REGULAR ARTICLE

Spatiotemporal expression pattern of DsRedT3/CCK gene construct during postnatal development of myenteric plexus in transgenic mice

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Abstract Cholecystokinin (CCK) is an early marker of both neuronal and endocrine cell lineages in the developing gastrointestinal tract. To determine the quantitative properties and the spatial distribution of the CCK-expressing myenteric neurones in early postnatal life, a transgenic mouse strain with a CCK promoter-driven red fluorescent protein (DsRedT3/CCK) was established. The cell-specific expression of DsRedT3/CCK was validated by in situ hybridization with a CCK antisense riboprobe and by in situ hybridization coupled with immunohistochemistry involving a monoclonal antibody to CCK. A gradual increase in the DsRedT3/CCK-expressing enteric neurones with clear regional differences was documented from birth until the suckling to weaning transition, in parallel with the period of rapid intestinal growth and functional maturation. To evaluate the proportion of myenteric neurones in which DsRedT3/CCK transgene expression was colocalized with the enteric neuronal marker peripherin, immunofluorescence techniques were applied. All DsRedT3/CCK neurones were peripherin-immunoreactive and the proportion of DsRedT3/CCK-expressing myenteric neurones in the duodenum was the highest after the third week of life, when the number of peripherin-immunoreactive myenteric neurones in this region had decreased. Nearly all of the DsRedT3/CCK-expressing neurones also expressed 5-hydroxytryptophan (5-HT). Thus, by utilizing a new transgenic mouse strain, we have demonstrated a small number of CCK-expressing myenteric neurones with a developmentally regulated spatiotemporal distribution. The coexistence of CCK and 5-HT in the majority of these neurones suggests their possible regulatory role in feeding at the suckling to weaning transition.

Keywords Cholecystokinin · Myenteric neurones · 5-Hydroxytryptophan · In situ hybridization · Immunofluorescence · Transgenic mice

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Introduction

Cholecystokinin (CCK) is a neuropeptide that is distributed widely throughout the central nervous system and the gastrointestinal tract. In the central nervous system, CCK is present in several brain regions (Dockray 1976; Muller et al. 1977; Larsson and Rehfeld 1979) and functions as a neurotransmitter or neuromodulator. The cellular expression of CCK in the intestine changes during development. The primary site of expression in adults is in the endocrine cells of the proximal small intestine (Crawley and Corwin 1994), whereas in fetal mice, neuronal CCK expression is widespread throughout the gastrointestinal tract (Lay et al. 1999, 2004). Immunohistochemical studies (Larsson and Rehfeld 1979; Hökfelt et al. 1980; Schultzberg et al. 1980; Furness et al. 1984, 1995) and intracellular recordings (Schutte et al. 1997) have led to CCK also being considered as a putative neurotransmitter in the enteric nervous system (ENS). However, several observations have indicated that the

actions of CCK on gastrointestinal motility are complex. In one tissue, the effects of CCK are often inhibitory (Storr et al. 2003; Giralt and Vergara 2000), whereas in another tissue, its effects are both excitatory and inhibitory (Scarpignato et al. 1993; Schutte et al. 1997). Such conflicting findings may be attributable to differences between species, differences between intestinal regions or muscle layers and differences in the neurochemical code of the CCK-expressing enteric neurones. CCK-immunoreactive neuronal structures have been found in most parts of the gastrointestinal tract but in small numbers relative to other peptides (Schultzberg et al. 1980; Furness et al. 1995). In general, the visualization of peptides in the enteric neuronal somata has frequently proven difficult, possibly because of low peptide levels in this part of the neurone. Earlier work (Schultzberg et al. 1980; Messenger and Furness 1990) has revealed marked increases in the number of CCK-immunoreactive cells after treatment with colchicine or vinblastine. Such treatments, however, make quantitative evaluation unreliable.

Whereas the patterns of CCK-expressing neuronal precursors in fetal life (Lay et al. 1999) and also of endocrine cells in adult intestines (Chandra et al. 2010) have been well characterized, nothing is known about the CCK-expressing enteric neurones in the early postnatal period. Data from the literature allow us to suppose, at the same time, that CCK has an important role in the functional maturation of the ENS (Weller 2006; Washington et al. 2011). Therefore, we have utilized a transgenic mouse strain with CCK promoter-driven DsRedT3, developed in our laboratory. Concurrently with the use of this DsRedT3/CCK expression in the myenteric neurones, we have applied immunofluorescence techniques to investigate the quantitative properties and the spatiotemporal distribution of the CCK-expressing myenteric neurones during the postnatal period ranging from birth up until the suckling to weaning transition and also in adults. We have verified the specificity of the expression pattern by in situ hybridization of intestinal whole-mounts with a CCK antisense riboprobe and also by in situ hybridization with a DsRedT3 antisense riboprobe coupled with immunocytochemistry involving a CCK-specific antibody.

In the past few years, substantial evidence has accumulated to indicate that the interaction between CCK and 5-hydroxytryptophan (5-HT) represents a key point in the peripheral mechanism of feeding control (Raybould et al. 2003; Powley and Phillips 2004; Hayes and Covasa 2006; Hayes et al. 2006). Accordingly, we have applied immunohistochemical staining with an antibody specific to 5-HT to determine whether DsRedT3/CCK-expressing myenteric neurones overlap 5-HT-immunoreactive neurones at the suckling to weaning transition, when the diet changes from milk to food pellets and at which time the mice begin to seek food independently of their mother (Curley et al. 2009).

Materials and methods

The mice used in this study were housed in the SPF animal facility of the Institute of Experimental Medicine, Budapest, Hungary. All experiments with animals were approved by in-house and national committees and were carried out in full compliance with institutional, NIH (NIH Publication #85-23, 1985) and EC (86/609/EEC/2) guidelines.

Production of BAC/DsRedT3/CCK transgenic mouse line

To generate transgenic mice that expressed the T3 variant of the Discosoma red fluorescence protein (Bevis and Glick 2002) under the control of the CCK promoter and regulation region (DsRedT3/CCK), we used BAC engineering technology. The DsRedT3 expression cassette was inserted in the first coding exon at the translation initiation site of the CCK gene by using homologous recombination in *Escherichia coli* (Lee et al. 2001). A bacterial artificial chromosome (BAC) clone (RP-23 60I1) containing the entire CCK gene and roughly 60 kb of upstream and downstream regions was used for BAC modification. In the targeting vector, the DsRedT3 cDNA (subcloned from the DsRed-MST-B vector, provided by Dr. Andras Nagy, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada, with the permission of Dr. Josh Huang, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., USA; Vintersten et al. 2004) with the SV40 polyadenylation signal and the neomycin selection marker flanked by flippase recognition target sites were inserted between the CCK homology arms (Fig. 1a). Recombination between the targeting vector and the BAC was carried out as described by Lee et al. (2001). Transgenic mice were derived by standard pronuclear injection of the linearized CCK-BAC/RedT3 DNA into FVB/N fertilized eggs. For this study, homozygous transgenic mice on the FVB/N background were used from the line CCK-BAC/RedT3 no. 47.

Tissue handling

Mice of either sex were killed by cervical dislocation between postnatal days 1.5 and 23.5 (P1.5–P23.5) and at adult ages between 110 and 130 days. All observations were repeated on specimens from at least three mice. Tissue samples were taken from identical segments of the duodenum, jejunum, ileum and colon. For whole-mount preparations, the intestinal segments were cut along the mesentery, pinched flat and fixed overnight at 4°C in 4% paraformaldehyde (PFA) solution buffered with 0.1 M phosphate buffer (PB; pH7.4). The mucosa, submucosa and circular muscle were
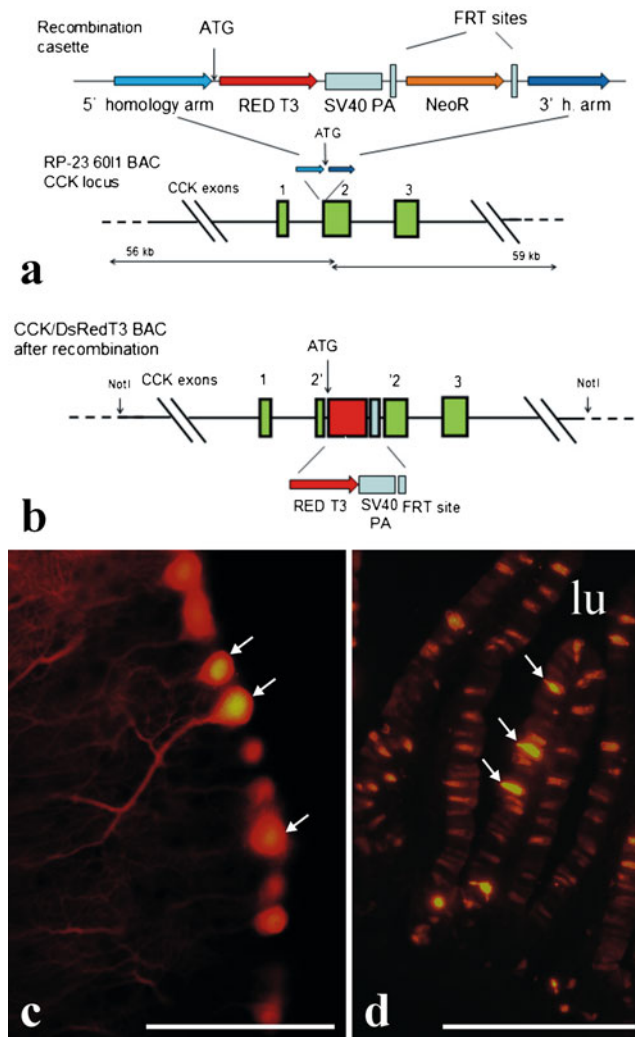
Fig. 1 a–d Representations of the mouse cholecystokinin (CCK)/ DsRedT3 gene transfer vector elements, the final transgene structure and fluorescence photomicrographs of the cerebellum and proximal small intestine of the transgenic mice. a DsRedT3 cDNA (RED T3) with an SV40 polyadenylation signal (PA) and a Neomycin selection marker (NeoR) flanked by flippase recognition target (FRT) sites. FRT sites were cloned between homology arms that flanked the translation initiation site (ATG) of the CCK gene. This cassette was inserted via homologous recombination into the CCK locus of the RP-23 60I1 bacterial artificial chromosome (BAC). b After the recombination and the Neomycin gene removal by the FLPe recombinase, the BAC insert (120 kb) was released by NotI digestion. The fragment was purified and used for microinjection into fertilized eggs. c Fluorescence photomicrograph of a cryosection prepared from the cerebellum of DsRedT3/CCK transgenic mice, with endogenously expressed DsRedT3 in the Purkinje cells (arrows). d Fluorescence photomicrograph of a cryosection prepared from the proximal small intestine of DsRedT3/CCK transgenic mice, with endogenously expressed DsRedT3 in the mucosal endocrine cells (arrows). Bars 100 μm

removed and whole-mounts with the myenteric plexus adhering to the longitudinal muscle were prepared. Whole-mounts were further processed for in situ hybridization and immunocytochemistry.

In situ hybridization

CCK and DsRedT3 antisense riboprobes were used to identify CCK-positive enteric neurones in the whole-mounts. To construct the pGEM/CCK template for probe synthesis, the CCK cDNA was generated via real time polymerase chain reaction (RT-PCR) amplification on adult brain RNA by using the primers 5′-CAGCCTTCTCCGCTGGAAC-3′ and 5′-CATAGCAACATTAGGTCTGGGAG-3′. The 615-bp fragment was then cloned into the pGEMT-easy vector according to the manufacturer's protocol (Promega, Madison, Wis., USA) and the orientation was verified by restriction digestion. The DsRedT3 fragment for probe synthesis was isolated from the DsRed MST-B vector by digestion with Ecl136II and BspTI restriction enzymes, then blunted by the Klenow fill-in reaction and subcloned into the SmaI and Ecl136II sites of the pBluescript-SK + (Stratagene, La Jolla, Calif., USA) vector. Orientation was verified by restriction digestion. For antisense RNA synthesis, both plasmids were linearized by NdeI and BamHI restriction enzyme digestion. The DNA fragments were purified on a 0.8% TRIS-acetate-EDTA (TAE) agarose gel by using a DNA Extraction Kit (Fermentas, Vilnius, Lithuania). In vitro transcription was carried out with 2 μg linearized templates, anti-dioxigenin (DIG) RNA labelling Mix (Roche, Basel, Switzerland) and T7 RNA polymerase (Promega). This was followed by treatment with RQDNase (Promega) to remove the template DNAs, which were precipitated with ammonium acetate and isopropanol and then dissolved in 20 μl RNAse-free water. The integrity of the RNA probes was checked on RNAse-free 1.2% TAE agarose gels. To detect hybridization signals, a method modified from Xiang and Burnstock (2004) was used. Briefly, whole-mounts were rinsed with PB saline (PBS) containing 0.1% Tween20 and then postfixed with 4% PFA in PBS. Prehybridization was performed in Hyb + buffer (50% formamide, 5× standard sodium citrate, 0.1% Tween20, 100 μg/ml yeast tRNA, pH6) at 60°C for 2 h. Hybridization was carried out in the same buffer with the added RNA probes overnight at 60°C. The remaining probes were removed by intensive washing. Preparations were subsequently incubated in blocking solution containing 5% bovine serum albumin and 2% fetal bovine serum followed by incubation in anti-DIG antibody conjugated to alkaline phosphatase (Roche, Basel, Switzerland) diluted 1:4000 in blocking solution overnight at 4°C and then washed intensively. Colour development was performed in a mixture of nitro-blue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate and levamisole in 0.1 M TRIS–HCl solution, in the dark, at room temperature, overnight. The preparations were rinsed in 10 mM TRIS–HCl to terminate colour development, mounted with Mowiol (Calbiochem-Merck, Darmstadt, Germany) and photographed by means of a Zeiss Axioscope2 microscope equipped with an integral cooled camera.

Immunocytochemistry

The immunocytochemical methods applied here were modified from our earlier reports (Bagyánszki et al. 2011). Briefly, whole-mounts were incubated overnight with rabbit polyclonal anti-peripherin antibody (Millipore, Billerica, Mass., USA; 1:400) or rabbit polyclonal anti-5-HT antibody (Sigma, St. Louis, Mo., USA; 1:500) at room temperature, then washed in PBS and incubated with fluorescein isothiocyanate (FITC)-labelled species-specific secondary antibody (Sigma; 1:100) for 4 h. Negative controls were performed by omitting the primary antibody, when no immunoreactivity was observed. Preparations were subsequently washed in PB, mounted in S3023 non-fluorescent mounting medium (Dako, Carpinteria, Calif., USA) and photographed randomly with an Olympus DP70 camera attached to an Olympus BX51 light microscope.

In situ hybridization coupled with immunocytochemistry

To improve the validation of the cell-specific expression of the transgene, a mouse monoclonal anti-CCK antibody (provided by CURE, University of California, Los Angeles, Calif., USA; 1:5000) was studied by immunocytochemistry on the same specimens upon which DIG-labelled in situ hybridization had been performed. Immunocytochemistry was carried out as described previously (Izbéki et al. 2008) by using a biotin-streptavidin-peroxidase procedure. Biotinylated goat anti-mouse immunoglobulin (Vector Lab, Burlingame, Calif., USA; 1:1000) was used as a secondary antibody. Negative controls were performed as described above.

Specimen analysis

For quantitative analysis of DsRedT3/CCK and peripherin-expressing myenteric neurones, 20 digital photographs identical in magnification, size and resolution were taken from each intestinal segment at each age. Data on 50 ganglia per intestinal segment per animal were included in the present study. Cells with red and green fluorescence were counted in each digital photograph through the use of Plexus Pattern Analysis software (Román et al. 2004). Statistical analysis was performed by means of a one-way analysis of variance with GraphPad Prism 4.0 software. Data were expressed as means ± SEM. The level of statistical significance was set at $P<0.05$. The raw data gained after counting 50 ganglia per intestinal segment per animal per age were also used to determine the mean ratio of the DsRedT3/CCK myenteric neurones. The ratio of the number of red-fluorescing cells over the green-fluorescing peripherin-immunopositive neurones in each ganglia was determined. The mean percentage was calculated as the mean of these data (±SEM).

Results and discussion

Widespread, but transient, CCK expression has previously been reported in embryonic intestines but is extinguished in the mouse at around embryonic day 15 (Lay et al. 1999). However, nothing is known about the developmental expression of CCK in the ENS in the early postnatal period, which is of a key importance in the development of intestinal barrier function and motility. Therefore, we have utilized a new transgenic mouse strain with CCK promoter-driven DsRedT3 (Fig. 1a, b). In addition to the DsRed-expressing cells in the brain (Fig. 1c) and in the intestinal endocrine cells (Fig. 1d) as published earlier in a transgenic CCK-green fluorescent protein mice (Chandra et al. 2010), DsRed-expressing enteric neurones (Fig. 2a, b) are also visible throughout the intestines in our newly developed DsRedT3/CCK transgenic mice.

The spatial distribution of CCK-expressing myenteric neurones was readily determined by fluorescence microscopy on whole-mounts prepared from the intestines of these transgenic mice. Endogenously expressed DsRedT3/CCK filled the cytoplasm of the myenteric neurones (Fig. 2a). The intensity of the fluorescence observed in the different DsRedT3/CCK neurones varied but was always strong enough to identify the red cells with certainty. The cell-specific expression of DsRedT3/CCK was validated by in situ hybridization with a CCK antisense riboprobe (not
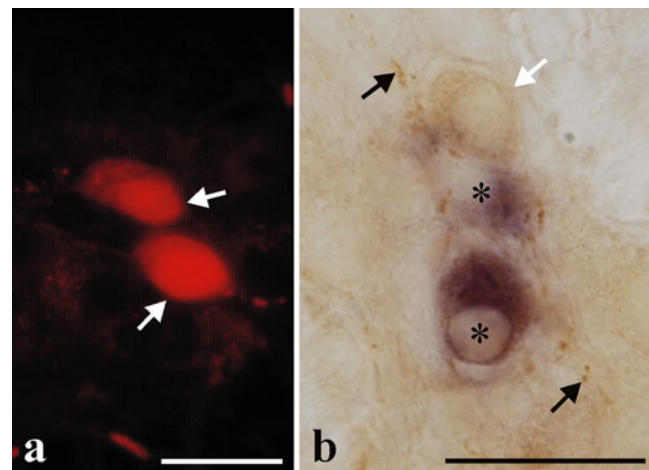

Fig. 2 a, b Identification of cell-specific expression of transgene in the myenteric neurones of the transgenic mice. Photomicrographs of myenteric ganglia exposed in whole-mounts prepared from the duodenum of DsRedT3/CCK transgenic mice. **a** Endogenously expressed DsRedT3 fills the cytoplasm of the myenteric neurones. Transgene expression is limited to two ganglionic cells here (*arrows*). **b** DsRedT3 mRNA expression and CCK immunoreactivity overlap in ganglionic cells (*asterisks*), after in situ hybridization with a DsRedT3 antisense riboprobe, coupled to immunocytochemistry with an antibody specific to CCK. CCK-immunopositive varicosities (*black arrows*) and some of the cell bodies (*white arrow*) do not display DsRedT3 mRNA expression. *Bars* 100 μm

Fig. 3 a–c Density and percentage of neurones in the myenteric ganglia on the postnatal days between P1.5 and P23.5 along the proximo-distal axis of the gastrointestinal tract of DsRedT3/CCK mice. a Number of DsRedT3-expressing myenteric neurones is limited to 0–4 neurones per ganglion. Starting from P3.5, the number of red cells is always the highest in the duodenum. After a gradual increase, the density of red cells peaks on P21.5. b Density of peripherin-immunoreactive myenteric neurones also increases gradually in the early perinatal period and peaks on P17.5. c Ratio of myenteric neurones with endogenously expressed DsRedT3 is low; however, it is always the highest in the duodenum, with a peak at P21.5. Data are expressed as means ± SEM; *$P<0.05$, **$P<0.01$, ***$P<0.001$
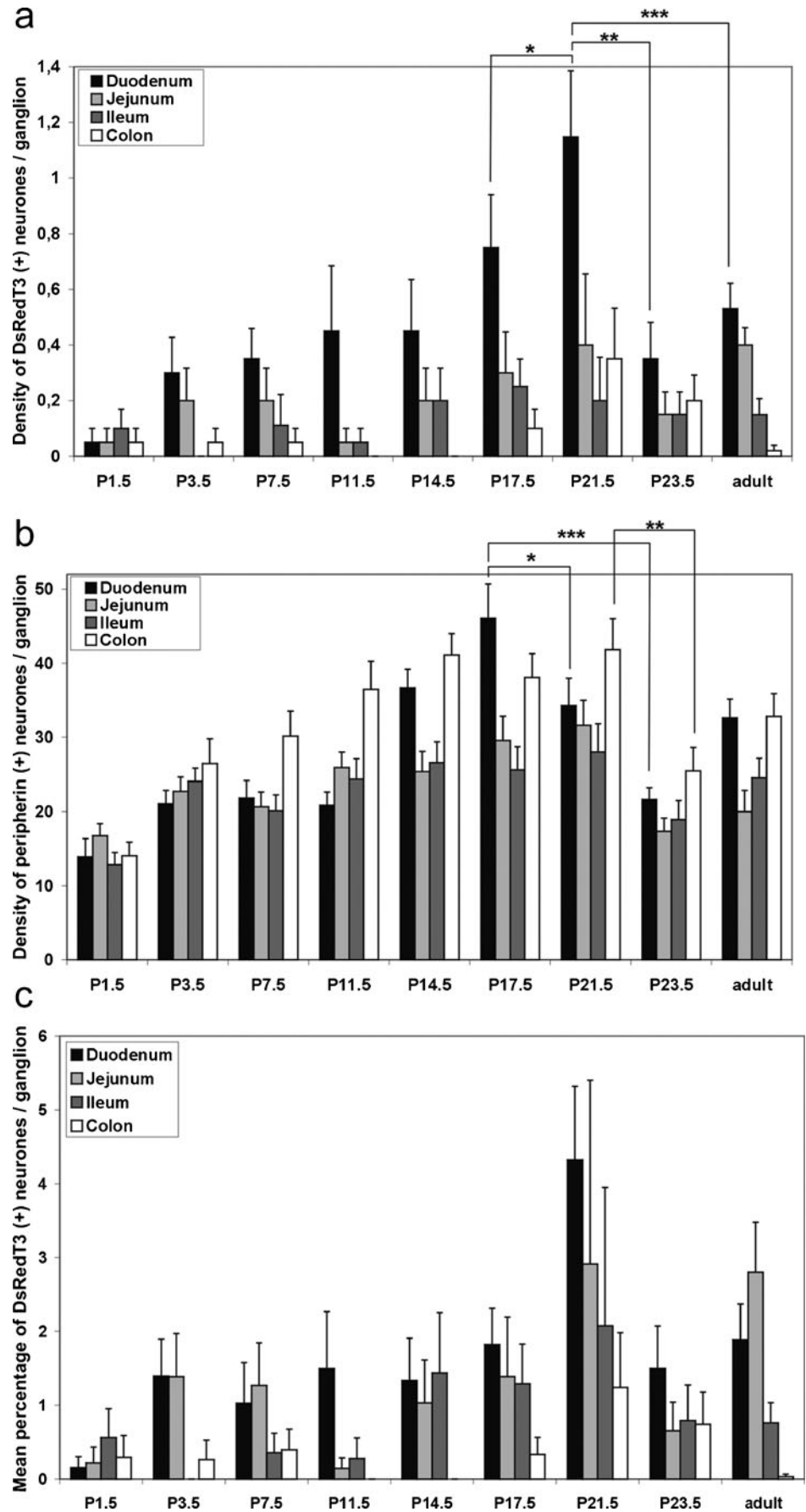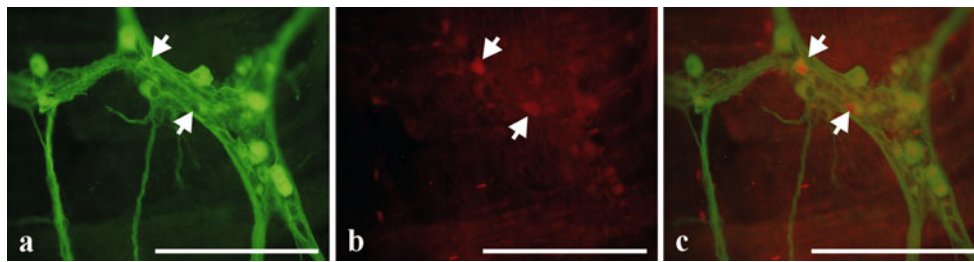
Fig. 4 a–c Photomicrographs of myenteric plexuses exposed in whole-mounts prepared from the duodenum of DsRedT3/CCK mice. **a** Peripherin immunoreactivity (*green*). **b** Native fluorescence of DsRedT3 (*red*). **c** Merged image (*yellowish*). After immunostaining of the whole-mounts with fluorescein-isothiocyanate-labelled anti-peripherin antibody, all the transgene-expressing red cells were immunoreactive to peripherin (*arrows*). *Bars* 1 mm

shown). In situ hybridization was also performed with DsRedT3 antisense riboprobes, when it was coupled with immunocytochemistry and the use of a mouse monoclonal anti-CCK antibody (Fig. 2b). In the vast majority of the transgene-expressing cells, DsRedT3 was revealed by in situ hybridization to be colocalized with CCK mRNA and, similarly to the transgene expression, its expression was limited to several ganglionic cells. However, when in situ hybridization was coupled with immunocytochemistry, not all the CCK-positive cells were labelled with the DsRedT3 antisense riboprobe (Fig. 2b), indicating that the transgene expression is highly specific, but not complete. Several studies on transgenic mice have reported variable levels of transgene expression (Lay et al. 2004; Recillas-Targa et al. 2004; Emery 2011). This is mostly the consequence of the incompleteness of the regulatory region included in the transgene and the effect of the integration site.

The number of myenteric neurones with red fluorescence was limited to 0–4 neurones per ganglion throughout the proximo-distal axis of the gastrointestinal tract on all post-natal days between P1.5 and P23.5 and also in the adult intestines (Fig. 3a). Even though the number of cells was low, the regional specificity was clear. After P3.5, the number of red fluorescent myenteric neurones was always the highest in the duodenum and the lowest in the distal part of the small intestine and the colon. A gradual increase in the number of red cells was documented in all intestinal segments between P1.5 and P21.5, when a sharp increase was noticed. The density of the transgene-expressing red fluorescent-myenteric neurones increased significantly between P17.5 and P21.5; although the increase was transitory, there was a pronounced increase in the duodenum. The cell number then dropped to one-third by P23.5 and was maintained at the same or a similar level in the adults. The relative sizes of the transgene-expressing CCK neurone populations were determined by staining the whole-mounts with FITC-labelled anti-peripherin antibody, when all the transgene-expressing cells proved to be immunoreactive to peripherin (Fig. 4a–c). The regionality and the dynamics in the quantitative changes in the peripherin-immunoreactive neurones were similar to those documented in the case of the transgene-expressing red cells. However, the density of the peripherin-immunoreactive cells reached a maximum on P17.5 and subsequently decreased significantly by day 21.5, i.e. at the time when the transgene-expressing myenteric neurones reached their maximum density (Fig. 3a, b). Accordingly, the ratio of transgene-expressing CCK myenteric neurones between P17.5 and P21.5 was temporarily more than doubled in the duodenum (Fig. 3c).

We think that the decrease in the density of the peripherin-immunoreactive myenteric neurones in this period reflects the age-related neuronal loss, as is consistent
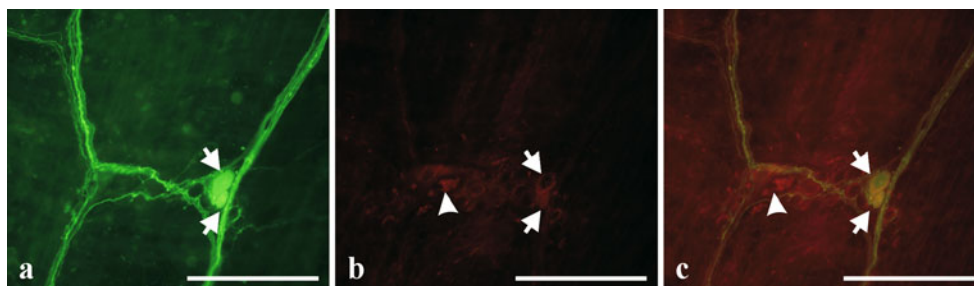

Fig. 5 a–c Photomicrographs of myenteric plexuses exposed in whole-mounts prepared from the duodenum of DsRedT3/CCK mice. **a** 5-HT immunoreactivity (*green*). **b** Native fluorescence of DsRedT3/CCK. **c** Merged image (*yellowish*). After immunostaining of the whole-mounts with 5-HT-specific antibody, the majority of myenteric neurones endogenously expressing DsRedT3 overlapped with 5-HT-immunoreactive neurones (*arrows*), whereas others did not (*arrowhead*). *Bars* 1 mm

with previous reports from various species (Gabella 1989; Santer 1994). However, the sharp increase in CCK neuronal density on P21.5 might be of physiological significance. This is the time of the suckling to weaning transition, when the diet changes from milk to food pellets (Curley et al. 2009), a possible trigger changing the control of feeding behaviour. The quantitative changes presented here lead us to suggest an important role of CCK neurones in this triggering. The peculiar role of CCK in the intestinal function from this particular age on was previously proposed when the ontogeny of neuronal activation by exogenously administered CCK-8 was investigated in the myenteric plexus of the rat duodenum (Washington et al. 2011). Despite the existence of both CCK receptors in 4-, 14-, 21-, and 35-day-old rats, exogenous CCK is only able to activate the myenteric neurones of 21- and 35-day-old rats, indicating a delayed role for these neurones in CCK-mediated gastrointestinal functions. To study neuronal activation, Gulley et al. (2005) chose the duodenum, because this intestinal segment contains the majority of CCK_1 receptors, which mediate the activation of the enteric neurones by CCK. Interestingly enough, in our mice, the duodenum was also the gut segment that contained the highest number of CCK myenteric neurones in all age groups after P3.5 (Fig. 3a). In the past few years, CCK and 5-HT have been demonstrated as synergistically interacting in the peripheral mechanism of feeding control (Raybould et al. 2003; Powley and Phillips 2004; Hayes and Covasa 2006; Hayes et al. 2006). Accordingly, we applied immunohistochemical staining with an antibody specific to 5-HT and found that, at P21.5, nearly all of the transgene-expressing myenteric neurones overlapped with the 5-HT-immunoreactive neurones (Fig. 5a–c).

Confirmation that the coexistence of CCK and 5-HT in these myenteric neurones has functional significance and does indeed contribute to the successful transition from suckling to weaning in mammals awaits further experimental investigations. However, the results of the present work have convinced us that the DsRedT3/CCK mice used in this study can serve as a suitable model for such studies.

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