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Brain Research 1058 (2005) 109 – 119
BRAIN RESEARCH
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Research Report

Strain differences in myenteric neuron number and CCK_1 receptor mRNA expression may account for differences in CCK induced c-Fos activation

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Accepted 28 July 2005
Available online 19 September 2005

Abstract

We utilized a diaminobenzidine reaction enhanced with nickel to compare dorsal vagal complex (DVC) and myenteric neuronal Fos-Like immunoreactivity (Fos-LI), in response to sulfated cholecystokinin-8 (CCK-8) (5, 10, 20, 40 μg/kg), among Sprague–Dawley (SD), Standard Long–Evans (SLE), Otsuka Long-Evans Tokushima Fatty (OLETF), and Long–Evans Tokushima Otsuka (LETO) rats. All rat strains but OLETF expressed Fos-LI in response to CCK-8. In addition, SD rats expressed more Fos-LI in the area postrema and myenteric neurons than SLE and LETO rats. To investigate the basis for these differences, we utilized cuprolinic blue staining, which stains neuronal cell bodies, to quantify the number of myenteric neurons, and a reverse transcriptase chain polymerase reaction to measure the gene expression of CCK_1 receptor in the gut. We found that SD rats have significantly more duodenal myenteric neurons than the other strains. In addition, this strain expressed significantly higher levels of the CCK_1 gene in both the duodenum and jejunum than the other strains. In conclusion, SD rats may express more myenteric Fos-LI in response to CCK due to increased numbers of myenteric neurons or more intestinal CCK_1 receptors than the other strains of rats.

Theme: Endocrine and autonomic regulation
Topic: Neuroendocrine regulation

Keywords: Sprague–Dawley; Long–Evans; LETO; OLETF; Fos; Myenteric plexus; Dorsal vagal complex; Cholecystokinin; CCK_1 receptor; NTS; Area postrema; Immunohistochemistry

1. Introduction

Cholecystokinin (CCK) (reviewed recently in [14]) is a hormone secreted by the I cells of the gastrointestinal (GI) tract, and neurons in the central and enteric nervous systems (CNS and ENS, respectively). CCK plays a major role in the digestive process, evoking functions such as gallbladder contraction, the inhibition of gastric emptying, the stimulation of pancreatic secretion, and satiety. The actions of CCK are mediated through its interactions with two G-protein coupled receptors, CCK_1 and CCK_2. These receptors are widely distributed throughout the GI tract and central and peripheral neurons.

Recently, a line of rats lacking the CCK_1 receptor has been identified. From a spontaneous occurrence of diabetes and obesity in an outbred colony of Long–Evans rats, a line of rats that is characterized by diabetes, polyuria, polydipsia, and obesity was produced by selective mating. These rats are known as Otsuka Long–Evans Tokushima Fatty (OLETF) rats. These rats show increased rates of weight gain, hyperglycemia, and non-insulin dependent diabetes mellitus [5]. A control strain of rats, known as Long–Evans Tokushima Otsuka (LETO) does not show any of the clinical signs discussed above and these have been considered normal Long–Evans rats.

In characterizing the pancreatic function of OLETF rats, it was noted that they did not respond to exogenous CCK [1]. This led to the demonstration that these rats lack CCK_1 receptors because of a 6.8 kb deletion in their CCK_1 receptor

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doi:10.1016/j.brainres.2005.07.074

gene, a deletion that spans the promoter region and the first and second exons. This deletion results in the absence of a functional CCK_1 receptor. Due to this mutation, the OLETF rats provide a useful model for studying the various functions of CCK.

To demonstrate a possible role for the ENS in physiological responses evoked by CCK-8, we provided the following data [2,3,8–15,17,18]. Exogenous CCK-8 and endogenous CCK, secreted in response to intestinal nutrient infusion of oleate, increased Fos-Like immunoreactivity (Fos-LI), a marker for neuronal activation [7], in the myenteric neurons of the rat small intestine and the dorsal vagal complex (DVC), which also receive afferent input from the vagus nerve, the main ex-trinsic innervation of the gastrointestinal (GI) tract. Increased DVC, but not myenteric, Fos-LI, was vagaly dependent.

Furthermore, in studies characterizing the CCK receptor subtype underlying the ability of CCK to activate hindbrain and enteric neurons, we demonstrated what appeared to be a strain difference in the responsiveness of enteric neurons to CCK. Utilizing Fos-LI as a marker of neuronal activation, we found that although CCK potently induced c-Fos expression in the enteric nervous system of Sprague–Dawley rats, 40 μg/kg, a supraphysiological dose of CCK, was the only dose that increased Fos-LI in the LETO rats. Since there were a number of differences in the design of the studies, we could not be sure whether the difference was age related (6–9 weeks LETO rats may not have a fully functional CCK_1 receptor in their myenteric neurons) or whether there was something specific about the LETO control strain in the distribution of their CCK_1 receptors. The current study was designed to investigate the reasons for this apparent strain difference. We compared Fos-LI in the myenteric and brainstem neurons in response to CCK-8 in four strains of 14–20 week old rats—Sprague–Dawley (SD), LETO, OLETF, and Standard Long–Evans rats (SLE).

2. Materials and methods

2.1. Animals

We used 47 male rats from each strain. Weights ranged between 280 and 380 g. Rats were housed singly in wire-mesh cages, in a controlled environment (lights were on from 0600 to 1800 and temperature was maintained at 21.5 °C). Rats had ad libitum access to water and pelleted rodent chow (Teklad, WI). To enhance adaptation to the laboratory, we handled each rat for 10 min/day for the first 7 days.

2.2. Experimental procedures

Rats from each of the tested strains were assigned to one of 5 treatment groups: saline ($n = 5$), 5 ($n = 9$), 10 ($n = 8$), 20 ($n = 16$), and 40 ($n = 9$) μg/kg CCK-8, and deprived of food, but not water, beginning at 6:00 PM on the day prior to an experiment. At 8:00 AM, the rats received an IP injection of 0.5 ml saline or 1 of 4 doses of CCK-8 (μg/kg, Bachem, CA): 5, 10, 20, 40.

Ninety minutes after the IP injection, rats were anesthetized with sodium pentobarbital (10 mg/kg, IP) and perfused transcardially in two stages. First, the rats were perfused with 500 ml of Krebs solution (Krebs saline formula in mM: 119 NaCl, 4.7 KCl, 1.2 $NaH_2PO_4 \cdot H_2O$, 25 $NaHCO_3$, 1.2 $MgSO_4$, 11.1 glucose, and 1 M $CaCl_2$.) to collect the small intestine, and second with 500 ml of 4% formaldehyde (made in 0.1 M phosphate buffered saline [PBS]) to collect the brainstems. The small intestine was exposed through a midline abdominal incision and the duodenum and jejunum were collected. On the basis of our previous experiments [2,3,8–15,17,18], the duodenal sample was 5–10 cm aborad from the pylorus and the jejunal sample was 20–25 cm aborad from the pylorus. The lumens of the removed segments were rinsed with Krebs solution, and the segments were placed in Sylgard-coated Petri dishes (World Precision Instruments, FL), opened along the mesenteric attachment, stretched and pinned with the mucosal side up, and stored overnight in Zamboni's fixative at 4 °C.

On the next day, the tissues were unpinned and cleared three times in 100% dimethyl sulfoxide (DMSO), 10 min each time, followed by three 10-min rinses with 0.1 M PBS, pH 7.4. Whole mounts (approximately 1 cm^2) of longitudinal smooth muscle with adhering myenteric plexus from the duodenum, jejunum, and ileum were prepared using a dissecting microscope and processed for immunohistochemical detection of Fos-LI.

After collection, the brainstems were postfixed with 4% formaldehyde for 2 h, and placed in 25% sucrose overnight at room temperature. The brainstems were sectioned at 40 μm on a cryostat at −20 °C. Consistent with previous work done in Sprague–Dawley rats, the areas cut included the following levels according to the Paxinos and Watson rat brain atlas [6]: the area postrema (AP, −4.5 mm caudal to interaural plane), nucleus of the solitary tract (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). Sections were taken at multiple levels of the NTS to insure sampling from sites receiving both gastric and intestinal vagal afferent innervation.

2.3. Immunohistochemistry

Based on our previous methods [3,8–15,18], the whole mount preparations and brainstem sections were incubated for 24 h at room temperature in primary antiserum raised in rabbit against a peptide representing amino acids 4–17 of human Fos (Oncogene, Ab-5, San Diego, CA, [1:12,000 dilution]). After a subsequent overnight incubation in biotintylated donkey anti-rabbit serum (Jackson Immuno-Research Laboratories, PA [1:500 dilution]), the tissues

were incubated for 3 h in avidin conjugated to horseradish peroxidase (HRP) then washed with 0.01 M tris PBS and processed to reveal HRP activity using diaminobenzidine (DAB, Sigma, MO) intensified with nickel.

2.4. Counting procedure

Two observers, blinded to the treatments, counted fos positive cells from all of the sections. The fos positive cells at each intestinal level (duodenum and jejunum) of every animal were counted. The final count for each intestinal level represents the average from 10 non-overlapping, 40× microscopic fields. An automated computer software (ImagePro Plus, Media Cybernetics) was utilized to count Fos-LI in the brainstem sections. Fos-positive cells within the AP, NTS, and DMV were counted at the −4.5 mm caudal to interaural plane, and within the NTS and DMV were counted at the −4.8 mm caudal to interaural plane according to the rat brain atlas [6].

2.5. Cuprolinic blue staining

Duodenum and jejunum whole mounts were obtained from 16 untreated rats (n = 4 rats per strain). All whole mounts underwent cuprolinic blue staining according to the protocol of Holst, Phillips, and Powley [4], and the stained cell bodies were counted in 10, non-overlapping, 40× fields.

2.6. Reverse transcriptase polymerase chain reaction (RT-PCR)

Approximately 200 mg of fresh whole, tissues from the duodenum and jejunum of 12 animals (n = 3 rats per strain) was utilized. RNA isolation was done immediately after tissue collection, homogenization, and cleaning with Trizol (Invitrogen-Life Technologies, Inc., Carlsbad, CA). The RNA was isolated using the TRIZOL method (Invitrogen-Life Technologies, Inc., Carlsbad, CA) according to the manufacture's protocol.

Total RNA concentration was determined by ultraviolet absorbance at 260 nm. RNA from each sample was assessed for purity by determining the $A_{260/280}$ ratios (ratios of 1.5 to 2 were used) and the integrity of each sample was assessed by running samples on 2% agarose gels stained with ethidium bromide. Samples were selected based on bright staining of the 18 s and 28 s ribosomal bands with the latter showing twice the concentration.

First-strand cDNA was synthesized from 2 μg total RNA using RETROscript reverse transcriptase Protocol (Ambion, Inc., Austin, TX) according to the manufacturer's protocol.

The oligonucleotide primers used for amplification of CCK_1 receptor were designed from previously published CCK_1 receptor sequence in rats (Accession number NM_012688) using MacVector 7.0 software. The sequences of the primers for CCK_1 were as follows: 5′-TGA ACT CGG ACT GGA AAA TGA GAC-3′ for the forward primer and 5′-GCA TAG CGT CAC TTG GCA ACA G-3′ for the reverse primer. The primers were synthesized by Integrated DNA Technology (Coralville, IA). The expected amplification product size was 563 bp. Total RNA from known positive control duodenal tissue that expresses CCK_1 receptor was included. Negative control reaction that contained all the PCR components, except the target cDNA, was included in each PCR assay. Positive control primers for amplification of a conserved, constitutively expressed mRNA (*rig/S15*, ribosomal subunit protein) were used as a control for PCR efficiency (RETRO script kit, Ambion Inc., Austin, TX) and in the concurrent amplification of target template (CCK_1 receptor) and *rig/S15* gene.

The primers for the *S15* internal control gene are (5′-ttc cgc aag ttc acc tac c-3′) for the forward primer and (5′-cgg gcc ggc cat gct tta cg-3′) for the reverse primer and the expected product size is 361 bp. An aliquot of 5 μl of cDNA product was used for PCR which was performed on a Robocycler (Stratagene, La Jolla, CA) in 50-μl reaction volume containing 1× *Taq* DNA reaction buffer, 1.5 mM $MgCl_2$, 0.125 mM of each dNTP, 0.5 μM of each primer, and 1 unit of Super *Taq* DNA polymerase (Ambion). The PCR conditions included an initial cycle of 95 °C for 3 min, followed by 30 cycles each at 95 °C for 30 s (denaturing), 56 °C for 30 s (annealing), and 72 °C for 40 s (extension). The final extension step was executed at 72 °C for 7 min. The PCR final products were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining and UV illumination using Flour-s Imager (Bio-Rad). PCR markers (Promega) were included in the gel for determination of product size.

The band intensities for CCK_1 receptor density were determined for each animal by Quantity one software (Bio-Rad) and normalized to those of ribosomal S15 housekeeping gene.

2.7. Statistical analysis

To compare the counts of Fos-LI in the different brainstem levels and the myenteric neurons in response to the various doses of CCK-8, we performed two-way ANOVAs (four strain—by five CCK-8 doses) for each brain and intestinal site. To compare the numbers of cell bodies, which are stained by cuprolinic blue, in the four strains of rats, we performed a one-way ANOVA (strain—two intestinal levels). To compare the level of CCK_1 gene expression among the various strains of rats, we performed a one way ANOVA at each intestinal level.

All post hoc analyses were done with Bonferroni t test. Differences were considered significant at $P < 0.05$. All summary data are given as mean ± SEM.

3. Results

3.1. Intestine

3.1.1. Duodenum

Two-way ANOVA revealed significant effects of strain ($F(3) = 197.549$, $P < 0.001$), and treatment ($F(4) = 47.769$, $P < 0.001$). Also there was a significant strain by treatment interaction ($F(12) = 22.775$, $P < 0.001$). CCK induced significant increases in Fos-LI in SD, SLE, and LETO rats but not in OLETF rats. In response to 5, 10, and 40 μg/kg CCK, Fos-LI was increased in the SD rats more than SLE ($P < 0.001$ for each comparison) and LETO rats ($P < 0.001$ for each comparison). Twenty μg/kg CCK increased Fos-LI more in the SD rats than the LETO rats ($P < 0.001$), but not more than in the SLE rats ($P = 0.270$). In addition, there was a significant difference between the SLE and LETO rats at this dose ($P = 0.008$). Finally, there was no significant difference in the counts of Fos-LI neurons in response to 40 μg/kg CCK between the SLE and the LETO rats (Fig. 1).

3.2. Jejunum

Two-way ANOVA revealed a significant effects of strain ($F(3) = 63.341$, $P < 0.001$), and treatment ($F(4) = 27.877$, $P < 0.001$), and a significant strain by treatment interaction ($F(12) = 8.693$, $P < 0.001$). Fos-LI was significantly greater in CCK-treated SD and SLE but not the LETO and OLETF rats. In response to 5, 10, and 40 μg/kg CCK, Fos-LI was increased in the SD rats more than SLE ($P = 0.011$, > 0.001, > 0.001, respectively) and LETO rats ($P < 0.001$ for each comparison). There were no significant differences in Fos-LI between SD, SLE, and LETO in response to 20 μg/kg CCK. SLE also expressed more Fos-LI than LETO rats only in response to 5 μg/kg CCK-8 ($P = 0.013$) (Fig. 2).

3.3. Dorsal vagal complex

3.3.1. Area postrema

Two-way ANOVA revealed significant strain ($F(3) = 47.792$, $P < 0.001$) and treatment effects ($F(4) = 19.143$, $P < 0.001$). In addition, there was a significant strain by treatment interaction ($F(12) = 6.217$, $P < 0.001$). CCK induced significant increases in Fos-LI in SD, SLE, and LETO rats but not in OLETF rats. In response to 5, 10, 20, and 40 μg/kg CCK, Fos-LI was increased in SD rats more than SLE ($P = 0.006$, < 0.001, < 0.001, < 0.001, respectively). Only 5 and 10 μg/kg CCK increased Fos-LI in SD rats more than LETO ($P = 0.023$, < 0.001). Twenty and 40 μg/kg CCK did not increase Fos-LI in SD rats more than LETO (0.056, 0.912, respectively). Finally, there was more Fos-LI in LETO than SLE rats in response to 10 and 40 ($P < 0.001$ each), but not 20 μg/kg CCK ($P = 0.203$) (Fig. 3).

3.3.2. NTS/DMV (−4.5 mm)

Two-way ANOVA revealed significant strain ($F(3) = 57.673$, $P < 0.001$) and treatment effects ($F(4) = 25.916$, $P <$

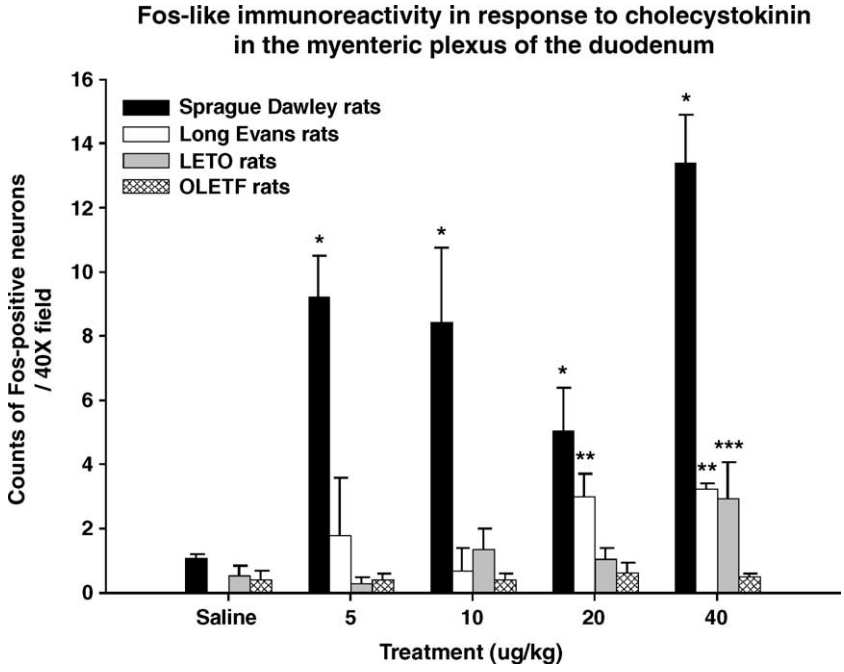

Fig. 1. Mean ± SEM of Fos-LI counts in the myenteric neurons of the duodenum in Sprague–Dawley (SD) (black bars), Standard Long–Evans (SLE, white bars), LETO (gray bars), and OLETF rats (striped bars) in response to four doses of CCK-8 (please refer to text for number of animals per treatment group). CCK-8 increased Fos-LI in the myenteric plexus of the duodenum. The counts of Fos-positive neurons were SD > SLE = LETO. *, **, and *** indicate significant difference from the saline injection ($P < 0.001$). Myenteric neurons of OLETF rats did not increase Fos-LI significantly.

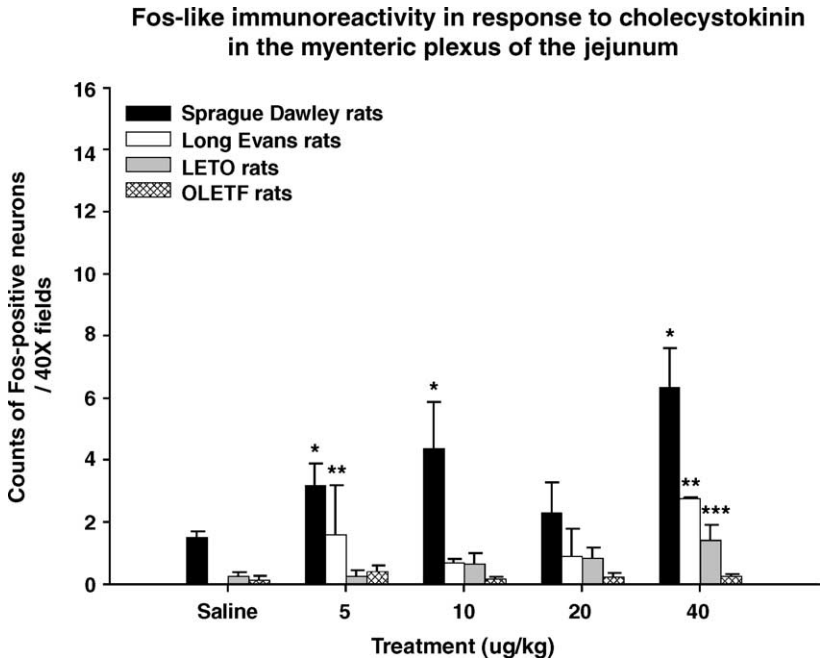

Fig. 2. Mean ± SEM of Fos-LI counts in the myenteric neurons of the jejunum in Sprague–Dawley (SD) (black bars), Standard Long–Evans (SLE, white bars), LETO (gray bars), and OLETF rats (striped bars) in response to four doses of CCK-8 (please refer to text for number of animals per treatment group). CCK-8 increased Fos-LI in the myenteric plexus of the jejunum. The counts of Fos-positive neurons were SD > SLE = LETO. *, **, and *** indicate significant difference from the saline injection ($P < 0.001$). Myenteric neurons of OLETF rats did not increase Fos-LI significantly.

0.001). In addition, there was a significant strain by treatment interaction ($F(12) = 7.526$, $P < 0.001$). CCK induced increases in Fos-LI in SD rats more than SLE at 5 and 10 μg/kg doses ($P = 0.002$, < 0.001, respectively). Only 5 μg/kg CCK increased Fos-LI in LETO rats ($P = 0.029$ and 0.156, respectively) (Fig. 4).

There were no significant differences in Fos-LI between SLE and LETO in response to 5, 10, and 20 μg/kg CCK

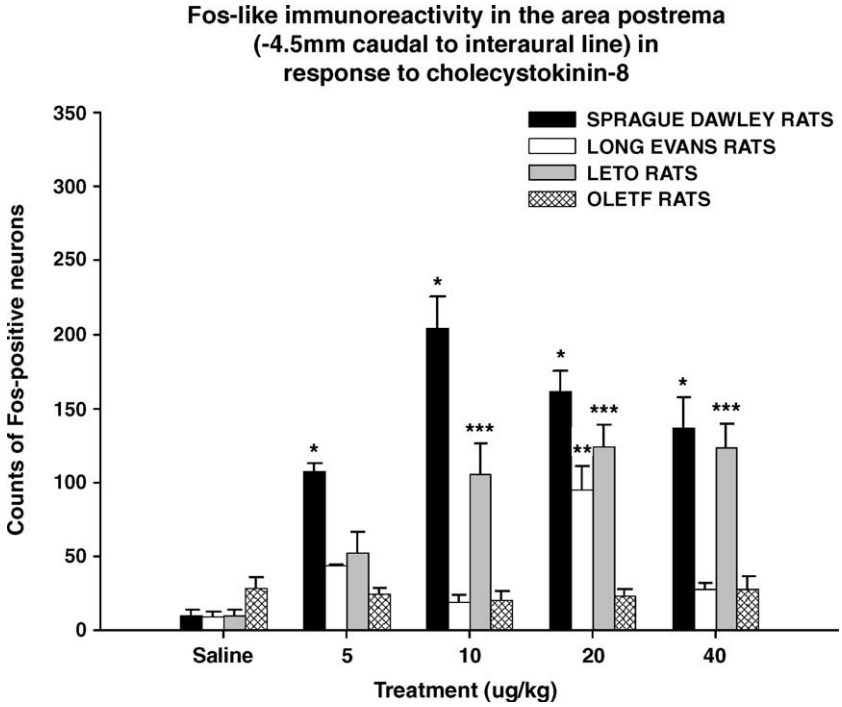

Fig. 3. Mean ± SEM of Fos-LI counts in the area postrema (AP) in Sprague–Dawley (SD) (black bars), Standard Long–Evans (SLE, white bars), LETO (gray bars), and OLETF rats (striped bars) in response to four doses of CCK-8 (please refer to text for number of animals per treatment group). CCK-8 increased Fos-LI in the AP. The counts of Fos-positive neurons were SD > LETO > SLE. *, **, and *** indicate significant difference from the saline injection ($P < 0.001$). The AP of OLETF rats did not increase Fos-LI significantly.

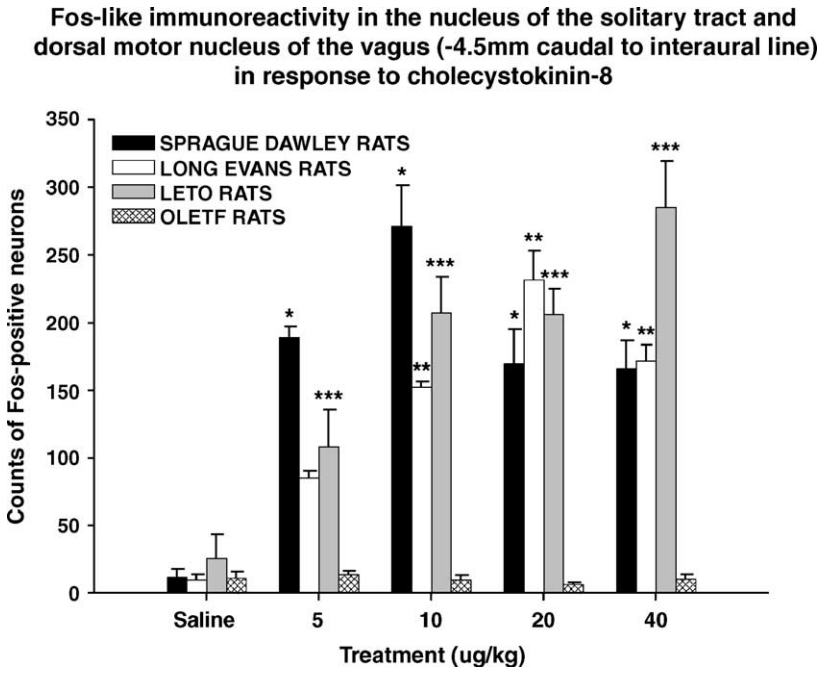

Fig. 4. Mean ± SEM of Fos-LI counts in the nucleus of the solitary tract (NTS) and dorsal motor nucleus of the vagus (DMV) in Sprague–Dawley (SD) (black bars), Standard Long–Evans (SLE, white bars), LETO (gray bars), and OLETF rats (striped bars) in response to four doses of CCK-8 (please refer to text for number of animals per treatment group). CCK-8 increased Fos-LI in the NTS/DMV. The counts of Fos-positive neurons were LETO > SD = SLE. *, **, and *** indicate significant difference from the saline injection ($P < 0.001$). The NTS/DMV of OLETF rats did not increase Fos-LI significantly.

(P = 0.848, 0.283, 0.648, respectively). LETO rats also expressed more Fos-LI than SLE only in response to 40 μg/kg CCK ($P < 0.001$).

3.3.3. NTS/DMV (−4.8 mm)

Two-way ANOVA revealed significant strain ($F(3)$ = 70.540, $P < 0.001$), and treatments effects ($F(4)$ = 34.576, $P < 0.001$). Additionally, there was a significant strain by treatment interaction ($F(12)$ = 5.789, $P < 0.001$). CCK induced significant increased in Fos-LI in SD, SLE, and LETO but not in OLETF rats. In response to 5 and 40 μg/kg CCK, Fos-LI was increased more in the SD rats than SLE (P = 0.003 and < 0.001, respectively), but not LETO rats (P = 0.267 and 0.151, respectively). In the LETO rats, 40 μg/kg increased Fos-LI more than SLE rats (P = 0.014). Finally, there was no significant difference in the counts of Fos-LI neurons in response to 10 and 20 μg/kg CCK between any of the rat strains (P values not reported) (Fig. 5).

3.4. Cuprolinic blue

3.4.1. Duodenum

One-way ANOVA revealed a significant strain effect ($F(3)$ = 2.895, P = 0.038). The counts of cell bodies stained with cuprolinic blue in SD rats were higher than LE (P = 0.042). There was no difference in these counts between SD and LETO and SD and OLETF (Ps = 0.261 and 1.000, respectively). Finally, there were no differences in the counts of cell bodies between OLETF/LE, OLETF/LETO, and LETO/LE (Ps = 0.480, 1.000, and 1.000, respectively) (Fig. 6).

3.4.2. Jejunum

One-way ANOVA revealed no significant effects of strain ($F(3)$ = 0.602, P = 0.615). There was no difference in the counts of myenteric neurons between the four strains of rats.

3.5. Levels of CCK_1 receptor gene expression

As expected, CCK_1 receptor band of 563 bp was detected in our positive control sample. The Rig-S15 housekeeping gene band of 361 bp was equally expressed in all strains of rats including OLETF. This confirms that our PCR assay conditions were sufficient to generate a semi-quantitative data. Equally true is the confirmation of the lack of CCK_1 receptor gene expression in OLETF, where the housekeeping gene was detected in each sample from this group but not CCK_1 receptor (Figs. 7, 8A and B).

In the duodenum and jejunum, one-way ANOVA revealed a significant strain effect ($F(3)$ = 635.270, $P >$ 0.001 and $F(3)$ = 4815.205, $P < 0.001$, respectively). In the duodenum and jejunum, the level of CCK_1 receptor gene expression in SD rats was significantly higher than in LE (Ps = 0.021 and 0.01, respectively), LETO (Ps = 0.022 and 0.01, respectively), and OLETF (Ps < 0.001 each). In addition, the level of expression in LETO and LE rats was

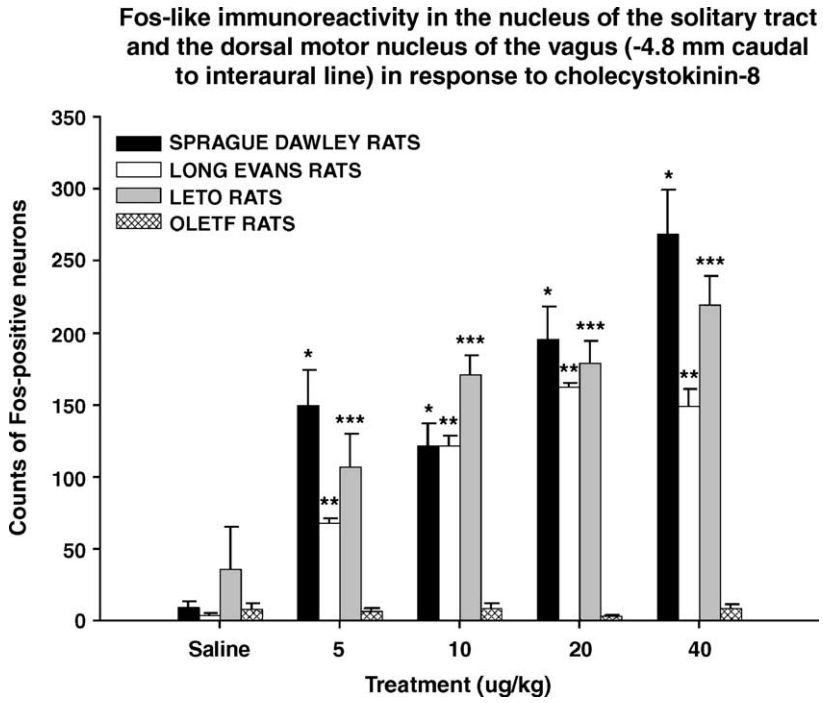

Fig. 5. Mean ± SEM of Fos-LI counts in the nucleus of the solitary tract (NTS) and dorsal motor nucleus of the vagus (DMV) in Sprague–Dawley (SD) (black bars), Standard Long–Evans (SLE, white bars), LETO (gray bars), and OLETF rats (striped bars) in response to four doses of CCK-8 (please refer to text for number of animals per treatment group). CCK-8 increased Fos-LI in the NTS/DMV. The counts of Fos-positive neurons were SD = LETO > SLE. *, **, and *** indicate significant difference from the saline injection ($P < 0.001$). The NTS/DMV of OLETF rats did not increase Fos-LI significantly.

significantly higher than OLETF ($P < 0.001$ each). There were no differences in the levels of CCK_1 receptor gene expression between LE and LETO rats in both the duodenum and jejunum ($P = 1.000$ each). Finally, as expected, OLETF rats did not demonstrate any CCK1 receptor gene expression.

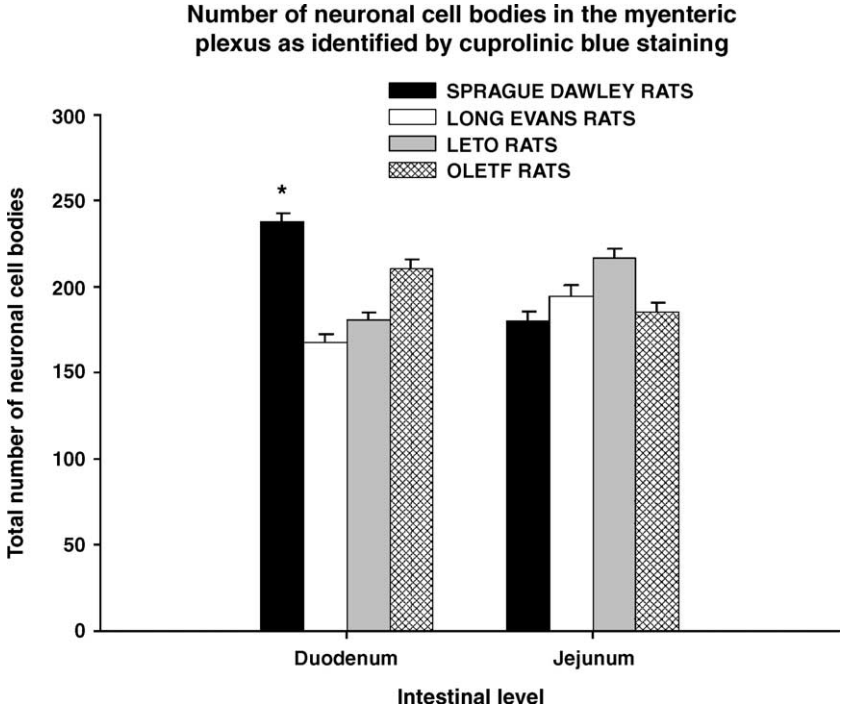

Fig. 6. Counts of neuronal cell bodies in the myenteric plexus of the duodenum and jejunum stained by cuprolinic blue in Sprague–Dawley (SD, black bars), Standard Long–Evans (SLE, white bars), LETO (gray bars), and OLETF rats (striped bars) (please refer to text for number of animals per treatment group). SD has more myenteric neurons in the duodenum than the other strains. * signifies the difference at $P < 0.05$.

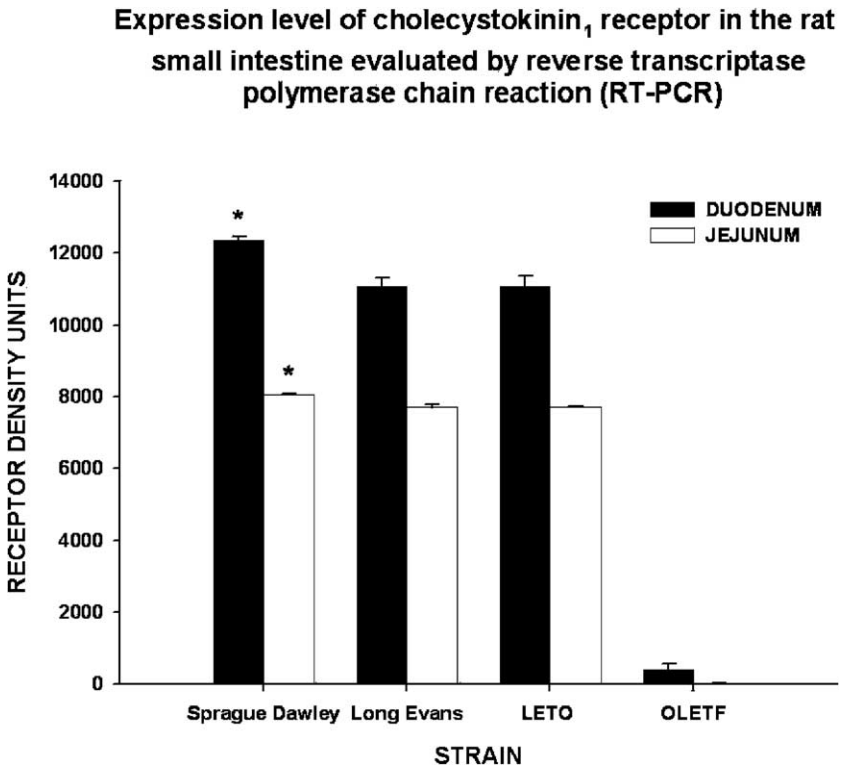

Fig. 7. Semi-quantitative RT-PCR of the expression level of CCK_1 receptor in the duodenum (black bars) and jejunum (white bars) of four normal rat strains (n = 3 rats per strain): Sprague–Dawley, Long–Evans, Long–Evans Tokushima Otsuka (LETO), and Otsuka Long–Evans Tokushima Fatty (OLETF). The level of CCK_1 receptor gene expression was significantly higher in Sprague–Dawley rats than the other three strains of rats. * signifies statistical difference at $P < 0.05$.

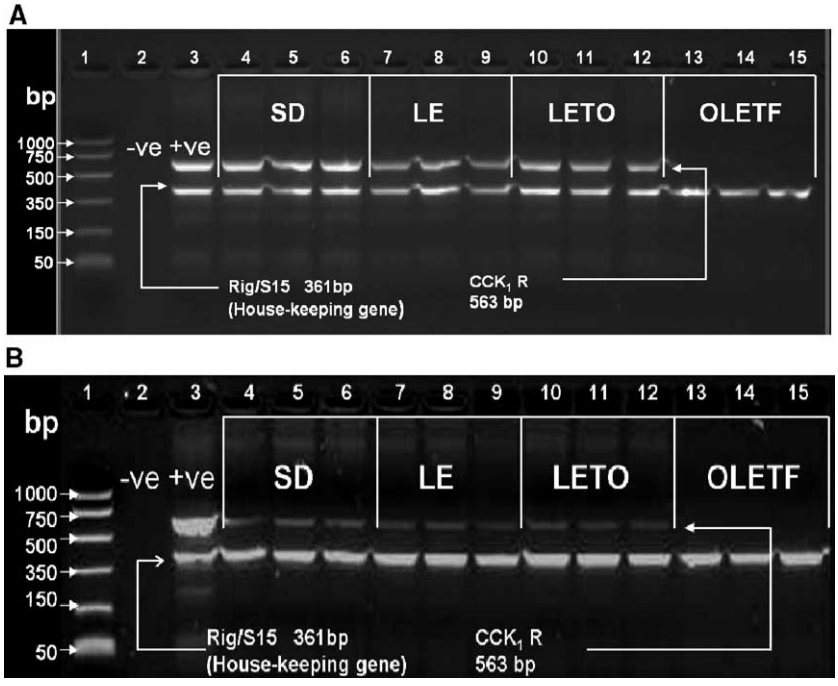

Fig. 8. Semi-quantitative RT-PCR showing the expression level of CCK_1 receptor in the duodenum (A) and jejunum (B) of four normal rat strains: Sprague–Dawley (SD), Standard Long–Evans (SLE), Long–Evans Tokushima Otsuka (LETO), and Otsuka Long–Evans Tokushima Fatty (OLETF). Lane 1, molecular weight markers; lane 2, negative control (−ve); lane 3, positive control (+ve); lanes 4 to 6, Sprague–Dawley (SD); lanes 7 to 9, Long–Evans (LE); lanes 10 to12, Long–Evans Tokushima Otsuka (LETO); lanes 13 to 15, Otsuka Long–EvansTokushma Fatty (OLETF). Rig/S15 is a constitutively expressed (housekeeping) mRNA from a ribosomal subunit protein and was amplified as an internal reference control for data normalization.

4. Discussion

Most immunohistochemical studies that have examined the various functions of CCK-8 have utilized SD rats. However, the recent availability of the OLETF and LETO strains of rats provided ideal models to study the functions of this peptide. Following our recent finding that CCK-8 increased Fos-LI in DVC and myenteric neurons via CCK_1 receptor [3], we identified significant differences in DVC and myenteric Fos-LI in response to CCK-8 between the tested strains of rats. Therefore, the present study was aimed at identifying the basis for such differences. The result of the current comparative study found that SD rats expressed more Fos-LI than SLE and LETO rats in only the AP of the DVC and in the myenteric neurons in response to CCK-8. As expected, OLETF rats, lacking CCK_1 receptors, did not express significant Fos-LI in response to CCK (Fig. 9).

The strain differences in the levels of c-*fos* expression suggest at least four different possibilities, which may explain the different counts of Fos-positive neurons in response to CCK-8 in the various strains of rats that we tested. First, SD rats may have more neurons than the other rat strains. Second, SD rats may have more CCK_1 receptors

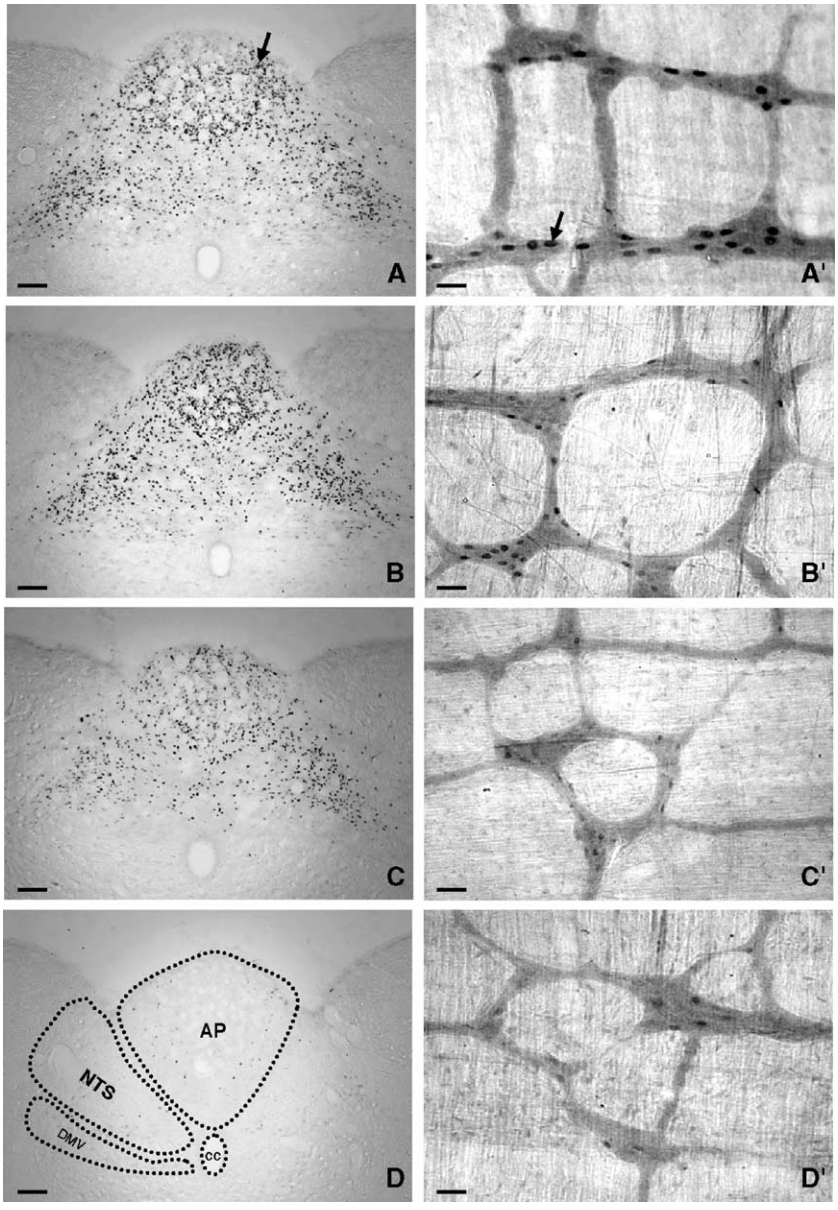

Fig. 9. Photomicrographs of the dorsal vagal complex (DVC) at −4.5 mm caudal to the interaural plane (panels A through D), and myenteric neurons of the duodenum (panels A′ through D′), in Sprague–Dawley (SD) (A and A′), Standard Long–Evans (SLE) (B and B′), LETO (C and C′), and OLETF rats (D and D′) in response to CCK-8 (40 μg/kg). CCK-8 increased Fos-LI (arrows) in the myenteric plexus of the duodenum of SD rats more than the other strains (please refer to the Results section in the text for exact comparisons). The DVC and myenteric neurons of the OLETF rats did not express Fos-LI in response to CCK-8. Abbreviations: AP = area postrema; DMV = dorsal motor nucleus of the vagus; NTS = nucleus tracti solitarii; CC = central canal. Scale bar = 10 μm.

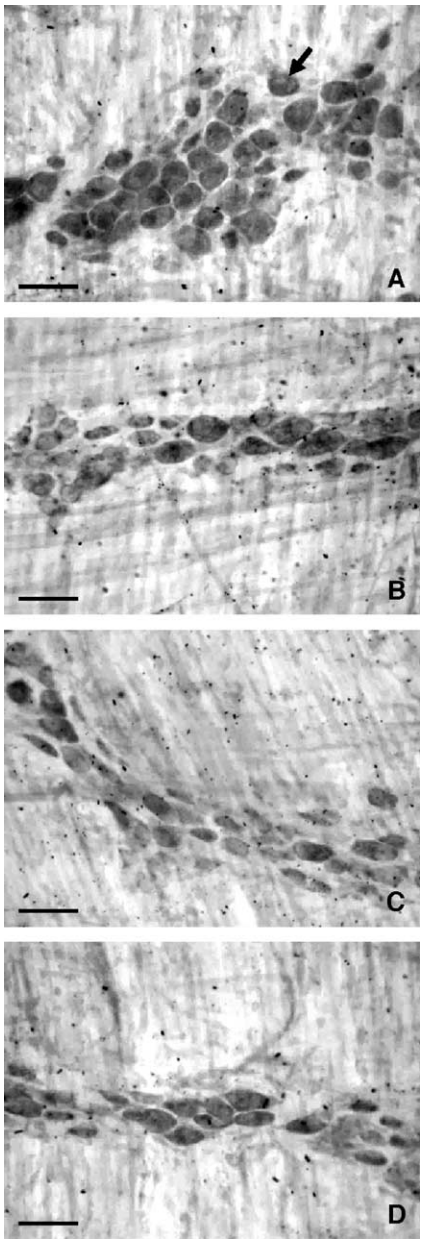

Fig. 10. Photomicrographs of duodenal myenteric neuronal cell bodies (arrow, panels A through D), in Sprague–Dawley (SD) (A), Standard Long–Evans (SLE) (B), LETO (C), and OLETF rats (D) stained with cuprolinic blue. The number of myenteric cell bodies in SD rats was significantly more than the number of myenteric neuronal cell bodies in the other strains of rats. Please refer to the text for exact comparisons. Scale bar = 10 μm.

than the other strains. Third, CCK_1 receptors in SD rats may be more readily functional than in SLE and LETO rats. Fourth, activation of CCK_1 receptors may evoke different outcomes in the different strains of rats. In the work presented here, we only explored two possibilities, the number of myenteric neurons and the level of CCK_1 receptor gene expression in the GI tract of these rat strains. Other studies are required to examine the other possibilities.

We found that the number of myenteric neurons, as revealed by cuprolinic blue staining (Fig. 10), in the duodenum is higher in SD rats than in the other strains. Therefore, the increase in myenteric Fos-LI by CCK-8 in SD rats compared with the other strains may be due, at least in part, to the increase in the number of these neurons in SD rats compared to LE, LETO, and OLETF. However, the lack of a strain-related difference in the number of myenteric neurons in the jejunum argues against a cell body basis for the differences in CCK induced c-*fos*.

This study also presents a systemic comparison of the CCK_1 receptor expression in the upper small intestine of four strains of rats. A prior immunohistochemical study [16] demonstrated that CCK_1 receptor is localized to the myenteric neurons of the stomach and small intestine of SD rats. However, no strain comparisons or quantification of this receptor distribution were attempted. Our data demonstrated that the level of CCK_1 receptor gene expression is higher, in both duodenum and jejunum, in SD rats than in the other three strains of rats. Therefore, a difference in the magnitude of CCK_1 receptor expression may explain the increase in CCK induced Fos-LI in the myenteric neurons in SD.

Only in the NTS/DMV at the −4.5 mm caudal to the interaural plane was Fos-LI more in the LETO compared to the SD and the SLE rats. We cannot explain this result. However, this study did not evaluate the level of CCK_1 receptor gene expression in the central nervous system. Such studies, as well as functional and CCK receptor binding studies, may be required to explain this finding.

In conclusion, the myenteric plexus of the duodenum and jejunum express more Fos-LI in response to CCK in SD rats than LE, LETO, and OLETF. This result can be partially explained by the increased number of myenteric neurons and increased CCK_1 receptor gene expression in this strain of rats compared to the others.

Acknowledgments

We thank Otsuka Pharmaceutical Co. (Tokushima, Japan) for providing the OLETF and LETO rats, and Dr. Robert J. Phillips, Department of Psychological Sciences, Purdue University for providing the cuprolinic stain protocol, and for his expert guidance in the quantification process of the cell bodies. Grants NIH S06/GM08091-31, The Birmingham Racing Commission, and Pfizer Student Funds Program supported this work.

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