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Long-term role of nitric oxide in the enteric nervous system of the transplanted rat intestine

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Abstract We investigated the long-term changes of the nitric oxide (NO)-related neural component after syngeneic total small bowel transplantation in rats. In the present study, the NO-related neural component was examined using the electrophysiological and NADPH-diaphorase histochemical technique. The rats were divided into four groups: an untreated young adult control group, an untreated 2-year-old control group, a group killed 1 month after transplantation, and a group killed 2 years after transplantation. A superfusion apparatus was used to evaluate the response of jejunal strips to electrical transmural stimulation. In the presence of adrenergic and cholinergic blockade, the inhibitory effect of L-N^G-nitro arginine (L-NNA; a nitric oxide synthesis inhibitor) on nonadrenergic, noncholinergic (NANC) relaxation was expressed as a L-NNA-sensitive component. The L-NNA-sensitive component accounted for 41.6 ± 4.6% (mean ± SE), 43.1 ± 3.5%, 54.6 ± 4.1%, and 55.8 ± 3.5% in the young control group, 2-year control group, 1-month transplant group, and 2-year transplant group, respectively, being significantly higher in the transplant groups ($p < 0.05$). The actual strength of the L-NNA-sensitive component was 0.24 ± 0.03 (mean ± SE), 0.26 ± 0.02, 0.44 ± 0.04, and 0.46 ± 0.04 mg of tension per mg of wet weight, respectively, also being significantly higher in the transplant groups ($p < 0.001$). In addition, the percentage of NADPH-diaphorase-positive fibers was 24.1 ± 1.1% (mean ± SE), 25.5 ± 1.4%, 31.0 ± 1.6%, and 30.9 ± 2.0%, respectively, being significantly higher in the transplant groups ($p < 0.05$). These results suggest that NO neurons in the intrinsic jejunal nervous system have an adaptive role in maintaining intestinal graft motility.

Key words Nitric oxide – Small bowel transplantation – L-NNA-sensitive component

Abbreviations *ETS* Electrical transmural stimulation · *L-NAME* L-N^G-nitro-arginine methylester · *L-NNA* L-N^G-nitro-arginine · *NADPH* Reduced nicotinamide-adenine dinucleotide phosphate · *NANC* Nonadrenergic, noncholinergic · *NO* Nitric oxide · *NOS* Nitric oxide synthase

Introduction

Since the introduction of the new immunosuppressant FK506, intestinal transplantation has attracted clinical interest. Actuarial 2-year patient and primary-graft survival rates are still only around 60% and 50%, respectively, making bowel transplantation a challenging procedure [21]. In addition, impaired motility of the transplanted small bowel can lead to various complications such as diarrhea and malabsorption [10, 21]. Both extrinsic and intrinsic nerves normally regulate the motility of the small intestine [2]. The surgical procedure of small bowel transplantation results in extrinsic denervation with complete transection of the connections between the intestine and the central nervous system. This extrinsic denervation may impair the function of the myenteric plexus after grafting. For example, abnormal release of excitatory and inhibitory neurotransmitters in the transplanted intestine after complete extrinsic denervation may alter bowel motility. In our previous physiological studies, we demonstrated that the cholinergic excitatory component of the enteric nervous system changes to a nonadrenergic, noncholinergic (NANC) component after grafting [11, 13]. In addition, we showed that substance P appeared to be the dominant NANC excitatory neurotransmitter in the transplanted intestine [12]. Nitric oxide (NO) is a major NANC inhibitory neurotransmitter in the gastrointestinal tract. We recently found that L-NNA-sensitive NANC relaxation was significantly increased in the small intestine at 4 weeks after grafting, suggesting enhanced NO production in the myenteric plexus [15]. It is also important to determine how the NO-related neural component is modified over the long term after small bowel transplantation. Accordingly, we investigated the role of NO in the intrinsic nervous system of the small bowel in rats surviving for 2 years after grafting.

Materials and methods

Experimental design

To assess the long-term effects of transplantation, rats surviving for 2 years after surgery ($n = 4$) were compared with a young adult control group ($n = 6$), a transplant group of rats that were killed after 4 weeks ($n = 6$), and control rats at least 2 years old ($n = 6$). The young adult and 2-year-old control groups did not undergo sham operations or receive any other treatment. To evaluate the physiologic properties of smooth muscle and nerves, eight tissue samples were obtained from each group. For histochemical evaluation, we also used eight tissue samples from each group. These samples of jejunal grafts or normal jejunum were rapidly cut into 15-mm and 10-mm lengths, and the 10-mm segments were processed for light microscopy with sectioning at an appropriate thickness.

Animals

Male Lewis rats (from the LEW/Crj strain) weighing 250–300 g were obtained from Charles River Japan. These rats were used as both small bowel donors and recipients since syngeneic transplantation avoids the risk of rejection and the need for immunosuppression. Animals were fasted for 12–18 h before operation.

Surgical procedure

Orthotopic total small bowel transplantation was performed using a microsurgical technique similar to that described previously [12, 15]. Briefly, anesthesia was induced with pentobarbital (40 mg/kg intraperitoneally). The entire small bowel of the donor rat was harvested from the ligament of Treitz to the terminal ileum, along with a vascular pedicle consisting of the superior mesenteric artery and the portal vein. The intestinal lumen was flushed with 0.5% neomycin, and the superior mesenteric artery was flushed with iced saline containing 10 U/ml heparin. In the recipient rat, the infrarenal aorta and vena cava vein were clamped, and end-to-side anastomosis was performed between the recipient aorta and the donor superior mesenteric artery along with the aortic cuff, as well as between the recipient vena cava and the donor portal vein. The entire small bowel was resected from the recipient, and the graft was anastomosed end-to-end with the duodenum and the remnant terminal ileum.

Recording of mechanical activity

The bowel strips were suspended in a superfusion apparatus [11, 13, 15] equipped with two platinum electrodes and were superfused with Krebs solution of the following composition: NaCl, 118 mM; KCl, 4.8 mM; $CaCl_2$, 2.5 mM; $NaHCO_3$, 25 mM; KH_2PO_4, 1.2 mM; $MgSO_4$, 1.2 mM; and glucose, 11 mM. The medium was maintained at 37 °C, adjusted to pH 7.4, and constantly aerated with a mixture of 95% O_2 and 5% CO_2. To study NO-dependent relaxation of the NANC component, we used atropine, guanethidine, and L-NNA. In all experiments, the solution also contained atropine and guanethidine (1.0 and 5.0 μM, respectively). Motor activity was recorded isometrically on a recticoder (RJG-4128; Nihon Kohden, Tokyo, Japan). The resting tension (1.0 g) was kept constant by readjustment during the equilibration period. Experiments were started after a 60- to 90-min equilibration period, and antagonists were given 15 min before the application of electrical transmural stimulation (ETS). L-NNA was added 15 min prior to ETS. The superfusate flow rate was adjusted to 1 ml/min using a peristaltic pump (SJ-1220; Atto, Tokyo, Japan). Electric pulses were delivered from an electrical generator (S-7272B; Nihon Kohden) that provided pulse trains at 4-min intervals lasting for 10 s. A voltage of 20 V and a duration of 1.0 ms were used in all experiments, while the impulse frequency was altered from 1.0 Hz to 20.0 Hz. The L-NNA-sensitive component was assessed at 2.0 Hz because maximum L-NNA-sensitive NANC relaxation of the untreated adult control intestine was found at this frequency [15].

Drugs

The following drugs were used: Atropine sulfate and guanethidine sulfate were obtained from Wako Pure Chemical Industries, (Osaka, Japan), L-N^G-nitro-arginine (L-NNA) from Research Biochemicals (Natick, Mass.).

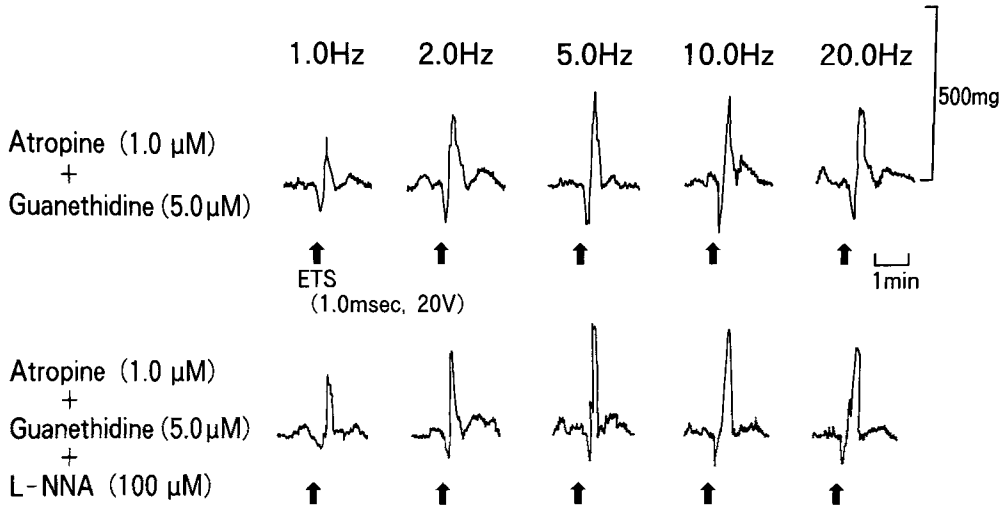

Fig. 1 Tracings of the response to electrical transmural stimulation (*ETS*, 20 V, 1.0 ms, 1.0–20.0 Hz, for 10 s) of young control adult rat jejunum. During ETS, longitudinal muscle strips showed a biphasic response comprising rapid relaxation followed by phasic contraction. The first phase of rapid relaxation was significantly antagonized by 100 μM L-N^G-nitro arginine (L-NNA)

NADPH-diaphorase histochemistry

Histochemical staining for NADPH-diaphorase was performed using the method previously described by Aimi et al. [1]. In brief, the strips were fixed overnight in 4% paraformaldehyde at 4 °C. NADPH-diaphorase activity was rendered visible by incubating the tissues in 0.1 M phosphate buffer (pH 8.0) – containing 0.01 mM β-NADPH, 0.02 mM nitroblue tetrazolium, and 0.3% Triton X-100 – at 37 °C for 2 h. Washing the whole mount preparations in 0.1 M phosphate buffer terminated the reaction. After several washings with 0.1 M phosphate buffer, the whole muscle layers were placed on glass slides, air-dried, and cover-slipped with gel-mount. As previously described [15], the number of NADPH-diaphorase-positive fibers was assessed using the IBAS 20 computer image analysis system (Zeiss, Germany). The total number of nerve fibers was expressed as the plexus area-to-frame area ratio.

Statistical analysis

Results shown are expressed as the mean ± SE. Statistical analysis was performed using a one-way analysis of variance (ANOVA). A p value of less than 0.05 was considered significant.

Results

Analysis of L-NNA-sensitive NANC relaxation

During 10 s of ETS, longitudinal muscle strips of the jejunum obtained from each group showed a biphasic response comprising rapid relaxation (the first phase) followed by phasic contraction (the second phase). Typical jejunal motility patterns are illustrated in Fig. 1. Tone was calculated as milligrams of tension per milligram of wet weight and was compared between each group. L-NNA markedly inhibited the first phase of the response to ETS and enhanced the second phase over the entire frequency range tested (1.0–20.0 Hz) (Figs. 2, 3). After pretreatment with atropine (1.0 μM) and guanethidine (5.0 μM), the inhibitory effect of L-NNA was assessed. The percentage of L-NNA-sensitive component in the young control group, 2-year control group, 1-month transplant group, and 2-year transplant group was 41.6 ± 4.6%, 43.1 ± 3.5%, 54.6 ± 4.1%, and 55.8 ± 3.5%, respectively (Table 1). The transplant groups had significantly higher L-NNA-sensitive component values than the control groups ($p < 0.05$). The strength of the L-NNA-sensitive component in these four groups was 0.24 ± 0.03, 0.26 ± 0.02, 0.44 ± 0.04,

Table 1 Changes of the L-N^G-nitro arginine *(L-NNA)* -sensitive component after small bowel transplantation. Values given as mean ± SE. (*C* Young adult control group, *2yC* 2-year-old control group, *1moT* 1-month transplant group, *2yT* 2-year transplant group)

	Group			
	C	2yC	1moT	2yT
Percentage of the L-NNA-sensitive component (%)	41.6 ± 4.6	43.1 ± 3.5	54.6 ± 4.1[a*,b*]	55.8 ± 3.5[a*,b*]
Absolute value of the L-NNA-sensitive component (mg/mg wet weight)	0.24 ± 0.03	0.26 ± 0.02	0.44 ± 0.04[a**,b**]	0.46 ± 0.04[a**,b**]

Values with the same letter are significantly different: *$P < 0.05$, **$P < 0.001$
[a] Compared with C
[b] Compared with 2yC

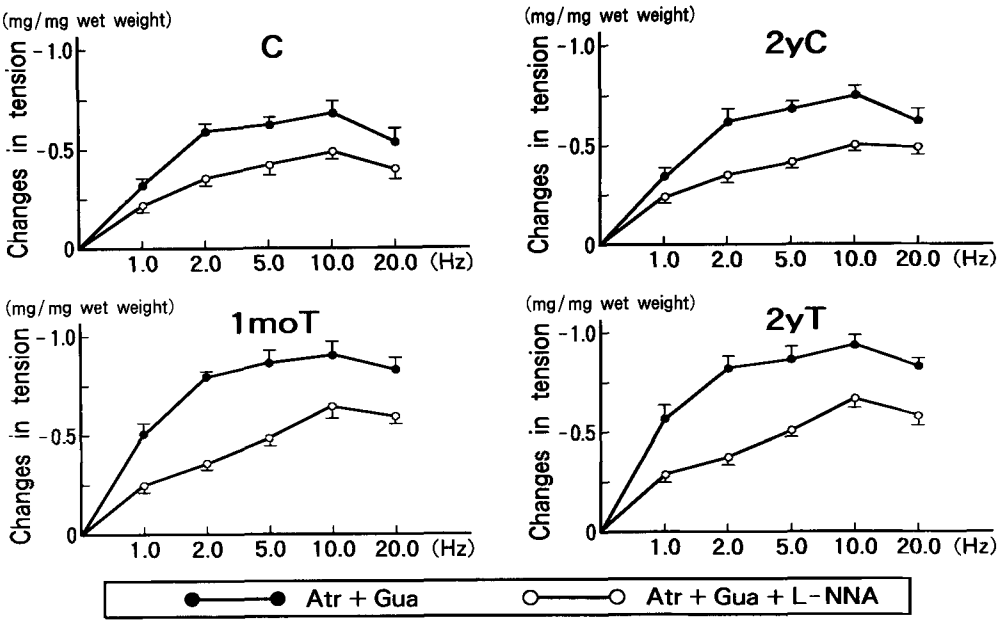

Fig. 2 Nonadrenergic noncholinergic (*NANC*) contraction in response to electrical transmural stimulation (20 V, 1.0 ms, 1.0–20.0 Hz, for 10 s) in the presence of atropine (*Atr*, 1.0 μM), guanethidine (*Gua*, 5.0 μM), and L-N^G-nitro arginine (L-NNA, 100 μM). Tension is given as mg per mg wet weight (*C* Young adult control group, *2yC* 2-year-old control group, *1moT* 1-month transplant group, *2yT* 2-year transplant group)

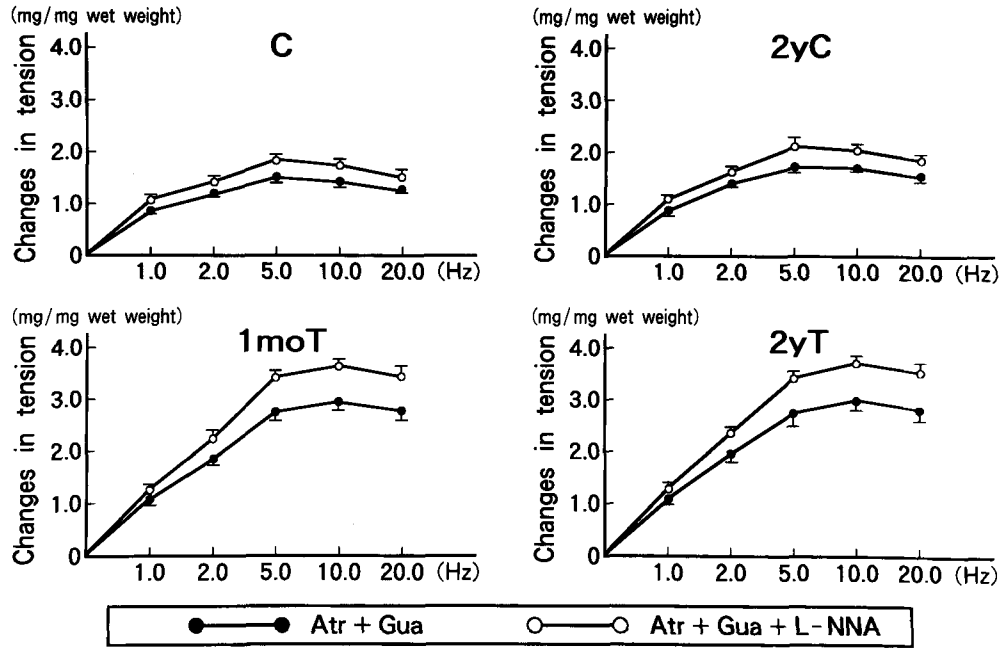

Fig. 3 Nonadrenergic noncholinergic (*NANC*) relaxation in response to electrical transmural stimulation (20 V, 1.0 ms, 1.0–20.0 Hz, for 10 s) in the presence of atropine (*Atr*, 1.0 μM), guanethidine (*Gua*, 5.0 μM) and L-N^G-nitro arginine (L-NNA, 100 μM). Tension is given as mg per mg wet weight. Abbreviations for groups as in Fig. 2

and 0.46 ± 0.04 mg/mg wet weight, respectively (Table 1). Therefore, the absolute value of the L-NNA-sensitive component was also significantly increased in the transplanted groups ($p < 0.001$).

NADPH-diaphorase histochemistry

The NADPH-diaphorase-positive fibers were clearly observed in the jejunal myenteric plexus of each group (Fig. 4). The positivity rate of the NADPH-diaphorase-positive fibers in these four groups was 24.1 ± 1.1 %, 25.5 ± 1.4 %, 31.0 ± 1.6 %, and 30.9 ± 2.0 %, respectively (Table 2). The rate was significantly higher in the transplant groups than in the control groups ($p < 0.05$).

Table 2 Changes of NADPH-diaphorase-positive fibers after small bowel transplantation. Values given as mean ± SE. (*C* Young adult control group, *2yC* 2-year-old control group, *1moT* 1-month transplant group, *2yT* 2-year transplant group)

	Group			
	C	2yC	1moT	2yT
Percentage of NADPH-diaphorase-positive fibers (%)	24.1 ± 1.1	25.5 ± 1.4	31.0 ± 1.6[a**,b*]	30.9 ± 2.0[a**,b*]

Values with the same letter are significantly different: $*P < 0.05$, $**P < 0.005$
[a] Compared with C
[b] Compared with 2yC

Discussion

Various morphological and functional changes of the transplanted small intestine have been reported. Histologically, it was shown that extrinsic innervation is totally lost in intestinal grafts including human grafts, whereas intrinsic innervation is preserved [9, 14, 18]. Impaired bowel motility was reported after small bowel transplantation. Schiller et al. found abnormal motility of the transplanted canine small intestine by measuring the intraluminal pressure and the intramural electrical activity [17]. Vane et al. also reported impaired electrical activity of transplanted intestinal grafts [22]. Extrinsic and intrinsic nerves regulate the motility of the small intestine [2], and transplantation causes complete disruption of the extrinsic innervation to the graft. The complete absence of extrinsic adrenergic innervation has been demonstrated after grafting [19]. In previous physiological studies, we have shown that the cholinergic excitatory component of the enteric nervous system changes to a NANC component, which becomes dominant after grafting [11, 13]. It has been suggested that substance P plays an important role in excitatory neurotransmission in grafts [12]. We also recently showed that the increase of NO-related neural component was associated with a marked increase of NO pro-

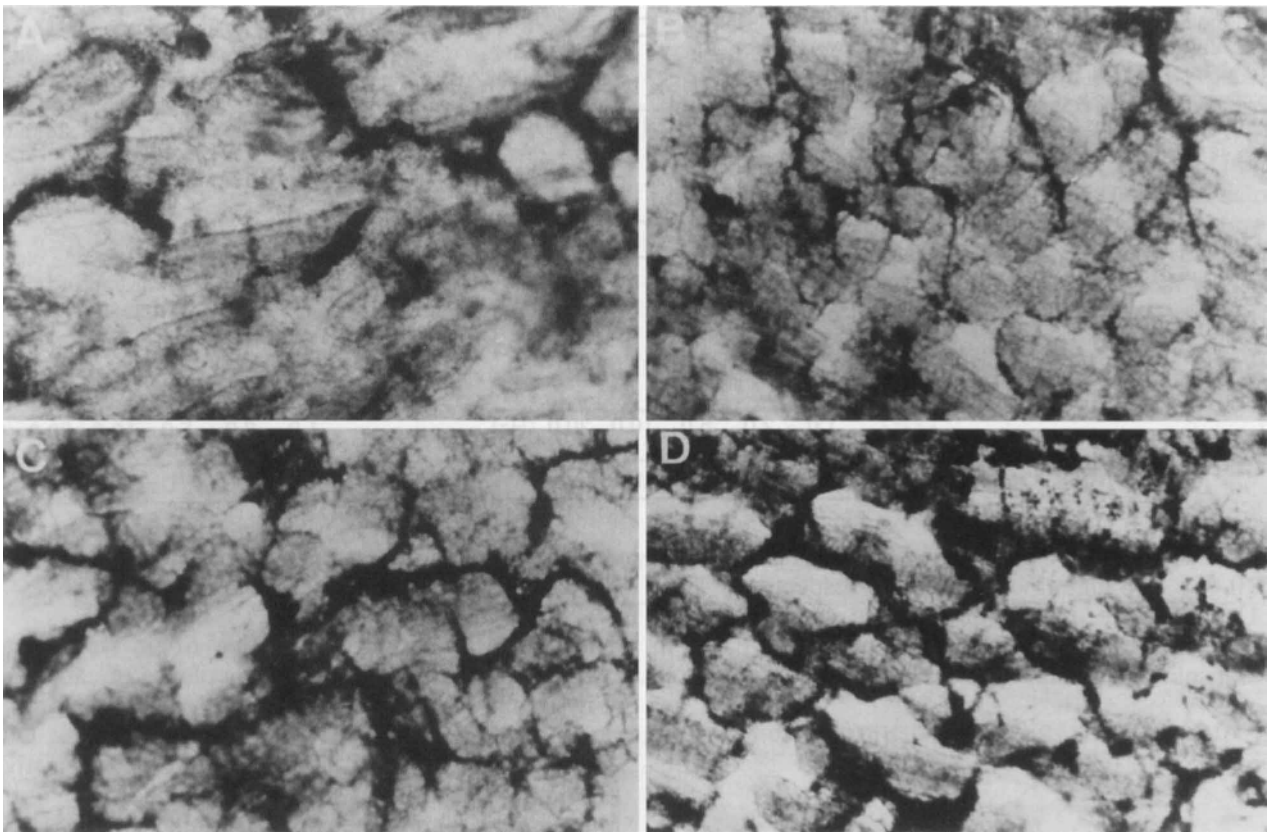

Fig. 4 NADPH-diaphorase histochemistry of the jejunal myenteric plexus in young adult control group (**A**), 2-year-old control group (**B**), 1-month transplant group (**C**), and 2-year transplant group (**D**)

duction in the graft soon after transplantation [15]. These findings suggest that extrinsic denervation may be a cause of intestinal dysmotility after small bowel transplantation. In the present study, we investigated the effect of the long-term role of the NO-related neural component in the transplanted intestine. We used a syngeneic rat model to allow assessment independently of immunologic factors. Recent studies have shown that NO is an inhibitory NANC neurotransmitter in the gastrointestinal tract, including the small intestine. NO released in response to stimulation of the myenteric plexus causes relaxation of gastrointestinal tract smooth muscle [5]. The rapid small bowel relaxation induced by NANC stimulation is significantly antagonized by NO synthesis inhibitors, such as L-NNA or L-N^G-nitroarginine methylester (L-NAME) [4, 7]. These observations suggest that NO neurons in the myenteric plexus are involved in mediating intestinal relaxation. It is generally accepted that NO is released by short impulse trains at lower stimulus frequencies, while peptidergic neurotransmitters are released during sustained stimulation or stimulation at high frequencies [6]. In this study, the rapid relaxation (first phase) evoked by ETS was frequency-dependent over the range 1.0–20.0 Hz. L-NNA (100 mM) significantly reduced the rapid relaxation induced at 2.0 Hz in each group, suggesting that muscle relaxation was primarily caused by the release of NO from the myenteric plexus. The relative contribution of the NO-related neural component was increased by transplantation. Aging itself did not affect the NO-related neural component since the L-NNA-sensitive component of NANC relaxation innervation was 41.6% in the young adult controls and 43.1% in the 2-year-old controls. In addition, the absolute value of the L-NNA-sensitive component remained high in the transplant groups. The NADPH-diaphorase histochemical technique was utilized to a specific histochemical marker for neurons producing NO. This technique specifically labels neurons containing nitric oxide synthase (NOS). As previously described [1, 3], NADPH-diaphorase-positive neuronal cell bodies and fibers were found throughout the entire rat gastrointestinal tract. Most of the myenteric neurons that were NOS-immunopositive in all regions of the entire gut also stained for NADPH-diaphorase [3]. NADPH-diaphorase-positive fibers were increased in the myenteric plexus of the transplant groups. These histochemistry findings are in agreement with our physiological observations in the present study. The effect of extrinsic denervation on NO pathways in the rat jejunum was first described by Nakao et al. [16]. They found that L-NAME-sensitive NANC relaxation induced by ETS was significantly increased in the jejunal longitudinal muscle strips obtained from rats treated with splanchnic ganglionectomy, but not from vagotomized rats. Furthermore, they showed that the number of NADPH-diaphorase-positive fibers in the myenteric plexus was significantly increased in the tissue obtained from rats treated with splanchnic ganglionectomy, but not in that of vagotomized rats. This strongly indicates that NO neurons in the myenteric plexus are independent of the vagus nerve and are negatively regulated by the splanchnic nerves in the rat small intestine. Increased activity of the NO pathways may be related to the absence of extrinsic adrenergic inhibitory innervation following transplantation. Recently, NO and various other neuropeptides have been shown to exist in different neurons [8, 20]. Thus, further assessment of the relationships between NO and neuropeptides is required using a syngeneic rat model. In summary, an increase in both NO-related neural component and NO staining of the strips was observed even 2 years after small bowel transplantation. NO neurons may play an important role in maintaining the intrinsic nervous system of the transplanted small bowel throughout the life of the graft.

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