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**Transient receptor potential vanilloid 1-immunoreactive signals in murine enteric glial cells**

Yamamoto M *et al*. TRPV1 and enteric glia

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**Abstract**

***AIM***

To investigate the possible involvement of transient receptor potential vanilloid 1 (TRPV1) in maturation of enteric glial cells (EGCs).

***METHODS***

Immunohistochemical and immunocytochemical techniques were used to analyze EGC markers in myenteric plexus (MP) as well as cultured MP cells and EGCs using TRPV1 knockout (KO) mice.

***RESULTS***

We detected TRPV1-immunoreactive signals in EGC in the MP of wild-type (WT) but not KO mice. Expression of glial fibrillary acidic protein (GFAP) immunoreactive signals was lower at postnatal day (PD) 6 in KO mice, though the difference was not clear at PD 13 and PD 21. When MP cells were isolated and cultured from isolated longitudinal muscle-MP preparation from WT and KO mice, the yield of KO EGC was lower than that of WT EGC, while the yield of KO and WT smooth muscle cells showed no difference. Addition of BCTC, a TRPV1 antagonist, to enriched EGC culture resulted in a decrease in the protein ratio of GFAP to S100B, another EGC/astrocyte-specific marker.

***CONCLUSION***

These results address the possibility that TRPV1 may be involved in the maturation of EGC though further studies are necessary to validate this possibility.

**Key words:** Enteric glial cells; Enteric nervous system; Glial fibrillary acidic protein; S100B; Smooth muscle cells

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**Core tip:** We report that immunosignals of glial fibrillary acidic protein (GFAP) in myenteric ganglia in transient receptor potential vanilloid 1 (TRPV1) knockout (KO) mice are weaker than in wild-type mice in the early postnatal period suggesting the possibility that the maturation of enteric glial cells (EGCs) might be retarded at least temporally in TRPV1 KO mice. Accordingly, in *in vitro* culture of isolated myenteric plexus cells/EGCs suggest that GFAP expression is affected by gene KO and an antagonist toTRPV1. The expression and function of TRPV1 in EGC merits further investigation.

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**INTRODUCTION**

The enteric nervous system (ENS), an integrative neuronal network that resides within the gut wall, autonomously controls gastrointestinal motility, secretion, and blood flow without major inputs from the brain[[1](#_ENREF_1),[2](#_ENREF_2)]. The ENS is composed of two main cell types, neurons and enteric glial cells (EGC), the latter being several fold more abundant than neurons[[3-5](#_ENREF_3)]. EGC share many phenotypical features with astrocytes, and were long believed to function mainly as support cells for neurons. However, emerging evidence has elucidated their regulatory role in a wide array of gastrointestinal (GI) physiological and pathophysiological processes[[6](#_ENREF_6)] including neurotransmission[[7](#_ENREF_7),[8](#_ENREF_8)], motility[[9-11](#_ENREF_9)], and inflammation[[8](#_ENREF_8)], as well as in secretory/absorptive[[12](#_ENREF_12),[13](#_ENREF_13)], barrier[[8](#_ENREF_8),[14-16](#_ENREF_14)] and repair[[17](#_ENREF_17)] functions of the intestinal epithelium and host defense against pathogens[[18](#_ENREF_18)].

Transient receptor potential vanilloid receptor 1 (TRPV1) is a nonselective cation channel activated by exogenous plant-derived vanilloid compounds such as capsaicin and resiniferatoxin as well as by endogenous membrane-derived lipid endocannabinoids such as anandamine, 2-arachidonoyl-glycerol, and N-arachidonoyl-dopamine[[19](#_ENREF_19)]. Moreover, TRPV1 is known to be a transducer channel activated by high temperature, low pH, and mechanical/osmotic stimuli. Although attention has been directed mainly to sensory neurons as the site of TRPV1 localization, TRPV1 expression has been detected in non-neuronal tissues/cells including keratinocytes of the epidermis, bladder urothelium, smooth muscles, liver, polymorphonuclear granulocytes, mast cells, and macrophages[[19](#_ENREF_19)].

TRPV1 has been reported to be present in astrocytes in brain[[20](#_ENREF_20)], spinal cord[[21](#_ENREF_21)] and retina[[22](#_ENREF_22)] and possibly to be involved in glial activation[[23](#_ENREF_23)], cell migration[[24](#_ENREF_24)], amyloid-β-induced inflammation[[25](#_ENREF_25)] and traumatic brain injury[[26](#_ENREF_26)]. However, it is unknown whether TRPV1 is present and functional in enteric glia. In the present study, using TRPV1-deficient (KO) mice and an acid-ethanol fixation protocol, specific TRPV1-immunoreactive (TRPV1-IR) signal was detected in wild-type (WT) EGC. In addition, the possible involvement of TRPV1 in the differentiation of EGC was investigated.

**MATERIALS AND METHODS**

***Antibodies***

Details of the primary antibodies used in the present study are shown in Table 1. The specificity of anti-TRPV1 antibodies is presented in Supplementary Figures S1 and S2. The secondary antibodies used were FITC-labeled donkey anti-mouse IgG antibody and Cy3-labeled donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) for intestinal tissues and Alexa488-conjugated goat anti-mouse antibody and Alexa568-conjugated goat anti-rabbit antibody (Molecular Probes, Eugene, OR) for isolated longitudinal muscle layer-myenteric plexus (LM-MP) and cultured cells.

***Animals***

C57Bl/6 mice were from Charles River Laboratories Japan Inc. (Kanagawa, Japan). TRPV1-deficient (B6.129X1-Trpv1<tm1Jul>/J) mice originally obtained from Jackson Laboratories (Bar Harbor, ME) were maintained at Charles River Laboratories Japan Inc. and transported to the animal facilities of Tsumura Laboratories on gestational day 14 (dams, 1 dam per cage) or at the age of 7 weeks (adult males, 4 mice per cage). The animals were allowed free access to water and standard laboratory food, and were housed at a temperature of 23 ± 2 °C with relative humidity of 55 ± 10%, and a 12:12-h light/dark cycle with lights on from 0700 to 1900 daily. All experimental procedures were performed according to the Guidelines for the Care and Use of Laboratory Animals of Tsumura & Co. Ethical approval for the experimental procedures used in this study was obtained from the Laboratory Animal Committee of Tsumura & Co. All animal procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

***Sample collection***

For the preliminary screening, 2 TRPV1-deficient (KO) and 2 C57/Bl6 wild-type (WT) dams were used. A total of 2 pups (each pup isolated from different dams) were randomly selected and sacrificed at each time point for both the KO and WT groups. Samples were collected on the day of birth (PD 0) and at around postnatal day 10 (PD 10-12), day 20 d (PD 20-21), day 30 (PD 30-33), day 60 (PD 61-62) and day 90 (PD 91-92). After anesthesia with isoflurane, animals were transcardially perfused with ice-cold normal saline followed by ice-cold acid-ethanol solution (a mixed solution of ethanol and acetic acid at a ratio of 20:1 v/v) to fix the tissues. Large and small intestines (LI and SI, respectively) were dissected and further fixed in acid-ethanol solution overnight at 4 °C, then cryoprotected and embedded in O.C.T. Compound (Sakura Finetech, Tokyo, Japan) for frozen sections according to standard procedures. For the confirmation analysis, 6 KO and 6 WT dams were used. A total of 6 pups (each pup isolated from different dams) were sacrificed at PD 6, PD 13 and PD 21. Intestinal tissue samples were collected as described above.

***Immunohistochemistry of intestinal tissue sections***

Ten μm-thick frozen sections were incubated in phosphate-buffered saline (PBS) for 10 min at room temperature, and then incubated with a mixture of primary antibodies overnight at 4 °C. After a thorough wash with PBS, sections were incubated with a mixture of secondary antibodies with nuclear counterstain (TO-PRO3, Molecular Probes, Eugene, OR) for 1 h at room temperature, washed and finally mounted with Vectashield (Vector, Burlingame, CA). Sections were observed and digital images were recorded with a confocal laser scanning microscope (C-1; Nikon, Tokyo, Japan).

***IHC of whole-mount preparation of longitudinal muscle layer-myenteric plexus***

LI segments were isolated from 5 week old mice and the layer-myenteric plexus (LM-MP) was peeled off. The peeled LM-MPs were stretched taut, pinned flat to a silicone ring and fixed with ice-cold acetone for 30 min. After fixation, each preparation was washed 3 times for 10 min each in PBS. The preparations were placed in Superblock (Thermo Fischer Scientific, Rockford, IL) containing 0.3% Triton X-100 overnight at 4 °C. The preparations were then placed in a mixture of primary antibodies diluted in antibody diluent (DAKO Japan, Tokyo, Japan) overnight at 4 °C. After removal from the primary antibody, the tissues were rinsed 3 times for 10 min per rinse with PBS and then incubated with a mixture of the relevant secondary antibodies overnight at 4 °C. After a final set of rinses, the preparations were mounted on microslides and coverslipped with Prolong Gold antifade reagent (Molecular Probes). The slides were observed using confocal laser microscopy FV-100D (Olympus, Tokyo, Japan).

***Co-culture of myenteric plexus cells and smooth muscle cells***

We prepared myenteric plexus cells (MPC)/ smooth muscle cells (SMC)mixture from SI, because a far smaller number of MPC/SMC were obtained from LM-MP of LI, presumably due to the short length of the LI tract and inefficient cellular liberation from the tissue. SI segments were isolated from 5-wk-old WT and KO mice and the LM-MP was peeled off. LM-MP was digested in digestion buffer containing 0.1% type II collagenase and 0.1% soybean trypsin inhibitor (Sigma-Aldrich) at 31 °C for 30 min. The buffer was then replaced with new digestion buffer and the mixture incubated at 31 °C for an additional 30 min. The remaining tissue pieces were dissociated by mechanical shear through micropipette tips. The cells were spun down at 200 g for 5 min and suspended in HuMedia-SG2 (Kurabo, Osaka, Japan). The cells were plated in type IV-collagen coated plates (BD Biosciences, San Jose, CA). The medium was replaced on the next day which led to discarding of almost all of the cells. The remaining attached cells, which were round and small, began to proliferate discernibly by around day 3. The cells were cultured for an additional 8 days. A representative image of the cells is shown in Supplementary Figure S3. Analysis by phase-contrast microscopy showed no apparent difference in the number and appearance of the cells between cultures derived from WT and KO mice.

***Enriched EGC culture***

Co-culture of MPC and SMC was initiated as described in the previous section. On day 5 of the co-culture, the cells were trypsinized, washed and labeled with anti-NGF receptor p75 rabbit polyclonal antibody[[27](#_ENREF_27)] for 5 min on ice. The cells were then washed and incubated with biotinylated anti-rabbit IgG antibody (BD Biosciences) for 5 min on ice. The cells were subsequently washed and mixed with magnetic beads conjugated with streptavidin. MACS system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) was used to enrich NGF-receptor p75-positive cells, which are derived from the neural crest. The cells were plated on poly-D-lysine-coated plate and cultured for an additional 6 days. Enriched EGC culture was performed using WT mice only, because the culture protocol has been found to be inapplicable to KO mice (the resultant culture contained only a trace number of surviving cells).

***Immunocytochemistry for enriched EGC cultures***

The cells were fixed with 4% phosphate-buffered paraformaldehyde for 15 min. The cells were then washed twice with PBS and permeabilized in 0.3% Triton-100 in PBS for 15 min. After being rinsed with PBS, sections were incubated overnight with in a mixture of the primary antibodies. The cells were incubated in a mixture of the relevant secondary antibodies (1:1000; Molecular Probes) for 60 min at room temperature. For TRPV1-staining, biotin-labeled anti-mouse IgG (BD Biosciences) and streptavidin-conjugated Alexa Fluor 647 (Molecular Probes) were used to amplify the signal intensity. Nuclei were stained with 2-(4-amidinophenyl)-1H -indole-6-carboxamidine (DAPI, Life Technologies). The cells were visualized, photographed and analyzed using Celaview RS100 (Olympus) or Image Xpress (Molecular Probes) cell imaging systems.

***Imaging analysis for IHC***

In IHC from intestinal sections, the green fluorescence of GFAP in MP was extracted and quantitated using Image J image analysis software (version 1.40g, National Institute of Health, Bethesda, MD, <http://rsbweb.nih.gov/ij/>) [[28](#_ENREF_28)]. The fluorescence intensity was normalized to the circumferential length of the intestinal tract.

***Imaging analysis for MPC and SMC co-culture system***

The MPC and SMC co-culture system contained a large number of α smooth muscle actin (αSMA)-positive cells (*i.e.,* SMC) and a small population of GFAP+ cells and GFAP- αSMA- cells. Virtually no PGP9.5+ cells were detected. After eliminating GFAP- αSMA- cells by imaging analysis, αSMA+ SMC and GFAP+ cells were easily distinguished by DAPI fluorescence; SMC have large nuclei with weak DAPI staining and EGC have small nuclei with bright DAPI staining (Supplementary Figures S3 and S4). We counted the number of small bright nuclei and large dim nuclei separately by setting the gate for size and brightness of the nucleus image (Supplementary Figure S4). The number of eliminated cells was then counted. The percentage of GFAP+ cells and SMC was defined as the ratio of the number of the cells in the respective populations to the total number of total MPC (DAPI-stained cells”).

***Imaging analysis for enriched EGC culture***

In enriched EGC culture, the majority of SMC was eliminated and most cells (> 90%) were stained with anti-GFAP, anti-S100B, or both antibodies. GFAP was stained with mouse monoclonal antibody and visualized with Alexa488-conjugated anti-mouse IgG. S100B was stained with rabbit polyclonal antibody and visualized with Alexa568-conjugated anti-rabbit IgG using Celaview (Supplementary Figure S5). For quantitation of fluorescence, we used a different laser source with a different wavelength (excitation wavelengths 488 and 568 nm, respectively) through different band path filters under different exposure setting optimal for each fluorescence. Therefore it was not possible to directly compare fluorescence intensity values obtained using different methods and measuring rules. As a result, in this analysis we measured the fluorescence of GFAP and S100B separately comparing the BCTC-treated wells and control wells. These cells had been isolated from a single preparation and dispensed into the wells at the same density.

***Statistical analysis***

Data are expressed as mean ± SEM. Unpaired Student’s t-test was used to analyze differences between 2 groups. For comparison among 3 groups, Dunnett test was performed. *P* < 0.05 was considered a significant difference.

**RESULTS**

Expression of TRPV1 and GFAP was analyzed in LI and SI, of WT and KO young adult mice by IHC (Figure 1). While a similar level of GFAP-IR signals was detected in both WT and KO mice, TRPV1-IR signal was abolished in KO mice. Several antibodies against TRPV1, including both monoclonal and polyclonal antibodies, gave essentially the same result (2 examples of which are shown in Supplementary Figures S1 and S2). In magnified view, TRPV1-IR signals were detected in a population of GFAP-positive cells (*i.e.,* EGC) mainly within the MP in both LI and SI (Figure 2). These data suggest that, at least some fractions of type I (intraganglionic) and/or type II (interganglionic) EGC[5] in the myenteric plexus express TRPV1. The observation was further supported by high magnification IHC using isolated LM-MP. As shown in Figure 3, TRPV1-IR signal co-existed with GFAP-IR signals but not with the enteric neuron marker PGP9.5-IR.

To investigate the functional role of TRPV1 in the postnatal development of EGC, we screened a series of LI and SI samples both from WT and KO mice at various postnatal days ranging from PD 0 to PD 92 by visual inspection. Only the samples obtained at PD 5 showed an apparent difference in GFAP-IR signals between WT and KO mice. Accordingly, image analysis was used to quantitated GFAP-IR signals at PD 6, PD 13 and PD 21 in WT and KO mice (*n* = 6 per time point). The results revealed that GFAP-IR signals at PD 6 were significantly weaker in KO mice compared with WT, both in LI and SI; however this difference was not observed at PD 13 nor PD 21 (Figures 4 and 5).

To obtain information about the possible influence of TRPV1 on EGC, we subsequently performed experiments using a co-culture system with MPC and SMC. Expression of GFAP and αSMA protein started at around culture day 5. At the end of the culture period, as described in Materials and Methods, most of the cells were αSMA+ SMC and the remainder was GFAP+ cells and αSMA-GFAP- cells. The results are in good accordance with those of previous studies[[29-32](#_ENREF_29)].

Using the same digestion solution and preparation procedure as described above, cells were prepared from LM-MP of WT and KO mice simultaneously. The cells were cultured for 11 d and the number of αSMA+SMC, GFAP+ cells and αSMA-GFAP- cells was counted and represented as the ratio of the number of each cell population to the total cell number. The experiment was repeated 4 times; the results are summarized in Figure 6. The yield of GFAP+ cells was significantly lower in KO than WT mice while the yield of SMC was not.

Enriched EGC were prepared from the 5-day co-cultures as described in Materials and Methods and the resultant culture contained EGC as the major cell population and a smaller population of SMC. The IHC results for the enriched EGC culture are shown in Figure 7. TRPV1-IR signal was present in GFAP+ and/or S100B+cells but not in αSMA+ cells.

Finally, we examined the effect of the TRPV1 agonist capsaicin and the TRPV1 antagonist BCTC on the expression of glial markers in enriched EGC cultures (Figure 8). GFAP-IR signals, but not S100B-IR signals, were significantly decreased by BCTC at 3 μmol/L. Capsaicin at concentrations of 33, 100, 3000 and 10000 nmol/L had no effect (data not shown).

**DISCUSSION**

Many researchers have reported the presence of functional TRPV1 in the sensory nerves of the GI tract[[33-36](#_ENREF_33)] and some have also reported its presence in intrinsic enteric nerves of the MP[[37-39](#_ENREF_37)]. It is controversial as to whether TRPV1 exists in GI structures other than extrinsic nerves, apart from in infiltrating inflammatory cells[[40](#_ENREF_40),[41](#_ENREF_41)]. The immunostaining pattern shown in the present study resembled that of previous studies[[37-39](#_ENREF_37)], demonstrating TRPV1-IR signals in intrinsic enteric nerves of the MP of guinea pig SI and LI. Thus, to our knowledge, our study is the first and only report addressing the possible presence of TRPV1 in EGC. The specificity of the antibodies used in this study was validated using TRPV1 KO mice (Figures 1 and 2) and recombinant TRPV1-expressing cells (Supplementary Figure S2). There are several possible reasons for the apparent discrepancies between our results and those of previous studies.

Firstly, the different antibodies may specifically detect different forms of TRPV1 protein. Buckinx and colleagues[[39](#_ENREF_39)] have reported that a guinea pig and a rabbit antibody raised against slightly different regions of the c-terminus of mouse TRPV1 yielded different staining patterns; *i.e.,* the former stained cytosolic IR signals and the latter stained fibrous IR signals. The antibodies used in the present study appeared to stain both cell bodies and fibers. TRPV1 protein is suggested to be present and functional in the cell membranes as well as intracellular organelles such as the endoplasmic reticulum, Golgi bodies and mitochondria[[42-48](#_ENREF_42)]. These different staining patterns might therefore result from conformational differences between intracellular and plasmalemmal TRPV1. Differences in interacting molecules also influence epitope recognition by antibodies. It should be noted the C-terminal region of TRPV1 contains several modulatory regions, such as phosphorylation sites and binding sites for calmodulin and phosphatidylinositol 4,5-bisphosphate[[49](#_ENREF_49), [50](#_ENREF_50)].

Secondly, we use acid ethanol fixation while the above-mentioned studies used methanol, Zamboni’s, and paraformaldehyde fixation. IHC of certain glial proteins has been known to provide different results depending on the fixation procedure. For example, certain anti-GFAP antibodies were reported to detect mainly fibrous astrocytes in brain white matter after acid-alcohol fixation while protoplastic astrocytes are detected in brain grey matter after aldehyde-based fixation[[51](#_ENREF_51)]. Comparison of the intensity of IHC with the amount of GFAP protein estimated by enzyme-linked immunosorbent assay has suggested that ethanol-based fixation provides better results than aldehyde-based fixation[[52](#_ENREF_52)]. Vimentin in astrocytes and Bergmann glial fibers in cattle, rabbit and rat brain[[53](#_ENREF_53)], and P-glycoprotein in rat brain astrocytes[[54](#_ENREF_54)] were also reported to be detected only after ethanol-acid fixation. Along these lines, in the course of our research on EGC ontogeny, we have found that acid-ethanol fixation provides stronger and more specific IHC for GFAP in EGC of the mucosal and smooth muscle layers of SI and LI.

Thirdly, it should be noted that the present data does not exclude the possibility of the presence of TRPV1 in enteric nerves. We did not detect the co-existence of TRPV1-IR signals with PGP9.5-IR signals, but this might have been due to the relatively poor sensitivity and resolution of the immunostaining patterns of PGP9.5 in the present study. Furthermore, previous studies demonstrating the existence of TRPV1-IR signals in intrinsic myenteric neurons used antibodies to choline acetyltransferase, calbindin and calretinin[[37](#_ENREF_37),[38](#_ENREF_38)]. Matsumoto and colleagues also reported that TRPV1-IR did not co-localize with PGP9.5 and NeuN in cell bodies of the MP[[40](#_ENREF_40)]. We found immunohistochemically-stained TRPV1+GFAP- areas in the LM-MP. Because the GFAP antibody appeared to mainly stain fibrous structures inside the cells, the TRPV1+GFAP- area could represent EGCs. However, it is possible that these areas are contained in structures other than EGCs. Extensive research, including studies on cellular and/or intracellular TRPV1-mediated calcium mobilization in these cell types, will be needed to clarify these issues.

TRPV1 has been reported to be expressed by astrocytes in mouse spinal cord[[21](#_ENREF_21)]; in mouse, rat and human brain[[20](#_ENREF_20),[23](#_ENREF_23),[55](#_ENREF_55)]; in rat retina[[22](#_ENREF_22)]; and in *in vitro* cultured rat astrocytes[[56](#_ENREF_56)]. Treatment with the TRPV1 agonist resiniferatoxin was reported to increase Fos expression by astrocytes in mouse brain. Furthermore, injection of capsaicin, another TRPV1 agonist, led to an increase in markers for microglia (ionized calcium-binding adapter molecule 1, Iba1) as well as astrocytes (GFAP) in the dorsal horn of the spinal cord after adjuvant-induced arthritis or partial sciatic nerve ligation[[23](#_ENREF_23)], and in the trigeminal nucleus caudalis[[57](#_ENREF_57)] Treatment with TRPV1 antagonist decreased the migration of reactive astrocytes isolated from the wounded retina[[24](#_ENREF_24)]. These data suggest that TRPV1 stimulation resulted in the activation of astrocytes and, presumably, microglia. TRPV1 in astrocytes has also been suggested to be involved in the pathogenesis and epileptogenesis of human mesial temporal lobe epilepsy[[55](#_ENREF_55)]. These reports suggest that TRPV1 in astrocytes is functional and plays certain roles in astroglial biology.

In the present study, firstly, TRPV1 KO mice showed weaker GFAP-IR signals only at PD 6, but not at PD 13 nor PD 21. Secondly, the number of GFAP-expressing cells developed from the isolated MPC was significantly lower in TRPV1 KO mice than in WT mice. Thirdly, treatment of isolated WT MPC with the TRPV1 antagonist BCTC resulted in a decrease in the expression ratio of GFAP to S100B; the latter is another frequently-used EGC/astrocyte-specific marker[[58](#_ENREF_58)]. S100B is a diffusible Ca+2/Zn+2-binding protein that is considered to be a “janus face” neurotrophin for neuron and astrocytes[[59](#_ENREF_59)] and to act as a proinflammatory cytokine involved in gut inflammation with specific relevance to nitric oxide production[[60](#_ENREF_60)]. It has been speculated that the intensity and differential intracellular localization of GFAP-IR and S100B-IR signals is related to the degree of differentiation and/or functional diversity of astrocytes[[39](#_ENREF_39),[61-64](#_ENREF_61)]. These data suggest that TRPV1 signaling may interfere with GFAP expression in EGC, at least during certain period of EGC maturation.

Because GFAP is widely recognized as an astrocyte differentiation marker, constituting the major intermediate filament protein of mature astrocyte[[65-67](#_ENREF_65)], the present finding suggest that TRPV1 might be involved in the differentiation/maturation of EGC. The ENS originates in the neural crest, which invades, proliferates, and migrates within the intestinal wall until the entire bowel is colonized with enteric neural crest-derived cells (ENCDC)[[68](#_ENREF_68)]. After initial migration, ENCDC differentiate into glia and neuronal subtypes and form a critical constituent for nervous system function. Although little is known about mechanisms controlling the development and differentiation of EGC, it has been suggested that Sox-10, Lgl4, ADAM22 and bone morphogenetic proteins are involved[[4](#_ENREF_4)]. GFAP appears later at the end of the mouse embryonic stage. Cells with functions similar to those of ENCDC exist in the bowel of adult and newborn humans and rodents[[69](#_ENREF_69),[70](#_ENREF_70)]. The present data indicate that TRPV1 might be involved in the regulation of GFAP expression in *in vitro* cultured MPC prepared from young adult animals as well as *in vivo* in the early postnatal period (around PD 6). Although the present study did not examined ENCDC markers, the methods similar to ours have been used to obtain enteric neural stem cells for enteric neural stem cell therapy[[71](#_ENREF_71),[72](#_ENREF_72)].

The following three points, however, should be noted. Firstly, we did not examine the functions of EGC. As reported for CNS astrocytes, deletion or alteration of TRPV1 signaling might influence EGC function. Further intensive investigation will be needed to clarify this point. Secondly, although a normal microenvironment around EGC can compensate for the effect of TRPV1 deletion, at least until PD 10 in the case of diseased intestines (*e.g.,* due to damage of neurons, SMCs and mucosa, the effect of TRPV1 deficiency might be more severe because of a lack of compensatory signals. Along these lines, we could not obtain enriched EGC from TRPV1 KO mice as described in Methods. Because enriched EGC cultures contain far smaller numbers of SMC compared to unenriched MPC culture, the signals from SMC may be important for compensation. Additional studies are needed to determine the possible effects of TRPV1 deletion on diseased intestine, such as intestinal inflammation, hyperalgesia after psychological or surgical stress, and functional dysregulation (*e.g.,* dismotility, nutrient malabsorption and diarrhea/constipation). It is possible that TRPV1 plays an important role in the repair/recovery/restore stage in GI tissues. Finally, the present study suggests the possible existence of TRPV1 in EGC mainly by immunohistochemical/immunocytochemical techniques. Biochemical isolation and identification of TRPV1 protein and functional validation of TRPV1 such as agonist-specific Ca2+ influx and Na+ current are necessary to establish the existence and function of TRPV1 in EGC.

In conclusion, a combination of immunohistochemistry, immunocytochemistry and isolated cell culture using TRPV1 KO mice addressed the possibility that EGCs express TRPV1 and play a role in cell maturation. Further extensive studies are needed to validate this possibility.

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**COMMENTS**

***Background***

The transient receptor potential vanilloid 1 channel (TRPV1) is a non-selective cation channel that is activated by a wide variety of exogenous and endogenous physical and chemical stimuli including heat, acidic condition and capsaicin. Enteric glial cells (EGCs) are one of major cell types comprising ENS. This study explored TRPV1 expression in mouse EGCs.

***Research frontiers***

Although the presence of TRPV1 in astrocytes in central nervous system has been reported, the presence in EGCs has not.

***Innovations and breakthroughs***

TRPV1-immunoreactive signal (TRPV1-IR) is detected in EGCs. The temporal retardation of postnatal maturation of EGCs in TRPV1 knockout mice is suggested.

***Applications***

The present results address possible involvement of TRPV1 in postnatal development/maturation of EGC. Dietary TRPV1 stimulation in the weaning period may affect postnatal ENS development. However the expression and biological function of TRPV1 in EGC requires further evaluation.

***Terminology***

TRPV1 is a non-selective cation channel that is activated by a wide variety of exogenous and endogenous physical and chemical stimuli including heat, acidic condition and various pungent materials.

***Peer-review***

The authors are to be commended for the work in the manuscript entitled " TRPV1-immunoreactive signals in murine enteric glial cells". This is an interesting paper highlighting the expression profiles of TRPV1 murine enteric glial cells.

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**Country of origin:** Japan

**Peer-review report classification**

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Grade B (Very good): 0

Grade C (Good): C, C

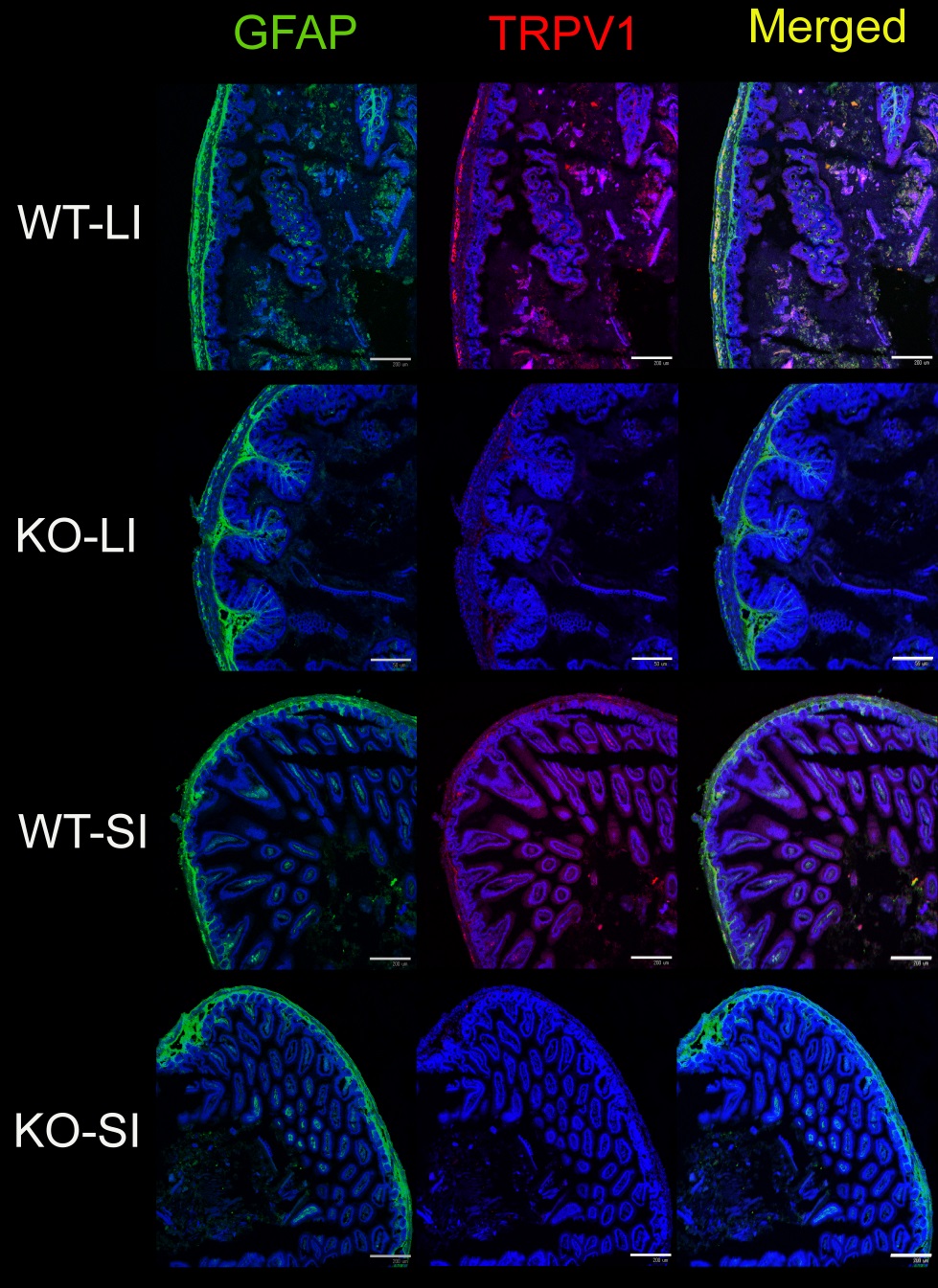
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Grade E (Poor): 0

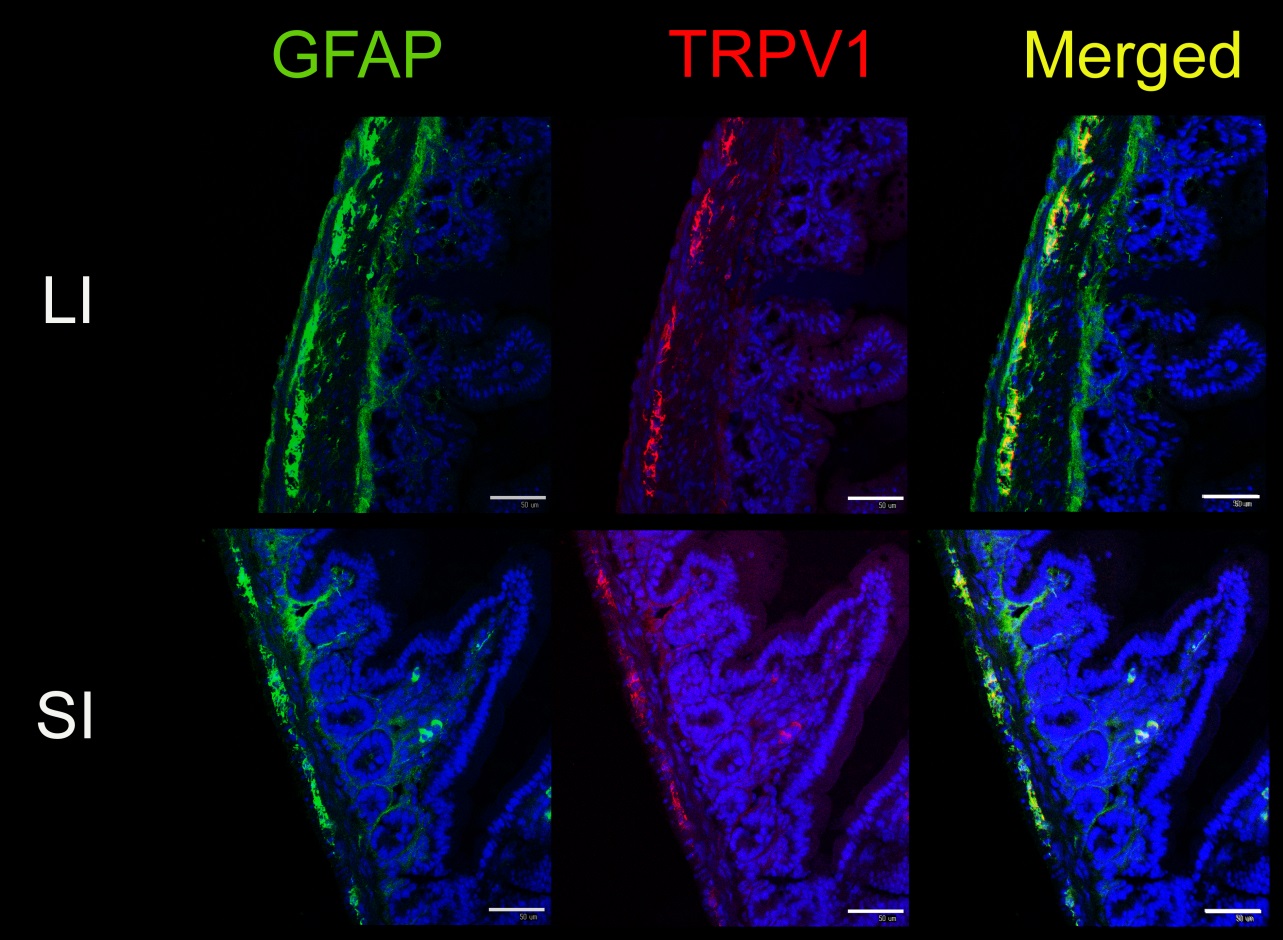
**Table1 Summary of the primary antibodies used in this study**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Antibody** | **Provider** | **Source and type** | **Product/Clone number** | **Immunogen** | **Usage** |
| TRPV1 | Bios | rabbit polyclonal | bs-1931R | a.a.825-835 of human TRPV1 (EDAEVFKDSMAPGEK) | Figures 1-3, Supple Figure S1 |
| LifeSpan | mouse monoclonal | LS-C122800 | a.a. 819-835 of rat TRPV1 (CGSLKPEDAEVFKDSMVPGEK) | Figure 6, Figure 7, Supple Figure S1 |
| GFAP | BD | mouse monoclonal | cocktail of 4A11, 1B4 and 2E1 | cow spinal cord homogenate (4A11, 1B4) or human /bovine GFAP (2E1) | Figures 1-3, Supple Figure S1 |
| DAKO | rabbit polyclonal | Z0344 | isolated GFAP from cow spinal cord | Figure 6, Figure 7 |
| CST | mouse monoclonal | GA5 | isolated GFAP from pig spinal cord | Supple Figure 2 |
| PGP9.4 | Abcam | Guinea pig serum | ab10410 | a.a. 175-191 of human PGP9.5 (GASSEDTLLKDAAKVCL) | Figure 6, Figure 7 |
| S100β | Proteintech | rabbit polyclonal | 15146-1-AP | recombinant human S100β | Figure 7, Figure 8, Supple Figure S3 |
| αSMA | Novus | rabbit monoclonal | E184 | synthetic peptides corresponding to N-terminus (human) | Supple Figure S2 |

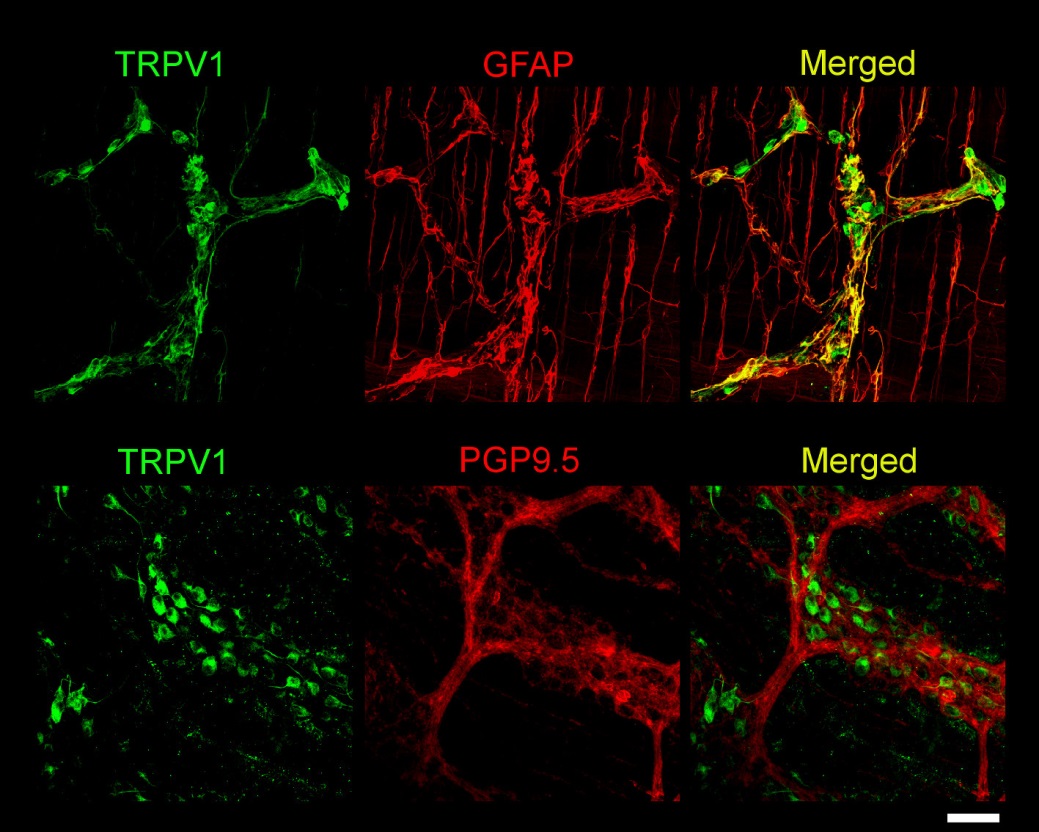
The negative controls of immunostaining performed in this study were shown in Supplementary Figure S6. Corresponding sequence of mouse TRPV1: TGSLKPEDAEVFKDSMAPGEK. The amino acid underlined is different from rat and the amino acid double-underlined is different from human. TRPV1 KO mice used in the present study preserved this sequence but the signal of immunohistochemistry in KO mice is faint, even in case detected, compared to that in WT mice, in the same staining and signal development protocols on the same slide on which the specimens from KO and WT mice were mounted together. Similar observations have been reported by Yamada *et al,* J Histochem Cytochem 2009; 57: 277-287.

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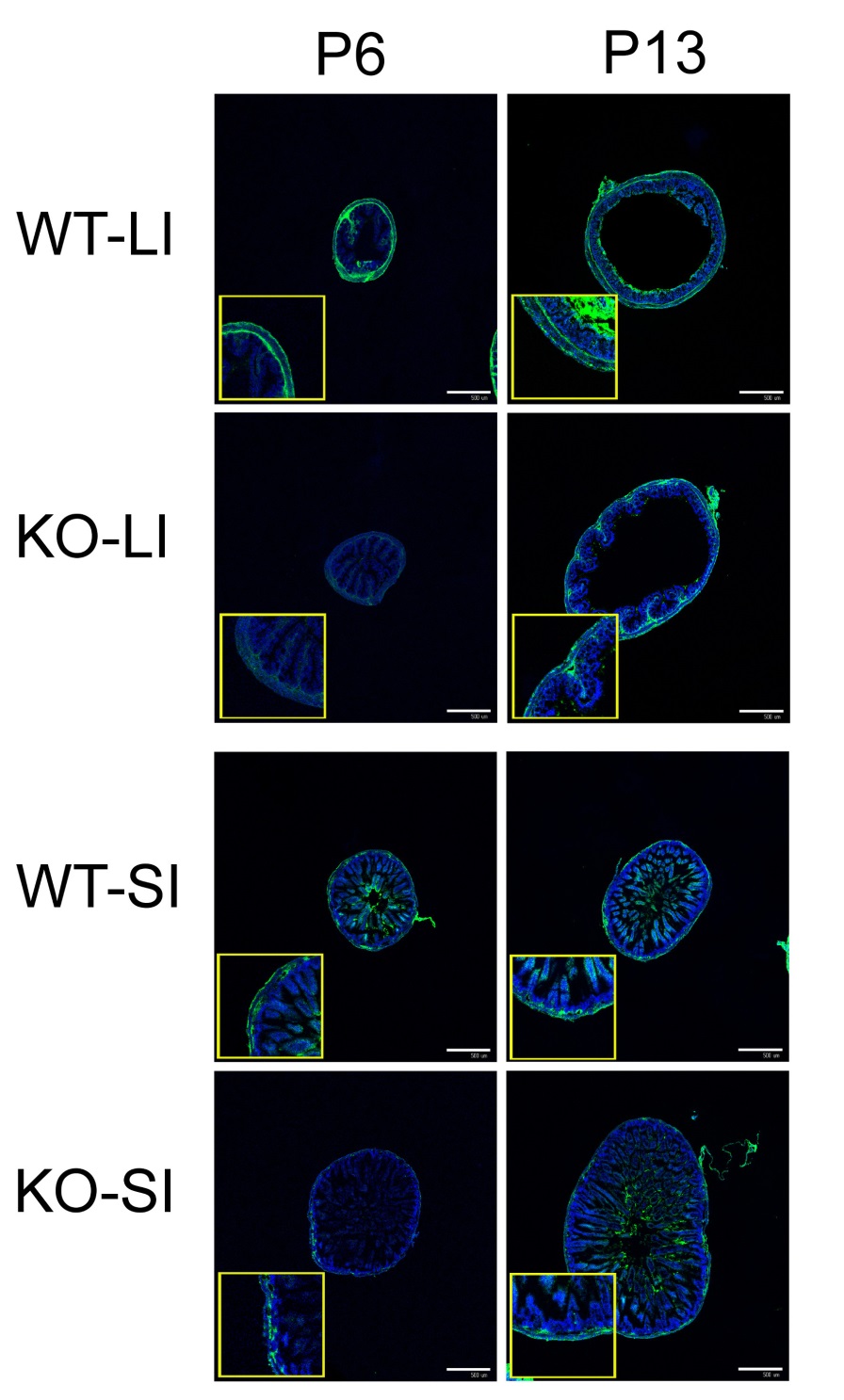
**Figure 1 Double immunostaining with glial fibrillary acidic protein and transient receptor potential vanilloid 1of small (SI) and large intestines of wildtype and TRPV1-deficient (KO) mice.**　The degree of GFAP-IR signal observed in the enteric nervous system was similar in WT and KO mice. TRPV1-IR signal was observed only in WT mice and was located in a confined area of the smooth muscle layer. Nuclei were stained with TO-PRO3. Representative data from 2 experiments using 2 mice per time point are shown. Scale bar represents 200 μm.

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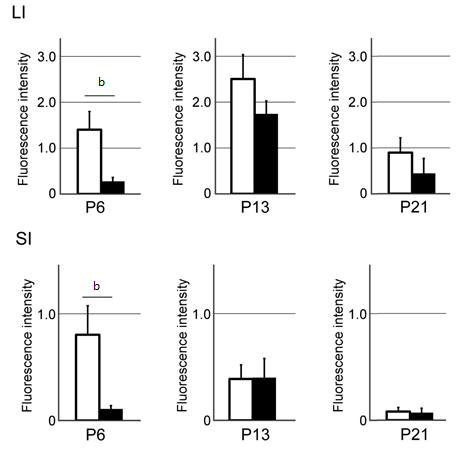
**Figure 2** **Enlarged view of glial fibrillary acidic protein/transient receptor potential vanilloid 1-stained SI and large intestines of wildtype mice.** Certain parts of Figure 1 were magnified.Glial fibrillary acidic protein **(**GFAP) is located in the myenteric plexus, submucosal plexus and fibrous structures penetrating into the circular muscle layer and mucosal layer (in WT LI, serosal membrane was also stained with GFAP; however, serosal membrane is known to show false-positive immunosignals with various antibodies). The location of TRPV1-IR signal coincided with some portions of GFAP-IR signal in the myenteric plexus. Nuclei were stained with TO-PRO3. Representative data from 2 experiments using 2 mice per time point are shown. Scale bar represents 50 μm.

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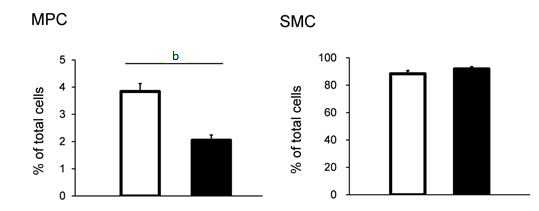
**Figure 3 Double immunostaining with transient receptor potential vanilloid 1and glial fibrillary acidic protein or PGP9.5 of LI-LMMP of wildtype mice**. LM-MP was isolated from 5 week old mice and whole-mount IHC was performed as described in Materials and Methods. Transient receptor potential vanilloid 1 (TRPV1) stained both cell bodies and fibers while GFAP stained mainly fibers and some portions of their IR signals were co-localized. Co-localization of PGP9.5 and TRPV1 IR signals was not observed. Nuclei were stained with DAPI. Representative data from 2 experiments using 2 mice per time point are shown. Scale bar represents 50 μm.

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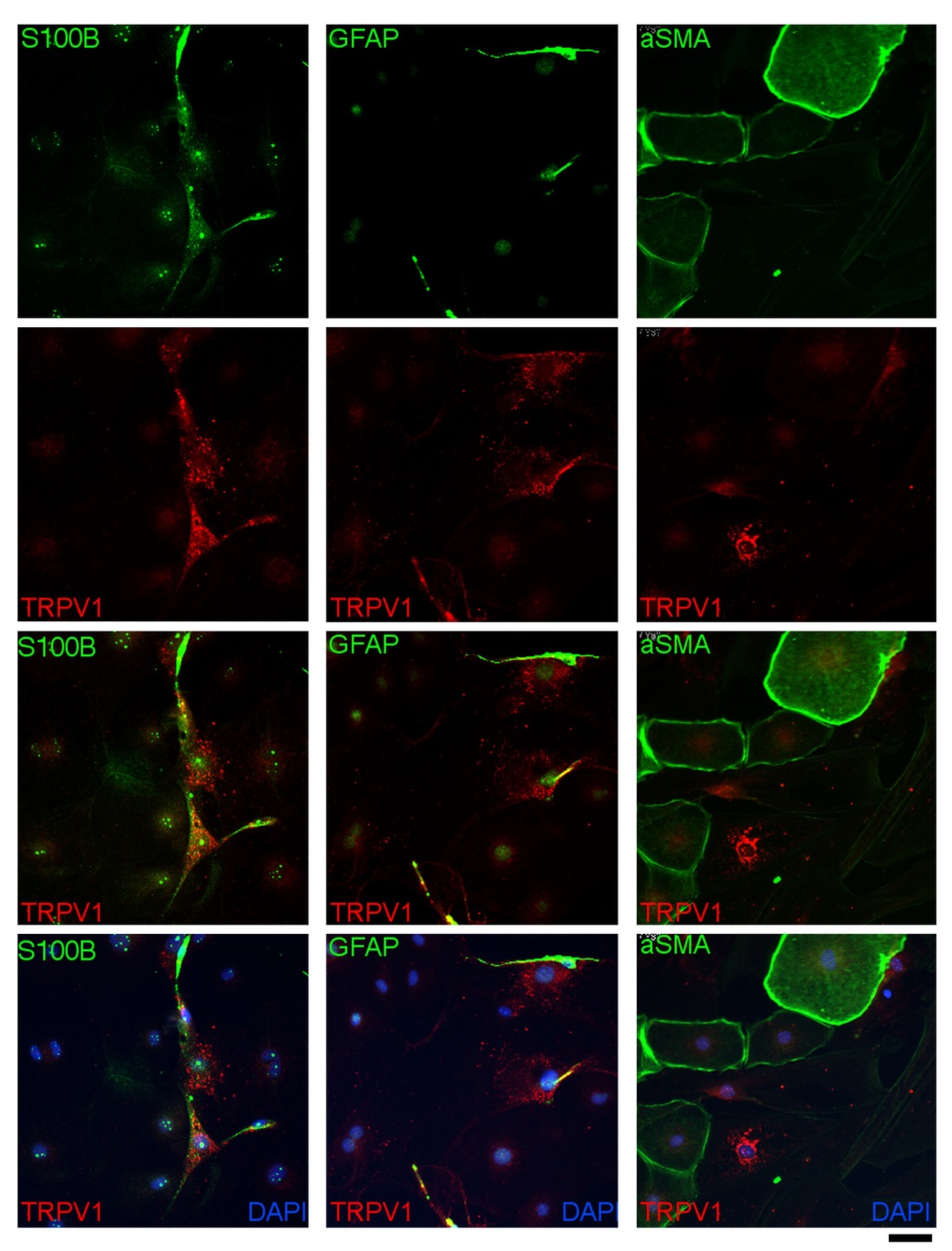
**Figure 4** **Difference of glial fibrillary acidic protein immunosignals between wildtype and KO mice (image).** LI and SI samples were isolated from wildtype (WT) and KO mice on PD 6 and PD 13. While the glial fibrillary acidic protein (GFAP)-IR signal was similar in WT and KO mice on PD 13, the signal on PD 6 was weaker in KO mice than in WT mice, both in SI and LI. Nuclei were stained with TO-PRO3. Representative data of an experiment using 6 mice per each time point/group. Scale bar represents 500 μm.



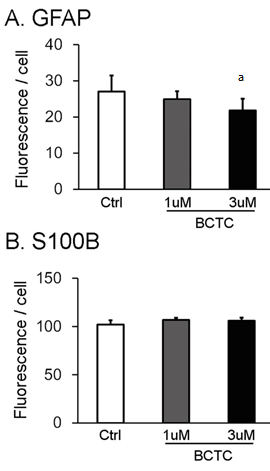
**Figure 5** **Difference in GFAP-IR signals between wildtype and KO mice.** The intensity of GFAP-IR signal for WT and KO mice on PD 6, PD 13 and PD 21 was quantitated by imaging analysis as described in Materials and Methods. The amount of GFAP-IR fluorescence was normalized to the circumferential length of the intestinal tract. Data represent mean ± SEM, *n* = 5-6. b*P* < 0.01.

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**Figure 6** **Specific decrease in the number of myenteric plexus cells in KO mice.** The cells were prepared from LM-MP of WT and KO mice simultaneously, using the same digestion solution in a single preparation. After 11-day culture, the numbers of αSMA+SMC, GFAP+ cells and αSMA- GFAP- cells were determined as described in Materials and Methods. Nuclei were stained with DAPI. The percentage of GFAP+ cells and SMC was calculated as the ratio of the number of each cell population to the number of total DAPI+ cells. The yield of EGC (GFAP+ cells) in KO mice was significantly lower than that in WT mice while the number of SMC obtained was similar for both types of mice. Data represent mean ± SEM, *n* = 4. b*P* < 0.01.

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**Figure 7** **Immunostaining of transient receptor potential vanilloid 1 in enriched enteric glial cells culture.** Enriched enteric glial cells (EGCs) were isolated and cultured as described in Materials and Methods. The cells were labeled with antibodies to GFAP, S100B, or SMC. The cultures contained many EGC and a small percentage of SMC. The location of TRPV1-IR signal is coincided with that of GFAP-IR signal and S100B-IR signal but not with αSMA-IR signal. Nuclei were stained with DAPI. Scale bar represents 20 μm.

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**Figure 8** **Effect of Transient receptor potential vanilloid 1agonist/antagonist on the signal ratio of glial fibrillary acidic protein -IR to S100B-IR.** Enriched enteric glial cells (EGCs) (prepared from day 5 MPC/SMC co-culture) were plated and cultured for an additional 5 d. The transient receptor potential vanilloid 1 (TRPV1) antagonist BCTC was added and after 24 h the cells were fixed. The cells were stained with antibodies and imaging analysis was performed using a Celaview system as described in Materials and Methods. BCTC (3 μmol/L) decreased the GFAP-IR signal but the S-100B-IR signal was unchanged. Data represent mean ± SEM, *n* = 5 - 8. a*P* < 0.05.