Cell and Tissue Research (2019) 378:555–558
https://doi.org/10.1007/s00441-019-03097-5

SHORT COMMUNICATION

Presence of MrgprD within the gastrointestinal wall: reality or fake?

Samuel Van Remoortel[1] · Jean-Pierre Timmermans[1]

Received: 22 April 2019 / Accepted: 13 August 2019 / Published online: 31 August 2019

Abstract

Due to their pivotal role in nociception and mast cell biology, the family of Mas-related G protein-coupled receptors (Mrgprs) has recently gained attention for their possible expression and role(s) in the gastrointestinal tract. In this context, based on immunocytochemical stainings using a commercial antibody, a recent study by Zhou et al. reported that the murine Mrgprd member is expressed in mouse gut lamina propria immune cells and in the outer smooth muscle layers pointing to a potential role for MrgprD in inflammatory responses and intestinal immunity. Immunohistochemical staining for G protein-coupled receptors (GPCRs), however, remains challenging and should be cautiously interpreted using appropriate specificity controls. Using the same antibody with an identical dilution, we did observe a similar staining in the same wild-type mouse strain, but an identical staining pattern was also found in mice lacking the MrgprD receptor, indicating that this antibody recognizes epitopes other than those of MrgprD. Moreover, in situ hybridization for MrgprD further indicated the absence of receptor mRNA expression in lamina propria immune cells and in the outer smooth muscle layers. Therefore, the results and conclusions regarding the presence of MrgprD at protein level within the GI wall as described in the study of Zhou and collaborators should be interpreted with strong caution and should be reconsidered in the light of the emerging possible roles of MrgprD and therapeutic perspectives in gastrointestinal pathophysiology.

Keywords Mas-related G protein-coupled receptor · Mrgprd · Immunohistochemistry · Nociception · Gastrointestinal tract

Introduction

The family of Mas-related G protein-coupled receptors (Mrgprs) is classified as rhodopsin-like G protein-coupled receptors (GPCRs), which is a superfamily of GPCR's that is currently exploited the most as drug target (Hauser et al. 2017). Mrgprs are expressed in rodents and humans and have emerged as novel players in nociception, mast cell biology, and neuroimmune communication (Dong et al. 2001; Lembo et al. 2002; Zylka et al. 2003; Burstein et al. 2006; Bader et al. 2014; Solinski et al. 2014). Based on these functional aspects, Mrgprs are highly relevant in gastrointestinal (GI) (patho)-physiology and thus prompted a more in-depth exploration of their expression and role(s) in the gut (Avula et al. 2011, 2013).

For one particular Mrgpr member, namely, the murine MrgprD member, a growing body of evidence has indicated an important role in gastrointestinal (GI) (patho)physiology. Last year, an elegant study by Bautzova and colleagues found that MrgprD plays a pivotal role in chronic abdominal pain disorders such as IBD and IBS (Bautzova et al. 2018). Moreover, in a recently published study, Zhou et al. claimed to provide experimental evidence that MrgprD is expressed in the mouse small and large intestine, more specifically in the outer intestinal muscle layers and in F4/80-positive macrophages and CD3-positive T cells in the lamina propria, as well as in distinct primary and continuous cell lines (Zhou et al. 2019). Based on these findings, the authors further suggest that the receptor could also be involved in motility and neuroimmune communication in the gut wall, which is an important finding for the field that warrants further exploration of its role in relation to GI disorders characterized by neuroimmune pathophysiology.

It is well known that immunohistochemical staining of GPCR's is challenging and that such staining results can be easily misinterpreted if appropriate specificity controls are not performed. Even though the study of Zhou et al. is commendable, we further verified receptor expression in the bowel with more robust and informative antibody specificity controls, such as MrgprD immunolocalization in the GI tract of wild-type mice and mice lacking MrgprD receptor expression.

✉ Jean-Pierre Timmermans
jean-pierre.timmermans@uantwerpen.be

[1] Laboratory of Cell Biology & Histology, Department of Veterinary Sciences, University of Antwerp, Universiteitsplein 1, BE-2610 Wilrijk, Antwerp, Belgium

Furthermore, in situ hybridization was performed to localize receptor mRNA expression in the bowel wall.

Material and methods

Animals

Experiments were performed on adult (3–5 months old) male C57BL/6N wild-type mice and Mrgprd^{tm1Mj} *(MrgprdΔ^{EGFPf}) mice (kindly provided by Dr Anderson, Howard Hughes Medical Institute).* In these mice, the MrgprD receptor gene is removed and replaced by an in-frame enhanced green fluorescent protein (eGFP) gene cassette, whereby these animals lack MrgprD receptor expression and thus serve as valid controls for antibody specificity (Zylka et al. 2005). Animal housing and handling procedures were conducted in accordance with the European Directive 86/609/EEC and approved by the Ethical Committee for animal experiments of the University of Antwerp (EC 2016-19).

Immunohistochemistry

After cervical dislocation, the ileum was isolated, flushed to remove fecal content, and immersion-fixed for 2 h in phosphate-buffered 4% paraformaldehyde solution at room temperature. Subsequently, tissues were processed for cryosectioning, and 12-μm-thick cryosections were mounted on poly-L-lysine-coated slides and further processed for immunohistochemistry. Sections were incubated for 1 h with 0.01 M phosphate-buffered saline (PBS) containing 1% Triton X-100 and 10% normal horse serum (NHS), followed by an overnight incubation at 4 °C with primary antiserum: rabbit anti-Mas-related G protein-coupled receptor D (MrgprD, 1/100, Abcam, ab155099). Visualization was achieved using Cy3-conjugated donkey anti-rabbit immunoglobulins (1/8000, Jackson ImmunoResearch, Cambridgeshire, UK). All antisera were diluted in 0.01 M PBS containing 10% NHS and 1% bovine serum albumin. High-resolution images were obtained on a Leica TCS SP8 laser scanning confocal microscope.

In situ hybridization

In order to localize receptor mRNA expression in dorsal root ganglia and ileum, in situ hybridization (ISH) was performed using the RNAScope 2.5 HD BROWN platform (Advanced Cell Diagnostics, Newark, CA, USA). All tissue preparations and experiments were carried out according to the manufacturer's protocol. For detection of murine MrgprD gene transcripts, a target probe directed against mouse *MrgprD* (Advanced Cell Diagnostics cat. No 417921) was used. Tissue sections were imaged on an Olympus BX41 brightfield microscope equipped with a Leica EC3 camera.

Results

Immunohistochemistry in the ileum of wild-type mice showed MrgprD immunoreactivity in the lamina propria and in the outer muscle layers (Fig. 1a, b). To further validate antibody specificity, the ileum of MrgprD knockout mice that lack receptor expression was immunostained with the MrgprD antibody. Unfortunately, the Mrgprd antibody resulted in a similar immunohistochemical staining pattern in the GI tract of these knockout mice compared with wild-types, indicating that the antibody recognizes epitopes other than those of MrgprD (Fig. 1c, d). Moreover, using the RNAscope in situ hybridization platform, we were able to observe MrgprD mRNA expression within cell bodies of DRG neurons but not within the gut wall (Fig. 2).

Discussion

A growing number of studies are exposing novel roles for distinct Mrgprs, including MrgprD, in the GI tract. In this context, a recent immunohistochemical study by Zhou et al. reported the expression of MrgprD in the mouse GI tract using a commercial MrgprD antibody (Zhou et al. 2019). In the latter study, only omission of the primary antibody was used to assume specificity of their immunohistochemical staining. Being frequently (mis)used as a control for antibody specificity, but in reality merely accounting for a method specificity control, omission of the primary antibody as a single control is insufficient to guarantee antibody specificity, especially in light of the challenging nature of immunohistochemistry for GPCR's.

In the current study, we probed for MrgprD expression within the gut using the same antibody as used in the study by Zhou et al., but further validated antibody specificity using more robust specificity controls. Our results show that the antibody indeed shows positive immunostaining at the level of the lamina propria and the outer muscle layers (Fig. 1a, b), but a similar staining pattern was observed in MrgprD knockout animals (Fig. 1c, d). To rule out any interference of the possible presence of eGFP in MrprD knockout mice, which however was not observed in the gut, the visualization of MrgprD-IR in both wild-type and knockout mice was performed using CY-3-conjugated secondary immunoglobulins. The observed similar staining pattern of the MrgprD antibody in wild-type mice and MrgprD knockout mice clearly indicates that the antibody does not specifically stains for MrgprD. These findings are even further strengthened by our in situ hybridization results. Whereas MrgprD mRNA was expressed in sensory neurons of the DRG, which is in

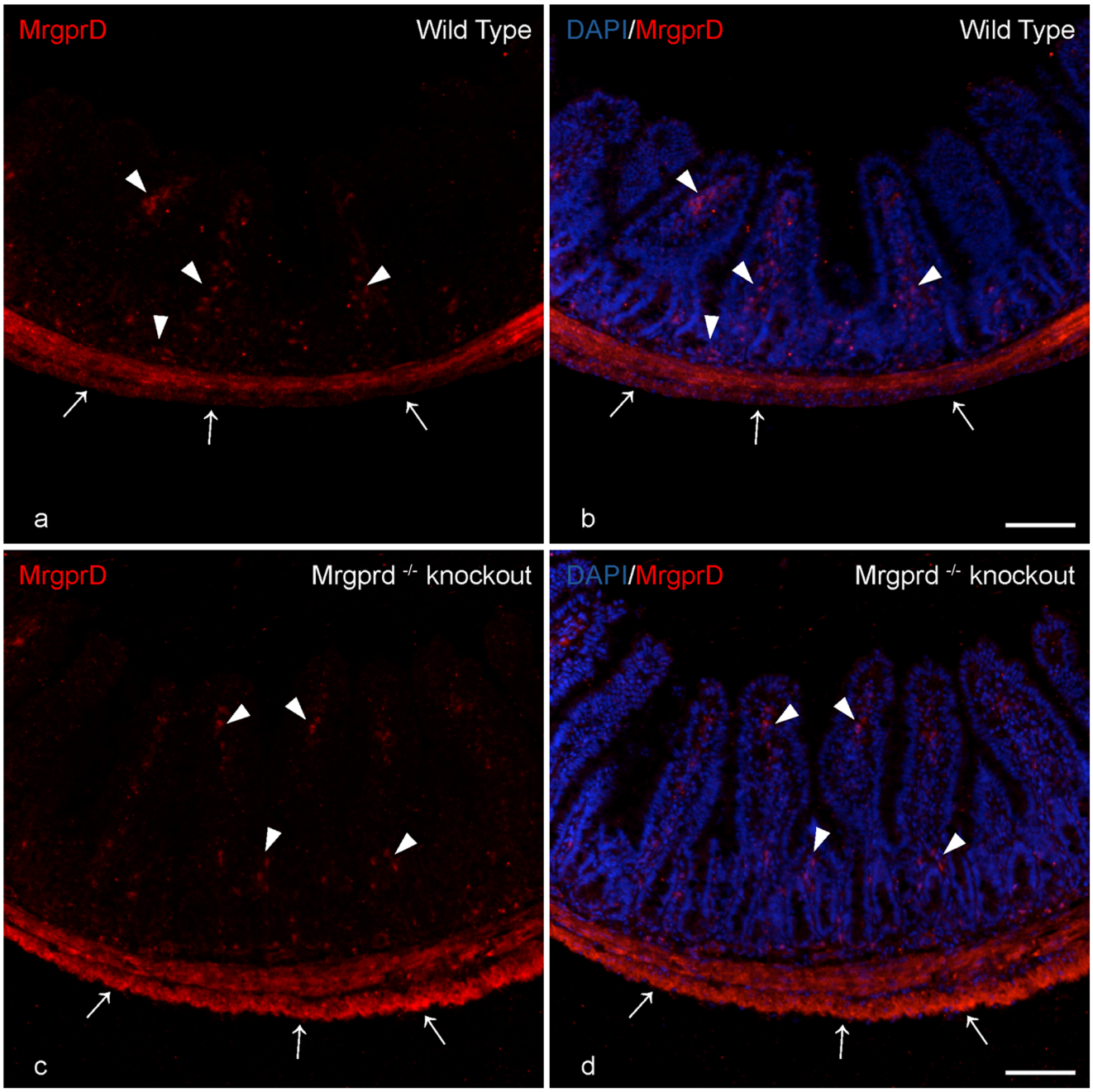

Fig. 1 MrgprD immunohistochemistry in the mouse ileum. **a-d** Representative images of 12-μm cryosections of the mouse ileum immuno-stained with the rabbit anti-MrgprD antibody (Abcam, ab155099, 1:100 dilution). In the ileum of C57/Bl6N wild-type mice (**a**, **b**), the antibody resulted in immunostaining (red) in the lamina propria (arrowheads) and in outer muscle layers (arrows). Unfortunately, in the ileum of MrgprD knockout mice (**c**, **d**), similar staining results (red) were obtained in both layers, i.e., cells in the lamina propria (arrowheads) and in the outer circular and longitudinal muscle layer (arrows). MrgprD: Mas-related G protein-coupled receptor D; DAPI (=nuclear counterstain): 4′,6-diamidino-2-phenylindole. $n = 3$ mice per group. Scale bars 100 μm

line with existing literature (Dong et al. 2001; Zylka et al. 2005; Liu et al. 2008), we did not observe MrgprD mRNA expression by cells that lie within the gut wall. Interestingly, in a recent study by Bautzova et al. that used another MrgprD antibody that was validated in MrgprD knockout mice, sparse MrgprD-immunopositive nerve fibers were observed in the mouse intestine, but no MrgprD immunoreactivity was reported in lamina propria immune cells or in the outer smooth muscle layers (Bautzova et al. 2018). The presence of sparse MrgprD-immunoreactive nerve fibers reported in that study, together with the absence MrgprD mRNA expression by cells within the gut wall as observed in our ISH experiments, indicates that MrgprD seems primarily to be expressed by extrinsic viscerosensory pathways.

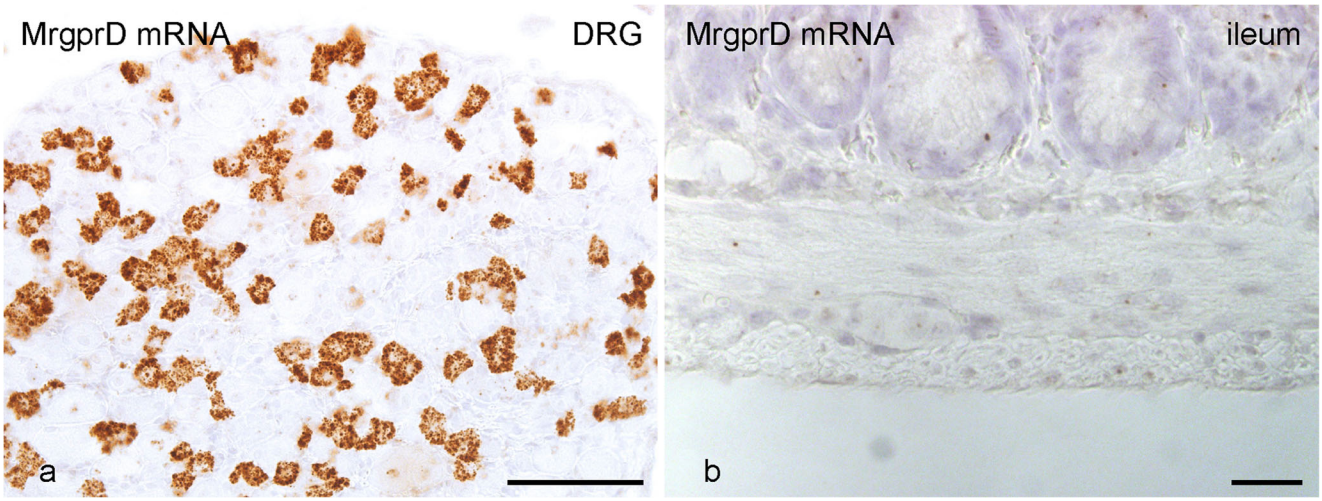

Fig. 2 Probing for MrgprD mRNA expression in the mouse DRG and ileum. **a**, **b** Representative images of 5-μm paraffin sections of the C57/Bl6N wild-type mouse DRG (**a**) and ileum (**b**) after MrgprD in situ hybridization (ISH). In DRG, MrgprD probe reactivity was observed in cell bodies of DRG neurons (**a**, brown), but *not* in the ileal outer muscle layers (**b**). $n = 3$ mice per group. Scale bars 100 μm

Overall, our findings unambiguously indicate that immunohistochemical staining of Mrgpr's, as for other GPCR's, should always be treated with care if appropriate specificity controls are lacking. Therefore, the results and conclusions presented in the study by Zhou et al. regarding the presence of MrgprD at protein level within the GI wall should be interpreted with strong caution and need to be reconsidered.

Acknowledgments The authors would like to express their gratitude to Sofie Thys, Elien Theuns, and Dominique De Rijck for technical and administrative assistance.

Funding information This study was supported by FWO grant G019314N to JPT.

Compliance with ethical standards

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All animal experiments were approved by the Ethical Committee for animal experiments of the University of Antwerp (EC 2016-19).

Conflict of interest The authors declare that they have no conflict of interest.

References

Avula LR, Buckinx R, Alpaerts K et al (2011) The effect of inflammation on the expression and distribution of the MAS-related gene receptors MrgE and MrgF in the murine ileum. Histochem Cell Biol 136: 569–585. https://doi.org/10.1007/s00418-011-0862-7

Avula LR, Buckinx R, Favoreel H et al (2013) Expression and distribution patterns of Mas-related gene receptor subtypes A-H in the mouse intestine: inflammation-induced changes. Histochem Cell Biol 139:639–658. https://doi.org/10.1007/s00418-013-1086-9

Bader M, Alenina N, Andrade-Navarro MA, Santos RA (2014) MAS and its related G protein-coupled receptors, Mrgprs. Pharmacol Rev 66: 1080–1105. https://doi.org/10.1124/pr.113.008136

Bautzova T, Hockley JRF, Perez-Berezo T et al (2018) 5-oxoETE triggers nociception in constipation-predominant irritable bowel syndrome through MAS-related G protein–coupled receptor D. Sci Signal 11: eaal2171. https://doi.org/10.1126/scisignal.aal2171

Burstein ES, Ott TR, Feddock M et al (2006) Characterization of the Mas-related gene family: structural and functional conservation of human and rhesus MrgX receptors. Br J Pharmacol 147:73–82. https://doi.org/10.1038/sj.bjp.0706448

Dong X, Han S, Zylka MJ et al (2001) A diverse family of GPCRs expressed in specific subsets of nociceptive sensory neurons. Cell 106:619–632

Hauser AS, Attwood MM, Rask-Andersen M et al (2017) Trends in GPCR drug discovery: new agents, targets and indications. Nat Rev Drug Discov 16:829–842. https://doi.org/10.1038/nrd.2017.178

Lembo PMC, Grazzini E, Groblewski T et al (2002) Proenkephalin A gene products activate a new family of sensory neuron–specific GPCRs. Nat Neurosci 5:201–209. https://doi.org/10.1038/nn815

Liu Y, Yang FC, Okuda T et al (2008) Mechanisms of compartmentalized expression of Mrg class G-protein-coupled sensory receptors. J Neurosci 28:125–132. https://doi.org/10.1523/JNEUROSCI.4472-07.2008

Solinski HJ, Gudermann T, Breit A (2014) Pharmacology and signaling of MAS-related G protein-coupled receptors. Pharmacol Rev 66: 570–597. https://doi.org/10.1124/pr.113.008425

Zhou C, Li J, Liu L et al (2019) Expression and localization of MrgprD in mouse intestinal tract. Cell Tissue Res. https://doi.org/10.1007/s00441-019-03017-7

Zylka MJ, Dong X, Southwell AL, Anderson DJ (2003) Atypical expansion in mice of the sensory neuron-specific Mrg G protein-coupled receptor family. Proc Natl Acad Sci U S A 100:10043–10048. https://doi.org/10.1073/pnas.1732949100

Zylka MJ, Rice FL, Anderson DJ (2005) Topographically distinct epidermal nociceptive circuits revealed by axonal tracers targeted to Mrgprd. Neuron 45:17–25. https://doi.org/10.1016/j.neuron.2004.12.015