shown. Base-pair (bp) ladders are at the right and left sides of the gel. NTC: no template control. Transcripts of predicted sizes were found in the extracts.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duod - WT
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c
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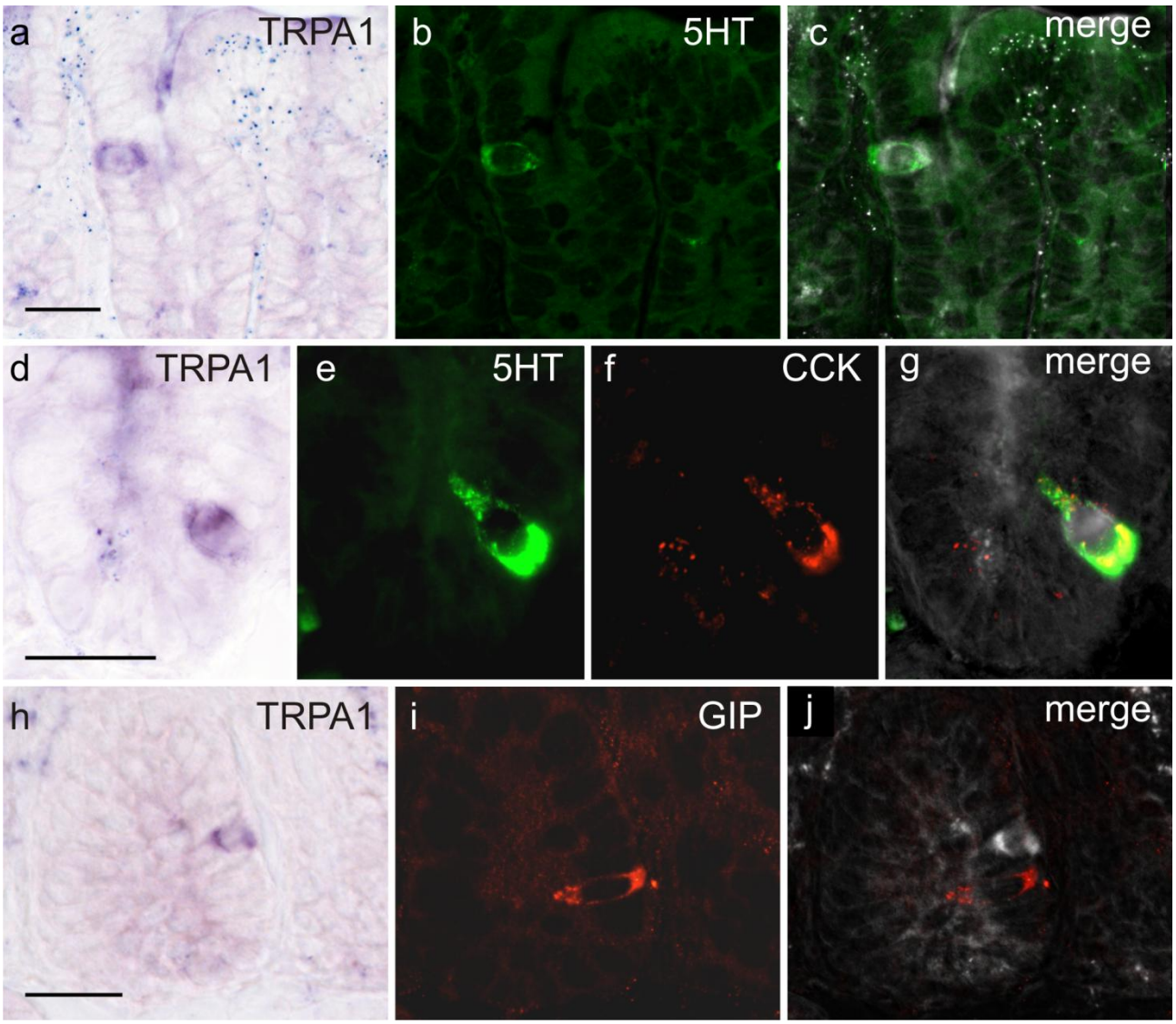
d
DRG - WT
e
DRG - KO
f
DRG - sense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b
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CCK
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h
TRPA1
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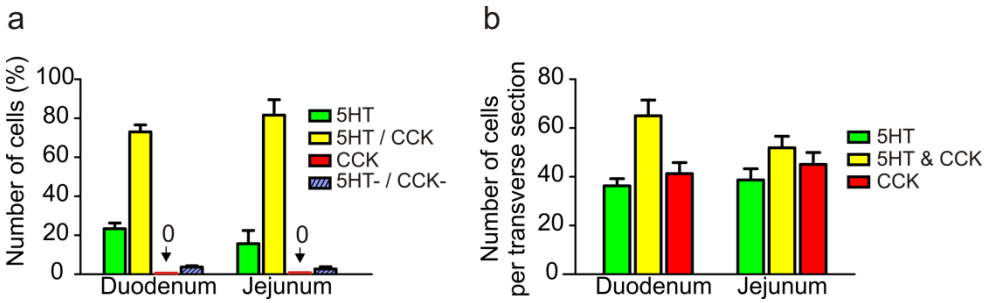
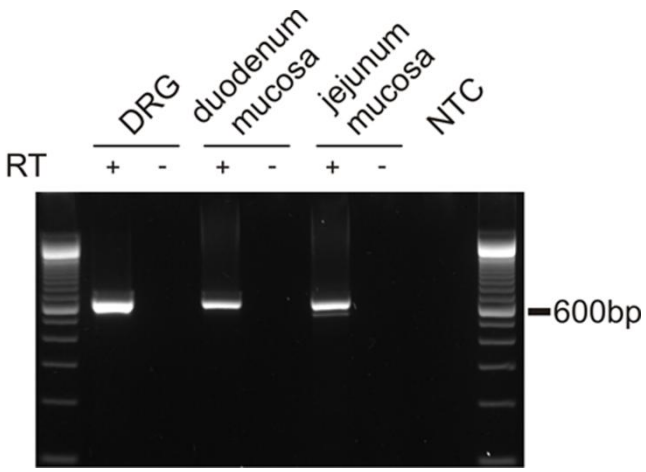

a
Number of cells (%)
100
80
60
40
20
0
0
0
Duodenum
Jejunum
5HT
5HT / CCK
CCK
5HT- / CCK-
b
Number of cells per transverse section
80
60
40
20
0
Duodenum
Jejunum
5HT
5HT & CCK
CCK


DRG
duodenum mucosa
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NTC
RT
+ - + - + -
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