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The enteric nervous system of $P2Y_{13}$ receptor null mice is resistant against high fat diet- and palmitic acid-induced neuronal loss

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ABSTRACT

Objective: 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 convey a range of short term and long term cellular effects. This study investigated the role of the ADP sensitive $P2Y_{13}$ receptor in lipid induced enteric neuropathy.

Design: Littermate $P2Y_{13}^{+/+}$ and $P2Y_{13}^{-/-}$ mice were fed either normal diet (ND) or high fat diet (HFD) for 6 months. Intestines were analysed for morphological changes as well as neuronal numbers and relative numbers of VIP- and nNOS-containing neurons. Primary cultures of myenteric neurons from small intestine of $P2Y_{13}^{+/+}$ or $P2Y_{13}^{-/-}$ mice were exposed to palmitic acid (PA), the $P2Y_{13}$ receptor agonist 2meSADP and the antagonist MRS2211. Neuronal survival and relative number of VIP-containing neurons were analysed.

Results: In $P2Y_{13}^{+/+}$, but not $P2Y_{13}^{-/-}$ 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 $P2Y_{13}^{-/-}$ mice compared to $P2Y_{13}^{+/+}$ mice. The relative numbers of nNOS-containing submucous colonic neurons increased in $P2Y_{13}^{+/+}$ HFD mice. HFD also caused ileal mucosal thinning in $P2Y_{13}^{+/+}$ and $P2Y_{13}^{-/-}$ mice, compared to mice fed ND. *In vitro* PA exposure caused loss of myenteric neurons from $P2Y_{13}^{+/+}$ mice while neurons from $P2Y_{13}^{-/-}$ mice were unaffected. Presence of MRS2211 prevented PA-induced neuronal loss in cultures from $P2Y_{13}^{+/+}$ mice. 2meSADP caused no change in survival of cultured neurons.

Conclusion: $P2Y_{13}$ 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 $P2Y_{13}$ receptor stimulation.

INTRODUCTION

The gastrointestinal (GI) tract is emerging a 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 to 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 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 (GPCR). [5, 9] $P2Y_{13}$ is a GPCR activated by ADP and coupled to G_i/G_o signalling.[10] It was initially identified in spleen and brain but is also expressed in dorsal root ganglia, microglia, pancreatic beta cells, hepatocytes and mast cells.[10-15] In central neurons $P2Y_{13}$ 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 $P2Y_{13}$ receptor reduces high density lipoprotein (HDL) levels and increases hepatic HDL uptake in mice.[13, 19-21] Research obtained, either by silencing the $P2Y_{13}$ receptors through targeted gene deletion or small interfering RNA, suggests a pro-apoptotic role of $P2Y_{13}$ receptor activation. In contrast, $P2Y_{13}$ receptor activation was found to elicit neuroprotection in cerebellar granule neurons.[18]

It was recently shown that feeding mice high fat diet (HFD) for 6 months results in a substantial loss of myenteric neurons in both 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 $P2Y_{13}$ receptor has been attributed

in apoptosis, neuroprotection and lipid transport initiated the current study. By using $P2Y_{13}^{+/+}$ and $P2Y_{13}^{-/-}$ mice fed either a normal diet (ND) or HFD we investigated the possible role of the $P2Y_{13}$ receptor in HFD-induced enteric neuropathy. In addition we used primary myenteric neuronal cultures from $P2Y_{13}^{+/+}$ and $P2Y_{13}^{-/-}$ mice to investigate survival and relative numbers of vasoactive intestinal peptide (VIP) expressing neurons in response to palmitic acid (PA), $P2Y_{13}$ 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

$P2Y_{13}^{-/-}$ mice were backcrossed into the C57BL/6J background generating $P2Y_{13}^{-/+}$ genotypes which subsequently were used to produce $P2Y_{13}^{+/+}$ (n=12) and $P2Y_{13}^{-/-}$ (n=13) littermates. [19] At 1 month of age the mice were divided into four groups; two ND groups ($P2Y_{13}^{+/+}$ n=6, $P2Y_{13}^{-/-}$ n=7) continuing on standard chow (Lactamin R36) and two HFD groups ($P2Y_{13}^{+/+}$ n=6, $P2Y_{13}^{-/-}$ 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 sacrificed by cervical dislocation, weighed and body composition determined by dual-energy x-ray absorpmetry [23] using Lunar PIXImus scanner (GE lunar, USA).

The abdominal cavity was opened and the gastro-intestinal tract from lower esophageal sphincter to the anal canal 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 0.1M phosphate buffer, pH 7.2) for 24h at 4 ˚C and rinsed 3 times in Tyrode's solution containing 10% sucrose at 4 ˚C. Segments from 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

$P2Y_{13}^{+/+}$ (n=10, 20-23g) and $P2Y_{13}^{-/-}$ mice (n=8, 20-23g) 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, anesthetized mice (i.p. injection with Ketalar/Rompun) had their small intestine exposed via midline incisions. The longitudinal muscle layer with attached myenteric ganglia was stripped from approximately 15 cm of the distal small intestine. Tissues were placed in Ca^{2+} and Mg^{2+} free HBSS containing 1.9 CDU/mL collagenase 1-A (1.5 mg/mL, Sigma-Aldrich, SE) and 4.7 µU/mL protease IX (1.5mg/mL, Sigma-Aldrich, SE) and enzymatically and mechanically separated. Trypsin (0.4 mg/mL, BioChrom AG) and EDTA (0.003%; Sigma-Aldrich) were added. Trypsin inactivation was by addition of 50% v/v fetal bovine serum (FBS, Life Technologies, SE). Cell suspension was centrifuged at 15.6 g for 10 min. Supernatant was removed and pellet washed three times in HBSS, centrifuged at 15.6 g for 10 min and diluted to 0.8 mL in NBA cell culture medium (Life Technologies, SE) containing 10% FBS, 0.5 mM L-glutamine (K0282, BioChrom AG), 50 U/mL penicillin and 50 µg/mL streptomycin (A2213, BioChrom AG). Cell cultures were prepared by adding 50 µL of a constantly mixed cell suspension into 8-well chambers (734-0402, BD Falcon, VWR) prefilled with 450 µL of the NBA cell culture medium. From each animal two 8-well chambers (69mm^2/well) were prepared. Suspensions from multiple animals were never pooled. Cultures were incubated 4 days prior to experimental treatments.

Pharmacological agents and experimental treatments

Stock solutions of PA (P9767, Sigma-Aldrich), 2-methylthioadenosine diphosphate trisodium salt (2meSADP; 1624, Tocris, UK) and MRS2211 (2402, Tocris UK) were prepared, aliquoted and stored at -20 ˚C.

PA was conjugated to BSA (Merck, SE) by adding PA stock (10^{-2} M) together with 20% BSA w/v to medium and mixed at 37 ˚C 1h prior to use. Final PA:BSA molar ratio was 4-5:1. Final BSA concentration was ≤ 2% v/v. Different concentrations (10^{-4} – 10^{-3} M) of albuminated PA were added to neuronal cultures from $P2Y_{13}^{+/+}$ and $P2Y_{13}^{-/-}$ 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 $P2Y_{13}^{+/+}$ 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 Stefaninis fixative for 30 min and rinsed 2x10 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 manufactures protocol. To estimate the relative numbers of neurons expressing neuronal nitric oxide synthetase (nNOS) a modified immunolabelling protocol was used allowing for 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 by 2x8min microwaving in citrate buffer (0.1M citrate, pH 6.5) followed by 10min cooling in water bath and 20min 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 40 min in 0.1mg/mL Streptavidin (Sigma, SE), washed 3x10 min in WB, incubated 120 min in 0.5mg/mL biotin (Sigma, SE) and washed 3x10 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, were performed on consecutive sections.

Neuronal cell cultures are devoid of mouse IgG thus allowing for visualisation of antigen-antibody complexes using indirect immunofluorescence also of monoclonal HuC/HuD antibodies. Double immunolabelling of cultures and sections were 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 (1:1000, S11223, Life Technologies, SE) were mixed and slides were incubated 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 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 1:1000 in PBS-T-B incubated 1h, washed in PBS-T and mounted in ProLong®gold (Life Technologies, SE).

Morphometic 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 enteric nerve cell bodies in submucous and myenteric ganglia was used to estimate neuronal number per mm section length. The relative numbers 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.

Neuronal survival *in vitro* was calculated by counting the total number of surviving neurons in the entire culture well (69 mm^2) 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 Bonferronis post hoc test (*in vivo* data), or one-way analysis of variance followed by Dunnet's post hoc test towards controls (*in vitro* data). A confidence level of 95% was considered significant.

RESULTS

***In vivo* findings**

$P2Y_{13}^{+/+}$ and $P2Y_{13}^{-/-}$ mice fed HFD had increased body weight ($p<0.001$, ND: $P2Y_{13}^{+/+}$ 32.1±2.8g, $P2Y_{13}^{-/-}$ 33.6±2.5g; HFD: $P2Y_{13}^{+/+}$ 46.2±2.0g, $P2Y_{13}^{-/-}$ 49.7±1.6g) and body fat % ($p<0.001$, ND: $P2Y_{13}^{+/+}$ 24.0±2.5%, $P2Y_{13}^{-/-}$ 21.7±1.8%; HFD: $P2Y_{13}^{+/+}$ 47.5±1.8%, $P2Y_{13}^{-/-}$ 44.8±1.7%) compared to $P2Y_{13}^{+/+}$ and $P2Y_{13}^{-/-}$ ND mice. Weights of the digestive tract did not differ between the four groups (ND: $P2Y_{13}^{+/+}$ 3.3±0.3g, $P2Y_{13}^{-/-}$ 3.7±0.3g; HFD: $P2Y_{13}^{+/+}$ 3.6± 0.3g, $P2Y_{13}^{-/-}$ 3.0±0.1 g). Morphometry on intestinal sections showed HFD to cause a modest ileal mucosal thinning, in both $P2Y_{13}^{+/+}$ and $P2Y_{13}^{-/-}$ mice that was absent in colon. No alterations in muscularis propria thickness were observed in ileum or colon in any of the groups. HFD caused a significant loss of myenteric neurons in ileum and colon of $P2Y_{13}^{+/+}$, but not in $P2Y_{13}^{-/-}$, mice. In colon from $P2Y_{13}^{-/-}$ mice, both on ND and HFD, a slight, but significant, increase in myenteric neurons per mm section was noted compared to $P2Y_{13}^{+/+}$. No change in the numbers of submucous neurons in ileum and colon was observed in any of the groups. Results are summarized in figure 1 and table 3.

Both ND and HFD fed $P2Y_{13}^{-/-}$ mice displayed lower relative numbers of colonic VIP-containing submucous neurons, compared to $P2Y_{13}^{+/+}$ mice. HFD did not cause any change in the relative numbers of VIP-containing enteric neurons, compared to ND mice. The relative numbers of myenteric neurons expressing nNOS was similar in ileum and colon of $P2Y_{13}^{+/+}$ and $P2Y_{13}^{-/-}$ mice irrespective of diet. $P2Y_{13}^{+/+}$ mice fed HFD had higher relative number of nNOS-containing submucous neurons in colon as compared to the other three groups. Results are summarized in table 3.

Bodipy staining of ileum and colon from $P2Y_{13}^{+/+}$ mice on HFD displayed a marked lipid droplet accumulation in muscularis propria while the other three groups ($P2Y_{13}^{+/+}$ ND, $P2Y_{13}^{-/-}$ ND and $P2Y_{13}^{-/-}$ HFD) showed no or low levels of droplet accumulation (fig 1,e-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 estimated as percent of control.

Survival of myenteric neurons from $P2Y_{13}^{+/+}$ and $P2Y_{13}^{-/-}$ mice.

Survival of cultured myenteric neurons from ND fed $P2Y_{13}^{+/+}$ and $P2Y_{13}^{-/-}$ 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). Control wells displayed a large number ($P2Y_{13}^{+/+}$ 6.0 ± 0.1 neurons/mm^2, n=31; $P2Y_{13}^{-/-}$ 5.9 ± 0.2 neurons/mm^2, n=20) of evenly distributed neurons. Exposing cultures from $P2Y_{13}^{+/+}$ mice to increasing concentrations of PA (10^{-4} – 10^{-3} M) caused a severe and concentration dependent loss of neurons. In contrast, in neuronal cultures from $P2Y_{13}^{-/-}$ mice no loss of myenteric neurons were 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 $P2Y_{13}^{+/+}$ cultures against PA induced neuronal loss. Addition of 2meSADP (10^{-7} – 10^{-5}M) did not affect neuronal survival in either $P2Y_{13}^{+/+}$ or $P2Y_{13}^{-/-}$ cultures. Neither did the combined exposure of 2meSADP (10^{-7} – 10^{-5}M) and MRS2211 (10^{-5}M) to $P2Y_{13}^{+/+}$ cultures cause any change in neuronal survival. MRS2211 (10^{-5}M) *per se* increased the number of surviving neurons in $P2Y_{13}^{+/+}$ cultures. Results are summarized in fig 2 and table 4.

VIP expression in cultured myenteric neurons from $P2Y_{13}^{+/+}$ and $P2Y_{13}^{-/-}$ mice.

The relative numbers of cultured myenteric VIP-containing neurons from $P2Y_{13}^{+/+}$ and $P2Y_{13}^{-/-}$ 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 were observed after exposure to PA (10^{-3}M) in both $P2Y_{13}^{+/+}$ and $P2Y_{13}^{-/-}$ cultures, compared to controls. In $P2Y_{13}^{+/+}$ cultures addition of MRS2211 (10^{-5} M) reversed PA-induced reduction of VIP-containing neurons at all PA concentrations except 10^{-3}M. No change in the relative numbers of VIP-containing neurons were observed in $P2Y_{13}^{+/+}$ or $P2Y_{13}^{-/-}$ cultures exposed to

2meSADP (10^{-4} – 10^{-3} M) compared to controls. $P2Y_{13}^{+/+}$ 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 $P2Y_{13}^{-/-}$ and $P2Y_{13}^{+/+}$ littermate mice. In addition, pharmacological *in vitro* studies on myenteric neurons from ND fed $P2Y_{13}^{-/-}$ and $P2Y_{13}^{+/+}$ were undertaken to reveal possible changes in neuronal survival after PA exposure and stimulation or inhibition of the $P2Y_{13}$ 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 ileum and colon in the $P2Y_{13}^{+/+}$ mice. Surprisingly the $P2Y_{13}^{-/-}$ mice showed a remarkable protection against HFD-induced neuronal loss. It is also notable that the $P2Y_{13}^{-/-}$ mice fed ND displayed an even higher number of neurons per mm section in colon compared to ND fed $P2Y_{13}^{+/+}$ littermates.

The finding that PA-induced loss of myenteric neurons from $P2Y_{13}^{+/+}$ 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 $P2Y_{13}^{-/-}$ 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 $P2Y_{13}$ receptor or presence of MRS2211 abolished PA-induced neuronal loss *in vitro*. This indicates an unpredicted contribution of $P2Y_{13}$ receptor activation in executing PA-induced loss of myenteric neurons. MRS2211-induced inhibition of $P2Y_{13}$ receptors resulted in increased survival of cultured $P2Y_{13}^{+/+}$ derived myenteric neurons, indicating constitutive activation of neuronal loss by way of $P2Y_{13}$ receptor stimulation. Such constitutive activation could possible explain the

finding that $P2Y_{13}^{-/-}$ mice *in vivo* display increased myenteric neuronal density in colon compared to $P2Y_{13}^{+/+}$ mice.

The neuroprotection observed in $P2Y_{13}^{-/-}$ 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 $P2Y_{13}^{-/-}$ mice does not show changed insulin sensitivity or glucose tolerance, inhibition of $P2Y_{13}$ 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 links PA exposure and neuronal $P2Y_{13}$ receptor activation with a pro-apoptotic outcome.

Curiously, exposing $P2Y_{13}^{+/+}$ cultured myenteric neurons to the stable and selective $P2Y_{13}$ 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. Exposure thereby decreases constitutive apoptotic $P2Y_{13}$ signalling (Olde B, et al unpublished). The absence of effect of 2meSADP exposure may indicate that the protective effect of the $P2Y_{13}^{-/-}$ phenotype is indirect. Complex links between $P2Y_{13}$ intracellular lipid- and energy metabolism and/or neuronal trans-membrane lipid transport may be operating. Altered lipid transport and metabolism in $P2Y_{13}^{-/-}$ mice has been highlighted in multiple studies. Activation of the $P2Y_{13}$ receptor in mice leads to augmented hepatic HDL uptake and bile acid secretion.[21] Silencing the $P2Y_{13}$ receptor results in reduced TG-HDL endocytosis and internalization.[13] Gene profiling and phenotypic analysis of $P2Y_{13}^{-/-}$ 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, $P2Y_{13}^{-/-}$ mice fed HFD did not accumulate lipid droplets in muscularis propria, which suggestively underline the possibility of an altered lipid metabolism. An interesting finding was the ileal mucosal thinning observed in both $P2Y_{13}^{+/+}$ and $P2Y_{13}^{-/-}$ mice fed HFD. Previous studies

have shown lipid infusions, unlike other nutrients, to cause a mucosa barrier impairment in rat small intestine.[29] 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 $P2Y_{13}^{-/-}$ mice than in the $P2Y_{13}^{+/+}$ mice, irrespective of diet. This probably reflects a neuronal VIP content below the immunocytchemical 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 low expression of VIP mRNA or excessive release of the peptide, not matched by VIP mRNA expression. It may also reflect a low expression of PACAP or PAC_1 receptors since PACAP by PAC_1 receptor activation increases VIP mRNA expression.[30] In addition present results suggest that $P2Y_{13}$ receptor activation influences VIP expression. VIP expression is under calcium response element binding protein (CREB) control and the $P2Y_{13}$ 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 $P2Y_{13}^{+/+}$ and $P2Y_{13}^{-/-}$ derived cultures and that it decreases after high PA exposure in both genotypes. In myenteric neurons from $P2Y_{13}^{+/+}$ cultures the selective $P2Y_{13}$ receptor antagonist reversed the PA-induced decrease of VIP -containing neurons at all PA concentrations, except the highest. Inactivating the $P2Y_{13}$ 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. [31-33]

With the exception of an increased number of colonic nNOS-containing submucous neurons in $P2Y_{13}^{+/+}$ mice on HFD the relative numbers of nNOS-containing enteric neurons were similar in both ileum and colon irrespective of diet. Why the relative numbers of submucous nNOS-containing neurons increases 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.[34] 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%,[35] 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 see.[36] Purinergic signalling has also been emphasized as highly associated with diabetes and its adverse effects.[37] In the gut ATP is mainly regarded a co-transmitter of NO and VIP eliciting non-nitrergic 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,[38] none of the purines or the purinergic receptors has so far been ascribed any protective or hazardous role in the ENS. With the present study we present strong evidence for the involvement of the $P2Y_{13}$ receptor in lipid-induced loss of myenteric neurons both *in vivo* and *in vitro*.

CONCLUSION

Myenteric neurons from mice lacking the $P2Y_{13}$ receptor or treated with a selective $P2Y_{13}$ 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 $P2Y_{13}$ receptors would better withstand injurious events involving lipotoxicity and metabolic stress. Also, $P2Y_{13}$ 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 INTERESTS

The authors declare no conflict of interest

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TABLES

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.

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	[26]
Vasoactive intestinal peptide (VIP), purified porcine	1:1.200	7852	Euro Diagnostica, SE	Rabbit	[27]
Neuronal nitric oxide synthase (nNOS), synthetic rat cerebellar	1:5.000	9223	Euro Diagnostica, SE	Rabbit	[28]
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	

Table 3 Neuronal survival and relative expressions of VIP and nNOS neurons in myenteric (MG) and submucous ganglia (SG) of $P2Y_{13}^{+/+}$ and $P2Y_{13}^{-/-}$ 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 mm cryosection. b. Relative numbers of VIP-immunoreactive neurons expressed in percentage of HuC/HuD immunoreactive ones. c. Relative numbers of nNOS-immunoreactive neurons expressed in percentage of HuC/HuD-immunoreactive ones. Data are shown as means ± SEM, * $p<0.05$, ** $p<0.01$, $p<0.001$ all comparisons against $P2Y_{13}^{+/+}$ ND; n=6-7 in each group

a HuC/HuD-immunoreactive neurons per mm

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 VIP-immunoreactive neurons (% of HuC/HuD)

Genotype	Diet	Ileum MG	Ileum SG	Colon MG	Colon SG
$P2Y_{13}^{+/+}$	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 nNOS-immunoreactive neurons (% of HuC/HuD)

Genotype	Diet	Ileum MG	Ileum SG	Colon MG	Colon SG
$P2Y_{13}^{+/+}$	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

Table 4 Neuronal survival of cultured myenteric neurons and relative numbers of VIP-immunoreactive neurons. Survival is expressed as % of the control run in parallel. Relative numbers of VIP-immunoreactive neurons are expressed as % of HuC/HuD-immunoreactive neurons. a. Shows cultures derived from $P2Y_{13}^{+/+}$ mice. b. Cultures derived from $P2Y_{13}^{-/-}$ mice. Data is given as means ±SEM * $p<0.05$, ** $p<0.01$, all comparisons against control; n=5-20 in each group

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
$4x10^{-4}$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
$4x10^{-4}$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

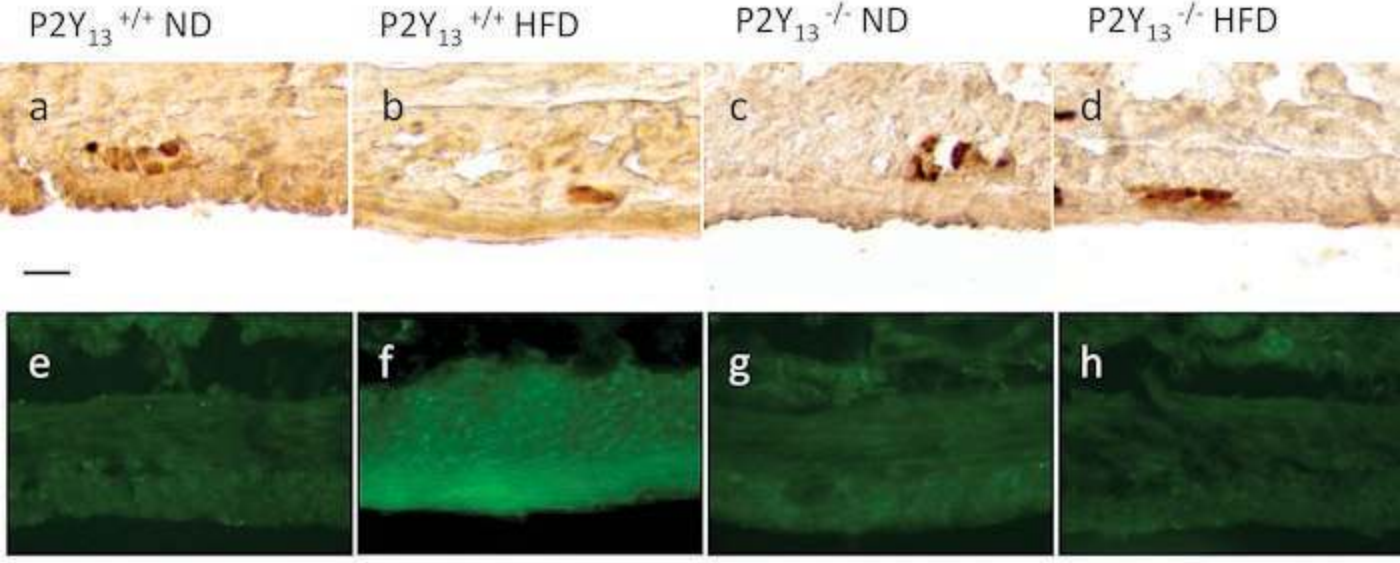
Treatment	Survival (% of control)	VIP neurons (% of HuC/HuD)
control	100	16.5 ± 1.0
2meSADP		
10^{-7}M	117.0 ± 10.0	12.5 ± 1.4
10^{-6}M	100.5 ± 3.5	12.0 ± 1.7
10^{-5}M	106.0 ± 12.5	12.3 ± 1.7
PA		
10^{-4}M	102.4 ± 4.6	16.0 ± 1.5
$4x10^{-4}$M	96.4 ± 4.1	14.8 ± 0.9
10^{-3}M	90.0 ± 7.5	9.0 ± 0.6 *

FIGURE LEGENDS

Fig. 1 Upper panel shows representative micrographs of ileum from $P2Y_{13}^{+/+}$ (a, b, e and f) and $P2Y_{13}^{-/-}$ (c, d, g and h) mice fed 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 ileum (i) and colon (j). a-d cryosections immunostained for enteric neurons using the pan neuronal marker HuC/HuD. $P2Y_{13}^{+/+}$ mice fed HFD (b) have markedly less myenteric neurons per mm compared to ND fed mice (a). HFD does not change neuronal density in $P2Y_{13}^{-/-}$ mice (d). e-h cryosections stained with Bodipy® to illustrate lipid droplets within muscularis propria. $P2Y_{13}^{+/+}$ mice fed HFD (f) have increased intramuscular lipid droplet formation, compared to $P2Y_{13}^{+/+}$ ND mice (e). $P2Y_{13}^{-/-}$ mice do not accumulate lipid droplets in muscularis propria (g, h). i and 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, * $p<0.05$, all compared to $P2Y_{13}^{+/+}$ ND; n=5-12 in each group. Bar represents 20µm.

Fig. 2 Representative micrographs of cultured myenteric neurons from $P2Y_{13}^{+/+}$ (a-d) and $P2Y_{13}^{-/-}$ mice (e and f) small intestine. Neurons are visualized using HuC/HuD immunostaining. Exposure to palmitic acid (PA) (b) causes loss of $P2Y_{13}^{+/+}$ neurons, compared to control (a). Exposure to the $P2Y_{13}$ antagonist MRS2211 increases survival of $P2Y_{13}^{+/+}$ neurons (c). MRS2211 also protects $P2Y_{13}^{+/+}$ neurons against PA-induced loss (d). Neurons from $P2Y_{13}^{-/-}$ mice are protected against PA-induced neuronal loss. Bar represents 20 µm.

Fig. 3 Representative micrographs of VIP immunoreactive cultured myenteric neurons from $P2Y_{13}^{+/+}$ (a, b) and $P2Y_{13}^{-/-}$ mice (c, d) small intestine. No difference in the relative number of neurons immunoreactive for VIP was observed between $P2Y_{13}^{+/+}$ (a) and $P2Y_{13}^{-/-}$ (c) derived cultures. Exposure to palmitic acid (PA, $4x10^{-4}$M) did not change percentage of neurons immunoreactive for PA in $P2Y_{13}^{+/+}$ (b) or $P2Y_{13}^{-/-}$ (d) derived cultures. But exposure to 10^{-3}M PA reduced the relative number of neurons immunoreactive for VIP. Bar represents 20 µm.


P2Y13 +/+ ND
P2Y13 +/+ HFD
P2Y13 -/- ND
P2Y13 -/- HFD
a
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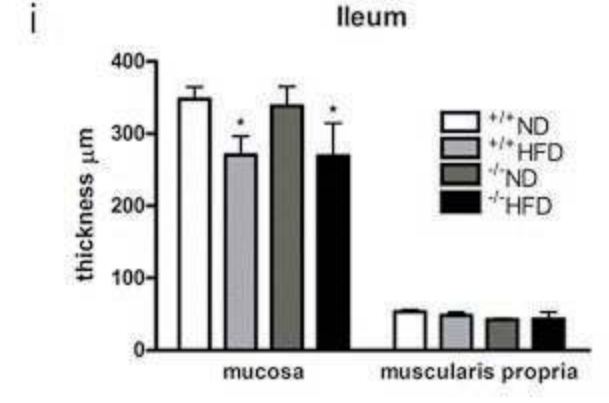
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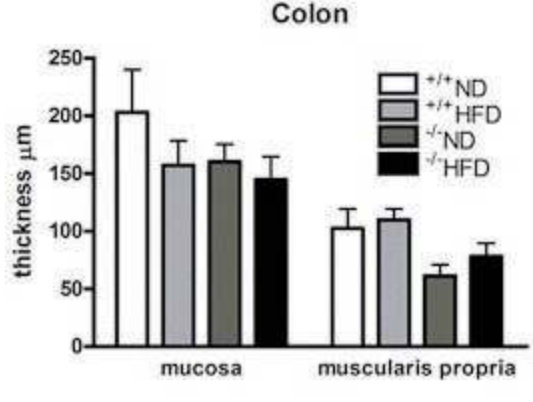
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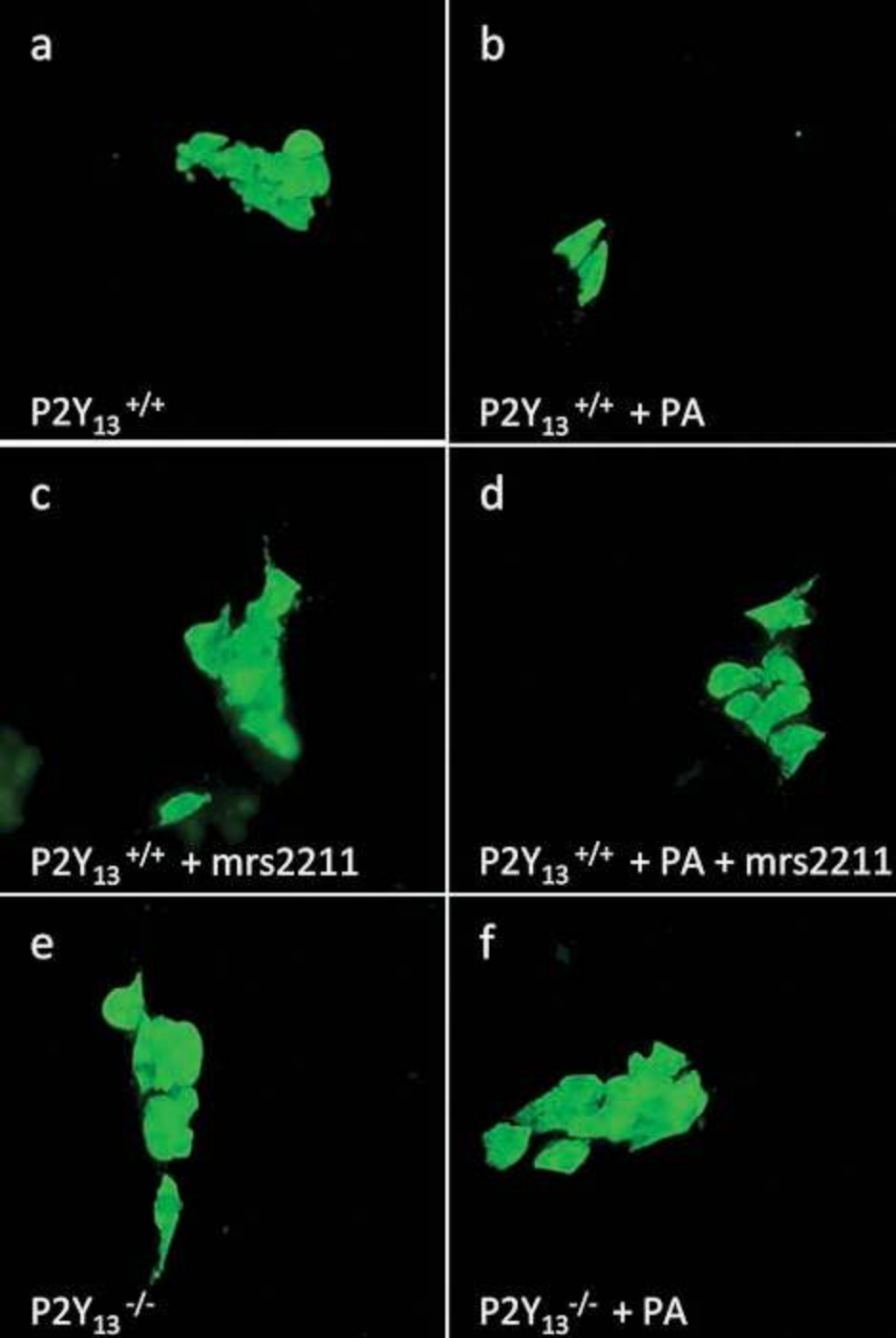
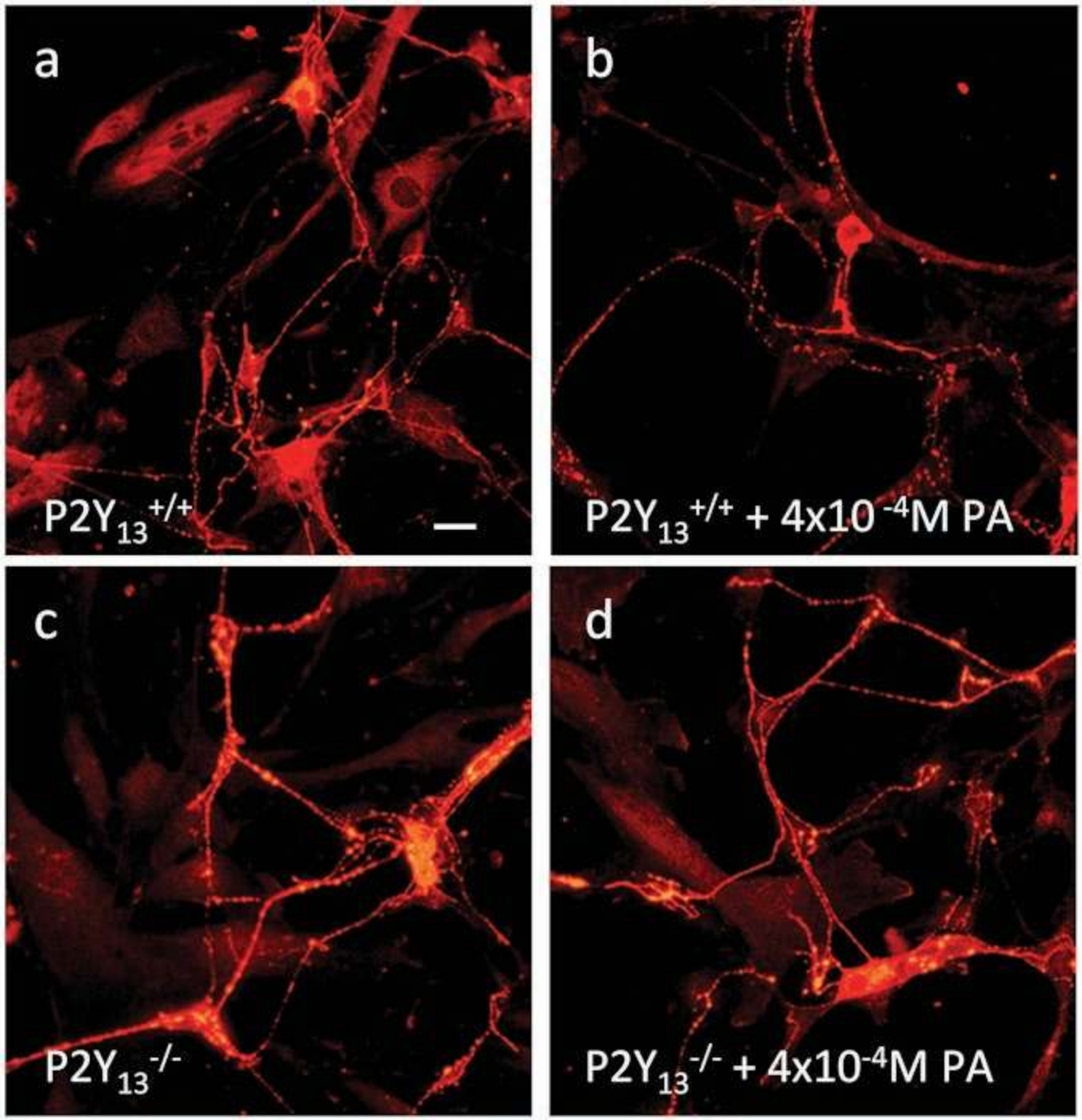

a
P2Y13 +/+
b
P2Y13 +/+ + PA
c
P2Y13 +/+ + mrs2211
d
P2Y13 +/+ + PA + mrs2211
e
P2Y13 -/-
f
P2Y13 -/- + PA


a
P2Y13+/+
b
P2Y13+/+ + 4x10-4M PA
c
P2Y13-/-
d
P2Y13-/- + 4x10-4M PA