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ORIGINAL ARTICLE

Decreased expression of TRAAK channels in Hirschsprung's disease: a possible cause of postoperative dysmotility

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Abstract

Aim of the study Potassium (K^+) channels with a two-pore domain (K2P) are a large family of hyperpolarising ion channels which play a key role in cell excitability. This family comprises three members: TREK-1, TREK-2 and TRAAK. TRAAK channels have previously been reported to be expressed in murine enteric ganglia. To date, no data exist regarding TRAAK channel expression in the human colon. Thus, we designed this study to investigate *TRAAK* gene expression in the normal human colon and in Hirschsprung's disease (HSCR).

Methods HSCR tissue specimens ($n=6$) were collected at the time of pull-through surgery, while control samples were obtained at the time of colostomy closure in patients with imperforate anus ($n=6$). qRT-PCR analysis was undertaken to quantify *TRAAK* gene expression, and immunolabelling of TRAAK proteins was visualized using confocal microscopy.

Main results Confocal microscopy revealed TRAAK protein expression within both neurons and interstitial cells of Cajal in the myenteric plexus, with a reduction in both ganglionic HSCR colon and aganglionic HSCR colon, compared to controls. qRT-PCR analysis revealed a significant downregulation of the *TRAAK* gene in both aganglionic and ganglionic HSCR specimens compared to controls ($p<0.05$).

Conclusions *TRAAK* gene expression is significantly downregulated in HSCR colon, suggesting a role for these ion channels in colonic neurotransmission. *TRAAK* downregulation within ganglionic specimens highlights the dysfunctional nature of ganglia in this region.

Keywords TRAAK channels · Hirschsprung's disease · Aganglionosis

Introduction

Potassium (K^+) channels are protein complexes that form K^+-selective pores in biological membranes, allowing the passive transport of K^+ across cell membranes [1]. K^+ channels play a major role in the control of K^+ homeostasis, but are also involved in physiological functions that are associated with modifications of the electrical membrane potential, such as neurotransmission and hormone secretion, neuronal and muscular excitability [1].

There are three main classes of K^+ channels in animal cells, and classification of these channels is based primarily on structural and functional criteria. These include voltage-gated K^+ channels, inward rectifier K^+ channels and two-pore-domain K^+ (K2P) channels [2]. TRAAK, TREK1 and TREK2 are K^+-selective channels of the K2P family. They are robustly mechanosensitive channels. Mechanical force, whether applied to cells by stretching, poking or swelling activates TRAAK and TREK currents [3]. In addition to being mechanosensitive, these channels are also modulated by chemical and physical stimuli including lipids, lysolipids, arachidonic acid and other polyunsaturated fatty acids.

Co-ordinated gut motility and function is not solely dependent on the form of innervation it receives, but also on the cells that regulate neurotransmission along with the enteric nervous system (ENS). Smooth muscle cells (SMC), interstitial cells of Cajal (ICC) and platelet-derived growth factor receptor α-positive (PDGFRα$^+$) cells make up the 'SIP syncytium', communicating with each other and the ENS to regulate peristalsis in the gastrointestinal tract. Hirschsprung's disease (HSCR) is a congenital condition, affecting 1:5000 live births, which is characterised by the absence of ganglia in the distal colon. The extent of the

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aganglionic segment varies between patients from a short segment to total colonic aganglionosis.

Our research team has previously reported altered expression of the TREK-1 channel in HSCR colon [4]. We found that TREK-1 expression was significantly decreased in both ganglionic and aganglionic HSCR colon. To date, no data exist regarding TRAAK expression within the human intestine. Thus, we designed this study to investigate *TRAAK* gene expression in the normal human colon and in HSCR.

Materials and methods

Tissue samples

This study was approved by the Ethics Medical Research Committee, Our Lady's Children's Hospital, Dublin, Ireland (Ref. GEN/292/12) and tissue samples were obtained with informed parental consent. HSCR specimens from six patients who underwent pull-through surgery were studied. These specimens were divided into aganglionic and ganglionic specimens. We compared the most distal aganglionic segments with the most proximal ganglionic segments. HSCR patients were aged 6 ± 3 months. No additional health issues existed in these patients. Colonic control samples included six specimens from patients who underwent colostomy closure following surgical correction of imperforate anus. Control samples were taken from patients who were 11 ± 4 months old. None of the imperforate anus patients had HSCR. Tissue specimens were either snap-frozen in liquid nitrogen and stored at − 80 °C for protein extraction or embedded in OCT Mounting Compound (VWR International, Leuven, Belgium) for immunofluorescence and stored at − 80 °C until use.

Immunofluorescence staining and confocal microscopy

Frozen blocks of HSCR colon and normal control samples were sectioned transversely at a thickness of 10 µm, mounted on SuperFrost® Plus slides (VWR International, Leuven, Belgium) and fixed with 10% buffered formalin for 5 min. Sections underwent cell membrane permeabilization with 1% TritonX-100 for 20 min at room temperature. After blocking with 10% normal goat serum (Sigma-Aldrich Ltd, Arklow, Ireland) for 30 min to avoid non-specific absorption, sections were incubated with primary antibodies; rabbit anti-TRAAK (Abcam, UK), mouse anti-c-kit (Santa Cruz), mouse anti PGP9.5 (Santa Cruz), all used at a dilution of 1:100 in PBS + 0.05% TritonX-100, overnight at 4 °C. Sections were then washed in PBS + 0.05% Tween and incubated with corresponding secondary antibodies; goat anti-rabbit Alexa Fluor® 488, dilution 1:200 and goat anti-mouse Alexa Fluor® 594 (Abcam, UK), dilution 1:200 for 1 h at room temperature. After washing, sections were counterstained with DAPI antibody, dilution 1:1000 (Roche Diagnostics GmbH, Mannheim, Germany) for 10 min, washed, mounted and coverslipped with Fluorescent Mounting Medium (DAKO Ltd, Cambridgeshire, UK). All sections were independently evaluated by two investigators with a LSM 700 confocal microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany).

qRT-PCR

TRIzol reagent (invitrogen) was used for the acid guanidinium–thiocyanate–phenol–chloroform extraction method to isolate total RNA from HSCR and control tissues ($n = 6$ for each group), according to the manufacturer's protocol. Spectrophotometrical quantification of total RNA was performed using a NanoDrop ND-1000 UV–Vis spectrophotometer (Thermo Scientific Fisher, Wilmington, USA). The RNA solution was stored at − 20 °C until further use. cDNA synthesis and quantitative polymerase chain reaction reverse transcription of total RNA were carried out at 85 °C for 3 min (denaturation), at 44 °C for 60 min (annealing) and at 92 °C for 10 min (reverse transcriptase inactivation), using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics, West Sussex, UK), according to the manufacturer's instructions. The resulting cDNA was used for quantitative real-time polymerase chain reaction (qRTPCR), using a LightCycler 480 SYBR Green I Master (Roche Diagnostics, Mannheim, Germany), in a total reaction mix of 20 µl per well. The following gene-specific primer pairs were used: Human TRAAK (Eurofins) sense primer 5′ TTC CCT CAC TTC CAT CCA TC and Human TRAAK (Eurofins) antisense primer 5′ AAG CAA TTC CAC ACC CAC TC. For normalization purposes, real-time RT-PCR was also performed for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). GAPDH sense primer 5′ ACA TCG CTG AGA CAC CAT GG and GAPDH antisense primer 5′ GAC GGT GCC ATG GAA TTT GC were used. After 5 min of initial denaturation at 95 °C, 55 cycles of amplification for each primer were carried out. Each cycle included denaturation at 95 °C for 10 s, annealing at 60 °C for 15 s, and elongation at 72 °C for 10 s. Relative mRNA levels of gene expression were determined using a LightCycler 480 System (Roche Diagnostics) and the relative changes in gene expression were normalized against the level of GAPDH gene expression in each sample. Experiments were carried out in duplicate for each sample and primer.

Statistical analyses

A one-way ANOVA was conducted to determine a statistically significant difference between aganglionic, ganglionic

and normal controls ($p < 0.05$). Data are presented as mean ± standard error. Specimens were classified into three groups: aganglionic ($n = 6$), ganglionic ($n = 6$) and normal controls ($n = 6$).

Results

Immunofluorescence staining

Immunofluorescence, in conjunction with confocal microscopy, revealed that the distribution of TRAAK-positive cells was decreased in both the aganglionic and ganglionic HSCR colon compared to normal controls. TRAAK immunoreactivity was found to be co-localised to PGP9.5-labelled neurons and c-kit-labelled ICCs (Fig. 1).

qRT-PCR

The relative mRNA expression level of the *TRAAK* gene was significantly downregulated in both aganglionic and ganglionic HSCR specimens, compared to normal controls ($p < 0.05$) (Fig. 2).

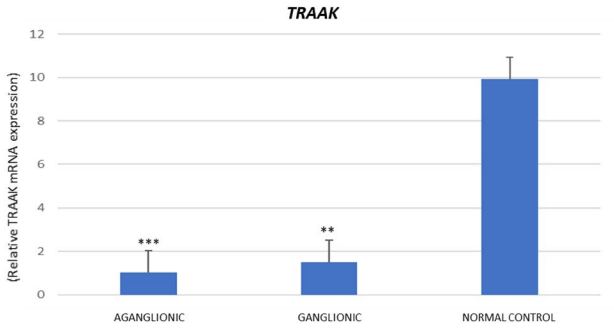

Fig. 2 qRT-PCR revealed significantly downregulated *TRAAK* gene expression in both the aganglionic and ganglionic HSCR specimens ($n = 6$) compared to normal control tissue ($n = 6$). Results are presented as mean ± SEM (**$p < 0.01$, ***$p < 0.001$)

Discussion

Gastrointestinal motility disorders, such as irritable bowel syndrome, can occur when coordinated smooth muscle contractility is disrupted. K^+ channels regulate gastrointestinal smooth muscle tone and are key to gastrointestinal tract relaxation [5]. TREK-1 and TRAAK have unique functional properties and represent the first cloned polyunsaturated fatty acids and stretch-activated K^+ channels. Both channels are activated by shear stress, cell swelling and negative pressure [1]. Disruption of the cytoskeleton by either biological or mechanical means potentiates the opening by

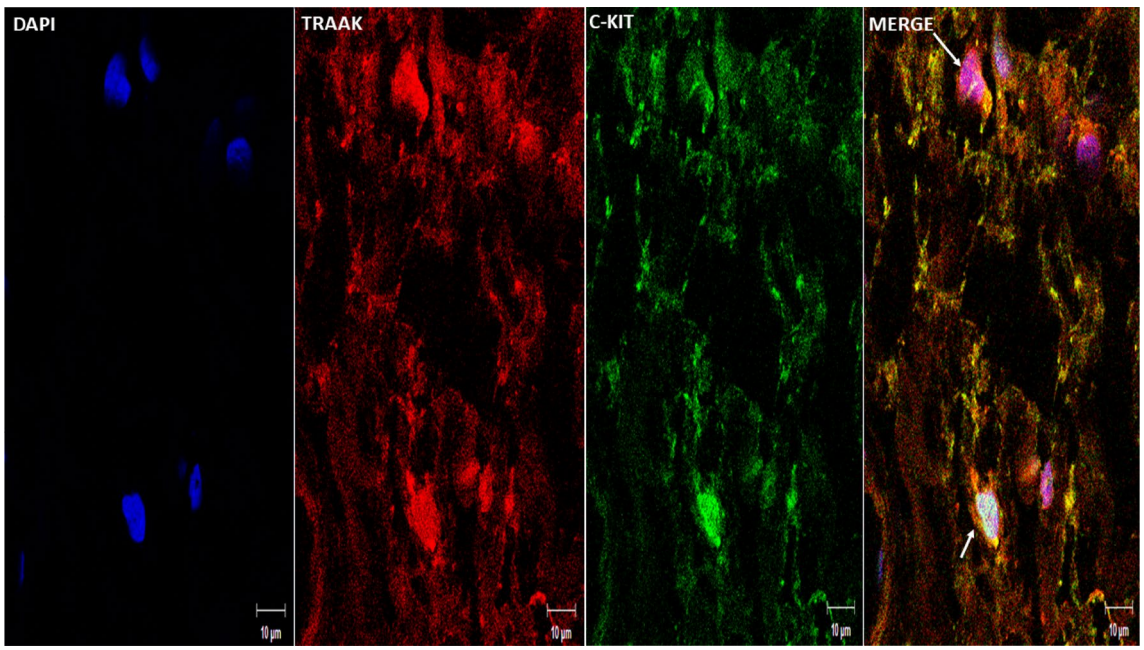

Fig. 1 Immunofluorescence staining of TRAAK-positive cells (red) in the ganglionic HSCR colon, found to be co-localised with c-kit-positive interstitial cells of Cajal (green). Nuclei were stained with DAPI (blue). Arrows show co-localisation

membrane stretch. This suggests that these channels are tonically repressed by the cytoskeleton, but that their mechanogating does not require the integrity of the cytoskeleton [1].

Our understanding of the expression of the K2P channel family in gastrointestinal tissues, however, is limited. K2P channels contribute to the generic background K^+ conductance in many cell types and can be regulated by a plethora of endogenous and environmental cues including changes in oxygen, temperature and PH levels or membrane tension [5]. TRAAK protein expression has previously been documented in the murine ileum and colon, where co-localisation was evident within neurons, but not in smooth muscle cells as is the case with TREK channels [5]. TRAAK expression has been thought to be essentially neuronally restricted, but in our current study, we report TRAAK expression in ICCs throughout the gut wall, in addition to ganglia within the ganglionic region of HSCR colon and in normal control samples.

Although the most striking histological feature in HSCR is the absence of ganglion cells, it is unlikely that this is the only cause of functional intestinal obstruction. There are a number of other histopathological findings, both in the aganglionic segment and in the proximal ganglionic segment in HSCR, which may account for the frequent discrepancy encountered between the length of the non-functional bowel and the degree of obstruction. The mechanisms underlying persistent bowel symptoms in HSCR patients who have had a properly performed pull-through operation are poorly understood and understudied. The goal of surgical treatment for HSCR is to enable the affected child to have regular spontaneous bowel motions without soiling. Advances in the management of HSCR afford most patients a satisfactory outcome. However, 35–48% of HSCR patients unfortunately suffer from persistent dysmotility symptoms such as constipation, soiling and an inflammatory condition of the bowel known as enterocolitis [6–8]. While a proportion of these patients are found to have a treatable pathology such as strictures, residual aganglionosis or transition zone, the majority have no identifiable cause for their ongoing bowel dysfunction.

In recent years, our research group has published mounting evidence regarding abnormalities of ion channels within the ganglionic segment of HSCR colon. These include HCN channels [9], TREK-1 [4], ryanodine receptors [10], KCNG3 and KCNG4 channels [11], SCN1B and FXYD1 [12]. In this current study, we have shown that the *TRAAK* gene is significantly downregulated in HSCR colon, and TRAAK protein expression was co-localised with neuronal and ICC markers, suggesting a role for TRAAK channels in neurotransmission of the colon. The marked decrease in TRAAK expression within ganglionic specimens highlights the physiologically abnormal nature of this segment in HSCR patients. We believe that these issues are partly responsible for the bowel dysmotility experienced by many HSCR patients, following a properly performed pull-through operation. It is no longer sufficient to use the presence of ganglia as a marker of normal bowel in the ganglionic region. Cumulatively, these findings also suggest that HSCR is not merely a neuronal condition, as was once thought. Our many studies have highlighted the abnormal nature of the smooth muscle layers in HSCR also, in addition to abnormalities in ICCs and PDGFRα^+ cells in both the ganglionic and aganglionic segments. Our hope is that this information will improve the future therapies for this condition and lead to more successful surgical techniques going forward.

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