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Gastrointestinal phenotype of GAD67lacZ transgenic mice with early postnatal lethality

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Summary. It has been proposed that γ-aminobutyric acid (GABA) in the gut may function as a neurotransmitter, hormone and/or paracrine agent. Our aim was to examine transgenic mice of the GAD67-lacZ line with impaired postnatal growth and early postnatal lethality for gastrointestinal abnormalities. The gastrointestinal tract was dissected and processed for histology, immunohistochemistry, electron microscopy, western blotting and measurement of GAD activity. Homozygous mice of both sexes displayed an intestinal phenotype characterized by a fragile and haemorrhagic intestinal wall, a reduced number of villi, epithelial lesions and the occasional appearance of pseudostratified epithelium. The number of GABA-immunoreactive enteroendocrine cells and mucin-secreting goblet cells increased significantly relative to wild-type epithelium. The appearance of GABA-immunopositive neuronal perikarya and the lack of GABA-immunoreactive varicose fibres were observed in the enteric plexuses of transgenic mice. Tissue homogenates of transgenic mice showed higher levels of expression of GAD67 and GAD65 as compared with wild-type mice. Our results suggest that the possible reason underlying the growth impairment and postnatal lethality observed in GAD67 transgenic mice is a functional impairment of GABAergic enteric neurons and disintegration of intestinal epithelium.

Key words: GAD67-lacZ transgenic mice, GABA-immunohistochemistry, Electron microscopy, Western blot analysis

Introduction

Gamma-aminobutyric acid (GABA) and its synthetic enzyme glutamate decarboxylase (GAD) are not limited to the nervous system, but are also found in non-neural tissues. During embryonic development, GABA appears long before the onset of inhibitory synaptogenesis (Katarova et al., 2000), suggesting its involvement in development and plasticity (reviewed in Varjú et al., 2001). In consequence of its extensive enteric neural and endocrine distribution, it has been proposed that GABA in the gut may function as a neurotransmitter and hormone and/or paracrine agent (Krantis and Harding, 1986; Sanders and Ward, 1992; Davanger et al., 1994). GABAergic neurons have been observed throughout the nerve layers of the rat, guinea pig and human gut (Tanaka, 1985; Hills et al., 1987). $GABA_A$ receptors are localized on submucosal and myenteric preparations of the rat distal colon, providing anatomical evidence for the GABAergic innervation of the enteric ganglion cells (Krantis et al., 1995). GABA, acting through GABA receptors, can modify gut motility (Kerr and Ong, 1986) and may influence the mucosal function, acting as a paracrine/hormone agent. Furthermore, GABA has been found in enteroendocrine cells of the rat antrum, small intestine and colon (Gilon et al., 1990; Krantis et al., 1994), where it is thought to influence the mucosal function (Varro et al., 1996). GABA released from neurons or non-neuronal cells could target $GABA_A$ receptors that occur on mucosal cells of the rat stomach, which mediate the stimulation of mucus secretion (Erdö and Wolff, 1990), and on enterochromaffin cells of the intestine, where GABA modulates serotonin release (Schworer et al., 1989). In addition, GABA can stimulate acid secretion via the local modulation of somatostatin and gastrin release (Harty and Franklin, 1983; Lloyd and Pichat, 1987). Little is known about GABAergic neural control of the submucosal and mucosal functions. However, the extensive GABAergic innervation of these layers, the localization of $GABA_A$ receptors on submucosal neurons, and the ramification of GABAergic fibres near the crypt epithelial cells strongly suggest a role for enteric GABAergic neurons in secretomotor pathways.

Molecular cloning studies have demonstrated that GABA synthesis is catalysed by 65-kDa and 67-kDa forms of GAD (Martin and Rimvall, 1993; Varjú et al., 2001), which are also responsible for the synthesis of the intestinal GABA (Williamson et al., 1995).

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In the present report, we examined the dissected gastrointestinal tract of homozygous Tg(GAD67-lacZ5.0)69 mice showing a postnatal lethal phenotype by histology, immunohistochemistry, electron microscopy, western blotting and measurement of GAD activity. We have found that GAD and GABA expression is altered in both the mucosa and enteric plexuses in 20% of all homozygous mice. This suggests that impaired GABA signalling during development and functional maturation of the mouse intestine may be responsible for the lethal intestinal phenotype of Tg(GAD67-lacZ5.0)69 transgenic mice, which may either be caused by an insertional disruption of an unknown gene and/or the transgene itself.

Materials and methods

Mice

The mice were maintained in a conventional animal facility and all experiments with these animals were conducted according to the guidelines for care and use of experimental animals issued by the National Institute of Health and the Society for Neuroscience.

Mice of the line Tg(GAD67lacZ5.0)69 have been previously described in detail (Katarova et al., 1998). Briefly, the transgene construct contains 5 kb of the 5'-regulatory region of the mouse GAD67 (Gad-1) gene, the first exon, the first intron and part of the second exon of GAD67 fused in frame with the coding sequence of the bacterial marker gene lacZ such that the N-terminal sixteen amino acids of the fusion protein are derived from the GAD67 protein. Mice heterozygous for the transgene GAD67-lacZ were originally bred with normal C57Bl/6 mice for multiple generations, and then intercrossed to obtain homozygous mice. When needed, heterozygous mice were derived by mating homozygous males with C57Bl/6 females.

Tissue preparation

The gastrointestinal tract (GIT) was dissected from 7-, 20- and 27-day-old mice deeply anaesthetized with Avertin (Polysciences). The intestine was flushed with phosphate buffer (PB; 0.05 M, pH 7.4), ligated and distended by filling with a mixture of 2% glutaraldehyde and 4% paraformaldehyde buffered with phosphate-buffered saline (PBS; 0.01 M) and immersed in the same fixative for 16 h at room temperature. After fixation, the samples were rinsed, and measured segments of the small intestine and colon were selected.

For electron microscopy, tissue segments of 20-day-old mice were postfixed in osmium tetroxide and embedded in Epon. Ultrathin cross-sections were contrasted with uranyl acetate and lead citrate and examined with a Philips CM10 electron microscope equipped with a MEGAVIEW II camera.

For paraffin embedding, Paraplast embedding media (Sigma) was used.

For wholemounts, the intestinal segments from 20-day-old control and transgenic mice were opened along the mesenteric attachment and submucosal and longitudinal muscle layers were prepared with the attached plexuses.

Histology and immunohistochemistry

Mayer's mucicarmine staining (Yu et al., 1999) was applied on 8-µm thick paraffin sections from the small intestines and colons of 7- and 27-day-old transgenic and wild-type animals (3-3 animals respectively). Immunohistochemistry was performed on paraffin sections and on wholemounts, using polyclonal anti-GABA (Sigma) and monoclonal anti-ß-Gal (Promega) antibodies. Diaminobenzidine was used to visualize the immunoreactivity. For double- labelling, a mixture of antibodies directed against GABA and ß-Gal was applied to the sections. Secondary antibodies labelled with fluorescein isothiocyanate or indocarbocyanine (Jackson Immunoresearch Laboratories) were used. Tissue preparations were viewed through an Axioskop fluorescence microscope equipped with an AxioCam camera.

Morphometric analysis

The thickness of the tissue layers around the intestinal lumen was measured on Epon-embedded toluidine-blue-stained semithin cross-sections taken from comparable regions of the small intestines and colons of 20-day-old control and transgenic animals. The Image-Pro Plus 3.0 morphometric program was utilized for measurements.

The number of mucicarmine-stained goblet cells was determined by counting the cells on digitalized images of sections at a 100-fold magnification. The counting of goblet cells and the measurement of the length of small intestinal epithelium was carried out by using the analySIS (SIS Extended Pro) software. The cells were counted on 50-50 images from wild-type and transgenic animals, respectively, by one person. The data were expressed as mucicarmine-stained cells/mm epithelium.

Statistical analysis

The thickness of the tissue layers and the number of mucin-secreting cells in 7- and 27-day-old control and transgenic mice were analysed statistically. After square root transformation and logarithmic transformation of the data, statistical analysis was performed, by using two-way ANOVA with SPSS software. A probability of $P<0.05$ was set as the level of significance in all analyses. Data were expressed as means±SE.

Western blot

27-day-old homozygous GAD67-lacZ transgenic mice of line 69 and age-matched controls were used. The

mice were killed by cervical dislocation, the GIT was dissected in ice-cold PBS and flushed with PBS. The mucosa was mechanically separated from the underlying plexus and muscle layers. The tissue was collected, centrifuged briefly and snap-frozen in liquid N_2. 1 g tissue was homogenized with a Polytron homogenizer in 6 ml ice-cold solubilizer buffer (0.2 mM pyridoxal phosphate, 1 mM amino-ethyl-thio-ammonium-bromide) containing COMPLETE proteinase inhibitors (Roche Diagnostics). The protein content was measured by the method of Bradford (Bradford, 1976). 30 mg protein from each fraction was applied on a 10% SDS-PAGE gel. The gel was blotted onto nitrocellulose (Schleicher and Schuel), blocked in 1% bovine serum albumin-TBST (10 mM Tris, pH 7.5, 0.15 M NaCl, 0.05% Tween 20) and incubated with the primary antibody overnight at 4 °C as follows: rabbit anti-GAD67 (Chemicon; 1:250); sheep anti-GAD65 serum 1440 (Katarova et al., 1990) (1:20,000); rabbit anti-GAD25 serum 8876 (1:1,000); and rabbit anti-GAD44 affinity purified (1:200). The blots were washed in TBST and incubated with anti-rabbit alkaline phosphatase-conjugated secondary antibody (Promega, 1:7,500) or with anti-sheep rabbit antibody (Boehringer, 1:10,000) followed by anti-rabbit alkaline phosphatase (1:10,000). Finally the blots were washed with TBST and stained in nitro blue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate (Promega). The blots were scanned and the relative intensities of the bands were measured with the NIH Image analysing program.

GAD activity

GAD activity in total homogenates from mucosa and underlying submucosal-muscle layers was measured exactly as described previously (Szabó et al., 1994) and the specific activity was calculated on the basis of protein content data.

Results

Postnatal lethal phenotype and intestinal abnormalities in GAD-lacZ transgenic mice

The transgenic mouse line Tg(GAD67lacZ5.0)69 has been estimated to carry more than 50 transgene copies arranged tandemly in a head-to-tail orientation. Approximately 20% of the progeny of all examined homozygous breeding pairs (n=50) was already visibly smaller at birth and exhibited a progressive runting and fasting phenotype, which was most severe at the end of the first postnatal month (Fig. 1). 20 days after birth transgenic mice, on average, had a 37.5% lower body weight than the heterozygous or wild-type mice. All mice exhibiting this phenotype died at 3-4 weeks of age. Upon macroscopic examination, the transgenic mice revealed intestinal obstruction and gross pathology in the gut, which was shorter and thinner with patchy inflammation and hemorrhages (Fig. 2A,B). The stomach-rectum distance in wild-type animals at 20 days

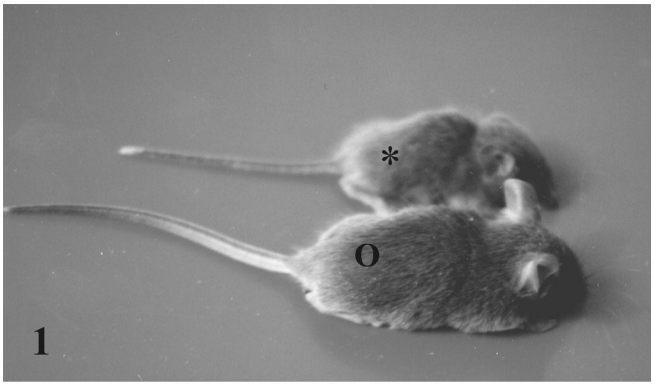

Fig. 1. Phenotypical appearance of 23-day-old transgenic (asterisk) and wild-type (circle) mice. The average weight of transgenic mice is reduced compared with that of wild-type animals.

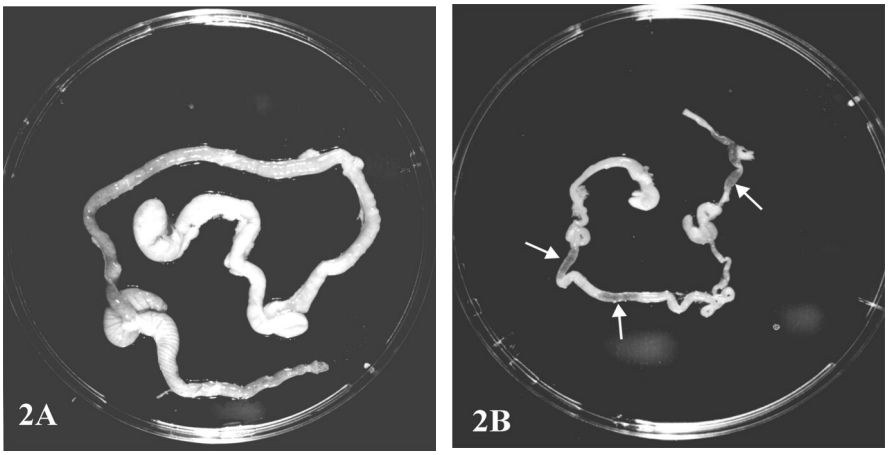

Fig. 2. Macroscopic appearance of the intestine of wild-type **(A)** and a GAD-67 transgenic mice **(B)** at 20 days of age. The intestine of the GAD-67 transgenic mouse is shorter and thinner, with patchy inflammation (arrows).

of age was on average 24 cm, while in transgenic animals the same distance was 15 cm.

Histological and morphometric analysis

Morphometric measurements revealed that with the exception of the circular muscle layer, all the tissue layers in the small intestine were significantly thinner (Fig. 3), whereas in the colon, only the longitudinal muscle layer was significantly thinner when compared to the wild-type (Fig. 3).

Electron microscopic examination of transgenic mice revealed a pronounced disruption of epithelial integrity in the small intestine, but not in the colon. The loss of junctional complexes, short and disorganized microvilli, the loss of basal/apical polarity and the occasional appearance of a multilayered epithelium were characteristic in the age-group examined by electron microscopy. The disintegration was pronounced at the villus tip epithelium (Fig. 4A), occasionally involving the entire villus and extending to the submucosa. The formation of intravascular microthrombi was frequently seen (Fig. 4A), though transmural perforation was not observed. The enteric nerves appeared unaffected (Fig. 4B). An exception was the enteric glia, which often displayed shrinkage and a vacuolized cytoplasm (Fig. 4B).

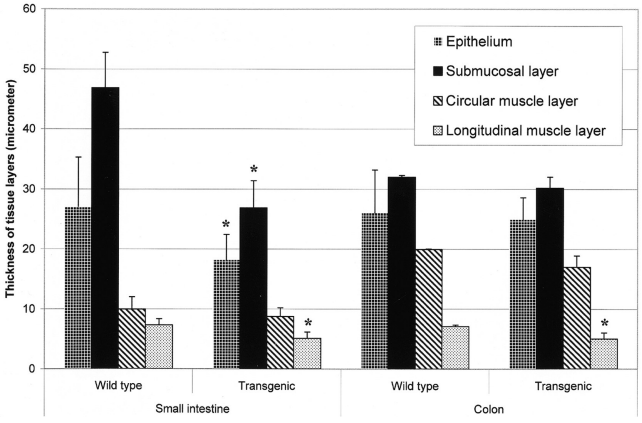

Fig. 3. The thickness of tissue layers in the small intestine and in the colon of wild-type and GAD-67 transgenic mice at 20 days of age. Data are expressed as means±SE. Significances are indicated by asterisks.

GABA immunohistochemistry revealed GABA-immunopositive nerves in both the submucosal and myenteric plexuses of wild-type animals. In the wild-type mice, the GABA-immunopositive varicose fibres were abundant and frequently formed baskets around non-immunoreactive perikarya (Fig. 5A), whereas the transgenic mice exhibited numerous immunoreactive perikarya and an almost complete lack of varicose fibres (Fig. 5B). In the small intestine of the GAD67 transgenic mice, a high number of GABA-immunoreactive enteroendocrine cells was observed. Double-labelling revealed that the GAD67-ß-Gal fusion protein detected with the ß-Gal-specific antibody colocalized with GABA in enteroendocrine cells (Fig. 6A,B), indicating that the expression of the fusion protein in the gut epithelium was correct.

Quantitative analysis showed that the mucin-secreting cells increased in number in the transgenic animals, and this increase was significant in the 27-day-

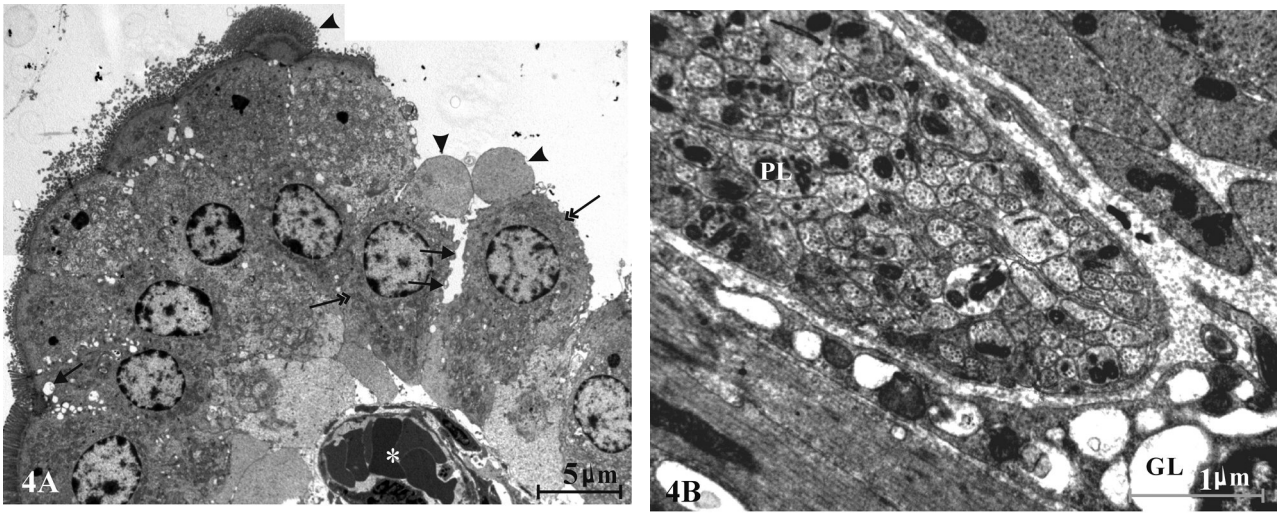

Fig. 4. Electron micrographs show the intestinal wall of a GAD-67 transgenic mouse at 20 days of age. The villus tip shows large intercellular spaces (arrows) within the epithelium. Dome- or circle-shaped protrusions (arrowheads) are seen with intact epithelial cells underneath (double arrows). Intravascular microthrombi (asterisks) are frequently observed **(A)**. The enteric plexuses (PL) appear unaffected, while the enteric glia (GL) displays vacuolized cytoplasm **(B)**.

old animals (Fig. 7).

GAD immunoblotting and enzyme activity

Our immunohistochemical results indicated that the production of GABA and/or its synthesizing enzyme GAD may be increased in GAD-lacZ transgenic mice. To verify this, we performed Western blot analysis and measured GAD activity in the mechanically-separated intestinal mucosa and submucosal-muscle layer fractions. With specific antibodies, we detected both GAD65 and GAD67 in the fraction containing the submucosal and muscle layers (Fig. 8), whereas only GAD67 was present in the mucosa (Fig. 8). In addition to the adult form, we also detected embryonic GAD25, which was more abundant in the mucosal fraction (data not shown). After staining, the blots were scanned and the relative intensities of the bands were measured. Both GAD65 and GAD67 consistently showed a 25-40% higher level of expression in the transgenic mice as compared with the non-transgenic controls. The data obtained from one such immunoblot, stained with two

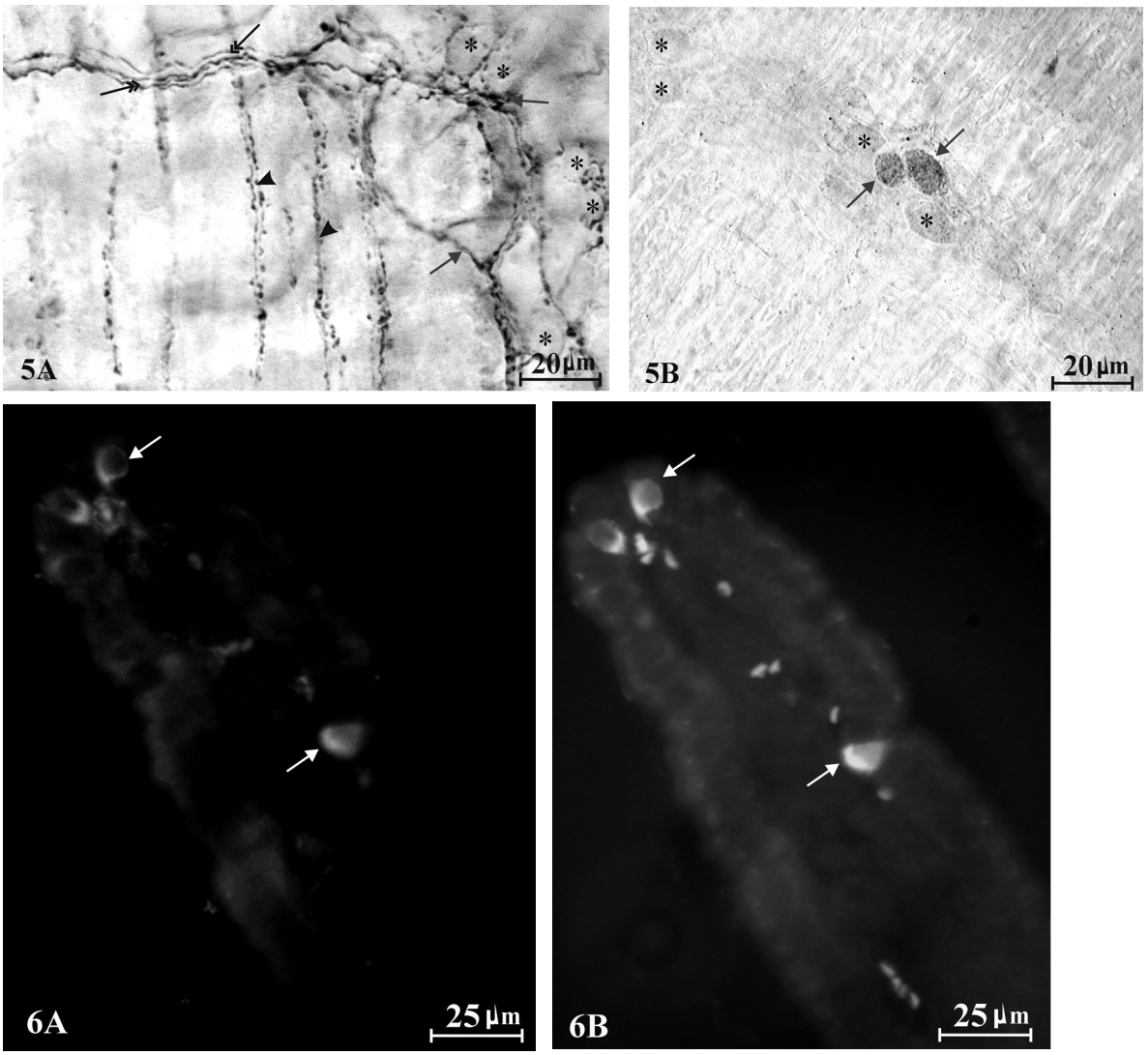

Fig. 5. Longitudinal muscle-myenteric plexus wholemount preparation from wild-type **(A)** and transgenic mice **(B)** at 20 days of age after GABA-immunostaining. Immunopositive varicose fibres **(A)** form baskets (arrows) around non-immunoreactive perikarya (asterisks) within the ganglia and run between ganglia (double arrows) and above the muscle layer (arrowheads). A complete lack of varicose fibres, an intense (arrows) and a pale (asterisks) staining of perikarya are characteristic in the myenteric plexus of transgenic mice **(B)**.

Fig. 6. After double-labelling with anti-GABA **(A)** and with anti-ß-Gal antibody **(B)**, the colocalization of these two antigens was revealed (arrows) in enteroendocrine cells in the small intestine of transgenic mice.

different antibodies, a GAD67-specific antibody and the sheep antiGAD65 serum 1440, which preferentially stains GAD65, are presented as a histogram above the blot (Fig. 8).

Independently, we measured the GAD activity in the same fractions and found that it increased by roughly 60% in the mucosal fraction (data not shown). The activity of the enzyme in this fraction was roughly 10% of that in the brain. We could not detect any changes in activity in the submucosal-muscle fraction, which was very low overall in both the transgenic and the control mice. This may be explained by the high level of degradation in this fraction.

Discussion

The unusual runted, postnatal lethal phenotype of transgenic mice of the GAD67-lacZ line 69 prompted us to study possible abnormalities of the gastrointestinal system in more detail. Approximately 20% of all homozygotes of both sexes displayed a severe phenotype 20 days after birth, when the average body weight was reduced relative to that of the wild-type mice, and the animals died before the age of 1 month. Homozygous mice exhibited an intestinal phenotype of varying severity, with a thinner intestinal wall, shorter and fewer villi, patches of inflammation and hemorrhages. Electron microscopic examination revealed a pronounced disruption of the epithelium. The shrinkage of the epithelium suggests an apoptotic rather than a necrotic process (Young and Cohn, 1986). The lack of junctional complexes, the impaired apical-basal polarity, the short and disorganized microvilli, the occasional appearance of pseudostratified epithelium, and the destruction of enteric glia resemble the situation in necrotizing enterocolitis (NEC), a human disease associated with premature birth (Cornet et al., 2001). Unlike NEC, transmural lesions were not observed in this case: the histological disintegration always started at the villous tip, often involved the entire villus and rarely extended to the submucosa, but not to the muscular layers or to the enteric plexuses. The structural integrity of the muscular and nerve tissues in the transgenic mice might ensure normal peristalsis during the survival period (roughly after the third week post-partum), as judged by the passage of food along the GIT. The restriction of the epithelial disruption to the small intestine suggests that the cytoprotective activities are even more effective in the distal segments of the intestine.

Our immunohistochemical results revealed profound changes in staining pattern of enteric plexuses of transgenic mice compared to control mice. The staining of neuronal perikarya for GABA in the submucosal and myenteric plexuses of the transgenic mice was accompanied by a dramatic decrease in the staining of

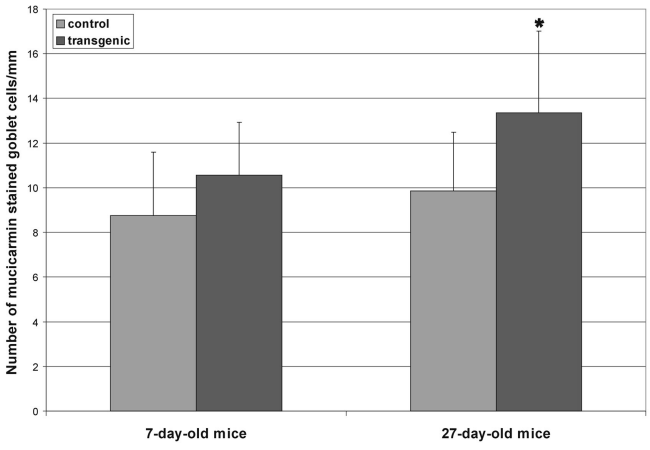

Fig. 7. The number of mucin-secreting cells increased in the transgenic animals as compared with that of the wild-type. The increase is significant in the case of the 27-day-old animals (asterisk).

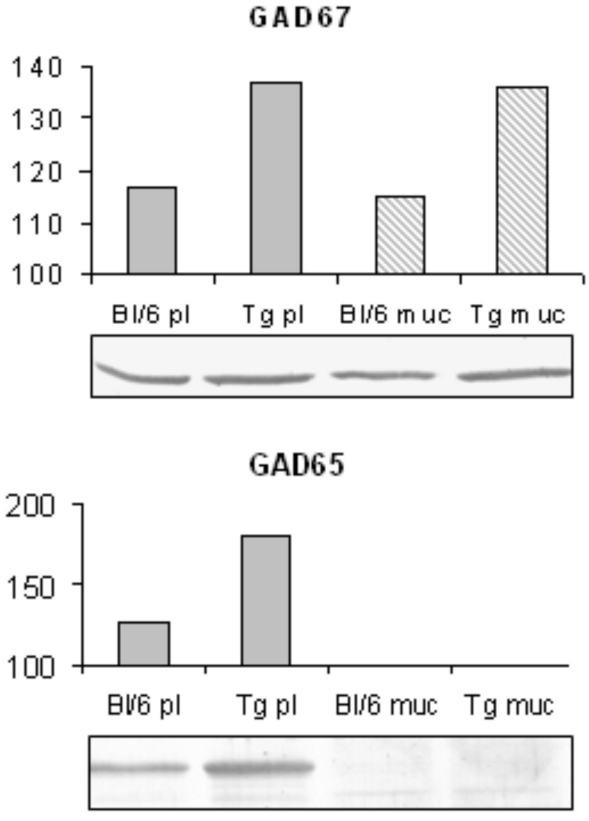

Fig. 8. Expression levels of adult GAD proteins in transgenic GAD67-lacZ and control C57Bl/6 mice at 27 days of age. Western blots of homogenates obtained from intestinal plexuses and mucosa from adult GAD67-lacZ or control mice were performed and scanned. The relative intensities of the bands measured with the NIH Image program are presented in a histogram, where the columns are placed above the respective bands. Bl/6 pl: plexus isolated from a C57Bl/6 control mouse; Tg pl: Total homogenate of plexus isolated from a transgenic GAD67-lacZ; Bl/6 muc and Tg muc: total homogenate of mucosa isolated from Bl/6 or normal GAD67-lacZ transgenic mouse.

varicose fibres, and the baskets formed by these fibres strongly suggest an impairment of the GABAergic transmission.

Western blot and enzyme activity studies showed that GAD65 and GAD67 was upregulated in both the mucosa and the submucosal layers. This finding is in agreement with the observed increase in number of GABA-positive cells in the epithelium. In the submucosal and myenteric plexuses, the upregulation of GAD seemed to be accompanied by the accumulation of GABA in the cytoplasm, rather than in the varicose fibres, which suggests that there is a depletion of the synaptosomal GAD/GABA pool at the expense of the cytoplasmic pool, resulting in an impaired GABAergic innervation of the mucosa. Since GABA is thought to play a crucial role in the secretomotor functions of the gut, acting as both inhibitory and excitatory transmitter (Poulter et al., 1999; Krantis, 2000), changes in the GABAergic transmission may account for the observed epithelial dysfunction and disintegration. Furthermore, it is possible that GABA secreted by GABAergic neurons and/or mucosal enteroendocrine cells (Poulter et al., 1999) acts as a trophic signal during the development and regeneration of the epithelium. The increased number of GABA-immunoreactive enteroendocrine cells in the epithelium with an enhanced GABA release might also elicit a multifactorial secretory response (Hardcastle and Hardcastle, 1996; Krantis, 2000), activating defence barriers protecting the intestinal wall against spontaneous lesions.

The distribution of the different GAD forms within the different layers of the gut deserves special attention. We found that of the two adult GAD forms only GAD67 was present in both the mucosal fraction and the underlying submucosal-muscle layer fraction. In contrast, GAD65 was expressed only in the submucosal-muscle layer fraction. In a similar study (Williamson et al., 1995) it was shown that the two GAD forms were unequally distributed in the different tissue layers of the guinea pig and rat ileum, with small amounts of GAD65 detected in the mucosal fraction. This discrepancy may be species- or strain-dependent or due to differences in preparation of the sample.

The enzymatically-inactive embryonic GAD25 was also detected and was more abundant in the mucosa. Interestingly, this form, which is derived from the N-terminus of GAD67 and contains the 16 amino acids included in the transgene, was down-regulated in the homozygous GAD67lacZ transgenics.

The molecular basis for the observed changes in GAD and GABA synthesis is still obscure. One possible explanation may be the overexpression of a fusion protein containing part of the N-terminal (presumed regulatory region) of GAD67, which might interfere with the synthesis and/or transport of other GADs through the formation of dimers (Martin and Rimvall, 1993; Soghomonian and Martin, 1998; Martin et al., 2000). Another possible explanation could involve the extremely high copy number of the transgene, which could trigger degeneration by a currently unknown mechanism.

In conclusion, our present results suggest that a possible explanation for the growth impairment of the GAD67 transgenic mice is a neurogenic-based epithelial disfunction. A compensatory increase in mucin secretion activated by the excess amount of GABA in the enteroendocrine cells may have a protective function (Abbas et al., 1998).

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