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ORIGINAL ARTICLE

Innervation of the entire internal anal sphincter in a mouse model of Hirschsprung's disease: a first report

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Abstract

Background Impaired function of the internal anal sphincter (IAS) may be implicated in postoperative obstructed defecation (POD) that may complicate Hirschsprung's disease (HD) patients. While innervation of part of the IAS in HD has been reported, accurate details based on anatomic landmarks that can explain the clinical morbidity seen in POD are lacking, and there appear to be no studies that specifically document the innervation of the "entire" IAS in HD. We used endothelin receptor-B knockout mice to represent HD (HD-mice) and C57B6 wild mice as controls (C-mice) to investigate the innervation of the entire IAS to assess the pathophysiology of POD experimentally.

Methods The end-point of the longitudinal muscle layer was used to define the border between the IAS and the circular muscle layer (CML). Specimens of anorectum from HD- and C-mice were immunostained with PGP 9.5 and S100 as general nerve markers, nNOS and VIP as parasympathetic nerve markers, TH as a sympathetic nerve marker, and calretinin as a reliable diagnostic marker for HD. Immunostained cells/fibers were quantified using ImageJ.

Results On fluorescence microscopy, PGP 9.5, nNOS, and calretinin were significantly lower in the IAS of HD-mice than in C-mice ($p < 0.05$, respectively), while there were no significant differences between HD-mice and C-mice for S100, VIP, or TH.

Conclusion We are the first to confirm that the expression of histochemical markers of innervation is abnormal throughout the "entire" IAS in HD-mice. Application of this finding may be beneficial for preventing POD and requires further research.

Keywords Hirschsprung's disease · Internal anal sphincter · Endothelin receptor-B knockout mice

Introduction

Accurate knowledge of the anatomy of the anal canal is vital for definitive surgical cure of Hirschsprung's disease (HD) and understanding its pathophysiology. Abnormal innervation of the internal anal sphincter (IAS) was studied "partially" in HD and allied disorders [1, 2], but postoperative obstructive defecation (POD) seen in HD patients cannot be fully explained based on these reports, although impaired function of the IAS [3] is obviously implicated. The incidence of POD as a surgical complication has drawn attention because it does not seem to follow any pattern or be predictable. In fact, the management of POD [3, 4], even including radical operative techniques [5], has been reported without a thorough understanding of its etiology. We believe thorough understanding of the histology of the "entire" IAS in HD would enhance the treatment of HD and possibly prevent POD.

In a recent study, Kinugasa et al. indicated that the border between the circular muscle layer (CML) of the rectum and the IAS could be distinguished by muscle innervation, and that the border corresponded approximately to what they term the squamous-columnar epithelial junction (SCJ), which we interpret as meaning the anorectal line (ARL) or the suprazonal line [6–11]. Based on this, the "partial" abnormal innervation reported in the literature, i.e., around the border between the CML and the IAS is obscure, and their assessments may even have been confined to the CML alone without actually assessing the IAS because there are no accurate details about specimens or anatomic landmarks

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recorded, meaning that innervation of the entire IAS has in fact, probably never really been studied in detail to date.

Because of these issues, we conducted this study to establish categorically, the exact nature of the innervation of the IAS in HD using a mouse model of HD as an experiment. To the best of our knowledge, we are the first to use a variety of histochemical markers to examine the "entire" IAS in a mouse model of HD, which only requires clinical confirmation to be accepted as a full assessment of the innervation of the "entire" IAS in HD.

Methods

We used endothelin receptor-B knockout mice to represent HD (HD-mice: $n = 3$; 18–21 g), and C57B6 wild controls (C-mice: $n = 3$; 18–21 g) to assess the innervation of the entire IAS. All animal procedures were reviewed and approved by the Institutional Review Board of the Juntendo University School of Medicine Animal Care and Use Committee (Institutional Review Board No. 280082).

Three mice in each group were sacrificed by cervical dislocation and the anal canal and peripheral tissues harvested. All tissues were fixed in 10% buffered formalin, embedded in paraffin, and cut in 10-μm thick sections and stained with hematoxylin and eosin (HE) conventionally. We defined the end-point of the longitudinal muscle layer (LML) as the border between the IAS and CML in this study.

All specimens of anorectum from C- and HD-mice were immunostained with anti-protein gene product 9.5 (PGP9.5) (rabbit polyclonal, 1:500, Enzo Life Science, USA) and anti-S-100 protein (S100) (rabbit polyclonal, 1:200, Leica Biosystems, UK) as general nerve markers, anti-neuronal nitric oxide synthase (nNOS) (rabbit polyclonal, 1:1000, Merck Millipore, Chandlers Ford, Hants, UK) and anti-vasointestinal peptide (VIP) (rabbit polyclonal, 1:20, Abcam, UK) as parasympathetic nerve markers, anti-tyrosine hydroxylase (TH) (rabbit polyclonal, 1:200, Merck Millipore, UK) as a sympathetic nerve marker, and anti-calretinin (calretinin) (rabbit polyclonal, 1:100, Zymed, USA) as a reliable diagnostic marker for HD [12].

The distribution of PGP 9.5, S100, nNOS, VIP, TH, and calretinin in the entire IAS and CML were analysed blindly in five random sections from each specimen using a Carl Zeiss LSM 780 microscope (Carl Zeiss Meditec, Oberkochen, Germany) and ImageJ (Rasband, W.S., ImageJ, US National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2016). The expression of each marker in the entire IAS between C- and HD-mice, the expression of each marker in the CML between C- and HD-mice, differences in expression between the IAS and CML in C-mice, and differences in expression between the IAS and CML in HD-mice were compared.

Statistical analysis

Statistical analysis of differences in expression of PGP 9.5, S100, nNOS, VIP, TH and calretinin in the entire IAS and CML between C- and HD-mice sections was performed using the Student's t test for unpaired comparisons using SPSS (IBM Corp. Released 2008. IBM SPSS Statistics for Windows, Version 17.0. Armonk, NY: IBM Corp). Statistical significance was defined as $p < 0.05$.

Results

On fluorescence microscopy, PGP 9.5, nNOS, and calretinin in the entire IAS of HD-mice were significantly lower than in C-mice ($p < 0.05$, respectively; Fig. 1). VIP and TH also appeared to be deceased in fluorescence microscopy images and on quantity analysis to the same extent as PGP 9.5, nNOS and calretinin, but were not significant ($p = 0.11$ and 0.87, respectively). Differences in S100 were also not significant ($p = 0.75$). See Fig. 2 and Table 1.

Results were the same for the CML, with PGP 9.5, nNOS, and calretinin in HD-mice being significantly lower than in C-mice ($p<0.05$, respectively; Fig. 3), while there were no significant differences between HD-mice and C-mice for S100, VIP, or TH ($p = 0.45$, 0.22 and 0.12, respectively; Fig. 4; Table 2).

On the other hand, TH was significantly lower in the entire IAS compared with the CML in C-mice ($p < 0.05$). For the other markers, PGP 9.5, S100, VIP, nNOS, and calretinin there were no significant differences between IAS and CML in C-mice ($p = 0.23$, 0.14, 0.31, 0.89 and 0.54, respectively). However, TH was significantly lower in the entire IAS compared with the CML in the HD-mice ($p < 0.05$). There were no significant differences between the entire IAS and the CML in HD-mice for the other markers, PGP 9.5, S100, VIP, nNOS, and calretinin ($p = 0.85$, 0.45, 0.49, 0.81and 0.61, respectively).

Discussion

We are the first to confirm there is abnormal innervation of the entire IAS in HD-mice. While this is valuable information in itself, it does not necessarily improve our approach to the anatomy of the anal canal, which is ambiguous and complex, and often poorly documented with no obvious definition of the border between the IAS and CML in most textbooks. Because of inconsistencies, we chose the endpoint of the LML as the border between the IAS and the

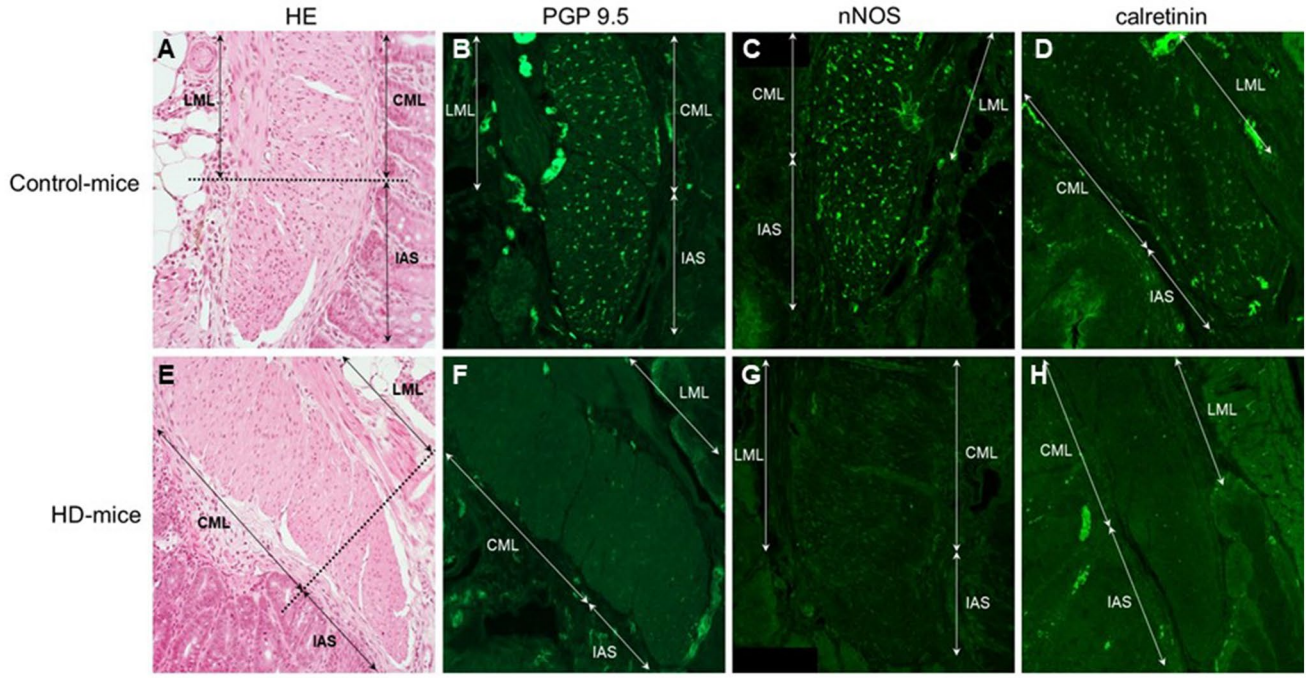

Fig. 1 Expression of hematoxylin and eosin (HE), PGP 9.5, nNOS, and calretinin in the internal anal sphincter (IAS) of control- and HD-mice. (**a, e** HE; **b, f** PGP 9.5; **c, g** nNOS; **d, h** calretinin; ×20, respectively). The expression profiles of PGP 9.5, nNOS, and calretinin in the entire IAS of HD-mice were significantly lower than in control-mice ($p < .05$, respectively)

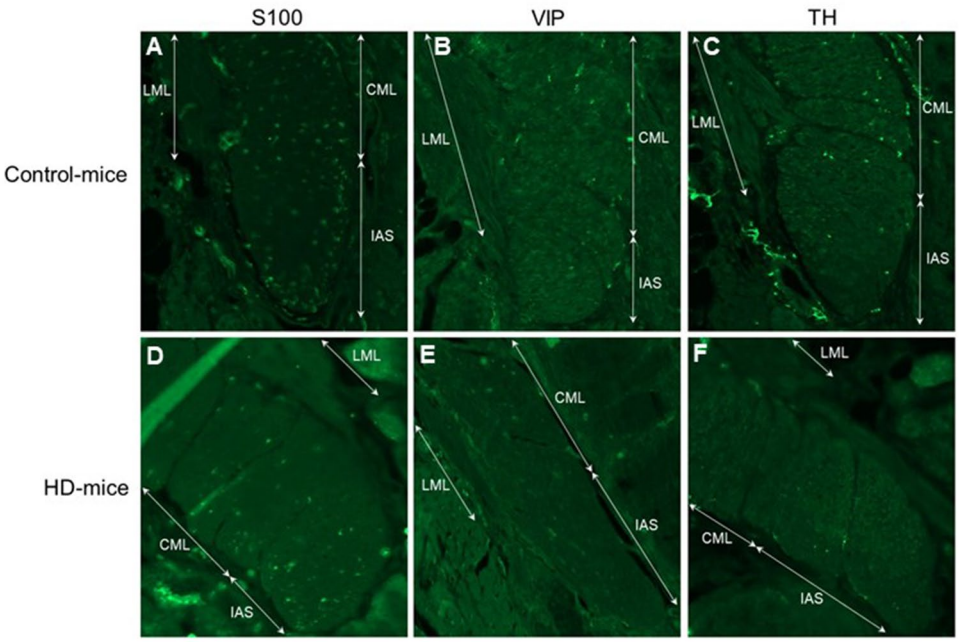

Fig. 2 Expression of S100, VIP, and TH in the internal anal sphincter (IAS) of control and HD-mice. (**a, d** S100; **b, e** VIP; **c, f** TH; ×20, respectively). There were no significant differences between control and HD-mice for S100, VIP, or TH ($p = .75$, 0.11 and 0.87, respectively)

CML to standardize our results and facilitate reproducibility and reliability.

In normal innervation of the IAS there is union of nerve fibers from Auerbach's nerve plexus of the most distal part of the rectum and the inferior rectal branches of the pelvic plexus running along the conjoint longitudinal muscle coat [10]. The observed decrease in PGP 9.5 and calretinin, as nerve markers, in the entire IAS in HD-mice is probably secondary to a decrease in nNOS, a parasympathetic nerve marker originating from the nerve plexus of the most distal part of the rectum, because it was the only other marker that was reduced in the entire IAS and the CML in HD-mice.

Table 1 Expression of each marker in the entire IAS. C-mice versus HD-mice

Marker	C-mice (mean %area)	HD-mice (mean %area)	*p* value
PGP 9.5	2.58 ± 1.18	0.77 ± 0.37	< 0.05
S100	1.83 ± 0.38	2.04 ± 1.91	0.75
nNOS	4.28 ± 1.02	0.41 ± 0.37	< 0.05
VIP	2.22 ± 1.49	1.62 ± 1.34	0.11
TH	0.54 ± 0.12	0.43 ± 0.13	0.87
calretinin	1.93 ± 0.91	0.38 ± 0.32	< 0.05

All data are mean ± standard deviation

IAS internal anal sphincter, *C-mice* control-mice, *HD-mice* model Hirschsprung's disease mice, *%area* immunostained area divided by the entire area

On the other hand, in C-mice, only TH was significantly lower in the entire IAS compared with the CML, which would appear to differ from the findings of Kinugasa et al. [10] who reported sympathetic nerves to be dominant in the IAS from cadavers, but there was no quantitative evaluation. Interestingly, we found the same expression profile for TH in HD-mice, with the entire IAS being significantly lower than in CML. Based on our findings, we hypothesize that the origin and composition of nerve fibers in the entire IAS is the same in HD- and C-mice.

Of note is the dissociation between PGP 9.5 and S100 observed. Although both markers are general nerve markers, PGP 9.5 is more specific for general nerve cells and S100 is more specific for glial cells. From other studies, S100 immunostaining has been shown to highlight neural hypertrophy in the muscularis mucosae in HD [13]. We think the dissociation we observed is related to differences in nerve and glial cell distribution.

Calretinin is a reliable immunohistochemical marker that specifically stains nerve cells and small intrinsic nerve fibers in both the myenteric and submucosal plexuses throughout the normal intestine with high sensitivity for the diagnosis of HD [12]. In this study, expression was significantly lower in HD-mice compared with C-mice in the CML, but also in the entire IAS. This finding hints at the histologic origin of innervation of the IAS; in other words diminished structural factors derived from CML intestinal nerves result in less innervation in the entire IAS.

When we assessed our results from a clinical perspective there was some confusion. We have achieved good postoperative continence in some cases of HD with pull-through alone without any surgical intervention to the IAS [7]. However, in hindsight we are not confident about this because the anatomic border between the CML and IAS is indistinct to the naked eye and we did not confirm the position of the IAS intraoperatively. Furthermore, based on the findings of our study, this would mean we

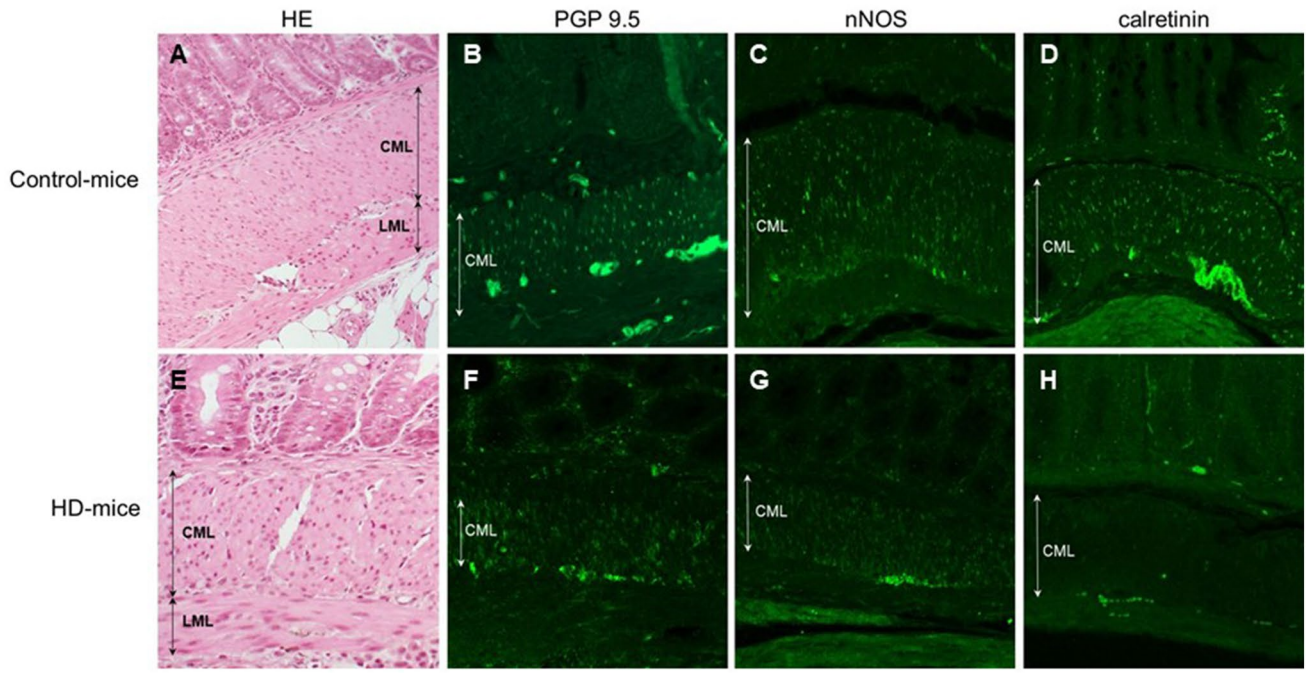

Fig. 3 Expression of Hematoxylin and eosin (HE), PGP 9.5, nNOS, and calretinin in the circular muscle layer (CML) of control and HD-mice. (**a, e** HE; **b, f** PGP 9.5; **c, g** nNOS; **d, h** calretinin; ×20, respectively). The expression profiles of PGP 9.5, nNOS, and calretinin in the CML of HD-mice were significantly lower than in control-mice ($p < 0.05$, respectively). *LML* longitudinal muscle layer

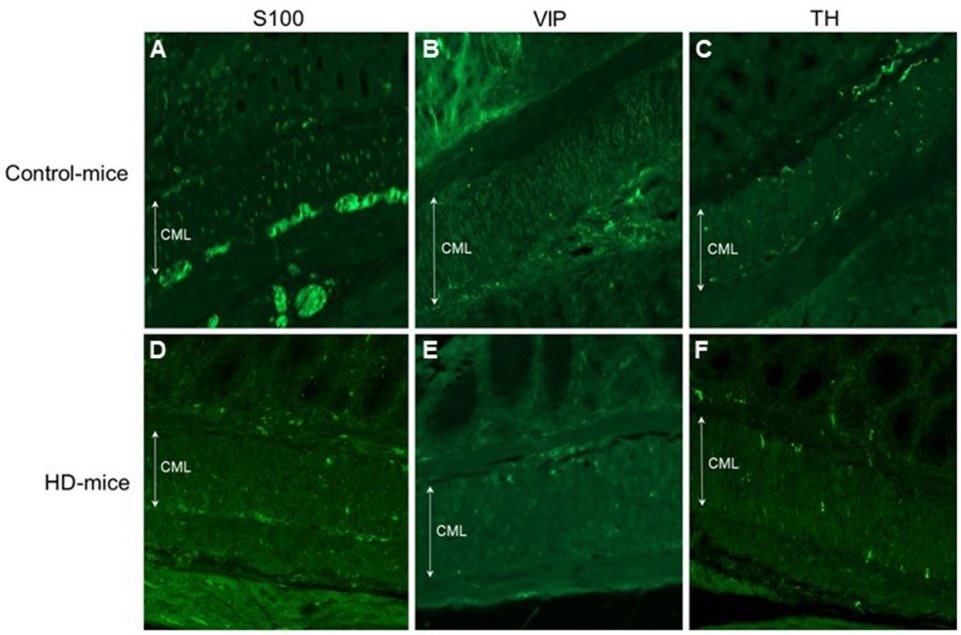

Fig. 4 Expression of S100, VIP, and TH in the circular muscle layer (CML) of control and HD-mice. (**a, d** S100; **b, e** VIP; **c, f** TH; ×20, respectively). There were no significant differences between control and HD-mice for S100, VIP, or TH ($p = .45$, 0.22 and 0.12, respectively)

Table 2 Expression of each marker in the CML

Marker	C-mice (mean %area)	HD-mice (mean %area)	*p* value
PGP 9.5	3.51 ± 1.62	0.61 ± 0.54	< 0.05
S100	0.29 ± 0.33	0.52 ± 0.45	0.45
nNOS	3.31 ± 1.79	0.79 ± 0.66	< 0.05
VIP	3.14 ± 3.75	0.52 ± 0.61	0.22
TH	3.98 ± 2.62	1.43 ± 1.14	0.12
calretinin	2.78 ± 1.14	0.45 ± 0.58	< 0.05

C-mice versus HD-mice. All data are mean ± standard deviation

CML circular muscle layer, *C-mice* control-mice, *HD-mice* model Hirschsprung's disease mice, *%area* immunostained area divided by the entire area

may leave an abnormal IAS intact. We are now in a position to assess the function of the abnormal IAS to determine the relevance of the overall abnormal innervation we identified experimentally in the "entire" IAS in HD-mice. Research on the clinical application of our findings is planned.

Our findings confirm that the anal canal is a complex structure reliant on sensory and motor innervation for normal bowel function. To further understand the apparent contradiction between good clinical outcome and abnormal innervation of the "entire" IAS, we plan to investigate the relationship between accurate anatomic landmarks for reliable and reproducible outcome of surgical intervention for HD.

Conclusions

Based on histochemical markers in C- and HD-mice, we are the first to confirm that the innervation of the entire IAS is abnormal in HD-mice. Innervation of the IAS and CML would also appear to be different between C- and HD-mice.

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