ORIGINAL ARTICLE

Effects of protein deprivation and re-feeding on P2X$_2$ receptors in enteric neurons

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Abstract

AIM: To investigate the effects of malnutrition and re-feeding on the P2X$_2$ receptor, nitric oxide synthase (NOS), calretinin, calbindin and choline acetyltransferase (ChAT) in neurons of the rat ileum.

METHODS: We analyzed the co-localization, numbers and sizes of P2X$_2$-expressing neurons in relation to NOS-immunoreactive (IR), calbindin-IR, ChAT-IR, and calretinin-IR neurons of the myenteric and submucosal plexus. The experimental groups consisted of: (1) rats maintained on normal feed throughout pregnancy until 42 d post-parturition (N); (2) rats deprived of protein throughout pregnancy and 42 d post-parturition (D); and (3) rats undernourished for 21 d post-parturition and then given a protein diet from days 22 to 42 (DR). The myenteric and submucosal plexuses were evaluated by double labeling by immunohistochemical methods for P2X$_2$ receptor, NOS, ChAT, calbindin and calretinin.

RESULTS: We found similar P2X$_2$ receptor immunoreactivity in the cytoplasm and surface membranes of myenteric and submucosal neurons from the N, D and DR groups. Double labeling of the myenteric plexus demonstrated that approximately 100% of NOS-IR, calbindin-IR, calretinin-IR and ChAT-IR neurons in all groups also expressed the P2X$_2$ receptor. In the submucosal plexus, the calretinin-IR, ChAT-IR and calbindin-IR neurons were nearly all immunoreactive for the P2X$_2$ receptor. In the myenteric plexus, there was a 19% increase in numbers per cm^2 for P2X$_2$ receptor-IR neurons, 64% for NOS-IR, 84% for calretinin-IR and 26% for ChAT-IR neurons in the D group. The spatial density of calbindin-IR neurons, however, did not differ among the three groups. The submucosal neuronal density increased for calbindin-IR, calretinin-IR and ChAT-IR neurons. The average size of neurons in the myenteric plexus neurons in the D group was less than that in the controls and, in the re-fed rats; there was a 34% reduction in size only for the calretinin-IR neurons.

CONCLUSION: This work demonstrates that expression of the P2X$_2$ receptor is present in inhibitory, intrinsic primary afferent, cholinergic secretomotor and vasomotor neurons. Undernutrition affected P2X$_2$ receptor expression in the submucosal plexus, and neuronal and size. These changes were rescued in the re-fed rats.

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INTRODUCTION

ATP is known to be a co-transmitter in the nervous system and a ligand of the P2X receptor family, which is made up of seven known receptor subunits (PX_{1-7})[1]. In the myenteric plexus, electrophysiological studies have found P2X receptors in 80%-90% of neurons[2]. P2X receptors play an important role in synaptic transmission within the neural pathways and mediate intestinal motility[3-5]. Immunohistochemical studies have documented the distribution of P2X receptors in the enteric nervous system of guinea pigs[6-11], rats[12-14] and mice[15,16]. There is only one earlier study in which the authors have reported expression of P2X2 receptor in calretinin and calbindin neurons in the ileal myenteric plexus of rats[12].

The effects of undernutrition on enteric and other autonomic neurons have been investigated[17-25]. In the enteric nervous system, a 27% decrease in the number of enteric neurons in the jejunum of rats submitted to severe prenatal malnutrition has been reported[18], and a mean neuronal loss of 13% in the myenteric plexus of the proximal colon has been observed after malnutrition[20]. Experiments that have examined the effect of re-feeding on enteric neuronal number suggest that, when neurons are reduced in number by undernutrition, they do not recover[18]. However, other reports have described a 15% decrease in the sizes of myenteric neurons from the large intestine of pre- and postnatally protein-deprived animals, as well as recovery of normal size after re-feeding[21]. A 45% size reduction in enteric neurons of the small intestine has also been observed following undernutrition[22].

The present work analyzed the effects of pre- and postnatal protein undernutrition and postnatal re-feeding on neurons immunoreactive for the P2X2 receptor, by specifically examining the expression of nitric oxide synthase (NOS), calretinin, calbindin and choline acetyltransferase (ChAT) in these neurons, as well as neuronal density and somatic size in the myenteric and submucosal plexuses in the rat ileum.

MATERIALS AND METHODS

Experimental animals

The study was conducted according to current legislation on animal experiments of the Biomedical Science Institute of the University of São Paulo. Young male and female Wistar rats (200-240 g body weight) were mated.

After conception, which was assumed to have occurred when vaginal sperm plugs were found, the females were placed in individual cages. During pregnancy, the nourished mothers received an AIN-93G normal protein diet (protein, 20%; fat, 7%; carbohydrate, 20% and fiber, 5%), and the undernourished mothers received the AIN-93G diet with low protein (protein, 5%; fat, 7%; carbohydrate, 20% and fiber, 5%) (Rhoster Indústria e Comércio Ltda, São Paulo, Brazil). The rats were maintained under standard conditions at 21℃, with a 12-h light/dark cycle, and all groups were supplied with water *ad libitum*. After parturition, the dams and pups received the same diet that the dam had during pregnancy. Only the male animals in the litters were used for experimentation. Females remained in the litters but were not investigated. There were three experimental groups. The first group of rats was maintained on normal feed throughout pregnancy until examined at 42 d (P42) (N, $n = 5$). The second group was protein-deprived throughout pregnancy and postnatally for 42 d (P42) (D, $n = 5$). The third group of rats was the deprived plus re-feeding group (DR, $n = 5$), in which animals were undernourished until P21, and then received the AIN-93G normal protein diet from P22 to P42[21,22]. At P42, animals were weighed and euthanized in a CO_2 chamber and the anterior abdominal wall was opened. The small intestine was removed and washed in PBS. The surface area of the small intestine was measured using a planimeter.

Immunohistochemistry

Fresh segments of ileum were removed from each animal of the N, D and DR groups and placed in PBS (0.15 mol/L NaCl in 0.01 mol/L sodium phosphate buffer, pH 7.2) that contained nicardipine (10^{-6} mol/L; Sigma, St Louis, MO, USA) to inhibit tissue contraction. The dissected pieces were opened 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 tissue was cleared of fixative with three 10-min washes in 100% DMSO, followed by three 10-min washes in PBS. All tissue was stored at 4℃ in PBS that contained sodium azide (0.1%). The fixed tissue was dissected and the mucosa, submucosa and circular layers were removed to obtain longitudinal muscle-myenteric plexus whole mounts. In the second type of preparation, the mucosa and muscularis externa were removed to reveal the intact submucous layer. Whole-mount preparations of the myenteric and submucosa of the ileum were preincubated in 10% normal horse serum in PBS that contained 1.5% Triton X-100 for 30 min at room temperature, to reduce non-specific binding and to permeabilize the tissue (Table 1). To localize P2X2 receptor immunoreactivity, we used a rabbit antiserum raised against amino acid sequence 457-472 of the rat P2X2 receptor, with a single Cys extension at the N-terminal (AB5244; Chemicon, Temecula, CA, USA). Incubation was for 48 h at 4℃ at a dilution of 1:120 in 10% normal horse serum in PBS that contained 1.5% Triton X-100. Double labeling was achieved using combina-

Table 1 Characteristics of primary and secondary antibodies			
Tissue antigen	**Host**	**Dilution**	**Code and reference**
NOS	Sheep	1:2000	H205
Calbindin	Mouse	1:500	Swant 300
Calretinin	Goat	1:100	CG1 Swant
ChAT	Goat	1:50	Chemicon
Donkey anti-rabbit IgG Alexa 488		1:500	Molecular probes
Donkey anti-sheep IgG Alexa 594		1:100	Molecular probes
Donkey anti-mouse IgG Alexa 594		1:200	Molecular probes

NOS: Nitric oxide synthase; ChAT: Choline acetyltransferase.

Table 2 Body weight and small-intestinal area of pre- and postnatal protein-deprived rats at 42 d postnatal			
	N	**D**	**DR**
Body weight (g)	160 ± 10	40 ± 18[a]	100 ± 22
Area of small intestine (cm^2)	20.41 ± 3.7	13.3 ± 0.6[a]	16.5 ± 0.4

[a]$P < 0.05$ *vs* protein deprived, Tukey's test for multiple comparisons.

tions of antisera (Table 1). Following incubation in primary antisera, tissue was given three 10-min washes in PBS and incubated in a mixture of secondary antibodies (Table 1). Further 10-min washes in PBS were made before tissue was mounted in glycerol buffered with 0.5 mol/L sodium carbonate buffer (pH 8.6).

Imaging
Preparations were examined on a Leica microscope equipped with the appropriate filters for Alexa 488 (450-490 nm excitation filter and 515-565 nm emission filter) and Alexa 594 (530-585 nm excitation filter and 615 nm emission filter). Images were recorded using an Image-Pro-Plus-coupled camera and Image-Pro Plus software (Media Cybernetics, Bethesda, MD, USA). Preparations were also analyzed using confocal microscopy on a Zeiss confocal scanning laser system installed on a Zeiss Axioplan 2 microscope (Carl Zeiss). The system had a krypton/argon laser for differential visualization of the fluorophores using a 488-nm excitation filter and a 522/535-nm emission filter for 488 and 568 nm excitation filters and a 605/632 nm emission filter for Alexa 594. The images were 512 × 512 pixels in size and the thickness of each optical section was 0.5 μm. Immunoreactive cells were scanned as a series of optical sections with a center spacing of 0.2 μm. Confocal images were collected using LSM 5 Image Zeiss processing software (Carl Zeiss MicroImaging, Germany). Images were further processed using Corel Photo Paint and Corel Draw software programs (Corel Corporation).

Quantitative analyses
The proportions of neurons in which antigen immunoreactivity was co-localized were determined by examining double-labeled neurons. Neurons were first located by the presence of a fluorophore that labeled one antigen, and then the filter was switched to determine whether the neuron was labeled for a second antigen, located with a second fluorophore of a different color. In this way, proportions of neurons labeled for pairs of antigens were determined. The cohort size was 100 neurons and data were collected from preparations obtained from at least four animals. The percentage of neurons immunoreactive to a second neurochemical was calculated and expressed as mean ± SE. The numbers of P2X$_2$-immunoreactive (IR), NOS-IR, calbindin-IR, calretinin-IR and ChAT-IR neurons and nerve cell perikarya were measured by examining the whole-mount preparations under a binocular microscope at a magnification of 100 ×. All neurons present in each 1 cm^2 were counted. The nerve cell perikarya profiles area, major axes, and minor axes of 50 nerve cell perikarya from each animal were obtained on a semiautomatic morphometry device, the Image-Pro Plus Program.

Statistical analysis
mean ± SE were calculated and compared by analysis of variance and Tukey test for multiple comparisons, as appropriate. The level of significance was set at $P < 0.05$.

RESULTS
The mean body weight of animals of the N group (160 ± 10 g) was approximately 400% greater than that of the D group (40 ± 18 g). The body weight of the DR group (100 ± 22 g) was restored to within 20% of normal at P42 ($P < 0.05$). The small intestine area of the D group was 34% less ($P < 0.05$) than that of the N group, and there was no statistical difference between the N and DR groups (Table 2).

The qualitative results demonstrated that P2X$_2$ receptor immunoreactivity was found in the myenteric and submucosal plexuses of the ileum of all groups. Positive labeling was seen in the cytoplasm and surface membranes of most nerve cells of the nourished, undernourished and refed groups (Figure 1). The labeling intensity of the P2X$_2$ receptor in the myenteric and submucosal ganglia of the N, D and DR groups was similar. Double-labeling studies were conducted to identify neurons that had P2X$_2$ immunoreactivity co-localized with NOS, calbindin, calretinin and ChAT in ileal myenteric neurons (Figure 1), and calbindin, calretinin and ChAT in the ileal submucosal plexus of the N, D and DR groups (Figure 2). In all groups, the cellular morphology of the myenteric plexus showed that NOS-IR neurons had a Dogiel Type I morphology, while calretinin-IR neurons exhibited Dogiel Type II morphology, and calbindin-IR neurons had both small and large Dogiel Type II neurons. In the submucosal plexus, calbindin-IR neurons had Dogiel Type II morphology. The intensity of ChAT immunoreactivity was reduced in some neurons of the myenteric and submucosal ganglia of undernourished rats.

Co-localization
The quantitative results revealed that, in the myenteric plexuses, the majority of NOS-IR, calbindin-IR, calretinin-
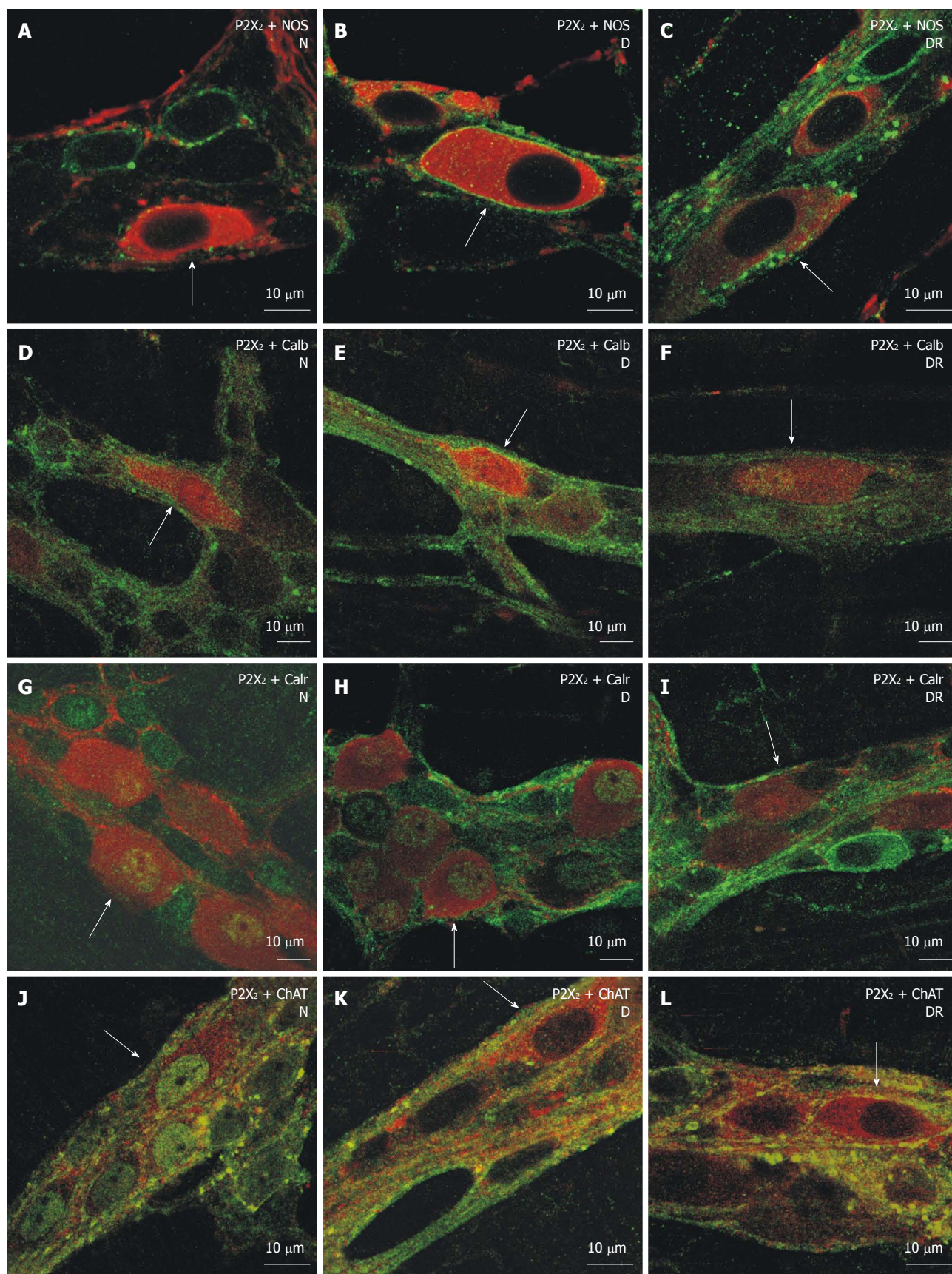
Figure 1 Co-localization of P2X$_2$ receptor immunoreactivity with nitric oxide synthase, calbindin, calretinin and choline acetyltransferase immunoreactivity in the ileal myenteric plexus in the N, D and DR groups. A-C: P2X$_2$ receptor-IR (green) co-localized with nitric oxide synthase (NOS)-IR (red); D-F: P2X$_2$ receptor-IR (green) co-localized with calbindin-IR (red); G-I: P2X$_2$ receptors (green) co-localized with calretinin (red); J-L: P2X$_2$ receptors (green) co-localized with choline acetyltransferase (ChAT) (red). Double-labeled neurons are indicated by arrows.
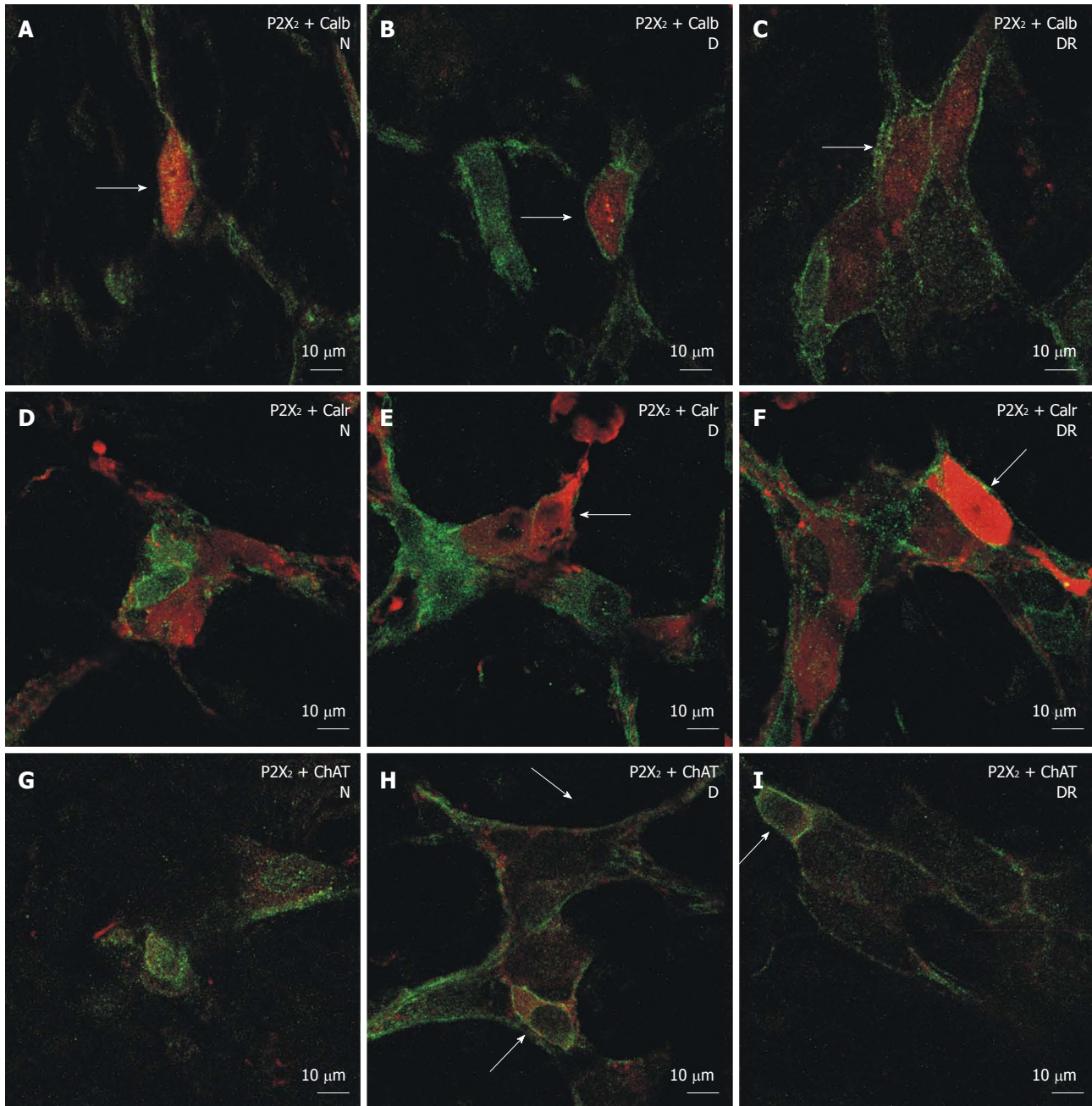
Figure 2 Co-localization of P2X$_2$ receptor immunoreactivity with calbindin, calretinin and choline acetyltransferase immunoreactivity in the ileal submucosal plexus in N, D and DR groups. A-C: P2X$_2$ receptor (green) co-localized with calbindin (Calb) (red); D-F: P2X$_2$ receptor (green) co-localized with calretinin (Calr) (red); G-I: P2X$_2$ receptor (green) co-localized with choline acetyltransferase (ChAT) (red). Double-labeled neurons are indicated by arrows.

IR and ChAT-IR neurons also were immunoreactive for the P2X$_2$ receptor. In the submucosal plexus of the ileum, P2X$_2$-IR neurons were also calbindin-IR, calretinin-IR, and ChAT-IR.

In the myenteric plexus, the majority of NOS-IR neurons were immunoreactive for the P2X$_2$ receptor (N group was 99% ± 0.6% co-localized, D group was 100%, and DR group was 99% ± 0.4%). Also, the majority of calbindin-IR neurons were IR for the P2X$_2$ receptor (N group was 98% ± 0.4%, D group was 100%, and DR group was 99% ± 1%). The majority of calretinin-IR neurons were also IR for the P2X$_2$ receptor (group

N was 100%, D group was 98% ± 0.6%, and DR group was 98% ± 1%). Most ChAT-IR neurons were also IR for the P2X$_2$ receptor in the N, D and DR groups (96.2% ± 2%, 96.2% ± 2%, and 97% ± 3%, respectively).

In the submucosal plexus, co-localization between calbindin-IR and P2X$_2$ receptor-IR neurons was complete in the N, D and DR groups. The co-localization between P2X$_2$ receptor-IR and calbindin-IR was 16% ± 0.7% in the N group, 31% ± 2% in the D group, and 24% ± 3% in the DR group ($P < 0.002$). In all three groups, calretinin-IR and ChAT-IR neurons co-localized 100% with P2X$_2$ receptor-IR neurons.
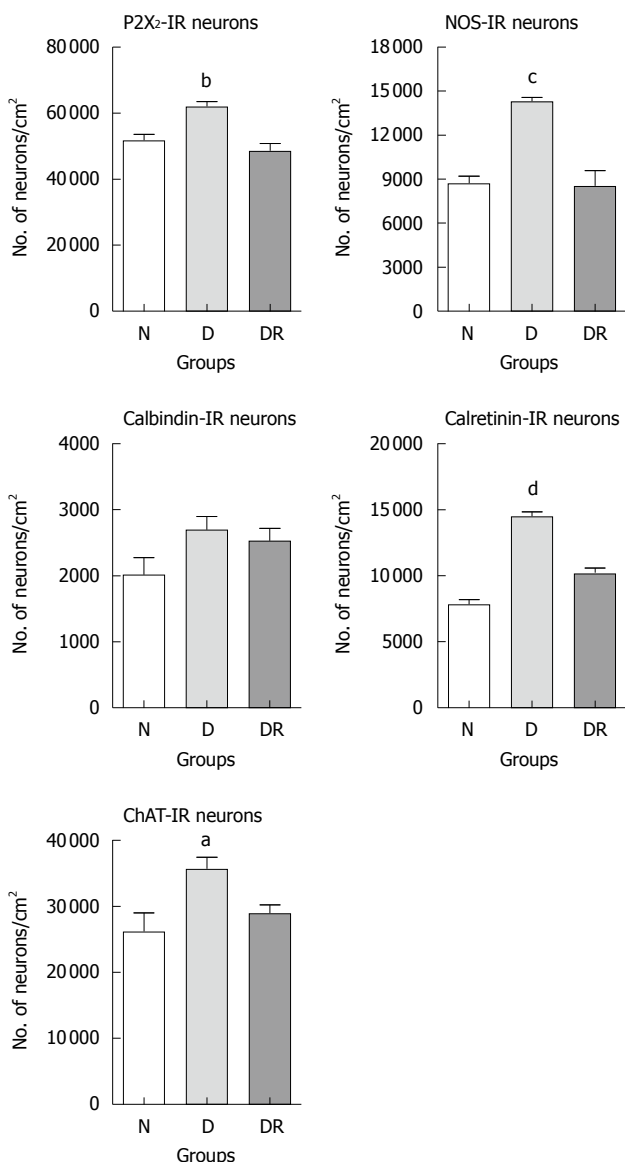
Figure 3 The density (neuron/cm^2) of neurons immunoreactive for the P2X$_2$ receptor, nitric oxide synthase, calbindin, calretinin, and choline acetyltransferase in the ileal myenteric plexus in the N, D and DR groups. [a]$P < 0.02$, [b]$P < 0.01$, [c]$P < 0.002$, [d]$P < 0.001$ *vs* N and DR groups. NOS: Nitric oxide synthase; ChAT: Choline acetyltransferase.
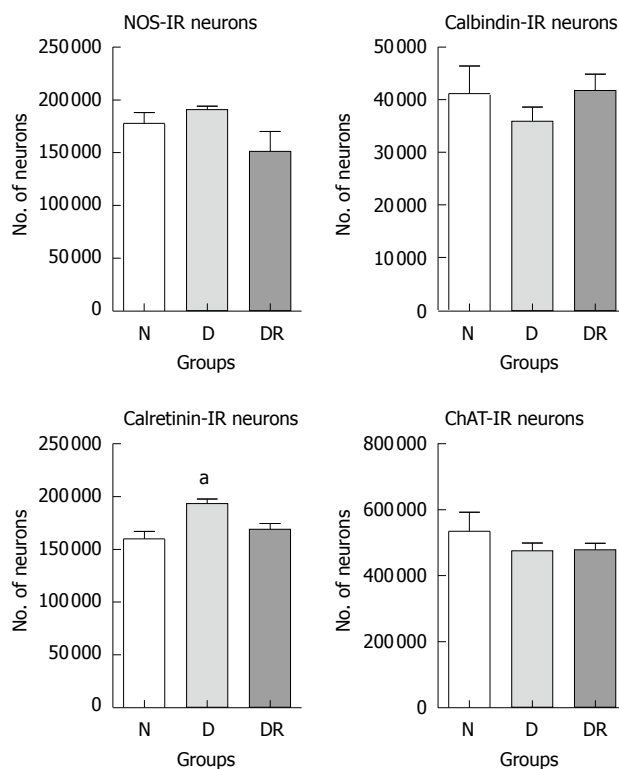
Figure 4 Calculated numbers of neurons immunoreactive for the P2X$_2$ receptor, nitric oxide synthase, calbindin, calretinin and choline acetyltransferase in the small intestine of the N, D and DR groups. [a]$P < 0.05$ *vs* N and DR groups. NOS: Nitric oxide synthase; ChAT: Choline acetyltransferase.

Neuronal density

In the myenteric plexus, the number of neurons per unit area was increased by 19% for P2X$_2$ receptor-IR neurons ($P < 0.01$), 64% for NOS-IR neurons ($P < 0.002$), 84% for calretinin-IR neurons ($P < 0.001$), and 26% for ChAT-IR neurons in group D ($P < 0.02$); calbindin-IR neuron density, however, did not differ among the three groups ($P > 0.05$, Figure 3). In the myenteric plexus, the total number of NOS-IR neurons, taking into account the change in intestinal surface area (Figure 4), calbindin-IR neurons and ChAT-IR neurons did not differ significantly between the three groups. There was, however, a 20% increase in the numbers of calretinin-IR neurons and decrease in P2X$_2$ receptor cells with undernutrition relative to controls ($P < 0.05$, Figure 4).

In the submucosal plexus, the density of P2X$_2$ receptor-IR, calbindin-IR and ChAT-IR neurons increased significantly in the undernourished group (67%, $P < 0.0003$; 189%, $P < 0.001$ and 42%, $P < 0.01$, Figure 5). Calretinin-IR neuron density did not differ among the three groups ($P > 0.05$, Figure 5). In the submucosal plexus, the total numbers of the calretinin-IR neurons decreased by 23% ($P < 0.05$), and this was accompanied by an 89% increase in the calculated numbers of calbindin-IR neurons. In this region, there was no change in the numbers of ChAT-IR neurons (Figure 6).

Nerve cell perikarya

Neuron size (nerve cell perikarya, the major and minor axes of the myenteric plexus neurons) of the calretinin-IR neurons were approximately 34% smaller in the protein-deprived rats ($P < 0.001$) than the control or re-fed rats. There was an increase of 35% in the nerve cell perikarya of calbindin-IR neurons and a 14% increase in the minor axes of the calbindin-IR neurons in the DR group, as well as a decrease of 15% in the major axes of the NOS-IR neurons (Table 3).

In the submucosal neurons, there were group differences ($P < 0.05$) with respect to the neuron size of calbindin-IR, calretinin-IR and ChAT-IR neurons. There was a 13% decrease in the major axes of calretinin-IR and ChAT-IR neurons ($P < 0.05$) and an 18% increase in the minor axes of calbindin-IR neurons ($P < 0.05$). Neu-
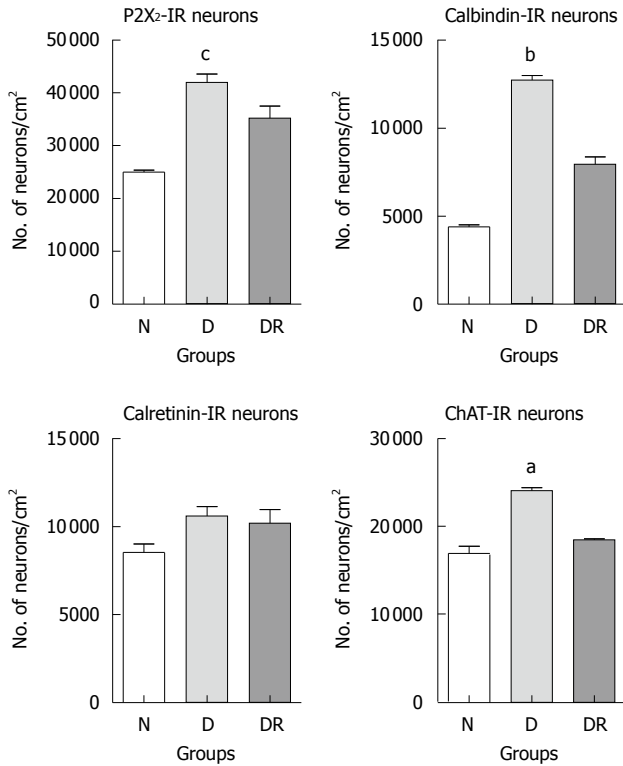
Figure 5 The density of neurons (neuron/cm²) immunoreactive for P2X₂ receptor, calbindin, calretinin and choline acetyltransferase in the ileal submucosal plexus in the N, D and DR groups. [a]$P < 0.01$, [b]$P < 0.001$, [c]$P < 0.0003$ *vs* N and DR groups. ChAT: Choline acetyltransferase.
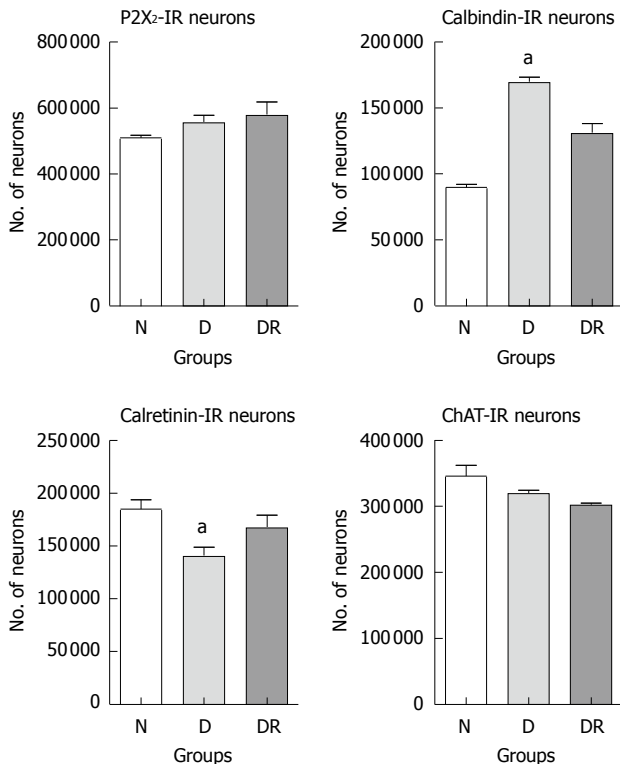

Figure 6 The calculated numbers of neurons immunoreactive for P2X₂ receptor, calbindin, calretinin and choline acetyltransferase in the ileal submucosal plexus of the N, D and DR groups. [a]$P < 0.05$ *vs* N and DR groups. ChAT: Choline acetyltransferase.

Table 3 Results of the profile area (μm^2), major and minor axes of nitric oxide synthase-IR, calbindin-IR, calretinin-IR and choline acetyltransferase-IR neurons in the ileal myenteric and submucosal plexuses of the N, D and DR groups

	N	D	DR
Myenteric plexus			
NOS			
Profile	240.4 ± 30.7	197.1 ± 25.8	225.8 ± 26.7
Major axes	25.2 ± 2.1	21.7 ± 1.6[a]	25.1 ± 1.9
Minor axes	12.1 ± 0.6	11.3 ± 0.8	11.4 ± 1.1
Calbindin			
Profile	227.7 ± 43.1	223.6 ± 26.3	307.5 ± 58[c]
Major axes	23.5 ± 2.8	24.1 ± 2.9	27.9 ± 4.6
Minor axes	12.2 ± 1.1	11.9 ± 0.3	14.0 ± 0.8[c]
Calretinin			
Profile	397.7 ± 39.6	259.2 ± 48.8[a]	331.5 ± 24.5
Major axes	29.3 ± 2.1	22.3 ± 3.5[a]	27.5 ± 2.7
Minor axes	16.7 ± 0.6	14.1 ± 0.5[a]	15.1 ± 0.5
ChAT			
Profile	229.4 ± 39.4	183.6 ± 39.3	198.7 ± 37.5
Major axes	21.8 ± 2.4	19.4 ± 2.4	20.1 ± 2.5
Minor axes	12.8 ± 1.0	11.7 ± 0.7	12.1 ± 0.8
Submucosal plexus			
Calbindin			
Profile	244.2 ± 47.6	256.7 ± 34.8	310.7 ± 46.2
Major axes	24.8 ± 1.7	27.4 ± 2.2	27.4 ± 1.7
Minor axes	12.4 ± 1.4	12.2 ± 0.8[b]	14.5 ± 1.2
Calretinin			
Profile	233.8 ± 51.5	200 ± 4.6	242.5 ± 41.6
Major axes	24.1 ± 2.1	20.8 ± 0.5[b]	25.7 ± 3.1
Minor axes	12.4 ± 1.7	12.1 ± 0.3	12.1 ± 1.4
ChAT			
Profile	185.5 ± 18.4	154.3 ± 22.4	175.3 ± 20.0
Major axes	20.1 ± 1.7	17.5 ± 0.8[b]	18.8 ± 0.7
Minor axes	11.4 ± 0.2	11.1 ± 0.9	11.5 ± 1.1

[a]$P < 0.05$, [b]$P < 0.001$ *vs* N and DR groups; [c]$P < 0.05$ *vs* N and D groups. Tukey's test for multiple values, mean ± SE, $n = 5$. NOS: Nitric oxide synthase; ChAT: Choline acetyltransferase.

ron size distributions in the myenteric and submucous plexuses of the N, D and DR groups are shown in the histograms of Figures 7 and 8.

DISCUSSION

Various methods have been used to induce experimental undernutrition[26]. The protocols of undernutrition and re-feeding employed in this study were effective, because malnourished animals lost weight, which was then recovered by re-feeding. These findings agree with those of other studies that have used similar protocols[21,22].

The antigen markers for different functional classes of neurons have been determined for guinea pig and mouse small intestine, and to a lesser extent in other mammals[27-31]. The expression patterns have been partly described in the rat[32,33]. NOS is expressed in inhibitory motor neurons in all species in the small and large intestine, whereas all other neuron types, such as excitatory motor neurons, interneurons, and intrinsic primary afferent neurons (IPANs) are immunoreactive for ChAT in the mouse and rat myenteric plexus[30,33-37]. Dogiel Type Ⅱ neurons, which are intrinsic primary afferent neurons in all species

Figure 7 Histograms showing the distribution of areas (μm²) of neurons immunoreactive for nitric oxide synthase (A), calbindin (B), calretinin (C) and choline acetyltransferase (D) in the ileal myenteric plexus of the N, D and DR groups. NOS: Nitric oxide synthase; ChAT: Choline acetyltransferase.

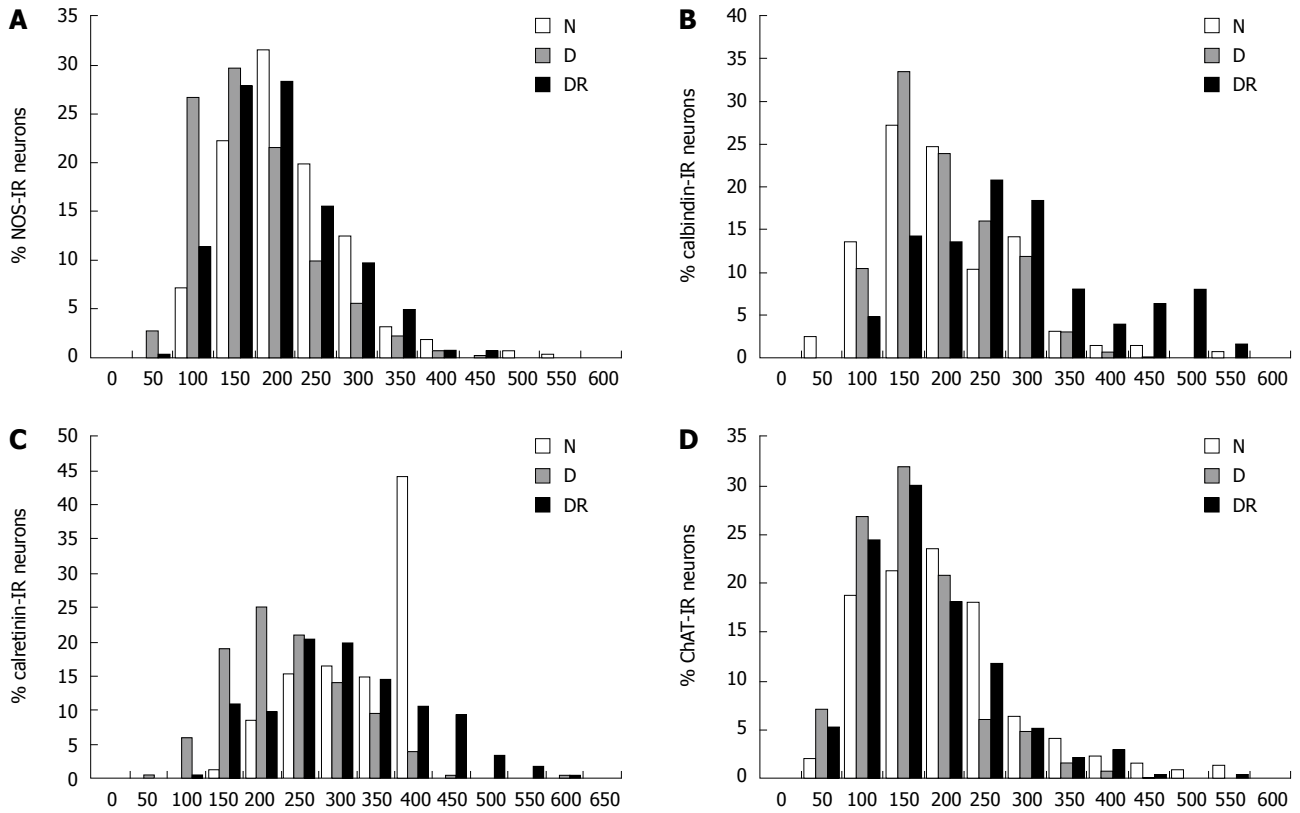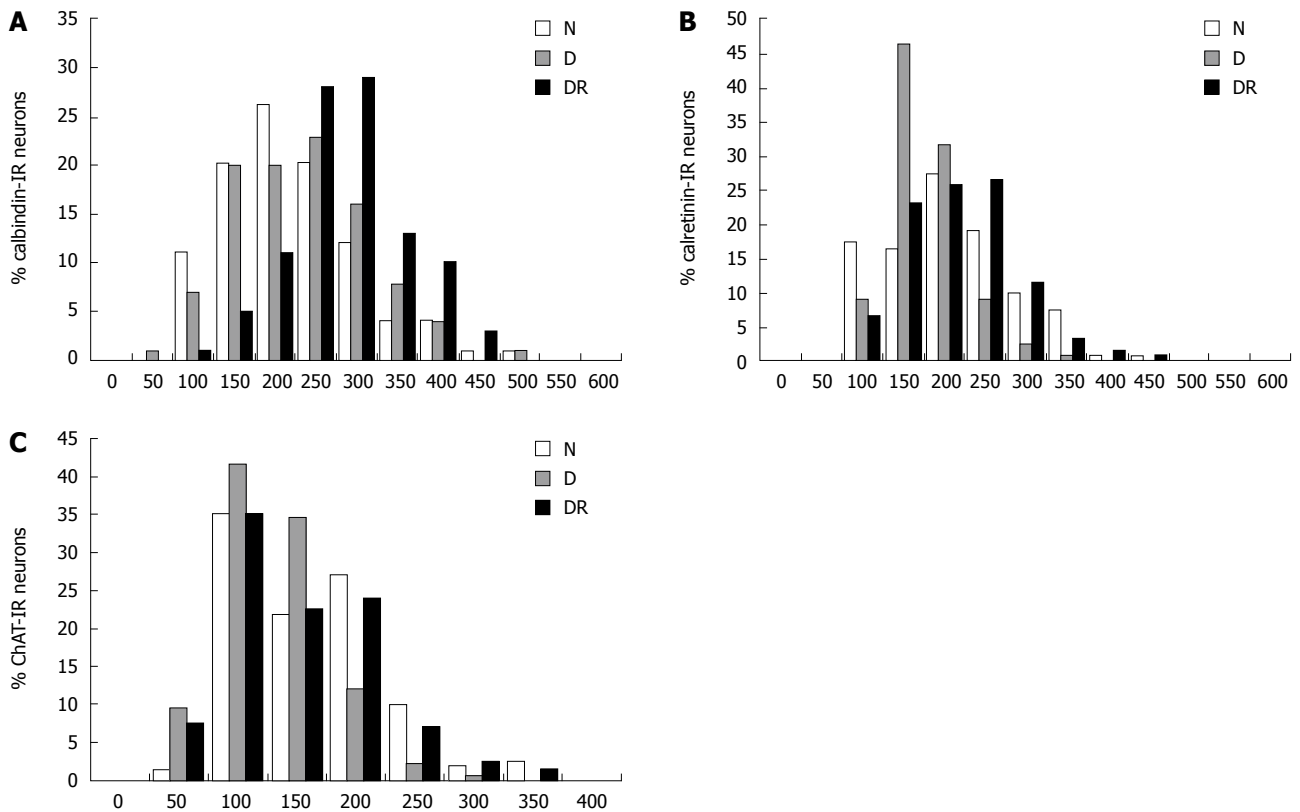**Figure 8 Histogram showing the distribution of areas (μm²) of neurons immunoreactive for calbindin (A), calretinin (B) and choline acetyltransferase (C) in the ileal submucosal plexus of the N, D and DR groups.** ChAT: Choline acetyltransferase.

studied, including rats[38], are immunoreactive for calretinin in the rat small intestine[33]. The subclasses of neurons in the submucosal ganglia of rat ileum have not been extensively studied but, by analogy with other small mammals, they are likely to include cholinergic and non-cholinergic secretomotor neurons and, possibly, IPANs[27,29,37]. In accordance with the data from rats and other small mammals, we chose the enzyme NOS to identify inhibitory motor neurons, ChAT to identify excitatory motor neurons and interneurons, calretinin to identify IPANs, and calbindin, which is a marker of many neurons in the rat small intestine. Within the three groups, the NOS-IR neurons had Dogiel Type I morphology and the calretinin-IR neurons in the myenteric and submucosal plexuses had Dogiel Type II morphology while presenting various sizes. Calbindin-IR neurons exhibited four distinct morphologies: the Dogiel type II neurons (large and small) and Dogiel type I neurons (small and elongated). These findings are consistent with the literature[37].

By qualitative analyses, there were no differences in neuron morphology between the N, D and DR groups. There was also no observed change in the labeling intensity of neurons immunoreactive for NOS, calretinin, and calbindin among the three groups. However, the intensity of ChAT immunoreactivity was reduced in some neurons of the undernourished group and increased in the re-fed group. These results are consistent with other studies in which a decrease in the intensity of ChAT immunoreactivity in the myenteric neurons of malnourished animals has been reported[22,39]. Other enzymes, such as NADH diaphorase, also show decreased immunoreactivity in malnourished and recovery in re-fed animals[21,22].

Previous studies have revealed the presence of P2X$_2$, P2X$_3$ and P2X$_7$ receptor-containing neurons in the enteric nervous system of guinea pigs[6-11], rats[12-14] and mice[15,16]. In the current work with the rat enteric nervous system, we found that P2X$_2$ receptors were present in both the cytoplasm and cytoplasmic membrane in neurons of the myenteric and submucosal plexuses.

The co-localization of different neuronal markers described in this work confirm the presence of P2X$_2$ receptors in NOS-IR, calretinin-IR, and calbindin-IR enteric neurons, as well as in ChAT-IR neurons of the myenteric and submucosal plexuses. ATP has been reported to depolarize 70%-90% of guinea-pig enteric neurons, which indicates that many enteric neurons have ionic P2X receptors[2,3]. In the myenteric plexus of rats, we found that the P2X$_2$ receptors exhibited complete co-localization with NOS-IR, calretinin-IR, calbindin-IR and ChAT-IR neurons in the three groups examined, without significant differences among them. This finding is consistent with the literature, which demonstrates the presence of the receptor in inhibitory neurons, as well as intrinsic excitatory and secretomotor/vasodilator primary afferent neurons in guinea pigs[7] and rats[12].

Our analyses of co-localization in the submucosal plexus showed that all calretinin-IR, ChAT and calbindin-IR neurons co-localized with P2X$_2$ receptor-IR neurons.

However, there was a significant increase ($P < 0.05$) in the co-localization of P2X$_2$ neurons with calbindin-IR neurons in the undernourished group, which recovered in the re-fed DR group. This result agrees with Xiang and Burnstock's[12] findings, in which they reported expression of P2X$_2$ receptor in calretinin and calbindin neurons in the ileal myenteric plexus of rats. The co-localization that we described in the myenteric and submucosal plexuses suggested that malnutrition did not change neurochemical coding, for the markers that were used, in the enteric nervous system.

Changes in the density of myenteric neurons have been observed in various regions of the gastrointestinal tract in models of undernutrition[20-23,25,39] and recovery is observed in re-fed rats[21,22,25]. The increase in neuronal density in undernourished protocols is likely due to decreases in the surface area of the small or large intestine[20-22,39]. In our work on the myenteric plexus, neuron densities were increased for P2X$_2$ receptor-IR, NOS-IR, ChAT-IR and calretinin-IR neurons in the D group, and went back to control levels in the DR group. This increase in neuron density was due to a reduction of approximately 34% in small-intestinal area in the D group. There was recovery of the intestinal area in the DR group. In contrast, the density of calbindin-IR neurons in the myenteric plexus did not differ among the three groups ($P > 0.05$). Moreover, the increases in neuron density in the myenteric plexus in the D group were dependent upon the neuronal class examined. NOS-IR neuron density increased by 64%, calretinin-IR neurons by 84%, and ChAT-IR neurons by 26%; these data suggest that undernourishment affects the neuronal subtypes differently. There was no change in the calculation of the total number of NOS-IR, calbindin-IR or ChAT-IR neurons in the small intestine of the three groups. However, the calretinin-IR neuron numbers were increased (20%) in the undernourished group and P2X$_2$ receptor-IR neurons were decreased by around 25% in the D and DR groups.

The density of P2X$_2$-receptor-expressing neurons in the myenteric plexus in group N was about 51 000/cm^2 in our study. This value is higher than the combined sum of the two major neuronal subtype populations of the myenteric neurons: NOS (8000/cm^2) + ChAT (26 000/cm^2). This discrepancy could be due to P2X$_2$ receptor staining in another neuronal class, which was not immunoreactive for NOS, calbindin, calretinin or ChAT. Also, P2X$_2$ receptor labeling could have also stained enteric glial cells. The presence of P2X and P2Y receptors has been described in astrocytes and microglia of the central nervous system[40,41] and in enteric glial cells[9,42]. In the mammalian enteric nervous system, the proportion of glial cells to neurons is about three to one[43-45].

The tonic release of ATP into the extracellular space without a particular stimulus is a widespread physiological process. However, the release of ATP into the extracellular environment is also caused by pathophysiological events like inflammation, ischemia, injury as a consequence of cell damage or acute cell death, and metabolic

stress[46]. All physiological effects of ATP including fast purinergic transmission and co-transmission, the secretion of neuropeptides, and mechanosensory transduction might be amplified by overtly increased extracellular concentrations of ATP[46].

Studies from the literature have reported changes in the expression of purinergic receptors in different dietary conditions in the central nervous system. A diet deficient in zinc, for example, increases expression of $P2X_6$ receptors in the hippocampus of rats[47], which suggests that dietary zinc levels also affect protein expression and could act as a modulator of the receptor function. Increased $P2Y_1$ receptor mRNA expression in the hypothalamus after food restriction has been reported in rats[48], and the data indicate that expression of ADP/ATP-sensitive $P2Y_1$ receptors in the hypothalamus is dependent on feeding conditions. The enhanced expression of the $P2Y_1$ receptor during the early and late interval of restricted feeding suggests an increased demand for purinergic signaling to enhance the activity of hypothalamic neurons. Also, there is an indication of $P2Y_1$ and A_{2A} that purinergic receptor mRNA expression is altered during acute and chronic food deprivation[49]. Some authors have suggested that ATP/ADP, acting as extracellular signal molecules in the rat brain, is involved in the regulation of food intake, possibly depending on $P2Y_1$-receptor-mediated nitric oxide production[50].

During metabolic stress, such as hypoglycemia or brain ischemia, activation of different P2 receptors has been demonstrated *in vivo* and *in vitro*. The $P2X_2$ and $P2X_4$ receptors are upregulated after oxygen and glucose deprivation in organotypical slice cultures and in CA1 and CA3 pyramidal cells after *in vivo* ischemia in gerbils[51]. During *in vivo* and *in vitro* ischemia, the $P2X_7$ receptor density is upregulated in microglia and on astrocytes and neurons[52]. Prenatal protein malnutrition might increase circulating concentrations of ATP, and this increases $P2X_2$ expression in cells. Enhancement of $P2X_2$ receptors in the D group suggests an increased demand for purinergic signaling. These changes were all reversed in re-fed rats, which demonstrated the effectiveness of re-feeding upon enteric neuron recovery.

Changes in neuronal expression of $P2X_{1-7}$ purinoceptors are frequently seen not only as a result of maturation and neuronal differentiation, but also after various types of acute insults to the central nervous system such as ischemia, hypoxia, mechanical stress, axotomy, and inflammation. Purinergic mechanisms are involved in the etiology of many neurodegenerative conditions, especially due to the large extracellular release of ATP, adenosine, and other neurotransmitters[46,53] upon neural damage. Prolonged stimulation of ATP receptors results in changes in the location and density of P2 receptors in the cell membrane[46]. Increased $P2X_3$ receptor expression has been observed in inflammatory bowel disease of the large intestine, which suggests that changes in this receptor can cause pain and dysmotility of the bowel[54].

Nitrergic neurons (using nitric oxide synthesized by NOS) and cholinergic neurons (those that use the acetyl-

choline synthesized by ChAT) represent two major subpopulations of myenteric neurons[55], although these patterns vary between guinea pigs[56], mice[30,57] and rats[33,36].

Our work implies that differences between groups in the total neuronal density in the myenteric plexus are comprised principally of changes in the NOS-IR-and ChAT-IR neuronal populations. The total neuron density in the myenteric plexus was approximately $34\,800/cm^2$ in the nourished group. This neuronal density is greater in comparison with that in previous studies, which have reported values of 15 000 to $20\,000/cm^{2[58]}$, $10\,000/cm^{2[59]}$ and $18\,000/cm^{2[60]}$. These differences could be, in part, due to methodology as well as the different ages and strains of rats used.

Marese *et al*[61] have quantified neuronal numbers in the myenteric plexus of the duodenum using the Giemsa histological method and myosin V pan-neuronal immunohistochemical labeling. These studies have demonstrated that the number of neurons/cm^2 decreases with animal age between 21 and 428 d. Our experiments used 42-d-old rats of the same lineage (Wistar), and the estimate of neuron density in our work is within the 21-60 d range reported by Marese *et al*[61] (21 d: Giemsa 89 335 neurons/cm^2; myosin V: 59 364/cm^2; 60 d: Giemsa: 47 814/cm^2; myosin V: 30 291/cm^2).

In the present work, the proportions of NOS-IR and ChAT-IR neurons were 32% and 68%, respectively, in the myenteric plexus of the malnourished group. Consistent with previous studies[35,56], we found that these proportions were maintained in the D and DR groups.

Submucosal plexus

ChAT-IR neurons comprised the majority of submucosal plexus neurons in the three groups. These findings agree with prior studies, which have described most neurons of the submucosal plexus as ChAT-IR[33,56]. We demonstrated, for the first time, an increase in the density of $P2X_2$ receptor-IR, calbindin-IR, calretinin-IR and ChAT-IR neurons in the deprived animals, which returned to control levels in the re-fed animals in the submucosal plexus. This increase was due to a 34% reduction in the area of the small intestine in the deprived animals.

The total number of calbindin-IR submucosal neurons increased in the small intestine of undernourished animals, in contrast to a decrease in calretinin-IR neurons and no change in the number of ChAT-IR neurons. These data indicate that the lack of protein nutrition can also have an impact on the chemical coding of the submucosal plexus. Differences in these measures between the myenteric and submucosal plexuses might reflect a differential effect of malnutrition or undernutrition on these two regions, as well as a differential effect of undernourishment on each neuronal subtype. In addition, the increase of calbindin-expressing neurons in the submucosal plexus could be a compensatory mechanism in response to the decrease in these neurons in the myenteric plexus.

Neuronal sizes

Previous immunohistochemical studies have shown that

undernutrition affects the neuron size profile of the gastrointestinal tract[20,21,39]. Analyses using the Giemsa technique[20,62] and histochemistry[22] have found no significant differences in the neuronal sizes in the small intestine in nourished, undernourished and re-fed animals. The present work, using an immunohistochemistry technique, was unable to verify exactly which neuronal class showed changes in size. In the myenteric plexus, there were decreases in size of the calretinin-IR neurons in groups D and DR. There was also an increase in the size of calbindin-IR neurons in the DR group, compared to the N and D groups. There was no change ($P > 0.05$) in the size of ChAT-IR neurons among the three groups. The size of NOS-IR neurons also did not change, consistent with previous reports[58]. In the submucosal plexus, the sizes of calbindin-IR, calretinin-IR and ChAT-IR neurons were not affected by undernutrition. However, the major axes of the calbindin-IR and minor axes of the calretinin-IR and ChAT-IR neurons decreased in group D, with recovery in group DR. The differences between the submucosal and myenteric plexuses suggest again that undernutrition affects the two plexuses differently. The distribution areas of NOS-IR, calbindin-IR, calretinin-IR and ChAT-IR neurons in our study ranged from 100 to 500 μm^2, in agreement with previous reports[22].

The current study demonstrates that both undernourishment and re-feeding has a different impact on neuronal subtypes. Undernutrition also differently affects the myenteric and submucosal plexuses; changes in calbindin-IR neuronal density in the submucosal plexus were not reflected in the myenteric plexus, where only the profile of the calretinin-IR neurons was affected by dietary restriction. These changes were all reversed in re-fed rats, which demonstrated the effectiveness of re-feeding upon enteric neuron recovery.

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COMMENTS

Background
The myenteric and submucous plexuses are affected in undernourished and re-fed rats.

Research frontiers
The chemical coding and P2X$_2$ receptor expression following malnourishment and re-feeding are unknown.

Innovations and breakthroughs
The present study showed the effects of undernourishment and re-feeding on the morphology of the P2X$_2$-immunoreactive (IR), nitric oxide synthase-IR, calbindin-IR, calretinin-IR and choline acetyltransferase-IR neurons of the myenteric and submucosal plexuses.

Applications
The present study suggests that re-feeding can restore almost all of the changes to inhibitory intrinsic primary afferent neurons and cholinergic neurons seen in undernourished animals.

Peer review
This is a study of the effects of deprivation and restoration of protein on neu-

ronal development in the submucosal and myenteric plexuses. The results describe changes induced by protein deprivation that are largely reversible by re-feeding.

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