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Cholecystokinin-8 activates myenteric neurons in 21- and 35-day old but not 4- and 14-day old rats

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ABSTRACT

Cholecystokinin (CCK) activates the myenteric neurons of adult rats. The goal of this work is to determine the ontogeny of this activation by CCK-8 in the myenteric plexus of the duodenum (2 cm immediately following the pyloric sphincter aborally) and compare it with that of the dorsal vagal complex (DVC) – which occurs in 1-day old pups. Despite the existence of both of the CCK receptors, CCK_1 and CCK_2, in 4, 14, 21 and 35 day old rats, CCK-8 (0, 5, 10, 20 and 40 μg/kg, i.p.) increased Fos-like immunoreactivity (Fos-LI, a marker for neuronal activation) in the myenteric neurons of 21- and 35-day old rats but in the DVC of all age groups. As such, this belated activation of myenteric neurons by CCK-8 compared to the DVC may reflect a delayed role for these neurons in CCK-related functions.

1. Introduction

Cholecystokinin (CCK) is a gut-brain peptide secreted by the I cells of the small intestine and evokes physiological responses such as gallbladder contraction, increased exocrine pancreatic secretion and inhibition of gastric emptying. These, as well as other responses by CCK, occur through activation of two G-protein coupled receptors, CCK_1 and CCK_2, which are distributed centrally and peripherally (reviewed in ref. [54]).

In 1973, Gibbs et al. initially reported that CCK-8 reduces food intake in rats [19]. This reduction requires an intact vagus nerve [31], activation of CCK_1 receptor [10,62] and activation of the satiety nuclei in the dorsal vagal complex (DVC), which include area postrema (AP), nucleus tractus solitarius (NTS) and dorsal motor nucleus of the vagus (DMV) [9,12,14].

Because CCK is a gut-brain peptide, we hypothesized that CCK may act first on the enteric nervous system (ENS; myenteric and submucosal plexuses [15]) of the gut, which then activates the DVC through vagal and/or sympathetic-spinal afferents. During the course of evaluating this hypothesis, we have shown that endogenous CCK – released by the non-nutrient trypsin inhibitor camostat [45,64] as well as the long chained fatty acid oleate [20,53] and exogenous CCK-8 [22,55,67] increase Fos-like immunoreactivity (Fos-LI; a marker for neuronal activation) in one or both plexuses in adult rats. The order of this activation was duodenum > jejunum > ileum [20,21,55,58,66]. In addition, we have also shown that oleate and CCK-8 activated nitric oxide synthase-containing neurons in the myenteric plexus (a marker for inhibitory motor neurons) and calbindin and calretinin-containing neurons (a marker for sensory neurons) in the submucosal plexus [53,56,57].

The goal of the current study is to determine the ontogeny of myenteric neuronal activation by CCK-8 and compare it with activation of the dorsal vagal complex by the same peptide. Here, we quantified this activation by counting myenteric and DVC neurons that express Fos-LI in response to supraphysiological doses of CCK-8 (5, 10, 20 and 40 μg/kg) in four age groups of rats, 4-, 14-, 21- and 35-day old. These ages represent early neonatal (4 days), late neonatal (14 days), weaning/young (21 days) and prepubertal (35 days). In addition, it is known that CCK-secreting cells are present at prenatal day 16 in rats [35].

The non-physiological doses of CCK-8 were selected, as others and we have utilized previously [21,22,48,67,68], to insure activation of the neurons in all age groups, and not to evaluate a physiological function. Furthermore, to minimize the discrepancy in tissue sampling between all age groups, we chose the first 2 cm segments of the duodenum immediately following the pyloric sphincter and two levels of the brainstem according to our previous work and the rat brain atlas [38]. This intestinal level contains

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the highest level of CCK_1 receptors [7,29,55,66] and vagal afferents [36,37], which are responsible for carrying the satiety signal to the brain.

Finally, in anticipation of detecting differences in myenteric Fos-LI between the previous age groups, we have also determined density and sizes of myenteric neurons and expressions of CCK_1 and CCK_2 receptors in those age groups. Although, the density of these neurons was evaluated prior to this work, this is the first study that reports expression of CCK receptors in these age groups of rats.

To compare density and sizes of myenteric neurons in a standardized area in the duodenum of these rats, we used a cuprolinic blue staining method. Cuprolinic blue is the Alcian blue analogue copper phthalocyanine dye known as quinolinic phthalocyanine [24,61]. When dissolved in $MgCl_2$, cuprolinic blue binds only to single-stranded RNA and stains neuronal Nissl substance in the myenteric neurons. Therefore, it is a sensitive, selective, specific, and reliable pan-neuronal stain, which has been used frequently in quantitative analysis of myenteric neurons [24,25,40–42].

Finally, we also determined expression of CCK receptors in an area located immediately following the pyloric sphincter (2 cm segments) in all age groups utilizing a real time polymerase chain reaction (rt-PCR) test. This area, contains the highest density of I cells that secrete CCK as well as the majority of the CCK receptors in the gut, particularly CCK_1 receptor [4,6,7,20,27,29,30,33,50,55,66], which mediates activation of the enteric neurons by CCK [20,22,55,66,67]. However, the jejunum was not chosen in this study because developmentally it will be less consistent in structure compared to the duodenum in the different age groups. In addition, it contains significantly fewer CCK receptors and less activation by CCK compared to the duodenum.

2. Materials and methods

The Tuskegee University Animal Care and Use Committee approved the protocols for these studies. Male Sprague–Dawley rats (9–180 g, Harlan, PA) housed individually in wire-mesh cages in a controlled environment (lights on from 0600 to 1800 h and temperature maintained at 21.5 °C) and given free access to water and pelleted rodent chow (Teklad, WI) were used in this study. The pups were chosen randomly from an in house breeding colony of Sprague–Dawley rats (eight females and three males).

To adapt the adult rats (21- and 35-day old) to our laboratory environment and experimental procedures each rat was handled 10 min daily for a total of 5–7days prior to the experiment and given a saline injection intraperitoneally (i.p.). All injections were made in a volume of 0.1–0.5 ml of saline depending on the age of the rat.

2.1. Experiment 1

Activation of myenteric and DVC neurons by CCK-8:

Twenty rats representing each age were divided into five groups ($n = 4$ rats each) were used in this study. Based on previous experiments for maximum Fos expression in neonatal [48,68,69] and adult [20,21,45,66] rats the 4 and 14-day old pups were separated from dams and fasted for 3 h prior to the experiment, while the 21- and 35-day old rats were fasted from food but not water overnight. These periods do not affect gastric emptying or endogenous levels of CCK in these ages. Following fasting, all rats received CCK-8 (Bachem, CA, 5, 10, 20 and 40 μg/kg) or saline vehicle i.p. The choice of doses was based on previous work for producing maximum Fos expression in the brain and intestine [20,21,45,48,66,68].

Ninety minutes following the injections, which is the optimum time for maximum Fos expression based on previous work [20,21,45,48,66,68], all rats were sacrificed with an overdose of sodium pentobarbital (10 mg/kg, i.p.). The rats were then perfused transcardially in two stages: first, with 35–500 ml of Krebs solution, depending on the size of the rat, to collect the intestine and second, with 35–500 ml of 4% formaldehyde solution dissolved in 0.1 M phosphate buffered saline (PBS) to collect and fix the brains *in situ*.

Duodenal wholemounts, 1–2 cm intestinal segments collected immediately caudal to the pyloric sphincter, were peeled under a dissecting microscope and prepared for detection of Fos-LI as described previously [44,66]. Due to their soft consistency, the brainstems of the 4- and 14-day old rats were collected and post-fixed with 4% formaldehyde for 24 h at 4 °C [48,68], while the brainstems of the 21- and 35-day old rats were collected and post-fixed with 4% formaldehyde for 2 h at 4 °C [21,55,67]. Following this step, all brainstems were placed in a 25% sucrose solution overnight at room temperature, sectioned (40 μm thickness) on a cryostat at −20 °C to collect the DVC, blocked with 50% ethyl alcohol for 30 min to reduce the production of free radicals and improve background staining, and processed for detection of Fos-LI.

The wholemounts and DVC sections were incubated in a primary antiserum (Oncogene, Ab-5, San Diego, CA, USA [1:12,500 dilution]) raised in rabbit against the representing amino acids 4–17 of human Fos. After overnight incubation in biotintylated donkey anti-rabbit serum (1:500 dilution, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) the tissues were incubated for 3 h in avidin conjugated to horseradish peroxidase (HRP), after which they were washed and processed to reveal HRP activity using diaminobenzidine (DAB, Sigma, St. Louis, MO, USA) intensified with nickel [44,66].

Consistent with our previous work [44,66], the areas of the DVC included area postrema (AP, −4.5 mm caudal to interaural plane), nucleus tractus solitarius (NTS, −4.5 and −4.8 mm caudal to interaural plane), and the dorsal motor nucleus of the vagus (DMV, −4.5 and −4.8 mm caudal to interaural plane) according to the Rat Brain Atlas [38]. The sections were taken from multiple levels of the NTS to ensure sampling from sites receiving both gastric and intestinal vagal afferent innervation.

Two examiners, blinded to the treatments of the experiment, counted and averaged the counts of Fos-positive neurons in the myenteric neurons in a total area of 0.8 mm^2 (40 ganglia/animal) using Q-Imaging software and a 40× lens. The criteria included only black, rounded, regular nuclei. All large, irregular, faintly stained shapes were considered cell bodies and were not counted. The agreement rate between the two examiners exceeded 98%. In the DVC, Fos positive neurons were counted by an automated computer software (ImagePro Plus, Media Cybernetics) in the AP, NTS, and DMV bilaterally by selecting the areas based on the rat rain atlas [38] and counting Fos.

The counts of Fos-positive neurons in the different age groups were compared using a two-way Analysis of Variance (ANOVA) (dose and age are the two independent variables) followed by the Holm-Sidak test for multiple comparisons. Results are displayed as mean ± SEM and counts were considered significant if $p < 0.05$.

2.2. Experiment 2

2.2.1. Density and sizes of myenteric neurons

Sixteen rats ages 4-, 14-, 21- and 35-days ($n = 4$ per group) were deeply anesthetized and perfused transcardially with 35–500 ml each of Krebs saline solution depending on the size of the rat. Due to the variable length of the gastrointestinal tract in the different age groups, we collected 1–2 cm of the small intestine immediately caudal to the pyloric sphincter from all rats. The intestinal wholemounts of these segments containing the myenteric plexus were prepared as described previously [44,66] and processed for cupro-

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linic blue staining according to a previously published protocol [24,25,40] (provided by Dr. Robert Phillips, Purdue University).

Cuprolinic blue (Quinolinic phthalocyanine, www.polysciences.com, catalog number 17052) stain solution (50 mg cuprolinic blue added to 10 ml 0.05 M sodium acetate buffer containing 1.0 M $MgCl_2 \cdot 6H_2O$) was prepared and filtered through a 50 μm syringe filter. The wholemounts were rinsed five times, 5 min each, with distilled water, submerged in 200 μl of the stain solution, placed on a warmer (40 °C) for 6 h, rinsed with distilled water three times, 5 min each, washed for 1 min with a sodium acetate buffer solution (20.3 g $MgCl_2 \cdot 6H_2O$ added to 100 ml 0.05 M CH_3COONa), mounted on positively charged slides, dehydrated and covered for counting.

Two examiners, blinded to the conditions of the experiment, counted myenteric neurons and measured their diameter in a total area of 0.8 mm^2 using Q-Imaging software and a 40× lens (40 ganglia). The agreement rate between the examiners exceeded 98%.

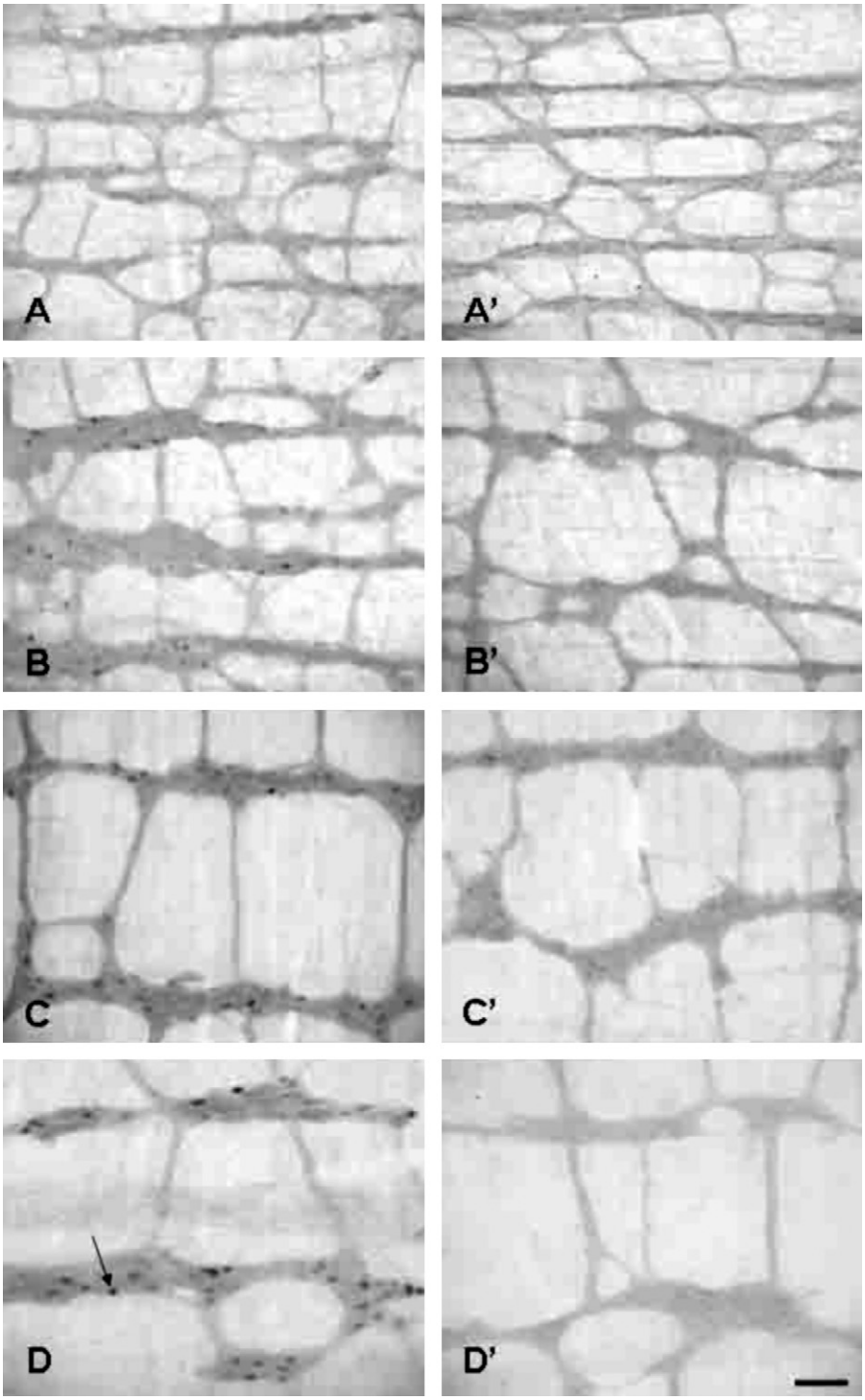

Fig. 1. Photomicrographs of myenteric plexuses activated by cholecystokinin-8 Intestinal sections were collected immediately caudal to the pyloric sphincter from 4-, 14-, 21- and 35-day old rats injected with cholecystokinin-8 (CCK-8; 40 μg/kg, A, B, C and D) or saline (A′, B′, C′ and D′). Wholemounts of these sections that contained the myenteric plexus underwent immunohistochemical detection of Fos-like immunoreactivity (Fos-LI; a marker for neuronal activation) 90 min following the injection for quantification. CCK-8 increased Fos-LI (arrow) in 21- and 35-day old rats (C and D) more than saline (C′ and D′). Scale bar = 200 μm.

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Data were analyzed by a one-way analysis of variance (ANOVA) (age or size as independent variables) followed by the Holm-Sidak test for multiple comparisons.

2.3. Experiment 3

2.3.1. Expression of CCK_1 and CCK_2 receptors in the duodenum of four age groups of rats

2.3.1.1. RNA isolation and reverse transcription. Sixteen rats ages 4-, 14-, 21- and 35-days ($n = 4$ per group) were sacrificed with an overdose of sodium pentobarbital (100 mg/kg, i.p.). From each rat, a 200 mg intestinal segment immediately following the pyloric sphincter was collected and homogenized. Total RNA was isolated using the TRIZOL method (Invitrogen-Life Technologies, Inc., Carlsbad, CA) according to the manufacturer's protocol, and the total RNA concentration was determined by ultraviolet absorbance at 260 nm (DU640, Beckman Coulter, Fullerton, CA). RNA from each sample was assessed for purity by determining the $A_{260/280}$ ratios (ratios of 1.5–2 were used) and the integrity of each sample was assessed by running samples on 2% agarose gels stained with ethidium bromide. All RNA samples were treated with RNase-free DNase (Ambion Inc, CA) to remove residual DNA. Samples were selected based on bright staining of the 18 and 28 s ribosomal bands, with the latter showing twice the concentration. First-strand cDNA was synthesized from 2 μg total RNA using Reaction Ready™ First Strand cDNA Synthesis (Super Array Bioscience Corporation, Frederic, MD).

2.3.2. Quantitative real-time PCR for CCK_1 and CCK_2 receptors

mRNA level was measured in 25 μl reaction mixture containing 12.5 μl RT^2 Real-Time SYBR/Fluorescein Green PCR master mix, 1 μl first strand cDNA, 1 μl RT^2 validated PCR primer sets for CCK_1, CCK_2 (Super Array Bioscience Corporation), and 10.5 μl PCR-grade water (Ambion Inc., CA). Samples were run in 96-well PCR plates (Bio-Rad, Hercules, CA) in duplicates, and the results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPD) housekeeping gene. The amplification protocol was set at 95 °C for 15 min, and 40 cycles each at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s followed by the melting curve determination between 55 and 95 °C to ensure detection of a single PCR product.

Relative difference in receptor expression was compared between the groups using a modification of the delta delta CT method ($\Delta\Delta C_T$) as described previously using a *t*-test [65]. Receptor gene expression was considered significant if the increase/decrease in the expression was equal to or more than two-fold from the baseline expression (control). This method has also been used previously [21].

3. Results

3.1. Activation of myenteric and DVC neurons by CCK-8

Fig. 1 shows Fos-LI in the myenteric plexuses in 4-, 14-, 21- and 35-day rats in response to CCK-8. CCK-8 increased Fos-LI relative to saline ($p < 0.001$, $F = 10.4$, DF = 4), and there was an effect of age ($p < 0.001$, $F = 15.3$, DF = 3) (Fig. 2). CCK-8 (40 μg/kg) increased Fos-LI in 21- and 35-day old ($p < 0.05$) and 20 μg/kg increased it in only 35-day old rats ($p < 0.05$) (Fig. 2).

Fig. 3 depicts Fos-LI in the DVC in four different age groups of rats, 4-, 14-, 21- and 35-day old. In the area postrema, CCK-8 increased Fos-LI ($p < 0.001$, $F = 47.7$, DF = 4) (Fig. 4) but there was no overall effect of age ($p = 0.4$, $F = 0.8$, DF = 3). In 4-day old pups, CCK-8 (20 and 40 μg/kg) increased Fos-LI ($p < 0.05$) and 40 μg increased it more than 20 μg. In 14- and 35-day old rats, CCK-8 (10, 20 and 40 μg /kg) increased Fos-LI significantly relative to saline ($p < 0.05$ for each dose) and 40 μg increased it significantly more than 20 μg in 35-day old rats. In 21-day old rats all doses of CCK-8 increased Fos-LI relative to saline ($p < 0.05$).

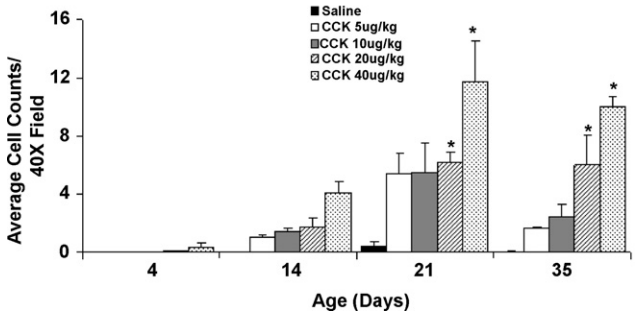

Fig. 2. Activation of myenteric neurons by cholecystokinin-8 Cholecystokinin-8 (CCK-8; 5, 10, 20 and 40 μg/kg,) or saline was given intraperitoneally to 4-, 14-, 21- and 35-day old, rats. Ninety minutes post injection, intestinal sections from each rat were collected immediately caudal to the pyloric sphincter, and the wholemounts of these sections were processed for detection of Fos-like immunoreactivity (Fos-LI). CCK-8 (40 μg/kg) increased Fos-LI in 21- and 35-day old rats (*) and 20 μg/kg increased it in 35-day old rats (*) compared to saline. Lower doses of CCK-8 (5 and 10 μg/kg) failed to increase Fos-LI. Each bar represents four rats.

In the nucleus tractus solitarius (−4.5 mm caudal to the interaural plane), CCK-8 increased Fos-LI ($p < 0.001$, $F = 56.7$, DF = 4) (Fig. 5), and there was no overall effect of age on this response ($p = 0.5$, $F = 0.6$, DF = 3). All ages, except 4-day old rats, expressed Fos-LI in response to all doses of CCK-8. In 4-day old rats CCK-8 (10, 20 and 40 μg/kg) increased Fos-LI.

In the nucleus tractus solitarius (−4.8 mm caudal to the interaural plane), CCK-8 increased Fos-LI ($p < 0.001$, $F = 42.4$, DF = 4), and there was no overall effect of age on this response ($p = 0.1$, $F = 1.6$, DF = 3) (Fig. 5). In 4-day old pups, CCK-8 (20 and 40 μg/kg) increased Fos-LI ($p < 0.05$), in 14- and 35-day old rats, 10, 20 and 40 μg/kg increased Fos-LI ($p < 0.05$); and, in 21-day old rats all doses of CCK-8 increased Fos-LI ($p < 0.05$). CCK-8 (40 μg/kg) increased Fos-LI in the 35-day old rats more than in 14-day old rats.

In the dorsal motor nucleus of the vagus (−4.5 mm caudal to the interaural plane), CCK-8 increased Fos-LI ($p < 0.001$, $F = 26.1$, DF = 4), and there was no overall effect of age on this response ($p = 0.2$, $F = 1.4$, DF = 3) (Fig. 6). In 4-day old rats, CCK-8 (20 and 40 μg/kg) increased Fos-LI ($p < 0.05$), in 14-day old rats CCK-8 (10 and 40 μg/kg) increased Fos-LI ($p < 0.05$), in 21 day old rats CCK-8 (5, 20 and 40 μg/kg) increased Fos-LI ($p < 0.05$) and in 35-day old rats CCK-8 (10, 20 and 40 μg/kg) increased Fos-LI ($p < 0.05$).

In the dorsal motor nucleus of the vagus (−4.8 mm caudal to the interaural plane), CCK-8 increased Fos-LI ($p < 0.001$, $F = 23.7$, DF = 4), and there was an age effect on this response ($p < 0.001$, $F = 11.4$, DF = 3) (Fig. 6). In 4-day old rats, CCK-8 (20 and 40 μg/kg) increased Fos-LI ($p < 0.05$); in 14-day old rats, only 10 μg/kg increased it ($p < 0.05$); in 21-day old rats only 40 μg/kg increased it ($p < 0.05$); and, in 35-day old rats, all doses of CCK-8 increased Fos-LI ($p < 0.05$). In addition, in 35-day old rats, CCK-8 (40 μg/kg) increased Fos-LI more than in 4-day old and 21-day old rats.

3.2. Density and sizes of myenteric neurons

Fig. 7 depicts the myenteric plexuses in 4, 14, 21 and 35-day old rats stained with cuprolinic blue. According to their shape, these neurons are most probably Dogiel type II, with large, rounded cell bodies and generally sensory in function [56,57]. With age, there was a gradual decrease in the number of myenteric neurons (per area, density) ($p < 0.001$, $F = 89.8$, DF = 3, and $p < 0.05$ for each individual comparison) (Fig. 8) and the order of this decrease was 4 > 14 > 21 > 35-day old rats. However, there was a

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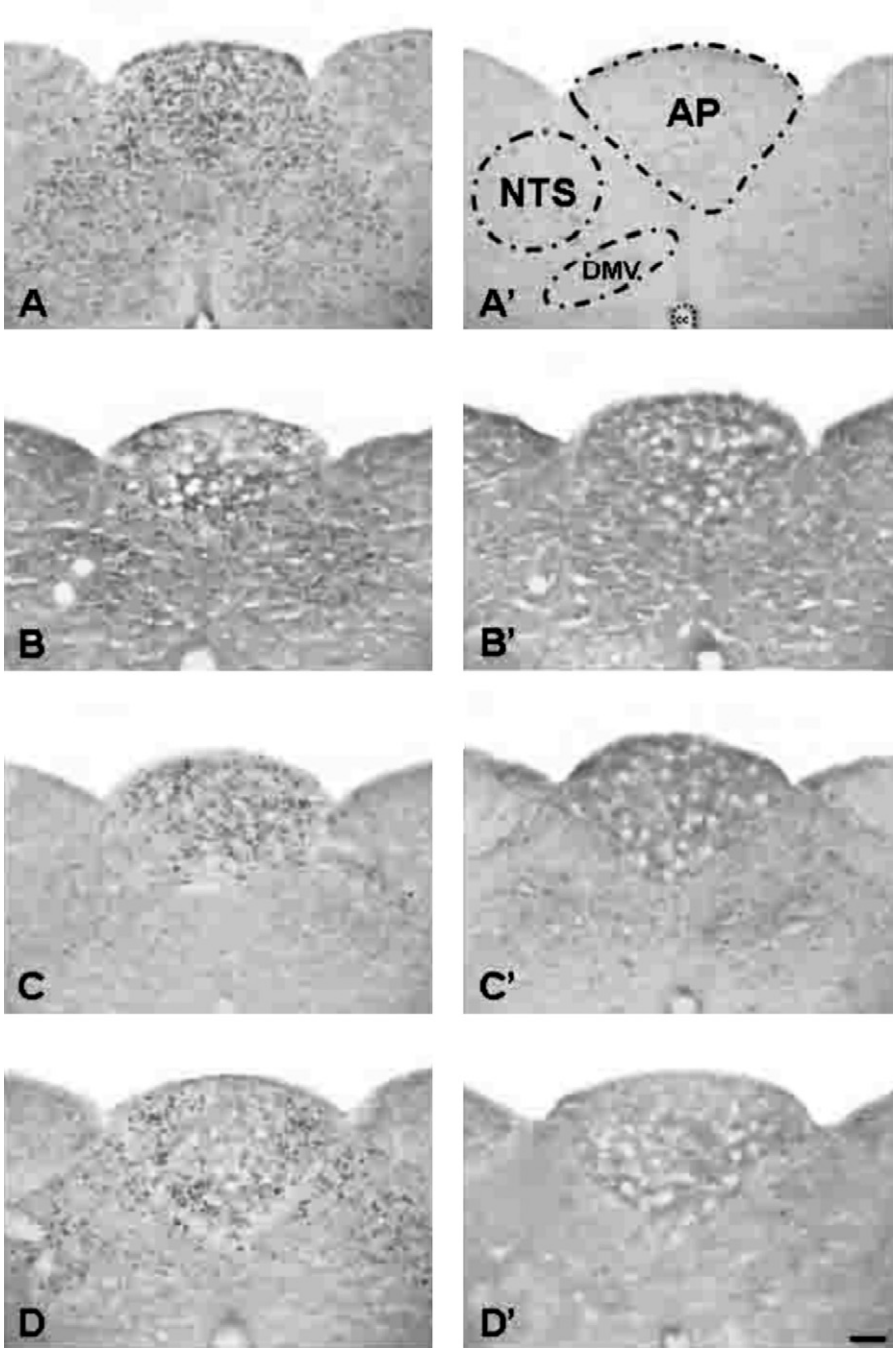

Fig. 3. Photomicrographs of dorsal vagal complex activation by cholecystokinin-8 Cholecystokinin-8 (CCK-8, 40 μg/kg) or saline was injected intraperitoneally in four groups of rats (4, 14, 21 and 35-day old) and the dorsal vagal complexes were processed for immunohistochemical detection of Fos-like immunoreactivity (Fos-LI) 90 min post injection. CCK-8 (A, B, C and D) increased Fos-LI (dark dots in A, B, C and D) in all age groups compared to saline (A′, B′, C′ and D′). (*Abbreviations*: AP: area postrema, NTS: nucleus tractus solitarius, DMV: dorsal motor nucleus of the vagus. Scale Bar: 100 μm.

gradual increase in the size of the myenteric neurons with age and the order of increase was 35 > 21 > 14 > 4-day old rats ($p < 0.001$, $F = 208.9$, DF = 3, $p < 0.05$ for each individual comparison) (Fig. 9).

3.3. Expression of CCK_1 and CCK_2 receptors

CCK_1 and CCK_2 receptors were present in the tested area in all age groups. CCK_1 receptors increased approximately two-fold in 4- and 14-day old rats and ten-fold in 21- and 35-day old rats (Fig. 10), while CCK_2 receptors increased one to two fold in 14- and 35-day old rats (Fig. 10).

4. Discussion

The current work found that CCK-8 activates the myenteric neurons in 21- and 35-day old rats, but not 4- and 14-day old rats. This delayed activation of myenteric neurons by CCK-8 compared to the DVC [48,68], despite using high, non-physiological, doses of the peptide and the existence of both receptors in all age groups, may reflect a delayed role for the myenteric neurons in CCK-related functions compared to the DVC.

In general, the inability of CCK-8 to activate the myenteric neurons in younger rats compared to older rats is related to one or more of the following factors e.g. c-*fos* gene expression, CCK receptors

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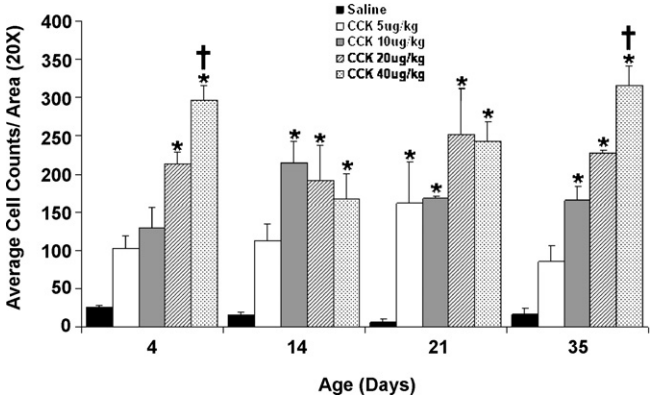

Fig. 4. Activation of the area postrema by cholecystokinin-8 Cholecystokinin-8 (5, 10, 20, 40 μg/kg) or saline were injected in 4-, 14-, 21-, 35-day old rats and Fos expression was quantified in the area postrema (−4.5 mm caudal to the interaural plane) 90 min following the injection. CCK-8 (10 μg/kg) increased Fos-LI more than saline in 14-, 21-, 35-day old rats, 5 μg/kg increased it in 21-day old rats, 20 and 40 μg/kg increased it in all age groups (*). CCK-8 (40 μg/kg) increased Fos-LI in the 4-day old rats more than 14-day old rats, and in the 35-day old rats more than 21-day old rats (†). Each bar represents four rats.

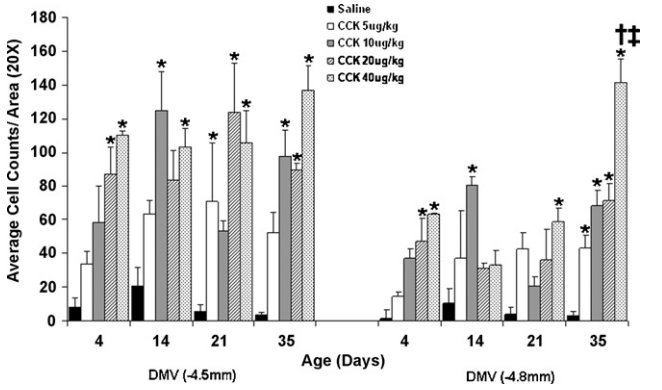

Fig. 6. Activation of the dorsal motor nucleus of the vagus by cholecystokinin-8 Cholecystokinin-8 (5, 10, 20,40 μg/kg) or saline were injected to overnight food deprived 4-, 14-, 21-, 35-day old rats and Fos-like immunoreactivity (Fos-LI) was quantified in the dorsal motor nucleus of the vagus (−4.5 and −4.8 mm caudal to the interaural plane) 90 min following the injection. In the −4.5 mm level, CCK-8 (40 μg/kg) increased Fos-LI in all age groups, 20 μg/kg increased it in all age groups except 14-day old, 10 μg/kg increased it in 14- and 35-day olds and 5 μg/kg increased it only in 21-day old rats (*). In the −4.8 mm level, CCK-8 (40 μg/kg) increased Fos-LI in all age groups except 14-day old rats. 20 μg/kg increased Fos-LI in 4- and 35-day old rats. 10 μg/kg increased Fos-LI in 14- and 35-day old rats and 5 μg/kg increased it in 35-day old rats (*). CCK-8 (40 μg/kg) increased Fos-LI in 35-day old rats more than in 21-, 4- (†) and 14-day old rats (‡). Each bar represents four rats.

or myenteric neurons. First, activation of the myenteric neurons by CCK-8 in 4 and 14-day old pups may involve activating immediate early genes other than c-*fos*. However, other stimuli, e.g. neurotrophin-3, increased this protein in similar age groups [8]. Therefore, *c-fos* can be stimulated in the myenteric neurons of younger rats, but not in response to CCK-8. As such, this possibility cannot explain our findings.

Second, CCK receptors in the gut of 4- and 14-day old rats may be immature, not functional, or located on mucosal or smooth muscle cells rather than on myenteric neurons. However, this study found that both receptors existed in the duodenum of rats from all age groups. In addition, many studies have shown that CCK receptors mediate multiple functions in the digestive system of rats as young as 1-day old, e.g., increased amylase secretion and smooth muscle contraction [23,26,49]. Therefore, since CCK receptors were present, fully developed and fully functional in all ages of rats, this possibility also does not explain our results.

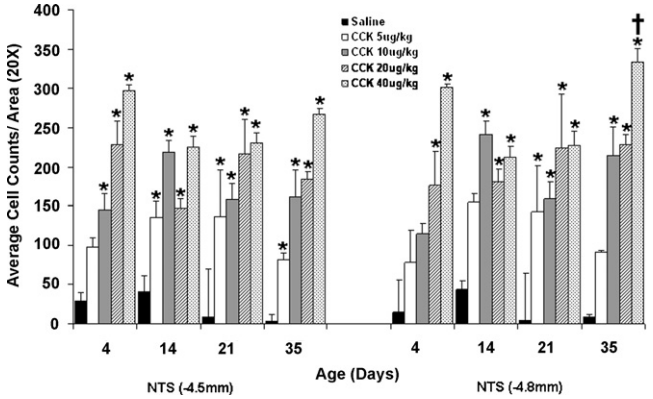

Fig. 5. Activation of the nucleus tractus solitarius by cholecystokinin-8 Cholecystokinin-8 (5, 10, 20, 40 μg/kg) or saline were injected to food deprived 4-, 14-, 21-, 35-day old rats and Fos-like immunoreactivity (Fos-LI) was quantified in the nucleus tractus solitarius (−4.5 and −4.8 mm caudal to the interaural plane) 90 min following the injection. In −4.5 mm, all doses of CCK-8 except 5 μg/kg increased Fos-LI in all age groups more than saline. 5 μg/kg increased Fos expression in all age groups except 4-day old rats groups (*). In −4.8 mm, CCK-8 (40 and 20 μg/kg) increased Fos-LI in all age groups, 10 μg/kg increased it in all age groups except 4-day old rats and 5 μg/kg increased it in 21-day old rats (*). CCK-8 (40 μg/kg) increased Fos expression in 35-day old rats more than 14- and 21-day old rats (†). Each bar represents four rats.

Although there are many sporadic studies that reported expression of one or both of the CCK receptors in whole tissue or enteric neurons in adult rats [34,46,47,49,60,63,64,66], this work represents the first demonstration of expression of both receptors in the duodenum of four different age groups of rats. In addition, due to incompatibility between the wholemount preparation process and the rt-PCR test it is not possible to test the expression of CCK receptors on the enteric neurons in wholemount preparations. Localization of CCK_1 receptor in this area follows the natural distribution of the endocrine I cells that secrete CCK and the majority of activation that we reported previously [1,2,36]. In comparison, the presence of CCK_2 receptor in the stomach parallels its role in mediating regulation of gastric acid secretion and pancreatic and gastric epithelial cell proliferation. The presence of both receptors in the gastrointestinal tract of rats as young as 4-days of age indicates relevance for these receptors.

Third, the myenteric neurons may be immature in young pups, which may explain why CCK-8 failed to activate them in the two younger ages. However, this also is not the case here. The ENS is well-developed and fully functional starting at 1-day of age [5,18,39]. Therefore, similar to the above two explanations, this possibility also cannot explain the inability of CCK-8 to activate the myenteric neurons of the younger rats in our study.

On the other hand, because the ENS consists of two plexuses, myenteric and submucosal, the role of the submucosal plexus in CCK-related functions requires testing. In fact, because the submucosal plexus resides closer to the lumen of the intestine, and therefore in more direct contact with the internal environment of the gut than the myenteric plexus [16], it is possible that submucosal neurons may have role in CCK-related functions at an earlier stage in life more than the myenteric neurons.

Finally, this study found that with age there is increase in the size of myenteric neurons and decrease in their density (number of cell bodies/area). This result is consistent with previous reports which demonstrated an age-dependent decrease in myenteric neuronal numbers in the duodenum of rats (1-, 7- and 14-day old [59]) and other species [17,32,43,51]. This age-related neuronal loss can be explained by two possibilities, method of staining and diet restriction.

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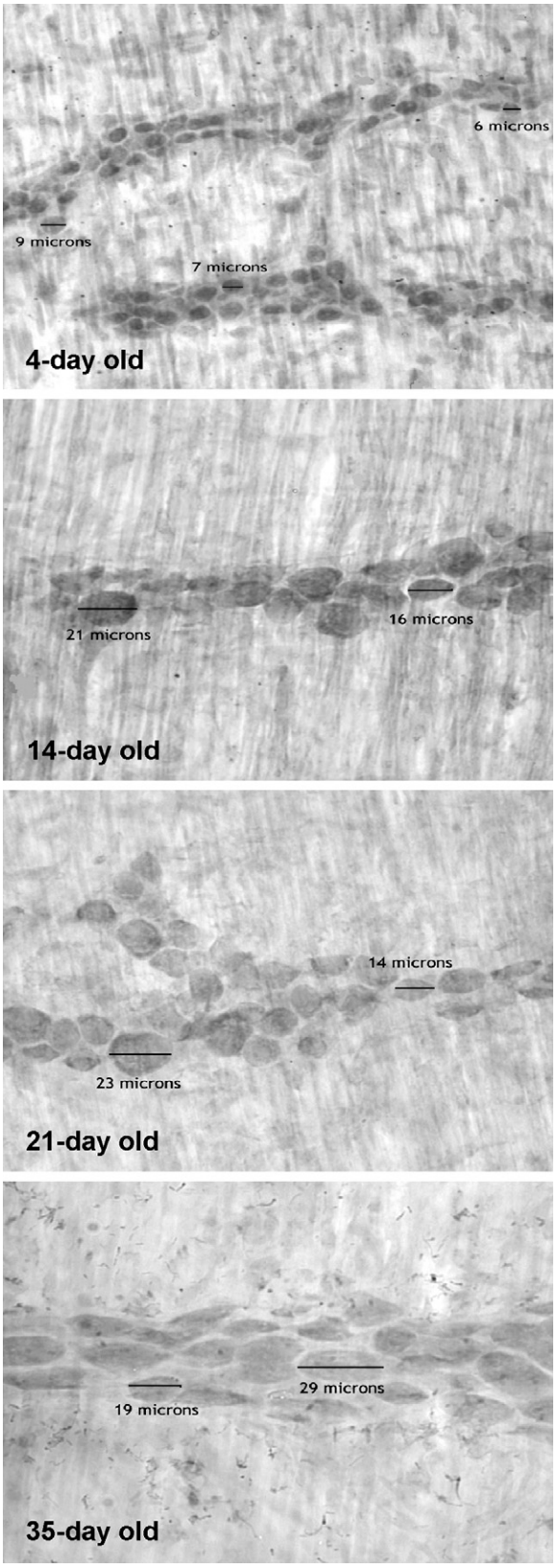

Fig. 7. Photomicrographs of myenteric neurons stained with cuprolinic blue The wholemounts of intestinal sections (1–2 cm each) from 4-, 14-, 21- and 35-day old rats ($n = 4$ rats per group) were collected immediately following the pyloric sphincter and the whole mounts were stained with cuprolinic blue to reveal the cell bodies of the myenteric neurons. With age, the average number of myenteric neurons was decreased, while the average size increased. Sections were counted in 0.8 mm^2 area under 40× magnification.

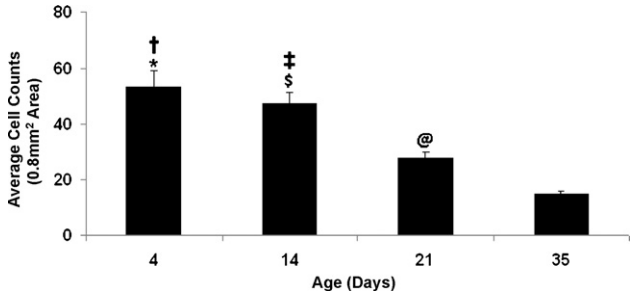

Fig. 8. Density of myenteric neurons Intestinal sections (1–2 cm each) from 4-, 14-, 21- and 35-day old rats ($n = 4$ rats per group) were collected immediately following the pyloric sphincter, and the wholemounts were stained with cuprolinic blue and the myenteric neurons were counted in a 0.8 mm^2 area by two independent examiners using 40× magnification. Counts in 4-day old rats were more than in 14-day old rats (*), 21-day old rats (†) and 35-day old rats (‡). There were more neurons in 14-day old rats compared to 21-day old rats ($) and 35-day old rats (@). There were more neurons in 21-day old rats compared to 35-day old rats (∧). Each bar represents 4 rats.

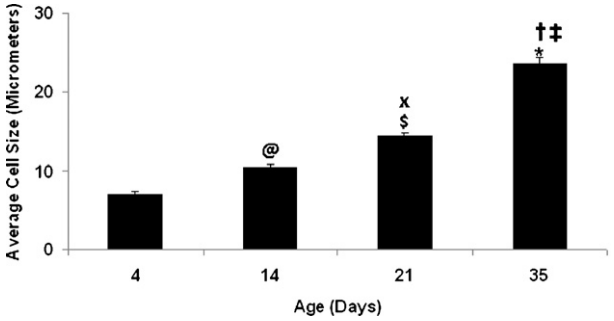

Fig. 9. Size of myenteric neurons Intestinal sections (1–2 cm each) from 4-, 14-, 21- and 35-day old rats were collected immediately following the pyloric sphincter, the wholemounts were stained with cuprolinic blue and the myenteric neurons were measured in a 0.8 mm^2 area by two independent examiners using 40× magnification. The size of myenteric neurons in 35-day old rats was larger than their counterparts in 21-day old (*), 14-day old rats (†) and 4-day old rats (‡), and the myenteric neurons of the 21-day old rats were larger than those in the 14-day old ($) and 4-day old rats (@), and the myenteric neurons in the 14-day old rats were larger than those in the 4-day old rats (∧). Each bar represents 4 rats.

There are three current methods used to stain myenteric neuronal cell bodies; immunohistochemical detection of the neuron specific enolase (NSE) [3], PGP 9.5 protein (a neuronal form of ubiquitin) [13,70] and visualization of single stranded nucleic acid with cuprolinic blue [24]. Many studies compared these techniques and found that staining neuronal cell bodies with cuprolinic blue

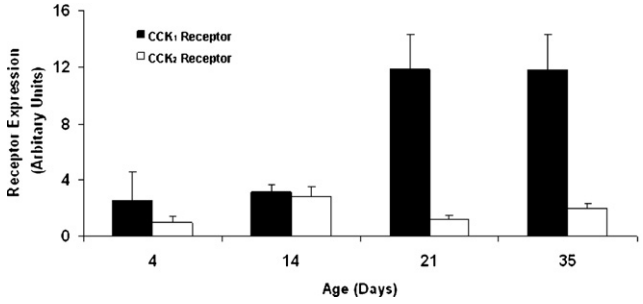

Fig. 10. CCK receptors expression in the duodenum of the rat Intestinal tissue (200 mg) was collected immediately following the pyloric sphincter from 4-, 14-, 21- and 35-day old rats and processed by real time polymerase chain reaction test for detection of CCK_1 and CCK_2 receptors. Both receptors existed in all age groups. The expression of CCK_1 receptors increased approximately two-fold in 4-and 14-day old rats and more than 10-fold in 21-and 35-day old rats. CCK_2 receptors increased approximately two-fold in 14-and 35-day old rats compared to the other age groups. Each bar represents 4 rats.

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is a preferred technique [25,28,40–42]. The previous studies have shown that PGP 9.5 and NSE failed to label 20% or more of the myenteric neurons and they had low staining intensity of the myenteric neurons compared to cuprolinic blue.

The other possibility that explains age-related neuronal loss is diet restriction. Santer et al. reported that malnutrition in pregnant rats causes decrease in the numbers of myenteric neurons [52]. However, Cowen and colleagues found that nutrient restriction, e.g., fat, rescues this loss [11]. The rats in the current work received *ad libitum* food and water. As such, reduction of myenteric neuronal density in the older rats is most probably due to normal aging.

In summary, despite using high doses of CCK-8 and the existence of both receptors in all age groups, unlike in the DVC, CCK-8 was only able to activate the myenteric neurons in the older rats. This delayed activation compared to the DVC may reflect a delayed role for these neurons in CCK-related functions.

Acknowledgments

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References

[1] Berthoud HR, Patterson LM. Anatomical relationship between vagal afferent fibers and CCK-immunoreactive entero-endocrine cells in the rat small intestinal mucosa. Acta Anat (Basel) 1996;156:123–31.
[2] Berthoud HR, Patterson LM, Neumann F, Neuhuber WL. Distribution and structure of vagal afferent intraganglionic laminar endings (IGLEs) in the rat gastrointestinal tract. Anat Embryol (Berl) 1997;195:183–91.
[3] Bishop AE, Carlei F, Lee V, Trojanowski J, Marangos PJ, Dahl D, et al. Combined immunostaining of neurofilaments, neuron specific enolase, GFAP and S-100. A possible means for assessing the morphological and functional status of the enteric nervous system. Histochemistry 1985;82:93–7.
[4] Bourassa J, Laine J, Kruse ML, Gagnon MC, Calvo E, Morisset J. Ontogeny and species differences in the pancreatic expression and localization of the CCK(A) receptors. Biochem Biophys Res Commun 1999;260:820–8.
[5] Branchek TA, Gershon MD. Time course of expression of neuropeptide Y, calcitonin gene-related peptide, and NADPH diaphorase activity in neurons of the developing murine bowel and the appearance of 5-hydroxytryptamine in mucosal enterochromaffin cells. J Comp Neurol 1989;285:262–73.
[6] Brand SJ. The post-natal development of cholecystokinin-like activity in the brain and small intestine of the rat. J Physiol 1982;326:425–33.
[7] Buffa R, Solcia E, Go VL. Immunohistochemical identification of the cholecystokinin cell in the intestinal mucosa. Gastroenterology 1976;70:528–32.
[8] Chalazonitis A, Rothman TP, Chen J, Lamballe F, Barbacid M, Gershon MD. Neurotrophin-3 induces neural crest-derived cells from fetal rat gut to develop in vitro as neurons or glia. J Neurosci 1994;14:6571–84.
[9] Chen DY, Deutsch JA, Gonzalez MF, Gu Y. The induction and suppression of c-fos expression in the rat brain by cholecystokinin and its antagonist L364,718. Neurosci Lett 1993;149:91–4.
[10] Corwin RL, Gibbs J, Smith GP. Increased food intake after type A but not type B cholecystokinin receptor blockade. Physiol Behav 1991;50:255–8.
[11] Cowen T, Johnson RJ, Soubeyre V, Santer RM. Restricted diet rescues rat enteric motor neurones from age related cell death. Gut 2000;47:653–60.
[12] Day HE, McKnight AT, Poat JA, Hughes J. Evidence that cholecystokinin induces immediate early gene expression in the brainstem, hypothalamus and amygdala of the rat by a CCKA receptor mechanism. Neuropharmacology 1994;33:719–27.
[13] Eaker EY, Sallustio JE. The distribution of novel intermediate filament proteins defines subpopulations of myenteric neurons in rat intestine. Gastroenterology 1994;107:666–74.
[14] Fraser KA, Davison JS. Cholecystokinin-induced c-fos expression in the rat brain stem is influenced by vagal nerve integrity. Exp Physiol 1992;77:225–8.
[15] Furness JB. The Enteric Nervous System. Malden, MA, USA: Blackwell Publishing; 2006.
[16] Furness JB, Kunze WA, Clerc N. Nutrient tasting and signaling mechanisms in the gut II. The intestine as a sensory organ: neural, endocrine, and immune responses. Am J Physiol 1999;277:G922–8.
[17] Gabella G. Fall in the number of myenteric neurons in aging guinea pigs. Gastroenterology 1989;96:1487–93.
[18] Gershon MD, Payette RF, Rothman TP. Development of the enteric nervous system. Fed Proc 1983;42:1620–5.
[19] Gibbs J, Young RC, Smith GP. Cholecystokinin decreases food intake in rats. J Comp Physiol Psychol 1973;84:488–95.
[20] Gulley S, Covasa M, Ritter RC, Sayegh AI. Cholecystokinin1 receptors mediate the increase in Fos-like immunoreactivity in the rat myenteric plexus following intestinal oleate infusion. Physiol Behav 2005;86:128–35.
[21] Gulley S, Sharma SK, Mansour M, Sullivan CN, Moran TH, Sayegh AI. Strain differences in myenteric neuron number and CCK(1) receptor mRNA expression may account for differences in CCK induced c-Fos activation. Brain Res 2005;1058:109–19.
[22] Gulley S, Sharma SK, Moran TH, Sayegh AI. Cholecystokinin-8 increases Fos-like immunoreactivity in the brainstem and myenteric neurons of rats through CCK1 receptors. Peptides 2005;26:1617–22.
[23] Harada E, Syuto B. Precocious alteration of digestive enzyme activities in small intestine and pancreas by chronic oral administration of protease inhibitor in suckling rats. Comp Biochem Physiol A Comp Physiol 1991;100:467–72.
[24] Heinicke EA, Kiernan JA, Wijsman J. Specific, selective, and complete staining of neurons of the myenteric plexus, using cuprolinic blue. J Neurosci Methods 1987;21:45–54.
[25] Holst MC, Powley TL. Cuprolinic blue (quinolinic phthalocyanine) counterstaining of enteric neurons for peroxidase immunocytochemistry. J Neurosci Methods 1995;62:121–7.
[26] Ichihara K, Eng J, Yalow RS. Ontogeny of molecular forms of CCK-peptides in rat brain and gut. Life Sci 1984;34:93–8.
[27] Johnson Jr EM, Cantor E, Douglas Jr JR. Biochemical and functional evaluation of the sympathectomy produced by the administration of guanethidine to newborn rats. J Pharmacol Exp Ther 1975;193:503–12.
[28] Karaosmanoglu T, Aygun B, Wade PR, Gershon MD. Regional differences in the number of neurons in the myenteric plexus of the guinea pig small intestine and colon: an evaluation of markers used to count neurons. Anat Rec 1996;244:470–80.
[29] Larsson LI, Rehfeld JF. Distribution of gastrin and CCK cells in the rat gastrointestinal tract. Evidence for the occurrence of three distinct cell types storing COOH-terminal gastrin immunoreactivity. Histochemistry 1978;58:23–31.
[30] Leung YK, Lee PC, Lebenthal E. Maturation of cholecystokinin receptors in pancreatic acini of rats. Am J Physiol 1986;250:G594–7.
[31] Lorenz DN, Goldman SA. Vagal mediation of the cholecystokinin satiety effect in rats. Physiol Behav 1982;29:599–604.
[32] Meciano Filho J, Carvalho VC, de Souza RR. Nerve cell loss in the myenteric plexus of the human esophagus in relation to age: a preliminary investigation. Gerontology 1995;41:18–21.
[33] Miyasaka K, Ohta M, Masuda M, Kawanami T, Matsumoto M, Funakoshi A. Sex difference in gene expressions of cholecystokinin (CCK) and CCK receptor in young and old rats. Arch Gerontol Geriatr 1995;21:157–65.
[34] Monstein HJ, Nylander AG, Salehi A, Chen D, Lundquist I, Hakanson R. Cholecystokinin-A and cholecystokinin-B/gastrin receptor mRNA expression in the gastrointestinal tract and pancreas of the rat and man A polymerase chain reaction study. Scand J Gastroenterol 1996;31:383–90.
[35] Ono E, Doi Y, Furukawa H, Hirata K, Fujimoto S. The differentiation of entero-endocrine cells of pre- and postnatal rats: light and electron microscopy and immunocytochemistry. Acta Anat (Basel) 1994;149:81–8.
[36] Patterson LM, Zheng H, Ward SM, Berthoud HR. Immunohistochemical identification of cholecystokinin A receptors on interstitial cells of Cajal, smooth muscle, and enteric neurons in rat pylorus. Cell Tissue Res 2001;305:11–23.
[37] Patterson LM, Zheng H, Ward SM, Berthoud HR. Vanilloid receptor (VR1) expression in vagal afferent neurons innervating the gastrointestinal tract. Cell Tissue Res 2003;311:277–87.
[38] Paxinos G, Watson C. The rat brain in sterotaxic coordinates. San Diego: Academic Press; 1997.
[39] Pham TD, Gershon MD, Rothman TP. Time of origin of neurons in the murine enteric nervous system: sequence in relation to phenotype. J Comp Neurol 1991;314:789–98.
[40] Phillips RJ, Hargrave SL, Rhodes BS, Zopf DA, Powley TL. Quantification of neurons in the myenteric plexus: an evaluation of putative pan-neuronal markers. J Neurosci Methods 2004;133:99–107.
[41] Phillips RJ, Kieffer EJ, Powley TL. Aging of the myenteric plexus: neuronal loss is specific to cholinergic neurons. Auton Neurosci 2003;106:69–83.
[42] Phillips RJ, Kieffer EJ, Powley TL. Loss of glia and neurons in the myenteric plexus of the aged Fischer 344 rat. Anat Embryol (Berl) 2004;209:19–30.
[43] Phillips RJ, Powley TL. As the gut ages: timetables for aging of innervation vary by organ in the Fischer 344 rat. J Comp Neurol 2001;434:358–77.
[44] Raboin SJ, Gulley S, Henley SC, Chan WC, Esdaile AR, Jackson CA, et al. Effect of sympathectomy and demedullation on increased myenteric and dorsal vagal complex Fos-like immunoreactivity by cholecystokinin-8. Regul Pept 2006;134:141–8.
[45] Raboin SJ, Reeve Jr JR, Cooper MS, Green GM, Sayegh AI. Activation of submucosal but not myenteric plexus of the gastrointestinal tract accompanies reduction of food intake by camostat. Regul Pept 2008;150:73–80.
[46] Reubi JC, Schaer JC, Waser B. Cholecystokinin(CCK)-A and CCK-B/gastrin receptors in human tumors. Cancer Res 1997;57:1377–86.
[47] Reubi JC, Waser B, Laderach U, Stettler C, Friess H, Halter F, et al. Localization of cholecystokinin A and cholecystokinin B-gastrin receptors in the human stomach. Gastroenterology 1997;112:1197–205.
[48] Rinaman L, Hoffman GE, Stricker EM, Verbalis JG. Exogenous cholecystokinin activates cFos expression in medullary but not hypothalamic neurons in neonatal rats. Brain Res Dev Brain Res 1994;77:140–5.
[49] Robinson PH, Moran TH, Goldrich M, McHugh PR. Development of cholecystokinin binding sites in rat upper gastrointestinal tract. Am J Physiol 1987;252:G529–34.
[50] Sankaran H, Goldfine ID, Deveney CW, Wong KY, Williams JA. Binding of cholecystokinin to high affinity receptors on isolated rat pancreatic acini. J Biol Chem 1980;255:1849–53.

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[51] Santer RM. Survival of the population of NADPH-diaphorase stained myenteric neurons in the small intestine of aged rats. J Auton Nerv Syst 1994;49:115–21.
[52] Santer RM, Conboy VB. Prenatal undernutrition permanently decreases enteric neuron number and sympathetic innervation of Auerbach's plexus in the rat. J Anat 1990;168:57–62.
[53] Sayegh AI, Covasa M, Ritter RC. Intestinal infusions of oleate and glucose activate distinct enteric neurons in the rat. Autonom Neurosci 2004;115: 54–63.
[54] Sayegh AI, Reeve Jr JR, Lampley ST, Hart B, Gulley S, Esdaile AR, et al. Role for the enteric nervous system in the regulation of satiety via cholecystokinin-8. J Am Vet Med Assoc 2005;226:1809–16.
[55] Sayegh AI, Ritter RC. CCK-A receptor activation induces fos expression in myenteric neurons of rat small intestine. Regul Pept 2000;88:75–81.
[56] Sayegh AI, Ritter RC. Cholecystokinin activates specific enteric neurons in the rat small intestine. Peptides 2003;24:237–44.
[57] Sayegh AI, Ritter RC. Morphology and distribution of nitric oxide synthase-, neurokinin-1 receptor-, calretinin-, calbindin-, and neurofilament-M-immunoreactive neurons in the myenteric and submucosal plexuses of the rat small intestine. Anat Rec A Discov Mol Cell Evol Biol 2003;271:209–16.
[58] Sayegh AI, Ritter RC. Vagus nerve participates in CCK-induced Fos expression in hindbrain but not myenteric plexus. Brain Res 2000;878:155–62.
[59] Schafer KH, Hansgen A, Mestres P. Morphological changes of the myenteric plexus during early postnatal development of the rat. Anat Rec 1999;256: 20–8.
[60] Schwartz GJ, Moran TH, McHugh PR. Autoradiographic and functional development of gastric cholecystokinin receptors in the rat. Peptides 1990;11:1199–203.
[61] Scott JE. Histochemistry of Alcian blue 3. The molecular biological basis of staining by Alcian blue 8GX and analogous phthalocyanins. Histochemie 1972;32:191–212.
[62] Smith GP, Tyrka A, Gibbs J. Type-A CCK receptors mediate the inhibition of food intake and activity by CCK-8 in 9- to 12-day-old rat pups. Pharmacol Biochem Behav 1991;38:207–10.
[63] Sternini C, Wong H, Pham T, De Giorgio R, Miller LJ, Kuntz SM, et al. Expression of cholecystokinin A receptors in neurons innervating the rat stomach and intestine. Gastroenterology 1999;117:1136–46.
[64] Sullivan CN, Raboin SJ, Gulley S, Sinzobahamvya NT, Green GM, Reeve Jr JR, et al. Endogenous cholecystokinin reduces food intake and increases Fos-like immunoreactivity in the dorsal vagal complex but not in the myenteric plexus by CCK1 receptor in the adult rat. Am J Physiol Regul Integr Comp Physiol 2007;292:R1071–80.
[65] Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genom Biol 2002;3 (RESEARCH0034).
[66] Webb T, Gulley S, Esdaile AR, Pruitt F, Sharma SK, Williams CS, et al. Effects of cholecystokinin-receptor antagonists on Fos-like immunoreactivity stimulated by sulfated cholecystokinin-8 in neurons of the myenteric plexus and hindbrain of rats. Am J Vet Res 2005;66:1308–13.
[67] Webb T, Gulley S, Pruitt F, Esdaile AR, Sharma SK, Cox JE, et al. Cholecystokinin-8 increases Fos-like immunoreactivity in myenteric neurons of the duodenum and jejunum more after intraperitoneal than after intravenous injection. Neurosci Lett 2005;389:157–62.
[68] Weller A. The ontogeny of postingestive inhibitory stimuli: examining the role of CCK. Dev Psychobiol 2006;48:368–79.
[69] Weller A, Smith GP, Gibbs J. Endogenous cholecystokinin reduces feeding in young rats. Science 1990;247:1589–91.
[70] Wilkinson KD, Lee KM, Deshpande S, Duerksen-Hughes P, Boss JM, Pohl J. The neuron-specific protein PGP 9.5 is a ubiquitin carboxyl-terminal hydrolase. Science 1989;246:670–3.

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