

The enteric nervous system of P2Y₁₃ receptor null mice is resistant against high-fat-diet- and palmitic-acid-induced neuronal loss

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Abstract Gastrointestinal symptoms have a major impact on the quality of life and are becoming more prevalent in the western population. The enteric nervous system (ENS) is pivotal in regulating gastrointestinal functions. Purinergic neurotransmission conveys a range of short and long-term cellular effects. This study investigated the role of the ADP-sensitive P2Y₁₃ receptor in lipid-induced enteric neuropathy. Littermate P2Y₁₃^{+/+} and P2Y₁₃^{-/-} mice were fed with either a normal diet (ND) or high-fat diet (HFD) for 6 months. The intestines were analysed for morphological changes as well as neuronal numbers and relative numbers of vasoactive intestinal peptide (VIP)- and neuronal nitric oxide synthase (nNOS)-containing neurons. Primary cultures of myenteric neurons from the small intestine of P2Y₁₃^{+/+} or P2Y₁₃^{-/-} mice were exposed to palmitic acid (PA), the P2Y₁₃ receptor agonist 2meSADP and the antagonist MRS2211. Neuronal survival and relative number of VIP-containing neurons were analysed. In P2Y₁₃^{+/+}, but not in P2Y₁₃^{-/-} mice, HFD caused a significant loss of myenteric neurons in both ileum and colon. In colon, the relative numbers of VIP-containing submucous neurons were significantly lower in the P2Y₁₃^{-/-}

mice compared with P2Y₁₃^{+/+} mice. The relative numbers of nNOS-containing submucous colonic neurons increased in P2Y₁₃^{+/+} HFD mice. HFD also caused ileal mucosal thinning in P2Y₁₃^{+/+} and P2Y₁₃^{-/-} mice, compared to ND fed mice. In vitro PA exposure caused loss of myenteric neurons from P2Y₁₃^{+/+} mice while neurons from P2Y₁₃^{-/-} mice were unaffected. Presence of MRS2211 prevented PA-induced neuronal loss in cultures from P2Y₁₃^{+/+} mice. 2meSADP caused no change in survival of cultured neurons. P2Y₁₃ receptor activation is of crucial importance in mediating the HFD- and PA-induced myenteric neuronal loss in mice. In addition, the results indicate a constitutive activation of enteric neuronal apoptosis by way of P2Y₁₃ receptor stimulation.

Keywords Palmitic acid · Enteric nervous system · Purinergic receptors · P2Y₁₃ knockout mice · Primary cell culture · High-fat diet

Introduction

The gastrointestinal (GI) tract is an emerging key organ in whole body homeostasis. The basis of optimal GI regulation is an intact enteric nervous system (ENS). The ENS innervates the entire digestive tract and plays pivotal roles in coordinating motility, secretion and blood flow. The prevalence of GI symptoms is high in the Western world, substantially affecting quality of life [1, 2]. Patients with overweight and/or diabetes report high incidences of GI symptoms compared with the normal population [3].

Purinergic signalling was first described 40 years ago [4], since then, the field has gained substantial interest [5]. ATP was the first nucleotide shown to act as a sole or co-transmitter in central and peripheral, including autonomic, nervous systems [5, 6]. ATP is not only freed from damaged or dying cells, but a physiological release of ATP can be triggered from

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both neuronal and non-neuronal cells [7]. Extracellularly, ATP is degraded by ectonucleotidases to ADP, AMP and adenosine rendering the system a high flexibility in the ensuing response [8]. A large number of selective purinergic receptors have been identified. P1 receptors are adenosine sensitive while P2 receptors are preferentially activated by ATP/ADP. P2 receptors are further subdivided into X, ionotropic and Y, G-protein-coupled receptors (GPCRs) [5, 9]. P2Y₁₃ is a GPCR activated by ADP and coupled to G_i/G_o signalling [10]. It was initially identified in the spleen and brain but is also expressed in the dorsal root ganglia, microglia, pancreatic beta cells, hepatocytes and mast cells [10–15]. In central neurons, P2Y₁₃ has been shown to be involved in neuronal differentiation, axonal elongation, oxidative stress and inflammatory pain behaviour [11, 16–18]. Peripherally, the receptor plays key roles in lipoprotein metabolism and reverse cholesterol transport [19]. Activation of the P2Y₁₃ receptor reduces high-density lipoprotein (HDL) levels and increases hepatic HDL uptake in mice [13, 19–21]. Research obtained, either by silencing the P2Y₁₃ receptors through targeted gene deletion or small interfering RNA, suggests a pro-apoptotic role of P2Y₁₃ receptor activation. In contrast, P2Y₁₃ receptor activation was found to elicit neuroprotection in cerebellar granule neurons [18].

It was recently shown that feeding mice with high-fat diet (HFD) for 6 months results in a substantial loss of myenteric neurons in both the ileum and colon [22]. The saturated free fatty acid (FFA), palmitic acid (PA), was put forward as a putative mediator of this effect, as PA exposure *in vitro* caused substantial neuronal loss. These findings and the role of P2Y₁₃ receptor that have been attributed in apoptosis, neuroprotection and lipid transport initiated the current study. By using P2Y₁₃^{+/+} and P2Y₁₃^{-/-} mice fed with either a normal diet (ND) or HFD, we investigated the possible role of the P2Y₁₃ receptor in HFD-induced enteric neuropathy. In addition, we used primary myenteric neuronal cultures from P2Y₁₃^{+/+} and P2Y₁₃^{-/-} mice to investigate survival and relative numbers of vasoactive intestinal peptide (VIP)-expressing neurons in response to PA, P2Y₁₃ receptor agonist and antagonist.

Methods

Ethics statement

Experimental design was approved by the animal ethics committee, Lund and Malmö, Sweden. All animals were used in accordance with the European Community Council Directive (86/609/EEC and 2010/63/EU) and the Swedish Animal Welfare Act (SFS 1988:534).

In vivo experiments

P2Y₁₃^{-/-} mice were backcrossed into the C57BL/6J background generating P2Y₁₃^{-/+} genotypes which were subsequently used to produce P2Y₁₃^{+/+} and P2Y₁₃^{-/-} littermates [19].

At 1 month of age, mice were divided into four groups: two ND groups (P2Y₁₃^{+/+} *n*=6, P2Y₁₃^{-/-} *n*=7) continuing on standard chow (Lactamin R36) and two HFD groups (P2Y₁₃^{+/+} *n*=6, P2Y₁₃^{-/-} *n*=6) changing to a chow containing elevated levels of fat (Research diets D12451, USA); see Table 1 for a survey on nutritional content. After 6 months on either ND or HFD, mice were killed by cervical dislocation, weighed and body composition determined by dual-energy X-ray absorptometry [23] using Lunar PIXImus scanner (GE lunar, USA).

The abdominal cavity was opened, and the GI tract from lower oesophageal sphincter to the anal canal was collected, opened along the mesenteric border, emptied and weighed before rinsed in cold saline. The intestines were fixed in Stefaninis fixative (0.2 % picric acid, 2 % formaldehyde in 0.1 M phosphate buffer, pH 7.2) for 24 h at 4 °C and rinsed three times in Tyrode's solution containing 10 % sucrose at 4 °C. Segments from the ileum and transverse colon were orientated for longitudinal and cross-sectioning, embedded in Tissue-Tek (Histolab, SE), frozen at -80 °C and sectioned (10 μm). Sections were processed for histochemistry and immunocytochemistry.

In vitro experiments

P2Y₁₃^{+/+} (*n*=10, 20–23 g) and P2Y₁₃^{-/-} mice (*n*=8, 20–23 g) on ND were used for *in vitro* experimentation. Primary cultures of myenteric neurons were prepared from the small intestine. Neurons were dissociated using a modification of a previously described method [24, 25]. In brief, anaesthetized mice (Ketalar[®]/Rompun[®]) had their small intestine exposed via midline incisions. The longitudinal muscle layer with attached myenteric ganglia was stripped from approximately

Table 1 Overview of nutritional content of the high-fat diet (HFD) and the normal diet (ND)

Nutritional content	HFD (g%)	ND (g%)
Protein	24	18.5
Nitrogen-free extract	46.2	55.7
Fat	24.0	4.0
Saturated	8.7	0.6
Monounsaturated	10.8	0.9
Polyunsaturated	4.4	2.6

HFD, Research diets D12451; ND, Lactamin R36; g%, gram percentages.

15 cm of the distal small intestine. The tissues were placed in Ca^{2+} - and Mg^{2+} -free Hank's Balanced Salt Solution (HBSS) containing 1.9 CDU/mL collagenase 1-A (1.5 mg/mL, Sigma-Aldrich, SE) and 4.7 $\mu\text{U/mL}$ protease IX (1.5 mg/mL, Sigma-Aldrich, SE) and enzymatically and mechanically separated. Trypsin (0.4 mg/mL, BioChrom AG) and EDTA (0.003 %, Sigma-Aldrich) were added. Inactivation of trypsin was by adding 50 % *v/v* fetal bovine serum (FBS, Life Technologies, SE). Cell suspension was centrifuged at 15.6g for 10 min. Supernatant was removed, and pellet was washed three times in HBSS, centrifuged at 15.6g for 10 min and diluted to 0.8 mL in Neurobasal A (NBA) cell culture medium (Life Technologies, SE) containing 10 % FBS, 0.5 mM L-glutamine (BioChrom AG), 50 U/mL penicillin and 50 $\mu\text{g/mL}$ streptomycin (BioChrom AG). Cell cultures were prepared by adding 50 μL of a constantly mixed cell suspension into 8-well chambers (BD Falcon, VWR) pre-filled with 450 μL of the NBA cell culture medium. From each animal, two 8-well chambers (69 mm^2/well) were prepared. Suspensions from multiple animals were never pooled. Cultures were incubated 4 days prior to experimental treatments.

Stock solutions of PA (Sigma-Aldrich, SE), 2-methylthioadenosine diphosphate trisodium salt (2meSADP, Tocris, UK) and MRS2211 (Tocris UK), were prepared, aliquoted and stored at -20°C .

PA was conjugated to bovine serum albumin (BSA, Merck, SE) by adding PA stock (10^{-2} M) together with 20 % BSA *w/v* to the medium and mixed at 37°C 1 h prior to use. Final PA/BSA molar ratio was 4–5:1. Final BSA concentration was $\leq 2\%$ *v/v*. Different concentrations (10^{-4} – 10^{-3} M) of albuminated PA were added to neuronal cultures from $\text{P2Y}_{13}^{+/+}$ to $\text{P2Y}_{13}^{-/-}$ mice; controls received medium containing 2 % BSA. In a separate set of experiments, neuronal cultures were grown in control or in 2meSADP (10^{-7} – 10^{-5} M)-enriched medium. Further, cultures from $\text{P2Y}_{13}^{+/+}$ mice were exposed to control or MRS2211 (10^{-5} M)-supplemented medium with or without PA (10^{-4} – 10^{-3} M) or 2meSADP (10^{-7} – 10^{-5} M).

All experimental treatments lasted for 4 days after which cells were fixed in Stefanini's fixative for 30 min and rinsed for 2×10 min in Tyrode's solution containing 10 % sucrose. To enhance antibody penetration, cultures were frozen and thawed prior to immunocytochemical processing.

Immunocytochemistry

For details on primary and secondary antibodies, see Table 2. All antibodies were diluted in phosphate buffered saline containing 0.25 % Triton X-100 and 0.25 % BSA (PBS-T-B). For visualization of submucous and myenteric neurons in mice on

ND or HFD, cryosections were immunostained with HuC/HuD-biotin and Vectastain ABC kit (Vector laboratories inc., USA) according to the manufacturer's protocol. To estimate the relative numbers of neurons expressing neuronal nitric oxide synthase (nNOS), a modified immunolabelling protocol was used allowing for a fluorescent HuC/HuD-biotin detection. Cryosections were washed in wash buffer (WB) consisting of PBS with 0.25 % Triton X-100 and 0.5 % BSA. Antigen retrieval was performed by 2×8 min microwaving in citrate buffer (0.1 M citrate, pH 6.5) followed by 10-min cooling in water bath and 20-min rinsing in running tap water. To block unspecific background staining occurring in sectioned mouse tissues when using monoclonal HuC/HuD antibodies, sections were washed in WB and incubated for 40 min in 0.1 mg/mL Streptavidin (Sigma-Aldrich, SE), washed for 3×10 min in WB, incubated for 120 min in 0.5 mg/mL biotin (Sigma-Aldrich, SE) and washed again for 3×10 min in WB. Antigenicity of VIP was destroyed by antigen retrieval; therefore, the protocol described above for double labelling with HuC/HuD could not be applied for estimating the relative numbers of VIP-containing neurons in cryosections. Instead, single immunolabelling of VIP and HuC/HuD, respectively, was performed on consecutive sections.

Neuronal cell cultures are devoid of mouse IgG thus allowing for visualization of antigen-antibody complexes using indirect immunofluorescence also of monoclonal HuC/HuD antibodies. Double immunolabelling of cultures and sections was performed by overnight incubation in a moist chamber at 4°C with a mixture of HuC/HuD and VIP (cultures only) or nNOS antibodies. Secondary antibodies and/or Alexa Fluor conjugated streptavidin (1:1,000, Life Technologies, SE) was mixed, and slides were incubated for 1 h at RT. All slides were mounted in PBS/glycerol 1:1 and analysed using fluorescence microscopy (Olympus BX43, LRI, SE) with appropriate filter setting.

Histochemistry

Morphometric analyses of the mouse intestine after ND or HFD were on toluidine-blue-stained cryosections. Cryosections were washed in PBS-T-B prior to immersion in 0.1 % toluidine blue in 60 % ethanol for 20 min, dehydrated, cleared and mounted (Pertex, Histolab, SE).

Intracellular lipid accumulations were visualized on intestinal cryosections from ND and HFD mice using Bodipy[®] 493/503 (Life Technologies, SE) diluted 1:1,000 in PBS-T-B, incubated for 1 h, washed in PBS-T and mounted on ProLong[®] Gold (Life Technologies, SE).

Table 2 Overview of primary and secondary antibodies used in immunocytochemistry

Raised against	Dilution	Code	Source	Host	References
Human neuronal protein, (HuC/HuD)	1:600	A21272	Life Technologies, SE	Mouse	[36]
Vasoactive intestinal peptide (VIP), purified porcine	1:1.200	7852	Euro Diagnostica, SE	Rabbit	[37]
Neuronal nitric oxide synthase (nNOS), synthetic rat cerebellar	1:5.000	9223	Euro Diagnostica, SE	Rabbit	[38]
Mouse IgG	1:1.000	115-485-166	Jackson Lab Inc, USA	Goat	
Rabbit IgG	1:1.000	711-515-152	Jackson Lab Inc, USA	Donkey	

Morphometric analyses and cell calculations

The heights of mucosa and muscularis propria were estimated using the mean from ten representative measurements from each animal (Aperio ScanScope CS/GL SS5082 and Aperio ImageScope platform; www.aperio.com). Immunocytochemical visualization of the enteric nerve cell bodies in submucous and myenteric ganglia was used to estimate neuronal number per millimetre section length. The relative numbers of nNOS-immunoreactive neurons were estimated using

double immunolabelling of HuC/HuD in combination with nNOS antibodies. All HuC/HuD-immunoreactive neurons were counted and checked for double labelling with nNOS; values are expressed in percentage. The relative numbers of VIP-immunoreactive neurons in cryosections were estimated by counting the numbers of VIP-immunoreactive and the number of HuC/HuD-immunoreactive neurons on consecutive sections. From each mouse, cryosections cut longitudinally at 3–4 different depths, comprising a total length of 25–30mm, were used.

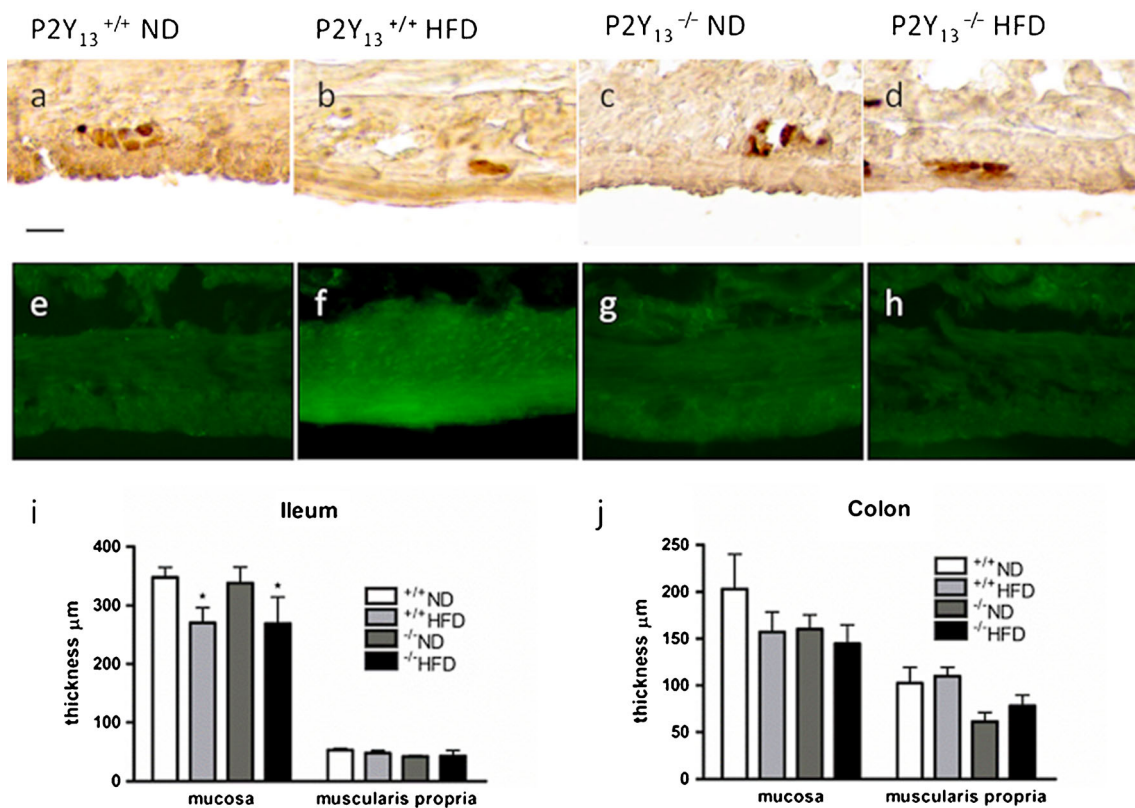


Fig. 1 Upper panel shows representative micrographs of the ileum from P2Y₁₃^{+/+} (a, b, e and f) and P2Y₁₃^{-/-} (c, d, g and h) mice fed with either normal diet (ND) or high-fat diet (HFD) for 6 months. Lower panel shows the effects of ND and HFD on the thicknesses of intestinal mucosa and muscularis propria in the ileum (i) and colon (j). a–d Cryosections immunostained for enteric neurons using the pan neuronal marker HuC/HuD. P2Y₁₃^{+/+} mice fed with HFD (b) have markedly less myenteric neurons per millimetre compared with ND-fed mice (a). HFD does not change neuronal density in P2Y₁₃^{-/-} mice (d). e–h Cryosections stained

with Bodipy® to illustrate lipid droplets within muscularis propria. P2Y₁₃^{+/+} mice fed with HFD (f) have increased intramuscular lipid droplet formation, compared with P2Y₁₃^{+/+} ND mice (e). P2Y₁₃^{-/-} mice do not accumulate lipid droplets in muscularis propria (g, h). i, j Except a slight but significant reduction in the ileal mucosa height of HFD-fed animals, no structural remodelling was noted in any of the groups. Data presented as means±SEM, asterisk denotes *p*<0.05, all compared with P2Y₁₃^{+/+} ND; *n*=5–12 in each group. Bar represents 20 μm

Neuronal survival *in vitro* was calculated by counting the total number of surviving neurons in the entire culture well (69 mm²) and expressed as percentage of the number of neurons in the control well run in parallel. The relative number of neurons expressing VIP *in vitro* was estimated using HuC/HuD and VIP double labelling.

Statistical analyses

Data are presented as means±SEM and analysed by GraphPad Prism (GraphPad Software Inc, USA). Statistical significance was determined using two-way analysis of variance followed by Bonferroni's post hoc test (in vivo data) or one-way analysis of variance followed by Dunnett's post hoc test towards controls (in vitro data). A confidence level of 95 % was considered significant.

Results

In vivo findings

P2Y₁₃^{+/+} and P2Y₁₃^{-/-} mice fed with HFD had increased body weight ($p < 0.001$, ND P2Y₁₃^{+/+} 32.1±2.8 g, P2Y₁₃^{-/-} 33.6±2.5 g; HFD P2Y₁₃^{+/+} 46.2±2.0 g, P2Y₁₃^{-/-} 49.7±1.6 g) and body fat percentage ($p < 0.001$, ND P2Y₁₃^{+/+} 24.0±2.5 %, P2Y₁₃^{-/-} 21.7±1.8 %; HFD P2Y₁₃^{+/+} 47.5±1.8 %, P2Y₁₃^{-/-} 44.8±1.7 %) compared with P2Y₁₃^{+/+} and P2Y₁₃^{-/-} ND mice. Weights of the digestive tract did not differ between the four groups (ND P2Y₁₃^{+/+} 3.3±0.3 g, P2Y₁₃^{-/-} 3.7±0.3 g; HFD P2Y₁₃^{+/+} 3.6±0.3 g, P2Y₁₃^{-/-} 3.0±0.1 g). Morphometry on intestinal sections showed that HFD causes a modest ileal mucosal thinning, in both P2Y₁₃^{+/+} and P2Y₁₃^{-/-} mice, that was absent in colon. No alterations in muscularis propria thickness were observed in the ileum or colon in any of the groups. HFD caused a significant loss of myenteric neurons in the ileum and colon of P2Y₁₃^{+/+}, but not in P2Y₁₃^{-/-} mice. In the colon from P2Y₁₃^{-/-} mice, both on ND and HFD, a slight but significant increase in myenteric neurons per millimetre section was noted compared with P2Y₁₃^{+/+} mice. No change in the numbers of submucous neurons in the ileum and colon was observed in any of the groups. Results are summarized in Fig. 1 and Table 3.

Both ND- and HFD-fed P2Y₁₃^{-/-} mice displayed lower relative numbers of colonic VIP-containing submucous neurons compared with P2Y₁₃^{+/+} mice. HFD did not cause any change in the relative numbers of VIP-containing enteric neurons compared with ND mice. The relative numbers of myenteric neurons expressing nNOS were similar in the ileum and colon of P2Y₁₃^{+/+} and P2Y₁₃^{-/-} mice irrespective of diet. P2Y₁₃^{+/+} mice fed with HFD had higher relative number of

Table 3 Neuronal survival and relative expressions of VIP and nNOS neurons in myenteric (MG) and submucous ganglia (SG) of P2Y₁₃^{+/+} and P2Y₁₃^{-/-} mice after normal diet (ND) or high-fat diet (HFD)

a Enteric neurons identified by immunostaining with the pan-marker HuC/HuD and expressed as number per millimetre cryosection						
Genotype	Diet	Ileum MG	Ileum SG	Colon MG	Colon SG	
P2Y ₁₃ ^{+/+}	ND	10.1±1.1	3.9±0.4	13.0±1.1	3.5±0.3	
P2Y ₁₃ ^{+/+}	HFD	6.7±0.4**	2.9±0.4	4.6±0.2***	2.1±0.2	
P2Y ₁₃ ^{-/-}	ND	9.5±1.2	5.2±0.9	18.9±2.4*	4.9±0.9	
P2Y ₁₃ ^{-/-}	HFD	10.2±1.1	4.0±0.8	20.5±2.7**	4.4±0.6	
b Relative numbers of VIP-immunoreactive neurons expressed in percentage of HuC/HuD immunoreactive ones						
P2Y ₁₃ ^{+/+}	ND	2.9±0.2	20.1±2.8	4.1±1.0	28.6±3.7	
P2Y ₁₃ ^{+/+}	HFD	2.7±0.8	22.6±3.1	9.1±3.5	30.4±7.9	
P2Y ₁₃ ^{-/-}	ND	3.0±0.8	14.3±1.3	2.0±0.4	15.9±1.4*	
P2Y ₁₃ ^{-/-}	HFD	3.3±0.6	27.9±4.9	1.6±0.2	16.7±3.0*	
c Relative numbers of nNOS-immunoreactive neurons expressed in percentage of HuC/HuD-immunoreactive ones						
P2Y ₁₃ ^{+/+}	ND	38.0±5.6	15.3±4.9	32.8±2.4	11.5±1.5	
P2Y ₁₃ ^{+/+}	HFD	38.25±2.4	12.8±2.5	40.3±5.9	26.0±5.5 *	
P2Y ₁₃ ^{-/-}	ND	39.4±3.6	18.0±6.6	43.4±1.2	17.4±3.6	
P2Y ₁₃ ^{-/-}	HFD	34.8±2.5	16.0±1.2	40.3±2.4	12.2±2.7	

Data are shown as means±SEM

$n=6-7$ in each group

* $p < 0.05$, ** $p < 0.01$, $p < 0.001$ all comparisons against P2Y₁₃^{+/+} ND

nNOS-containing submucous neurons in the colon as compared with the other three groups. Results are summarized in Table 3.

Bodipy staining of the ileum and colon from P2Y₁₃^{+/+} mice on HFD displayed a marked lipid droplet accumulation in muscularis propria while the other three groups (P2Y₁₃^{+/+} ND, P2Y₁₃^{-/-} ND and P2Y₁₃^{-/-} HFD) showed no or low levels of droplet accumulation (Fig. 1e-h).

In vitro findings

All *in vitro* investigations included a 4-day pre-treatment culture period followed by 4 days in treatment conditions. Untreated controls were always run in parallel, and neuronal survival was estimated as percent of control.

Survival of myenteric neurons from P2Y₁₃^{+/+} and P2Y₁₃^{-/-} mice

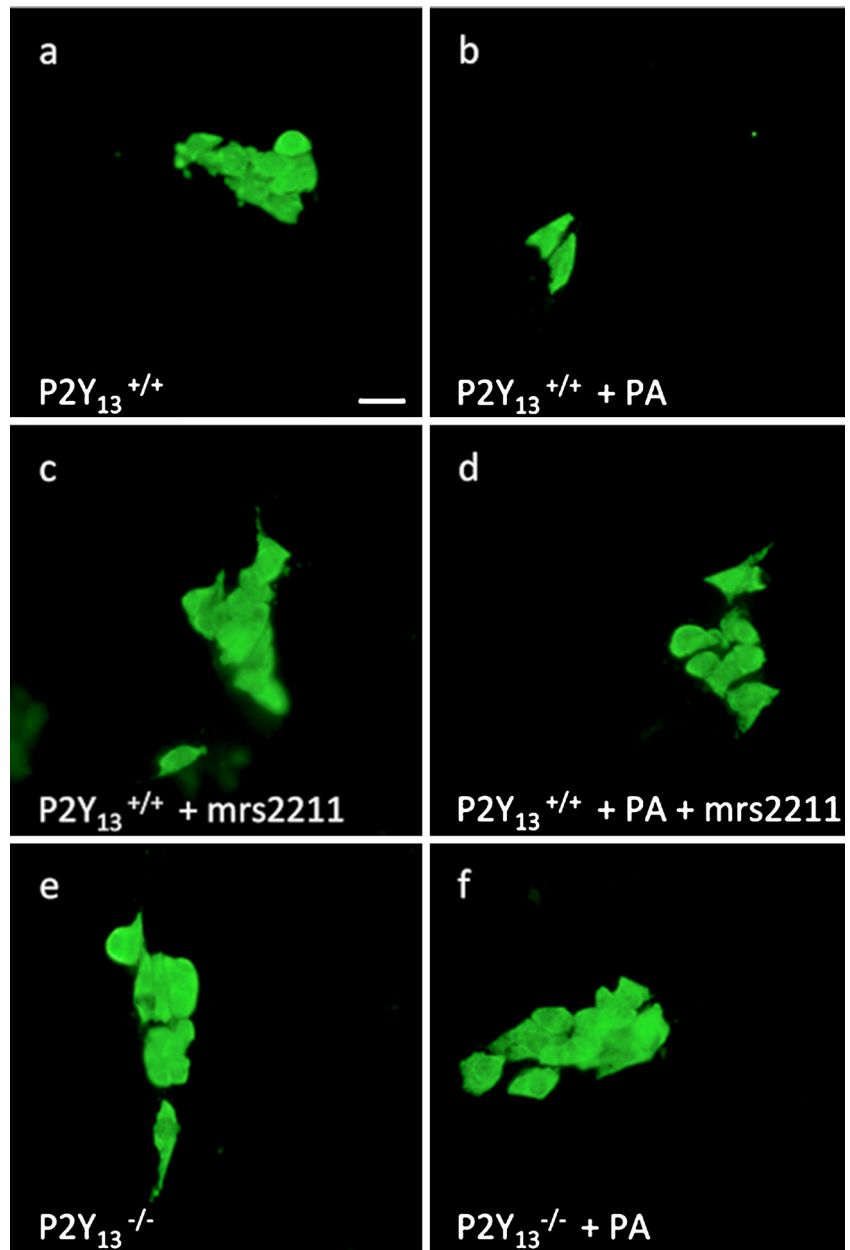
Survival of cultured myenteric neurons from ND-fed P2Y₁₃^{+/+} and P2Y₁₃^{-/-} mice was determined after exposure to PA (10⁻⁴–10⁻³ M) or 2meSADP (10⁻⁷–10⁻⁵ M) with or without MRS2211 (10⁻⁵ M). Control wells displayed a large number (P2Y₁₃^{+/+} 6.0±0.1 neurons/mm², $n=31$; P2Y₁₃^{-/-} 5.9±0.2 neurons/mm², $n=20$) of evenly distributed neurons. Exposing

cultures from P2Y₁₃^{+/+} mice to the increasing concentrations of PA (10⁻⁴–10⁻³ M) caused a severe and concentration-dependent loss of neurons. In contrast, in neuronal cultures from P2Y₁₃^{-/-} mice, no loss of myenteric neurons was observed after PA (10⁻⁴–10⁻³ M) exposure. The combined exposure of PA (10⁻⁴–10⁻³ M) and MRS2211 (10⁻⁵ M) protected P2Y₁₃^{+/+} cultures against PA-induced neuronal loss. Addition of 2meSADP (10⁻⁷–10⁻⁵ M) did not affect neuronal survival in either P2Y₁₃^{+/+} or P2Y₁₃^{-/-} cultures. Neither did the combined exposure of 2meSADP (10⁻⁷–10⁻⁵ M) and MRS2211 (10⁻⁵ M) to P2Y₁₃^{+/+}. MRS2211 (10⁻⁵ M) per se increased the number of surviving neurons in P2Y₁₃^{+/+} cultures. Results are summarized in Fig. 2 and Table 4.

VIP expression in cultured myenteric neurons from P2Y₁₃^{+/+} and P2Y₁₃^{-/-} mice

The relative numbers of cultured myenteric VIP-containing neurons from P2Y₁₃^{+/+} and P2Y₁₃^{-/-} mice were determined after exposure to PA (10⁻⁴–10⁻³ M) or 2meSADP (10⁻⁷–10⁻⁵ M) with or without MRS2211 (10⁻⁵ M). A reduction in the relative numbers of VIP-containing neurons was observed after exposure to PA (10⁻³ M) in both P2Y₁₃^{+/+} and P2Y₁₃^{-/-} cultures compared with controls. In P2Y₁₃^{+/+} cultures, addition of MRS2211 (10⁻⁵ M) reversed PA-induced reduction of VIP-containing neurons at all PA concentrations except at 10⁻³ M. No change in the relative numbers of VIP-containing

Fig. 2 Representative micrographs of cultured myenteric neurons from the small intestines of P2Y₁₃^{+/+} (a–d) and P2Y₁₃^{-/-} mice (e, f). Neurons are visualized using HuC/HuD immunostaining. Exposure to palmitic acid (PA) (b) causes loss of P2Y₁₃^{+/+} neurons, compared with control (a). Exposure to the P2Y₁₃ antagonist MRS2211 increases survival of P2Y₁₃^{+/+} neurons (c). MRS2211 also protects P2Y₁₃^{+/+} neurons against PA-induced loss (d). Neurons from P2Y₁₃^{-/-} mice are protected against PA-induced neuronal loss. Bar represents 20 μm



neurons was observed in P2Y₁₃^{+/+} or P2Y₁₃^{-/-} cultures exposed to 2meSADP (10⁻⁴–10⁻³ M) compared with controls. P2Y₁₃^{+/+} cultures exposed to MRS2211 (10⁻⁵ M) with or without 2meSADP (10⁻⁴–10⁻³ M) did not change the relative number of VIP-containing neurons. Results are summarized in Fig. 3 and Table 4.

Discussion

The current study investigated the effects of HFD on enteric neuronal survival in P2Y₁₃^{-/-} and P2Y₁₃^{+/+} littermate mice. In

Table 4 Neuronal survival of cultured myenteric neurons and relative numbers of VIP-immunoreactive neurons. Survival is expressed as percent of the control run in parallel. Relative numbers of VIP-immunoreactive neurons are expressed as percent of HuC/HuD-immunoreactive neurons

a Cultured myenteric neurons from P2Y ₁₃ ^{+/+} mice		
Treatment	Survival (% of control)	VIP neurons (% of HuC/HuD)
Control	100	17.0±0.6
10 ⁻⁵ M MRS2211	119±6.3*	14.4±1.2
2meSADP		
10 ⁻⁷ M	121.5±28.5	19.5±2.5
10 ⁻⁶ M	96.5±15.5	16.5±0.5
10 ⁻⁵ M	108.2±3.1	17.3±1.9
2meSADP+10 ⁻⁵ M MRS2211		
10 ⁻⁷ M	120.3±17.2	19.3±3.8
10 ⁻⁶ M	108.8±15.5	15.8±0.5
10 ⁻⁵ M	101.2±2.3	16.8±1.8
PA		
10 ⁻⁴ M	82.9±7.0*	18.0±0.6
4×10 ⁻⁴ M	57.4±3.4**	16.1±1.0
10 ⁻³ M	20.7±7.8**	12.4±1.2**
PA+10 ⁻⁵ M MRS2211		
10 ⁻⁴ M	116.0±14.0	17.5±2.5
4×10 ⁻⁴ M	100.4±8.0	16.8±2.2
10 ⁻³ M	92.5±2.5	12.0±1.6*
b Cultured myenteric neurons from P2Y ₁₃ ^{-/-} mice		
Control	100	16.5±1.0
2meSADP		
10 ⁻⁷ M	117.0±10.0	12.5±1.4
10 ⁻⁶ M	100.5±3.5	12.0±1.7
10 ⁻⁵ M	106.0±12.5	12.3±1.7
PA		
10 ⁻⁴ M	102.4±4.6	16.0±1.5
4×10 ⁻⁴ M	96.4±4.1	14.8±0.9
10 ⁻³ M	90.0±7.5	9.0±0.6*

Data is given as means±SEM

n=5–20 in each group

*p<0.05, **p<0.01 all comparisons against control

addition, pharmacological in vitro studies on myenteric neurons from ND-fed P2Y₁₃^{-/-} and P2Y₁₃^{+/+} were undertaken to reveal possible changes in neuronal survival after PA exposure and stimulation or inhibition of the P2Y₁₃ receptor using the agonist 2meSADP or the antagonist MRS2211.

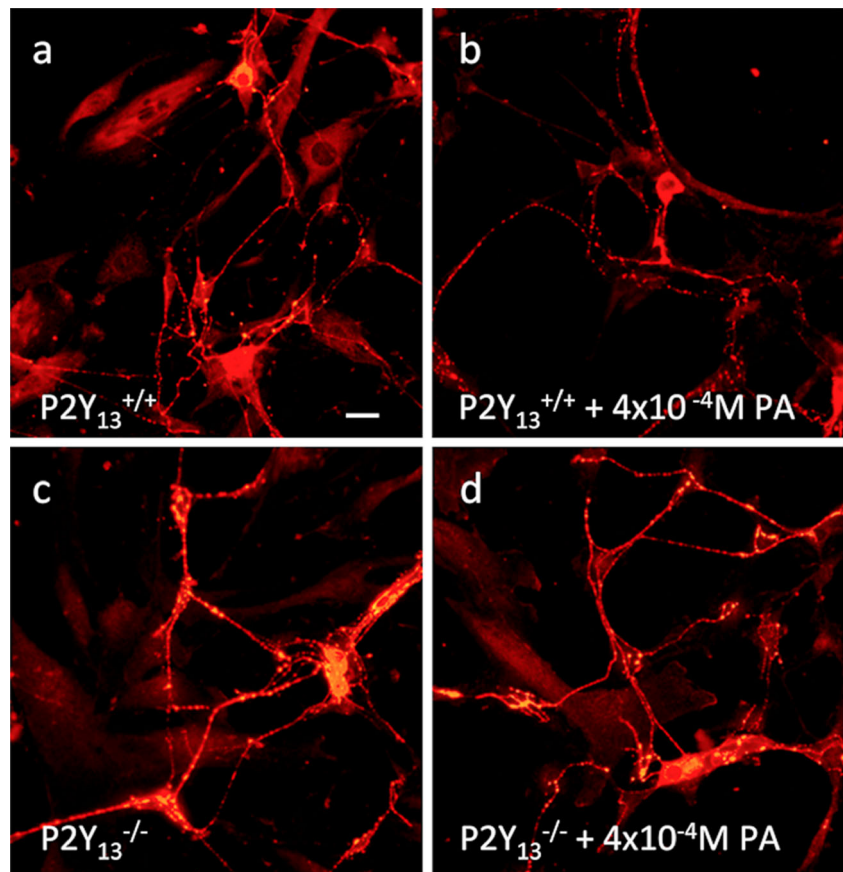
Lipid-induced neuronal loss

The study confirmed previous findings on HFD-fed C57BL/6 mice [22], in that HFD induced a significant loss of myenteric neurons in both the ileum and colon in the P2Y₁₃^{+/+} mice. Surprisingly, the P2Y₁₃^{-/-} mice showed a remarkable protection against HFD-induced neuronal loss. It is also notable that the P2Y₁₃^{-/-} mice fed with ND displayed an even higher number of neurons per millimetre section in colon compared with ND-fed P2Y₁₃^{+/+} littermates.

The finding that PA induces loss of myenteric neurons from P2Y₁₃^{+/+} mice in vitro is well in line with previous findings, describing PA-induced loss of cultured myenteric neurons from rat [22]. The current study also shows that cultured myenteric neurons from P2Y₁₃^{-/-} mice are resistant to PA-induced loss. In rat, the mechanisms behind PA-induced neuronal loss was found to be complex, but excessive palmitoylcarnitine formation and exhausted L-carnitine stores leading to energy depletion, low acetylcholine synthesis and oxidative stress were identified as important events [22]. The present study shows that absence of the P2Y₁₃ receptor or presence of MRS2211 abolished PA-induced neuronal loss in vitro. This indicates an unpredicted contribution of P2Y₁₃ receptor activation in executing PA-induced loss of myenteric neurons. MRS2211-induced inhibition of P2Y₁₃ receptors resulted in increased survival of cultured P2Y₁₃^{+/+}-derived myenteric neurons, indicating constitutive activation of neuronal loss by way of P2Y₁₃ receptor stimulation. Such constitutive activation could possibly explain the finding that P2Y₁₃^{-/-} mice in vivo display increased myenteric neuronal density in the colon compared with P2Y₁₃^{+/+} mice.

The neuroprotection observed in P2Y₁₃^{-/-} mice against the previously described, and here confirmed, PA- and HFD-induced loss of myenteric neurons is enigmatic. Compensatory mechanisms without any direct involvements of neurons may operate. The in vivo data have to be placed in light of a non-tissue-specific removal of the receptor, and the observed neuroprotection may be due to changes in lipid metabolism, altered insulin and glucose levels affecting whole body metabolism, or embryonic changes altering the prerequisites for PA-mediated neuronal loss. This is illustrated by the finding that though phenotypic analysis of P2Y₁₃^{-/-} mice did not show changed insulin sensitivity or glucose tolerance, inhibition of P2Y₁₃ receptor activity in mice using MRS2211 leads to increased insulin secretion and reduced plasma glucose [15, 20]. However, the here presented in vitro findings excitingly

Fig. 3 Representative micrographs of VIP immunoreactive cultured myenteric neurons from the small intestines of P2Y₁₃^{+/+} (a, b) and P2Y₁₃^{-/-} mice (c, d). No difference in the relative number of neurons immunoreactive for VIP was observed between P2Y₁₃^{+/+} (a) or P2Y₁₃^{-/-} (c) derived cultures. In contrast to the reduced relative numbers of VIP-immunoreactive neurons seen by exposure to 10⁻³M palmitic acid (PA), exposure to PA (10⁻⁴–4×10⁻⁴M) did not change the percentage of neurons immunoreactive for VIP in P2Y₁₃^{+/+} (b) or P2Y₁₃^{-/-} (d) derived cultures



link PA exposure and neuronal P2Y₁₃ receptor activation with a pro-apoptotic outcome.

Curiously, exposing P2Y₁₃^{+/+}-cultured myenteric neurons to the stable and selective P2Y₁₃ agonist 2meSADP did not hamper neuronal survival in vitro, while exposing cells to the antagonist MRS2211 enhanced the number of surviving neurons. This somewhat contradictory finding can partly be explained by MRS2211 being an inverse agonist rather than a pure antagonist (Olde B, et al. unpublished). Exposure thereby decreases constitutive apoptotic P2Y₁₃ signalling. The absence of the effect of 2meSADP exposure may indicate that the protective effect of the P2Y₁₃^{-/-} phenotype is indirect. Complex links between P2Y₁₃ intracellular lipid and energy metabolism and/or neuronal transmembrane lipid transport may be operating. Altered lipid transport and metabolism in P2Y₁₃^{-/-} mice have been highlighted in multiple studies. Activation of the P2Y₁₃ receptor in mice leads to an augmented hepatic HDL uptake and bile acid secretion [21]. Silencing the P2Y₁₃ receptor results in reduced TG-HDL endocytosis and internalization [13]. Gene profiling and phenotypic analysis of P2Y₁₃^{-/-} mice confirm that they hold altered lipoprotein, cholesterol and HDL metabolism [20]. HFD has previously been shown to increase lipid droplet accumulation in intestinal muscularis propria in C57BL/6 mice [22]. In contrast, P2Y₁₃^{-/-} mice fed with HFD did not accumulate lipid

droplets in muscularis propria, which suggestively underlines the possibility of an altered lipid metabolism. An interesting finding was the ileal mucosal thinning observed in both P2Y₁₃^{+/+} and P2Y₁₃^{-/-} mice fed with HFD. Previous studies have shown lipid infusions, unlike other nutrients, to cause a mucosa barrier impairment in the rat small intestine [26]. Whether the observed thinning of the mucosa is due to sustained lipid exposure when on HFD needs further investigations.

Lipid-induced alterations in VIP- and nNOS-containing neurons

Interestingly, the numbers of VIP-containing colonic neurons in submucous ganglia were significantly lower in the P2Y₁₃^{-/-} mice than in the P2Y₁₃^{+/+} mice, irrespective of diet. This probably reflects a neuronal VIP content below the immunocytochemical detection limit and may be a compensatory mechanism due to genetic modification causing, e.g. lack of growth/survival factors or altered functional demands. A low-VIP content may be due to a low expression of VIP messenger RNA (mRNA) or excessive release of the peptide not matched by VIP mRNA expression. It may also reflect a low expression of PACAP or PAC₁ receptors since PACAP by PAC₁ receptor activation increases VIP mRNA expression

[27]. In addition, present results suggest that P2Y₁₃ receptor activation influences VIP expression. VIP expression is under calcium response element-binding protein's (CREB) control, and the P2Y₁₃ receptor, being G_i coupled, may through cAMP modulation regulate the expression of VIP. Present in vitro results show that the relative numbers of VIP-containing neurons are equal in both P2Y₁₃^{+/+}- and P2Y₁₃^{-/-}-derived cultures and that it decreases after high-PA exposure in both genotypes. In myenteric neurons from P2Y₁₃^{+/+} cultures, the selective P2Y₁₃ receptor antagonist reversed the PA-induced decrease of VIP-containing neurons at all PA concentrations, except the highest. Inactivating the P2Y₁₃ receptor may thus change the expression of VIP through CREB pathway activation. VIP asserts, as has previously been described in several in vivo and in vitro systems, a potent role in neuroprotection [28–30].

With the exception of an increased number of colonic nNOS-containing submucous neurons in P2Y₁₃^{+/+} mice on HFD, the relative numbers of nNOS-containing enteric neurons were similar in both the ileum and colon irrespective of diet. Why the relative numbers of submucous nNOS-containing neurons increase to 26 % after HFD cannot easily be explained. A dramatic increase in the relative frequency of nNOS-containing submucous neurons has previously been reported to occur during culture [31]. After 8 days in culture conditions, 50 % of all submucous neurons were found to express nNOS. This suggests a role of nitric oxide (NO) in enteric neuronal plasticity and survival.

In contrast to a previous report on mice in which nNOS-containing submucous neurons were reported to be approximately 3 % [32], we detected high (15–30 %) relative numbers of nNOS-containing neurons. This could be explained by strain differences or by technical differences due to the antibodies used in the immunocytochemical detection of nNOS-containing neurons.

Purinergic transmission was first discovered and described in the GI tract by Professor G. Burnstock et al. in 1970 [4]. Since ATP was recognized as a non-adrenergic non-cholinergic (NANC) inhibitory transmitter, our knowledge on the importance of purine nucleotides and nucleosides in the GI tract has significantly expanded. Purinergic pathways have been elucidated in depth both concerning normal intestinal physiology and with focus on a number of GI diseases; for a review, Antonioli et al. [33]. Purinergic signalling has also been emphasized as highly associated with diabetes and its adverse effects [34]. In the gut, ATP is mainly regarded a co-transmitter of NO and VIP eliciting non-nitricergic NANC inhibitory motor responses. In addition, purines and purinergic receptors are important in regulating intestinal secretion as well as vasoconstriction. Both P2X and P2Y receptors are found to be expressed in the GI tract, and for most of the purinergic receptors, a functional role has been described. With the exception of a vague suggestion that purines may act

synergistically with glia-derived growth factors [35], none of the purines or the purinergic receptors has so far been ascribed any protective or hazardous role in the ENS. In the current study, we present strong evidence for the involvement of the P2Y₁₃ receptor in lipid-induced loss of myenteric neurons both in vivo and in vitro.

Conclusion

Myenteric neurons from mice lacking the P2Y₁₃ receptor or treated with a selective P2Y₁₃ receptor antagonist are resistant against HFD- and PA-induced loss. The cellular and molecular mechanisms behind, rendering them this unique feature, are at present not understood. Strong evidence supports that intestinal neuropathy and neurodegeneration are instrumental in several intestinal diseases. Consequently, it may be speculated that individuals with inactivated P2Y₁₃ receptors would better withstand injurious events involving lipotoxicity and metabolic stress. Also, P2Y₁₃ antagonism might constitute a novel therapeutic strategy to patients affected by intestinal dysmotility involving neuropathy. Further exploration of the purinergic pathways and possible enteric nerve/glia/immune cell interplays would bring on important clues on how to prevent or treat gastrointestinal dysfunction.

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Conflict of interest The authors declare no conflict of interest.

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