

Developmental anomalies of the enteric nervous system in normoganglionic segments of bowel from rats with total colonic aganglionosis

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Published online: 8 August 2007
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Abstract Polysialylated neural cell adhesion molecule (PSA-NCAM) is a marker for immature neurons and S100 beta is a known marker for enteric glia. The aim of this study was to determine the maturation of the enteric nervous system (ENS) in normoganglionic (Ng) bowel from rats with total colonic aganglionosis (TCA). Ng ileum was obtained from TCA rats (spotting lethal: mutant, *sl/sl*: $n = 15$) at 10, 19, and 24 days of age ($n = 5$ for each age). Normal control Ng ileum was obtained from wild type littermates (+/+, $n = 25$) at 10, 19, 24, 30, and 60 days of age ($n = 5$ for each age). Specimens were studied with immunohistochemistry for PSA-NCAM and S100 beta. On H-E staining, normal mature ganglion cells (GC) were identified in submucosa and myenteric plexuses in all specimens from TCA rats and normal controls. For PSA-NCAM, submucosa and myenteric GC in control ileum were strongly positive at 10 days old, weakly positive at 19 days old, and did not stain from 24 days old and after. However, in all ileum specimens from TCA rats, both submucosa and myenteric GC were strongly positive for PSA-NCAM regardless of age. For S100 beta, submucosa and myenteric

glial cells in control ileum were negative at 10 and 19 days old, but positive from 24 days old and after. However, in all ileum specimens from TCA rats, submucosa and myenteric glial cells were S100 beta negative regardless of age. Our results suggest that GC in the Ng segment of TCA rats remain immature beyond an age when they should be mature.

Keywords Polysialylated neural cell adhesion molecule (PSA-NCAM) · S100 beta · Hirschsprung's disease · Rats · Total colonic aganglionosis

Introduction

There are only a few reports in the literature about maturation of the enteric nervous system (ENS) in the normoganglionic (Ng) segment of bowel from cases of Hirschsprung's disease (HD) [1, 2]. We hypothesized that in HD (including long segment HD) as well as total colonic aganglionosis (TCA), developmental maturation of the ENS in the Ng segment may also be compromised, since even after Ng segment pull-through (PT) there is a subgroup of HD patients [3] whose post-operative outcome is unsatisfactory and some HD patients who tend to have diarrhea/enterocolitis [3].

Polysialic acid (PSA) is the carbohydrate portion of highly polysialylated neural cell adhesion molecule (PSA-NCAM) on the cell surface, appearing in the late embryonic and early postnatal brain, and contributes to various developmental events [8, 9]. The average chain length of PSA residues decreases with age and therefore, PSA-NCAM expression is regulated in the central nervous system (CNS) [8, 9]. It is expressed in newly generated neurons and is a marker for immature neurons [8–10]

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allowing the overall shape of immature neurons, including their dendrites and axons, to be visualized using immunohistochemistry for PSA-NCAM.

S100 beta proteins are a subfamily of EF-hand calcium-binding proteins found in enteric glia [16, 18]. S100 beta antibody, S100 beta immunoreactivity (IR) of Wistar rat bowel increases until the 10th day of life; during the first week of life, S100 beta IR is weak [19–21], indicating that S100 beta IR can be used as a maker for maturation of enteric glia.

The spotting lethal (*sl/sl*) rat with homozygous endothelin B receptor deficiency, an established animal model for HD, develops megadilatation of the bowel as a result of TCA [11, 12]. The aim of our study was to examine the maturation of the ENS in Ng segments from spotting lethal TCA rats using PSA-NCAM immunostaining patterns and compare them with expression patterns in controls.

Materials and methods

Congenital TCA rats and controls

Ng segments of bowel were obtained from congenital TCA rats (*sl/sl*, $n = 15$) at 10, 19 and 24 days of age ($n = 5$ for each age). No TCA rat survived longer than 24 days. Control Ng bowel was obtained from wild type litter mates (+/+, $n = 25$) at 10, 19, 24, 30, and 60 days of age ($n = 5$ for each age). All rats were obtained from the National Institute for Physiological Sciences, Okazaki, Japan. All specimens were obtained under pentobarbital (Nembutal[®]) anesthesia.

All animal experimental protocols were approved by the Institutional Animal Care and Use Committee at Juntendo University School of Medicine (Experimental Protocol Number: 150–134) and followed guidelines set forth in the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Tissue preparation

All specimens were fixed with phosphate buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 at 4°C overnight and were embedded in paraffin, and cut into serial 2.5 μm sections with a microtome. The sections were transferred to new sirane-coated slides and dried overnight.

All specimens obtained were examined with hematoxylin and eosin (H-E) staining to confirm that ganglion cells (GC) were present in both myenteric and submucosal plexuses. PSA-NCAM and S100 beta immunohistochemistry were used to confirm maturation of the ENS.

Immunohistochemistry

Immunohistochemical studies were performed using commercially available avidin–biotin–horseradish peroxidase complex (ABC) immunoperoxidase kits (Vector, CA, USA). Sections were immersed in methanol with 0.3% H_2O_2 to block endogenous peroxidase, then flooded with 1% bovine serum albumin and 1% normal goat serum to minimize nonspecific antibody binding and incubated with a mouse monoclonal anti-PSA-NCAM (IgM, 1:500; 12E3; [17]) [9] or a mouse monoclonal anti-S100 beta (IgG, 1:2,000, Sigma) overnight at 4°C. Sections were then incubated in secondary antibody preparations of biotinylated goat anti-mouse IgM (1:200, Vector, CA, USA) or biotinylated goat anti-rabbit IgG (1:200, Vector, CA, USA) for 60 min at room temperature, then washed in PBS and incubated in ABC preparation for 30 min at room temperature. After washing with PBS, development of peroxidase was achieved using freshly prepared 3-3'-diaminobenzidine tetrahydrochloride (Sigma, UK) 25 mg in 100 ml of 0.01 mol/l PBS containing 0.015% hydrogen peroxidase. Counter staining was performed with hematoxylin. To provide a negative control in immunohistochemical studies, the primary antibody was omitted. Light microscopy was used for all examinations.

Results

H-E staining

Mature normal GC were identified in the submucosa and myenteric plexuses in ileum specimens from all TCA and control rats. There was no evidence of concomitant intestinal neuronal dysplasia (IND), i.e., submucosal hyperganglionosis and presence of giant ganglia and hypoganglionosis in the transitional segment of bowel.

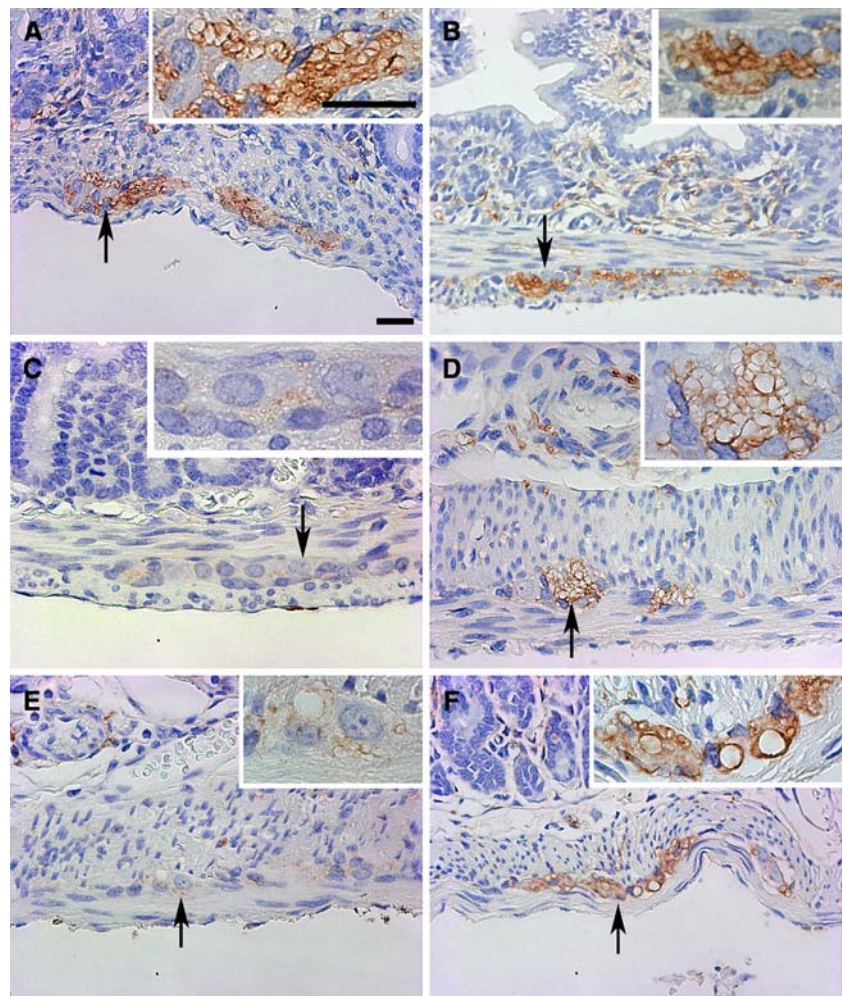
PSA-NCAM immunohistochemistry

In both TCA and control rats, strong PSA-NCAM IR was identified in all GC from ileum until day 10 of life (Fig. 1). However, in control rats, there was no PSA-NCAM IR identified in any GC from ileum from day 19 and after, and in TCA rats, strong PSA-NCAM IR was still identified in all GC from ileum on day 24.

S100 beta immunohistochemistry

In both TCA and control rats, all glial cells from ileum were negative for S100 beta IR until day 19 (Fig. 2). In contrast, in controls, S100 beta IR was identified in all glial

Fig. 1 **a** Control rat, day 10, **b** TCA rat, day 10, **c** control rat, day 19, **d** TCA rat, day 19, **e** control rat, day 24, **f** TCA rat, day 24 (original magnification $\times 40$). *Arrows* show ganglion cells. Strong PSA-NCAM IR was identified from day 10 to day 24 in TCA rats. In control rats, PSA-NCAM IR is identified on day 10. However, there was no PSA-NCAM IR on days 19 and 24



cells from ileum from day 24 and after, and in TCA rats, S100 beta IR was negative in all glial cells from ileum regardless of age.

Discussion

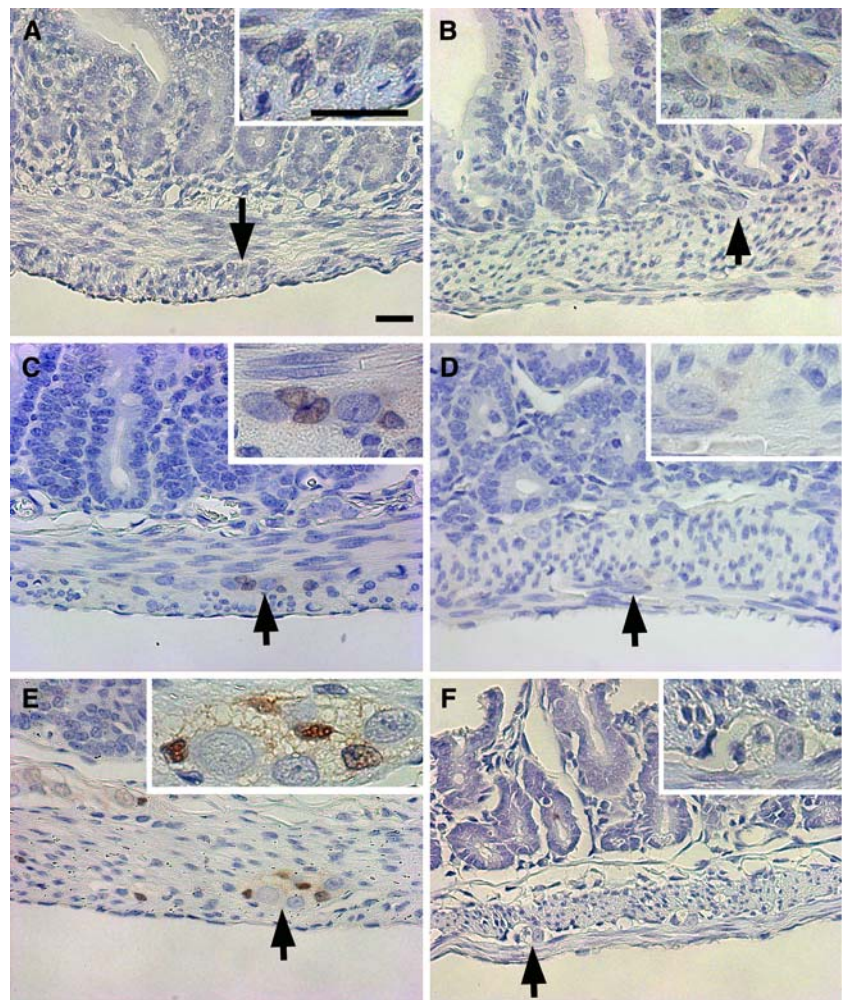
In the CNS, PSA-NCAM was expressed by immature neurons, and PSA-NCAM expression decreased as neurons mature. PSA-NCAM is also believed to play an inhibitory role in cell–cell interactions [8, 13, 14], and PSA-NCAM has been shown to have a negative regulatory effect on neuromuscular junction synaptogenesis in the chick ciliary ganglion system [15]. In the rat CNS, PSA-NCAM has been reported to be mainly expressed between the 18th day of gestation and the 11th day of life and is markedly reduced by day 22 [17]. In other words, PSA-NCAM is expressed mainly in immature neurons and decreased as neurons mature [8–10]. Therefore, we considered PSA-NCAM to be a reasonable marker for the immaturity of ganglion cells. This is the first time that PSA-NCAM

immunohistochemistry was used for investigating bowel neuronal maturation, and we found that in normal rats, GC mature before day 19.

This study confirmed that expression of PSA-NCAM in the GC of normal control rats decreased with age. The most striking finding of this study was that while all GC in Ng segments from TCA rats appeared mature on H-E staining, they were in fact strongly PSA-NCAM positive irrespective of age, indicating that in TCA rats, all GC in the Ng segment were immature; in other words, the ENS was not normal. Judging from PSA and S100 beta IR, GC in normal control rats would appear to mature by day 19, but in TCA rats, GC in Ng ileum are still immature on day 24 and probably continue to be immature, although we could not confirm this because TCA rats did not survive longer than 24 days.

Advances in management have allowed the majority of HD patients to have satisfactory outcome after Ng segment pull-through as well as technically adequate pull-through. However, a subgroup of HD patients continue to have persistent bowel dysfunction such as constipation

Fig. 2 **a** Control rat, day 10, **b** TCA rat, day 10, **c** control rat, day 19, **d** TCA rat, day 19, **e** control rat, day 24, **f** TCA rat, day 24 (original magnification $\times 40$). *Arrows* show ganglion cells. In TCA rats, glial cells were negative for S100 beta IR from day 10 to 24. S100 beta IR was identified from day 24 in control rats



and incontinence, or diarrhea when they get colds etc, or enterocolitis [4–7]. Concomitant IND [4], acquired aganglionosis [6], and transitional segment colon pull-through [7] have been raised as possible explanations for such post-operative problems. However, in the majority of HD cases with persistent bowel dysfunction, the exact cause remains unknown because biopsies of pulled-through colon in these patients usually contain normal and mature GC on H-E staining. A possible explanation for persistent bowel dysfunction in these patients is that the Ng segment seen on H-E staining is not actually normal; in other words, the ENS is abnormal as suggested by the findings of this study.

In conclusion, GC in TCA rats would appear to remain immature beyond an age when they should be mature, which may explain why a subgroup of HD patients continue to have abnormal bowel function even after Ng segment pull-through that is technically successful. Further studies are currently underway in a rat model for short segment HD and in Ng human HD bowel.

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