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ORIGINAL ARTICLE

Expression of the P2X$_2$ receptor in different classes of ileum myenteric neurons in the female obese *ob/ob* mouse

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Abstract

AIM: To examine whether the *ob/ob* mouse model of obesity is accompanied by enteric nervous system abnormalities such as altered motility.

METHODS: The study examined the distribution of the P2X$_2$ receptor (P2X$_2$R) in myenteric neurons of female *ob/ob* mice. Specifically, we used immunohistochemistry to analyze the co-expression of the P2X$_2$R with neuronal nitric oxide synthase (nNOS), choline acetyltransferase (ChAT), and calretinin (CalR) in neurons of the small intestine myenteric plexus in *ob/ob* and control female mice. In these sections, we used scanning confocal microscopy to analyze the co-localization of these markers as well as the neuronal density (cm^2) and area profile (μm²) of P2X$_2$R-positive neurons. In addition, enteric neurons were labeled using the nicotinamide adenine dinucleotide (NADH) diaphorase method and analyzed with light microscopy as an alternate means by which to analyze neuronal density and area.

RESULTS: In the present study, we observed a 29.6% increase in the body weight of the *ob/ob* animals (OG) compared to the control group (CG). In addition, the average small intestine area was increased by approximately 29.6% in the OG compared to the CG. Immunoreactivity (IR) for the P2X$_2$R, nNOS, ChAT and CalR was detectable in the myenteric plexus, as well as in the smooth muscle, in both groups. This IR appeared to be mainly cytoplasmic and was also associated with the cell membrane of the myenteric plexus neurons, where it outlined the neuronal cell bodies and their processes. P2X$_2$R-IR was observed to co-localize 100% with that for nNOS, ChAT and CalR in neurons of both groups. In the *ob/ob* group, however, we observed that the neuronal density (neuron/cm^2) of P2X$_2$R-IR cells was increased by 62% compared to CG, while that of NOS-IR and ChAT-IR neurons was reduced by 49% and 57%, respectively, compared to control mice. The neuronal density of CalR-IR neurons was not different between the groups. Morphometric studies further demonstrated that the cell body profile area (μm²) of nNOS-IR, ChAT-IR and CalR-IR neurons was increased by 34%, 20% and 55%, respectively, in the OG compared to controls. Staining for NADH diaphorase activity is widely used to detect alterations in the enteric nervous system; however, our qualitative examination of NADH-diaphorase positive neurons in the myenteric ganglia revealed an overall similarity between the two groups.

CONCLUSION: We demonstrate increases in P2X$_2$R expression and alterations in nNOS, ChAT and CalR IR in ileal myenteric neurons of female *ob/ob* mice compared to wild-type controls.

Key words: Obesity; P2X$_2$ receptor; Myenteric neurons; Chemical coding; Mouse

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INTRODUCTION

Obesity is a chronic heterogeneous disorder characterized by abnormal or excessive fat accumulation that presents a risk to health[1]. The leptin-deficient *ob/ob* mouse exhibits both obesity and peripheral diabetic neuropathy, traits characteristic of human patients, and thus represents a valuable animal model of obesity and type 2 diabetes-like pathology[2].

Obesity is associated with an increased risk of type 2 diabetes and is often accompanied by complications of the gastrointestinal tract such as gastroparesis, constipation, diarrhea, and fecal incontinence[1]. Disruptions in the function of the gastrointestinal tract have been associated with alterations in the enteric nervous system[3]. Enteric neurons comprise both the myenteric and submucosal plexuses, which control the motility of the intestine, the transport of fluids from the intestinal mucosa, and local blood flow[3]. The groups of neuronal cell bodies (ganglia) in these plexuses are interconnected by nerve fiber bundles[3]. The myenteric and submucosal plexuses also regulate gastrointestinal secretions important for the digestion of food particles, intestinal lubrication, nutrient uptake, regulation of pH, and the concentration of solutes and elimination of waste products[3]. Studies of chemical coding in neurons of the mouse enteric nervous system have been particularly important to our understanding of the function of enteric neurons[4,5]. Defects in enteric nervous system function have been reported in *ob/ob* mice, including alterations in intestinal motility[6] and in the enteric neurons[7,8]. Down-regulations in intestinal motility have been reported in obese humans[1]. Thus, the mouse is a valuable animal model for the study of obesity-related pathology in the enteric nervous system.

Adenosine 5'-triphosphate (ATP) has been well-established as a neurotransmitter and a ligand of the P2X receptor family, which is made up of seven known receptor subunits (PX_{1-7})[9]. These receptors play an important role in synaptic transmission within the neural pathways that mediate intestinal motility[10]. Previous immunohistochemical studies have documented the distribution of $P2X_2$ receptors ($P2X_2Rs$) in the enteric nervous system of the guinea pig[11-14], rat[15-18] and mouse[19-21]. However, the distribution of $P2X_2R$ expression in the enteric nervous system of obese mice remains unstudied.

It has been reported that the $P2X_2R$ is expressed in inhibitory, cholinergic, and intrinsic afferent neurons in the enteric nervous system[11]. The goal of the current study is to analyze the chemical coding, density, and area profile of neuron immunoreactivity (IR) for the $P2X_2R$, neuronal nitric oxide synthase (nNOS), choline acetyltransferase (ChAT), calretinin (CalR), and positivity for nicotinamide adenine dinucleotide (NADH)-diaphorase activity in the small intestine enteric nervous system of female *ob/ob* and control mice.

MATERIALS AND METHODS

Animals

Two groups of 11-mo-old female mice were compared in these studies. The obese group (OG) consisted of six homozygous (*ob/ob*) C57BL/6J mice, and the control group (CG) was comprised of six wild-type C57BL/6J mice. The animals were bred at the State University of Campinas Breeding Center. The animals were housed at five per cage in an artificially lit room (12 h/12 h, light/dark) and were fed a standard pellet diet (Nuvilab, São Paulo, Brazil) and water *ad libitum*. Animals were euthanized for experiments using a CO_2 chamber. This study was conducted according to current legislation on animal experiments at the Biomedical Science Institute of the University of São Paulo.

Collection of blood for biochemical analysis

Samples of blood were obtained *via* the puncture of the plexus axillary artery of mice previously anesthetized with xylazine (16 mg/kg) and ketamine (120 mg/kg). Following collection, the blood remained at rest for 2 h at room temperature for clot formation, and then was centrifuged at 2500 × *g* for 20 min at 4 ℃ to obtain the serum. The serum supernatant was maintained at -40 ℃ until being analyzed for glucose composition.

Immunohistochemistry

Following CO_2 euthanasia, fresh segments of distal ileum were removed from each animal and placed in phosphate-buffered saline (PBS: 0.15 mol/L NaCl in 0.01 mol/L sodium phosphate buffer, pH 7.2) containing nicardipine (10^{-6} mol/L; Sigma, United States) to inhibit tissue contraction. The dissected segments were sliced open along the mesenteric border and cleaned of their contents using PBS. They were then pinned out tautly, mucosa side down, onto a balsa-wood board and fixed overnight at 4 ℃ in paraformaldehyde in 0.2 mol/L sodium phosphate buffer (pH 7.3). The next day, the segments of ileum tissue were cleared of fixative with three 10-min washes in 100% dimethyl sulfoxide followed by three 10-min washes in PBS. All tissue was stored at 4 ℃ in PBS containing sodium azide (0.1%). The fixed tissue was then dissected and the mucosal, submucosal, and circular layers were removed to obtain whole-mounts of the longitudinal muscle-myenteric plexus. In a second type of preparation, the mucosa and muscularis externa were removed to leave the intact submucosal layer. Whole-mount preparations of the myenteric and submucosa of the ileum were then pre-incubated in blocking buffer, 10% normal horse

Table 1 Characteristics of primary and secondary antibodies

Antigen	Host	Dilution	Code and reference
$P2X_2$ receptor	Rabbit	1:120	AB5244, Chemicon
Nitric oxide synthase	Sheep	1:2000	H205 (Williamson *et al*[31], 1996)
Choline acetyltransferase	Goat	1:50	Chemicon
Calretinin	Goat	1:100	CG1 (Swant)
Secondary antibodies			
Donkey anti-rabbit IgG Alexa 488	Donkey	1:500	Molecular probes
Donkey anti-sheep IgG Alexa 594	Donkey	1:100	Molecular probes

serum in PBS containing 1.5% Triton X-100 for 45 min at room temperature to reduce non-specific antibody binding and to permeabilize the tissue, respectively (Table 1). To localize $P2X_2R$ IR, we used a rabbit antiserum raised against amino acids 457-472 of the rat $P2X_2R$, with a single Cys extension at the N-terminal (AB5244 from Chemicon, Temecula, CA, United States). Tissues were incubated with the antibody at a dilution of 1:120 in blocking buffer for 48 h at 4 ℃. Dual immunohistochemistry was achieved using combinations of antisera (Table 1). Following incubation in primary antisera, the tissue samples received three 10-min washes in PBS and were then incubated in a mixture of secondary antibodies (Table 1). Following three further 10-min washes in PBS, the tissue samples were mounted onto microscope slides in glycerol buffered with 0.5 mol/L sodium carbonate buffer (pH 8.6). Tissues preparations were examined using both Leica and Nikon epifluorescent microscopes. Images were captured using a digital camera coupled to Image-Pro Plus software. Preparations were also analyzed by confocal microscopy using a Zeiss confocal scanning laser system installed on a Zeiss Axioplan 2 microscope. The image dimensions were 512 × 512 pixels, with an optical section thickness of 0.5 μm. Immunoreactive cells were scanned as a series of optical sections, with a center spacing of 0.2 μm, and the images were collected using LSM 5 Image Zeiss processing software, and further processed using Corel Photo Paint and Corel Draw software programs.

Statistical analysis

Antigen colocalization was determined by examining preparations immunolabeled with two fluorescent secondary antibodies. Neurons of interest were identified by immunofluorescence for one antigen; the filter was switched, and labeling for the second antigen was determined. In this way, the proportion of neurons immunoreactive for both antigens was determined. For each antigen pair, the cohort size was 100 neurons. Co-IR for the $P2X_2R$ with that of the other antigens (nNOS, ChAT, or CalR) was assessed. The percentage of double-immunoreactive neurons was calculated and expressed as mean ± SE (n = number of tissue preparations). The number of neurons immunoreactive for the $P2X_2R$, nNOS, CalR, and ChAT, as well as the neuronal profile (area of cell body), were measured by examining the whole-mount preparations under a binocular microscope at 100 × magnification. All neurons present in each cm^2 were counted. The profile areas of 50 nerve cell perikarya from each animal were measured using Image-Pro Plus software. Data were compared by one-way analysis of variance (ANOVA) and Student's t-tests; $P < 0.05$ was considered to be statistically significant.

NADH diaphorase staining

A separate cohort of 12 animals was weighed and euthanized in a CO_2 chamber prior to the opening of the anterior abdominal wall. The distal ileum was removed and washed in Krebs solution. The surface area of the entire small intestine was measured using a planimeter. Each piece of ileum was then ligated with cotton thread at the proximal end and gently distended with Krebs solution introduced with a syringe into the distal end. When the intestine was sufficiently distended, the syringe needle was withdrawn and the ligature was simultaneously tightened. The following steps were then performed to label neurons using the NADH diaphorase histochemical technique[22,23]. Following incubation in Krebs solution at room temperature for 15-30 min, the small intestine was transferred to a permeabilizing agent (0.3% Triton-X 100 in Krebs solution) for 15-90 s and then submitted to three 10-min washes in Krebs solution. The specimens were incubated for 30-45 min at 20 ℃ in 20 mL of incubation medium that contained 0.5 mg/mL of nitro blue tetrazolium (Sigma Chemical Co, St. Louis, MO) in distilled water (25 parts), 0.1 mol/L sodium phosphate buffer, pH 7.3 (25 parts), distilled water (50 parts), and 0.5 mg/mL of the reduced form of β-NADH (Sigma, United States). The reaction was stopped by immersion in 10% buffered formalin solution, in which the tissue samples were fixed for a minimum of 24 h at room temperature. Whole mount preparations were then prepared as follows: the small intestine was opened, the mucosa was removed, and the longitudinal muscle, with the myenteric plexus attached, was lifted at one corner and gently removed from the entire strip. After several washes in distilled water, fragments (2 cm^2) of the proximal, middle, and distal portions of the large intestine were prepared as whole mounts in glycerol on microscope slides and sealed with Entellan (Merck).

Myenteric neurons were identified by the presence of intense formazan reaction product filling the perikaryon, as well as by large, round, and unstained nuclei. The number of neurons and the profile areas of the nerve cell bodies were measured by examining the whole mount preparations under a binocular microscope at a magnification of 400 ×. All labeled neurons in each fragment were counted. The profiles of 100 nerve cell perikarya from each portion were obtained using a semi-automatic morphometry device (Image-Pro Plus Program 3.1)[17,18].

Results are expressed as mean ± SE. Data were compared by ANOVA with Student's t-tests for multiple comparisons, as appropriate. $P < 0.05$ was considered statistically significant.

Table 2 Body weight, small intestine area and glucose levels in female control (n = 4) and *ob/ob* mice (n = 4)

	Control group	Obese group
Body weight (g)	25.62 ± 0.62	65.52 ± 1.08[a]
Small intestine area (cm^2)	26.6 ± 2.8	34.5 ± 3.21[a]
Glucose (mg/dL)	195.75 ± 58.09	322.2 ± 99.2[a]

Student's *t*-tests for multiple comparisons. [a]$P < 0.05$ *vs* control.

RESULTS

In the present study, we found a significant (29.6%, $P < 0.05$) increase in the body weight of the *ob/ob* animals (OG) compared to controls (Table 2). In addition, the average small intestine area was increased by approximately 29.6% in the OG as compared to the CG ($P < 0.05$; Table 2). Plasma glucose measurements revealed levels of 195.75 ± 58 mg/dL in control mice and 322.3 ± 58 mg/dL in the OG mice ($P < 0.05$). Mice with blood glucose levels above 300 mg/dL were considered to be diabetic (Table 2).

Immunolabeling

IR for the $P2X_2R$, nNOS, ChAT and CalR was detectable in the myenteric plexus, as well as in the smooth muscle, in both groups. This IR mainly occurred throughout the cytoplasm and on cell surface and nuclear membranes, and effectively outlined the neuronal cell bodies and their processes in the myenteric plexus (Figure 1). nNOS-IR neurons were detected in both groups. Some nNOS-IR neurons displayed short dendritic processes, characteristic of Dogiel type I morphology. The primary and secondary fiber tracts were also immunoreactive for nNOS (Figure 1). ChAT-IR neuronal cell bodies were abundant in the myenteric ganglia; ChAT-IR was present in the cytoplasm of neurons from both groups. Nearly all of the ChAT-IR neurons displayed Dogiel type I morphology. We also observed a small number of large, smooth-surfaced ChAT-IR cell bodies. In addition, varicose ChAT-IR fibers were observed surrounding the ganglia, and constituted the primary and secondary fiber tracts of the ileum myenteric plexus (Figure 2). IR for CalR was detected in neuronal cell bodies and axonal/dendritic processes in both the control and the OG. Some neurons showed typical Dogiel type I morphology, with short dendritic processes, while others displayed long axonal projections characteristic of Dogiel type Ⅱ morphology (Figure 2).

Statistical analysis

Double immunolabeling for the $P2X_2R$ in combination with anti-nNOS, ChAT, and CalR IR was conducted to identify different chemical classes (chemical coding) of neurons in the myenteric plexus. In the CG, 99.5% ± 0.6% of NOS-IR neurons exhibited $P2X_2R$-IR and, in the OG, the colocalization score was similar (99.1% ± 1.1%). The colocalization of ChAT-IR and CalR-IR with $P2X_2$-IR was 100% in neurons from both the control and the OGs (Table 3). In summary, these analyses did not show any statistically significant differences in the colocalization of $P2X_2R$-IR with that of the other antigens between the two groups (Table 3).

Table 3 Results of the quantitative analysis of double labeling studies of immunoreactivity against the $P2X_2$ receptor, neuronal nitric oxide synthase, choline acetyltransferase, and calretinin in the myenteric plexus of the ileum from control and *ob/ob* female mice (n = 4, mean ± SE) (%)

	Control group	Obese group
$nNOS\text{-}IR^+/P2X_2\text{-}IR^+$	99.5 ± 0.6	99 ± 1.0
$ChAT\text{-}IR^+/P2X_2\text{-}IR^+$	100	100
$CalR\text{-}IR^+/P2X_2\text{-}IR^+$	100	100
$P2X_2\text{-}IR^+/nNOS\text{-}IR^+$	19.5 ± 3.6	19.5 ± 3.6
$P2X_2\text{-}IR^+/ChAT\text{-}IR^+$	23.6 ± 4.2	24.6 ± 4.37
$P2X_2\text{-}IR^+/CalR\text{-}IR^+$	23.7 ± 3.75	21.6 ± 1.20

nNOS: Neuronal nitric oxide synthase; ChAT: Choline acetyltransferase; CalR: Calretinin; IR: Immunoreactivity.

Upon examination of neuronal density in the myenteric plexus, we found an increase of 34.3% in the density of $P2X_2R$-IR neurons in the OG compared to controls ($P < 0.05$). In addition, the density of nNOS-IR and ChAT-IR neurons decreased by 42.6% and 53.4%, respectively, in the OG compared to controls ($P < 0.05$). There were no differences between the groups in the density of CalR-IR neurons (Figure 3). These data suggest that the density of $P2X_2R$-IR neurons increases, while that of nNOS-IR and ChAT-IR neurons decreases, in the OG compared to controls. We then estimated the total number of neuron IR for the $P2X_2R$ and found an increase of 105% in the OG compared to controls, while the total number of nNOS-IR and ChAT-IR neurons was decreased 35% and 47%, respectively, compared to controls ($P < 0.05$). The total number of CalR-IR neurons was not significantly different between the two groups (Figure 3).

We then evaluated the number of $P2X_2R$-IR neurons per myenteric ganglion in both groups. The number of $P2X_2$-IR neurons was 19 ± 1.8 neurons/ganglion in the OG *vs* 18.6 ± 3.9 neurons/ganglion in the CG. In addition, the number of nNOS-IR neurons per ganglion did not significantly differ between the groups, and was 6.6 ± 1.4 neurons/ganglion in the CG, and 5.2 ± 0.7 neurons/ganglion in the OG. The number of ChAT-IR neurons per ganglion also did not differ between the groups (5.9 ± 1.8 for CG, 5.1 ± 1.4 for OG). The number of CalR-IR neurons per ganglion also did not differ between groups (6.5 ± 1.2 for CG, 5.0 ± 0.2 for OG). Therefore, these data show that there were no significant differences between the control and OGs in the numbers of $P2X_2R$-, nNOS-, ChAT- or CalR-IR neurons in the myenteric ganglia (Figure 3).

Neurons with typical Dogiel type I and Ⅱ morphologies were observed in whole mounts immunolabeled for CalR-IR. The density of Dogiel type I neurons in the CG and OG was 6.3 ± 1.4 neurons/cm^2 and 5.7 ± 0.3

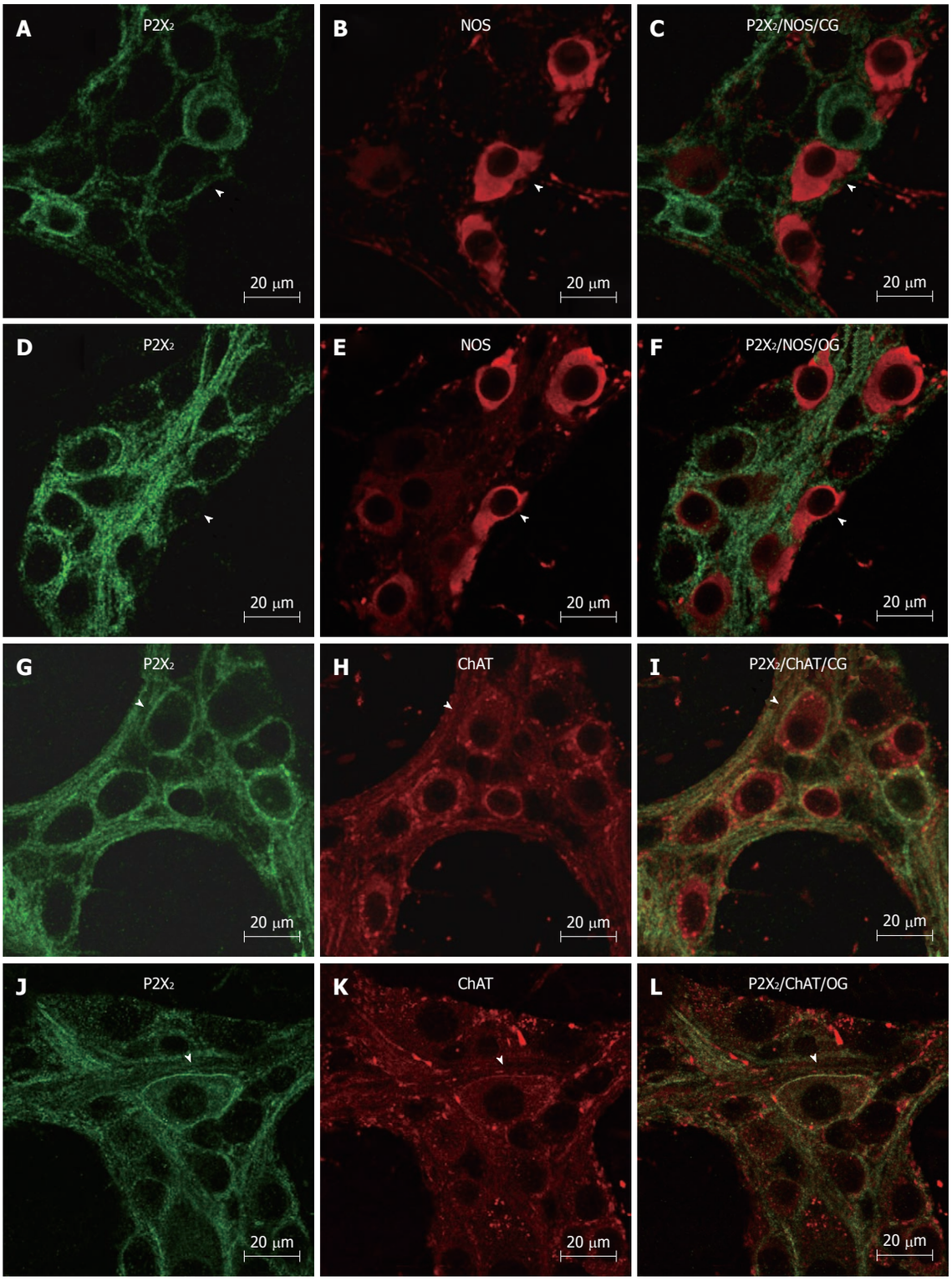

Figure 1 Immunoreactivity for $P2X_2$ receptor (A, D, G, J), nitric oxide synthase (B, E), choline acetyltransferase (H, K; merged images C, F, I, L) was examined in ileum myenteric plexus neurons from the control group (A-C, G-I) and the obese *ob/ob* group (D-F, J-L). Single arrows show double-labeled $P2X_2$ receptor ($P2X_2R$)- and nitric oxide synthase (NOS)-immunoreactivity neurons in the control group (CG) (A-C) and obese group (OG) (D-F) groups. Double-labeling for $P2X_2R$ and choline acetyltransferase (ChAT) in CG neurons (G-I) and OG (J-L) is indicated by arrowheads.

neurons/cm^2, respectively. The density of Dogiel type Ⅱ neurons was 1.3 ± 0.2 neurons/cm^2 in the CG and 1.5 ± 0.2 neurons/cm^2 in the OG. These data show that there were no significant differences in the density of CalR-IR Dogiel type Ⅰ and Ⅱ neurons between the CG and OG (Figure 3).

We then measured the cell body area of neurons in the myenteric plexus from both control and OGs.

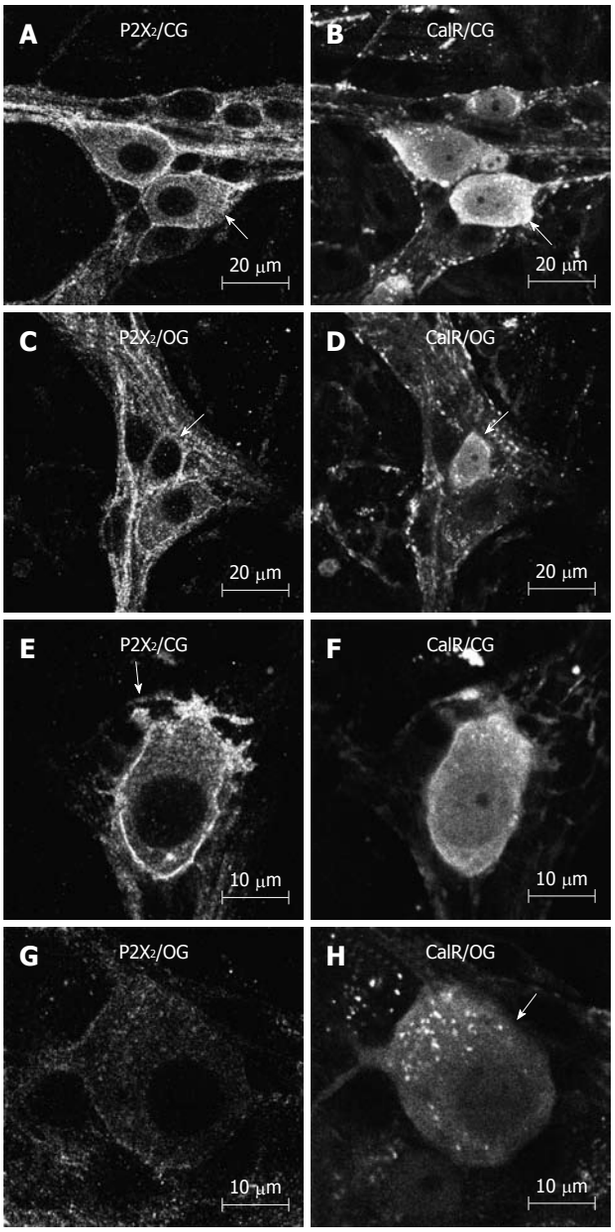

Figure 2 Immunoreactivity for P2X2 receptor (A, C, E, G) and calretinin (B, D, F, H) was examined in the ileum myenteric neurons of the control group (A, B, E, F) and the obese group (C, D, G, H). Single arrows show double labeling for P2X2 receptor and calretinin (CalR) in the neurons of the control group (CG) (A, B) and the obese group (OG) (C, D). E, F: **An example of a Dogiel type Ⅰ neuron with many lamellar and short dendrites; G, H: A neuron with** Dogiel type Ⅱ morphology with a round and smooth cell body and lipofuscin evident in the cytoplasm (arrow).

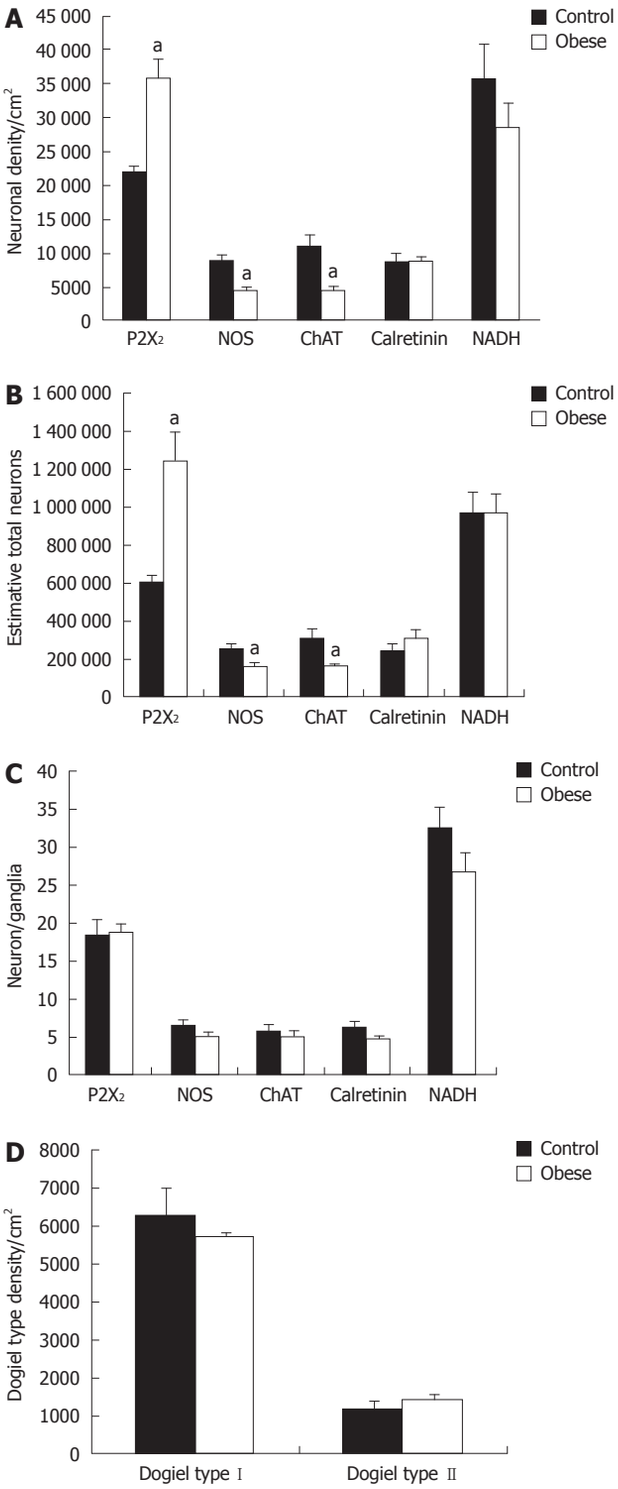

Figure 3 Neuronal density (neurons/cm^2) (A), the estimated total neuron number (B) and neurons per ganglion immunoreactive for P2X2 receptor, nitric oxide synthase, choline acetyltransferase, calretinin, and positive for nicotinamide adenine dinucleotide-diaphorase activity in the ileum myenteric plexus of control and obese mice (C), and densities of Dogiel type Ⅰ and Ⅱ neurons immunoreactive for calretinin (D) in control and obese mice. $^aP < 0.05$ *vs* control, mean ± SE, Student's *t*-test. NOS: Nitric oxide synthase; ChAT: Choline acetyltransferase; NADH: Nicotinamide adenine dinucleotide.

Although there was no change in the size of P2X2R-IR neurons between the groups, we observed a significant increase in the cell body areas of nNOS-IR (34%), ChAT-IR (17%), and CalR-IR neurons (35%) in the OG compared to controls ($P < 0.05$; Figure 4). The size distribution of neurons in the myenteric plexus of CG and OG mice are shown in Figure 5.

NADH-diaphorase analysis

Our qualitative examination of NADH-diaphorase positive neurons in the myenteric ganglia revealed an overall similarity between the two groups. With this method, the formazan reaction product labels the neuronal cy-

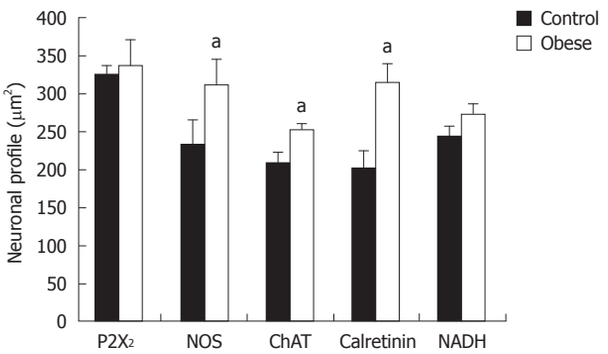

Figure 4 Cell body profile areas (μm²) of neurons immunoreactive for P2X2 receptor, nitric oxide synthase, choline acetyltransferase, calretinin and nicotinamide adenine dinucleotide-diaphorase in the ileum myenteric plexus of control and obese groups. $^{a}P < 0.05$ *vs* control, mean ± SE, Student's *t*-test. NOS: Nitric oxide synthase; ChAT: Choline acetyltransferase; NADH: Nicotinamide adenine dinucleotide.

toplasm with a varying intensity, while neuronal nuclei appear translucent. We did observe that the myenteric neurons appeared to be more widely-spaced (less dense, or grouped) in the myenteric ganglia of the OB group compared to controls (Figure 6).

This histochemical analysis provided some potentially useful general information about the myenteric neuronal population in the mouse ileum. The density of NADH-diaphorase-positive neurons was reduced by about 20% in the OG compared to controls; however, this reduction was not statistically significant (Figure 3). The total surface area of the small intestine in both groups was measured, and the total number of neurons was estimated to be 971 558 ± 205 000 in the CG and 974 042 ± 187 000 in the OG (Figure 3). In addition, an estimate of the number of neurons per myenteric ganglion did not reveal any significant differences between the groups (Figure 3). Cell body area profile measurements of NADH-diaphorase-positive neurons also showed no differences in overall neuronal size between the control and OGs (Figure 4).

DISCUSSION

Gastrointestinal disorders arising from alterations in intestinal motility in obese individuals have been related to alterations in the enteric nervous system[1]. In the current report, we demonstrate changes in the population of enteric neurons of the myenteric plexus in the *ob/ob* mouse that may explain, in part, impairments in intestinal motility suffered by obese individuals[1]. The current study demonstrates, for the first time, the presence of P2X2R IR in the myenteric plexus, and alterations in the chemical identity, or coding, of myenteric neurons in the ileum of obese female *ob/ob* mice compared to controls. Previous studies have revealed the presence of P2X2,3,7 receptor-containing neurons in the enteric nervous systems of various species[11-21]. In the current study, we show that IR for the P2X2R is present in both the cytoplasm and membrane of myenteric plexus neurons in control and obese animals. We confirm that IR for the P2X2R colocalizes with that for nNOS, ChAT and CalR in myenteric neurons. This finding is consistent with previous work demonstrating the presence of P2X2R in inhibitory neurons, as well as in intrinsic excitatory and secretomotor/vasodilator primary afferent neurons in the guinea pig[11] and rat[16-18]. We did not observe any differences in the co-localization of P2X2R with IR for these markers (nNOS, ChAT, CalR) between the control and obese mice, suggesting that the neurochemical coding of these neurons is not altered in the myenteric plexus of obese female *ob/ob* female mice compared to wild-type controls.

The creation of the *ob/ob* mouse on the C57BL/6J background, which followed the cloning and sequencing of the mouse *ob* gene and its human homologue, provides an excellent model for the study of obesity[2]. These mice present with obesity that is accompanied by the manifestation of motor and sensory nerve conduction deficits, small sensory nerve fiber neuropathy, intraepidermal sensory nerve fiber loss, and oxidative nitrosative stress in peripheral nerves, features that are characteristic of human subjects with obesity and diabetes[2]. Previous studies have demonstrated that mice with blood glucose levels above 300 mg/dL are useful as a mouse model of diabetes[2]. In the current study, the obese *ob/ob* mice had average blood glucose levels of 322.3 ± 58 mg/dL, clearly within the diabetic range. Consistent with previous reports, the body weights of the *ob/ob* mice were twice those of the control mice in our study. This increase in body weight was accompanied by a similar increase in the area of the ileum in the obese animals, in accordance with previous findings[6]. Obesity in humans is associated with increased risk of type 2 diabetes and is often accompanied by complications of the gastrointestinal tract. In the current work, we found high levels of glucose, well within the diabetic range, accompanied by alterations in the myenteric neurons in the obese *ob/ob* mice compared to controls, providing a possible explanation for the dysfunctions in intestinal motility that can accompany obesity. In keeping with this idea, animals with type 1 diabetes have been observed to have gastrointestinal complications[2].

We examined nNOS IR as a marker of inhibitory motor neurons, as well as ChAT and CalR-IR to identify excitatory motor neurons and interneurons, respectively. The results we obtained are consistent with the current literature[4]. For example, our morphological analysis showed that nNOS-IR neurons exhibit Dogiel type I morphologies, while CalR-IR neurons exhibit both Dogiel type I and II morphologies. Qu *et al*[4] in 2008 showed that about 15% of all myenteric neurons are Dogiel type II and are immunoreactive for CalR. Our current findings show that 14% of all the CalR-IR neurons in the myenteric plexus were Dogiel type II in the CG, and that a similar percentage (16%) existed in the OG.

ChAT is the synthesizing enzyme for the excitatory neurotransmitter acetylcholine, a transmitter not found in inhibitory motor neurons[3,4]. In the current work, we

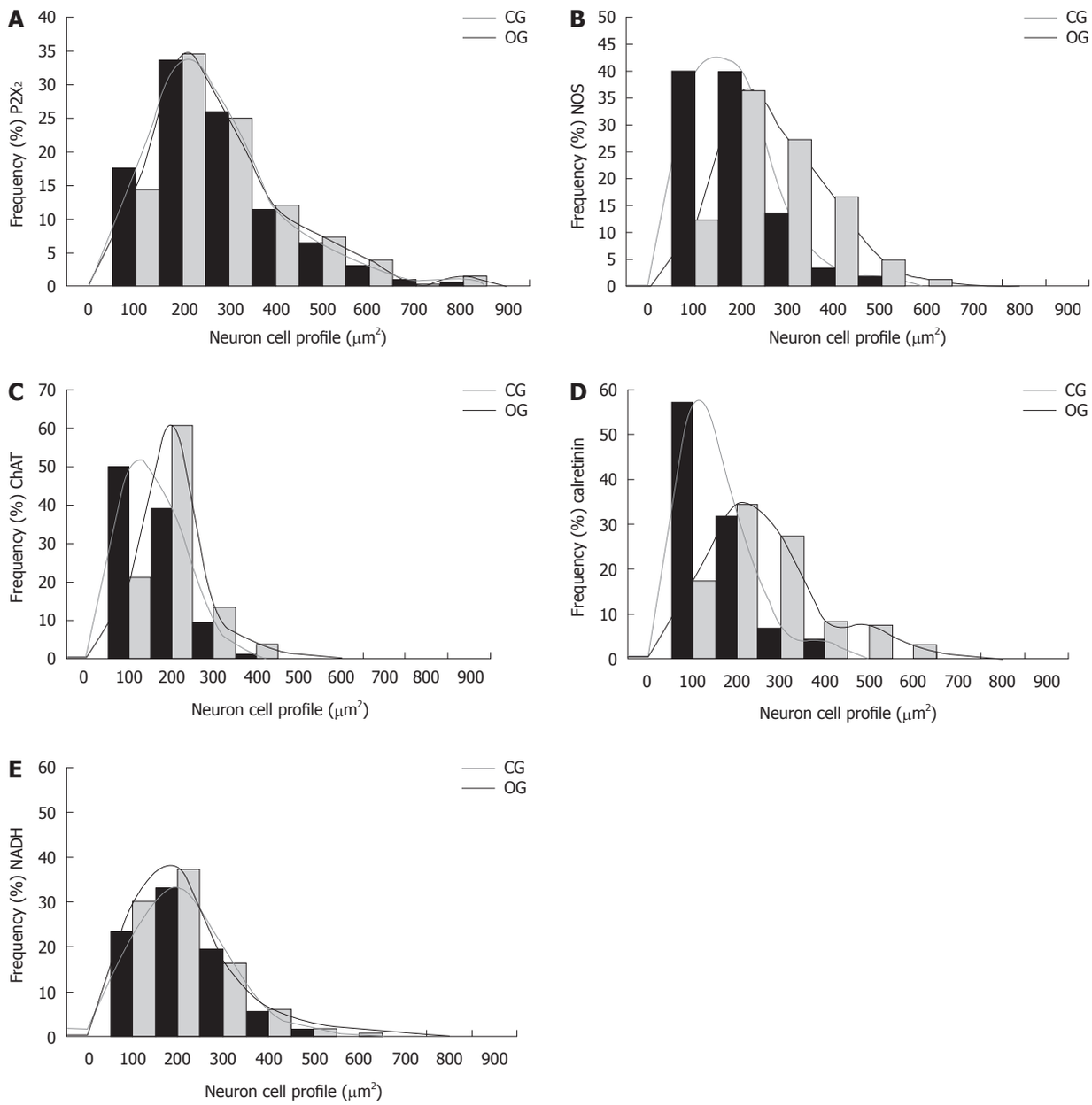

Figure 5 Frequency distribution of neuronal size profiles (μm^2) for neurons immunoreactive for $P2X_2$ receptor (A), nitric oxide synthase (B), choline acetyltransferase (C), calretinin (D), and positive for nicotinamide adenine dinucleotide-diaphorase (E) in the ileum myenteric plexus of control (black bars) and obese (grey bars) groups. NOS: Nitric oxide synthase; ChAT: Choline acetyltransferase; NADH: Nicotinamide adenine dinucleotide; CG: Control group; OG: Obese group.

show that numerous ChAT-IR neurons are present in the myenteric ganglia, as has been described in other species. In addition, we find that all the CalR-IR neurons are also immunoreactive for ChAT, confirming their identity as excitatory cholinergic motor neurons[4].

Changes in neuronal density have been observed in autonomic neurons[24], and various regions of the gastrointestinal tract in rat models of malnutrition and re-feeding[22,23,25] as well as following intestinal ischemia/reperfusion (I/R-i)[18]. In the *ob/ob* group of the current study, the density of nNOS-IR and ChAT-IR neurons was decreased by 42.6% and 53.4% compared to controls; in addition, the total number of nNOS-IR and ChAT-IR neurons was reduced by 35% and 47%, respectively, in the OG compared to controls. No statistically significant difference was found in the density of CalR-IR neurons between the groups. The observed decrease in neuronal density may be explained by the 29% increase in the area of the small intestine that we measured in the obese female mice. However, the estimated overall decrease in the number of neurons in the *ob/ob* intestine implies an actual loss of enteric neurons in these mice. Another enzyme, NADH-diaphorase, has been shown to decrease during malnourishment in neurons of the small and large intestines[22,23]. In the current study, however, we did not observe any alteration in the density of NADH-diaphorase-positive neurons in the OG compared to controls.

In the obese female mouse small intestine, we ob-

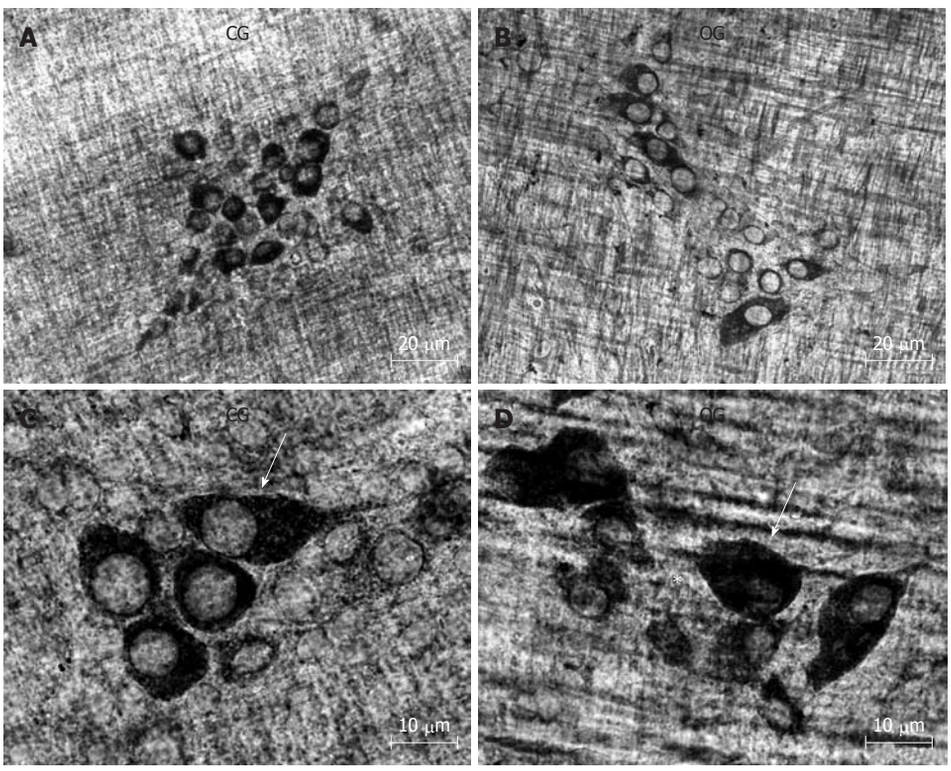

Figure 6 Ileum myenteric neurons labeled for nicotinamide adenine dinucleotide-diaphorase activity in the control group (A, C) and the obese group (C, D), and spaces between the neurons (asterisk) in the obese group group (B, D). Arrows show stained myenteric neurons. CG: **Control group**; **OG**: **Obese group**.

served increases in both the density and estimated number of $P2X_2$R-IR neurons compared to controls. This increase could be due to the presence of $P2X_2$R IR in another type of enteric nervous system cell, one which is not immunoreactive for nNOS, CalR or ChAT; namely, enteric glial cells. The presence of P2X and P2Y receptors has been described in astrocytes and microglia of the central nervous system (CNS)[9] as well as in enteric glial cells[26]. However, the increase of $P2X_2$R IR following undernourishment has been show to occur specifically in enteric neurons[17], making it likely that our observations of increased $P2X_2$R-IR also result from increased neuronal expression.

Changes in the central neuronal expression of $P2X_{1-7}$ purinoceptors frequently occur not only during maturation and neuronal differentiation, but also following various types of acute insults to the CNS. Prolonged stimulation of ATP receptors has been shown to result in changes in the location and density of P2 receptors in the cell membrane[27].

The NADH-diaphorase method is widely used in studies of alterations in the enteric nervous system and other autonomic plexus that relate to undernourishment, re-feeding, and age[22,23,28]. Our current data indicate that the density of NADH-diaphorase positive neurons showed a tendency to decrease in the obese mice compared to controls; however, these differences were not statistically significant.

Previous immunohistochemical and histochemistry studies have shown that under-nutrition[22,23] and I/R-i[18] affect the size profiles of neurons in the gastrointestinal tract. When using NADPH-diaphorase histochemistry to label myenteric neurons, we did not observe differences between the control and OGs, although the range of profile areas using this technique was consistent with that estimated from measurements of nNOS-, ChAT- and CalR-IR. Using an immunohistochemistry technique we were unable to verify exactly which neuronal classes demonstrated alterations in cell size in the obese mouse intestine. We show that cell area was increased for nNOS-, ChAT-, and CalR-IR neurons in the obese mouse, compared to controls. In agreement with these findings, modest (4.4%) increases in the profile area of myenteric neurons have been demonstrated in diabetic rats[29].

Epidemiological studies have suggested that excess body weight may be a major risk factor for colon and breast cancer. In an animal model of colon carcinogenesis, mice fed a high-fat diet exhibited a greater number of colon tumors than lean animals. Their increased abdominal fat was associated with higher concentrations of leptin, insulin, and insulin-like growth factor 1, which possibly mediate tumor growth. These data suggest that the metabolic burden created by excess adiposity accelerates uncontrolled cell growth and survival, thereby increasing the risk of developing breast and colon cancer[30].

Our understanding of the anatomical and molecular consequences of obesity becomes even more complex when we take into account the very complicated nature of the innervation of the gut. We believe that the current

study provides a positive step in our knowledge of the relationship between obesity, diabetes, and gastrointestinal disease. The results of this study suggest that there are significant changes in the intestinal ileum of obese mice, findings that further promote our understanding of obesity and the enteric nervous system.

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COMMENTS

Background

Obesity is a chronic heterogeneous disorder characterized by abnormal or excessive fat accumulation that presents a risk to health. Obesity is associated with increased risk of type 2 diabetes and is often accompanied by complications of the gastrointestinal tract, such as gastroparesis, constipation, diarrhea, and fecal incontinence. In addition, defects in enteric nervous system function have been reported in *ob/ob* mice, including alterations in intestinal motility and in the enteric neurons.

Research frontiers

The myenteric plexus is affected in obesity. The chemical coding of enteric neurons, as well as $P2X_2$ receptor ($P2X_2R$) expression, has not been characterized in the *ob/ob* mice.

Innovations and breakthroughs

The present study showed the effects of obesity in *ob/ob* mice on the morphology of the $P2X_2R$-, nitric oxide synthase-, calretinin- and choline acetyltransferase-immunoreactivity (IR) neurons of the myenteric plexus.

Applications

The present study suggests that obesity may change the chemical coding of the $P2X_2R$-IR neurons in the myenteric plexus, as well as that of inhibitory, cholinergic, and intrinsic primary afferent neurons on the enteric nervous system.

Terminology

The enteric nervous system is composed of the myenteric and submucosal plexuses, which control the motility of the intestine. Adenosine 5'-triphosphate is a well-known neurotransmitter and a ligand of the P2X receptor family, which is made up of seven known receptor subunits PX_{1-7}.

Peer review

This study fulfills its purpose and provides good results both qualitatively and quantitatively.

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