

Subdiaphragmatic Vagotomy Induces NADPH Diaphorase in the Rat Dorsal Motor Nucleus of the Vagus

Jeong Won Jahng¹, Dong Goo Kim¹, and Thomas A. Houpt²

¹Department of Pharmacology and Yonsei Brain Research Institute, Brain Korea 21 Project for Medical Science, Yonsei University College of Medicine Seoul, Korea;

²Department of Biological Science, The Florida State University, FL, USA.

Axotomy of the vagal motor neurons by cervical vagotomy induces NADPH diaphorase staining due to increased nitric oxide synthase expression in both the rat dorsal motor nucleus and nucleus ambiguus; furthermore, cervical vagotomy leads to cell death of the dorsal motor nucleus cells. Subdiaphragmatic vagotomy axotomizes the vagal motor cells further from the brainstem than cervical vagotomy, and cuts the fibers running only to the abdominal viscera. Here we report that subdiaphragmatic vagotomy is sufficient to induce NADPH diaphorase staining in the dorsal motor nucleus but does not induce staining in the nucleus ambiguus. Because the neurons of the dorsal motor nucleus do not undergo cell death after subdiaphragmatic vagotomy and are able to re-nerve the gut, the increased nitric oxide synthase expression after distal axotomy may be related more to regeneration than degeneration.

Key Words: Axotomy, motor neurons, nucleus ambiguus, nitric oxide synthase

INTRODUCTION

Axotomy of neurons often induces nitric oxide synthase (NOS) expression and hence NADPH diaphorase staining (NADPH-d). Increased NOS mRNA,¹ immunoreactivity,¹⁻⁶ or NADPH-d¹⁻⁹ has been observed in the dorsal motor nucleus of the vagus (DMN) and nucleus ambiguus (NA) 2-90 days after unilateral cervical vagotomy. Cervical vagotomy axotomizes the vagal motor fibers running to both the thoracic and abdominal viscera; furthermore, cervical vagotomy axotomizes the motor neurons of the DMN and NA relatively proximal to their cell bodies. As a result

of cervical axotomy, the cells within the DMN undergo degeneration and eventual cell death.^{6,10,11}

In this study, we examined NADPH-d in the DMN following subdiaphragmatic vagotomy. Subdiaphragmatic vagotomy axotomizes the motor fibers more distal to the cell bodies than cervical vagotomy, and cuts only the fibers running to the abdominal viscera. Unlike after cervical vagotomy, the cells of the DMN do not undergo cell death, and in fact regenerate the axons to the target organs of the abdomen.¹² The survival of the subdiaphragmatically axotomized DMN cells may be a combination of the distance of the fiber-cut, and the presence of trophic factors originating from the viscera.¹³ Thus, subdiaphragmatic vagotomy should reveal whether NADPH-d expression in the DMN requires proximal axotomy, in addition to revealing the NADPH-d-expressing motor neurons which project specifically to the abdominal organs.

MATERIALS AND METHODS

Adult male Sprague-Dawley rats (200-250g) were individually housed under a 12:12 light dark cycle with *ad lib* access to rodent chow and water. Unilateral subdiaphragmatic vagotomy (n = 4) was performed using the technique described previously.¹⁴ Under chloral hydrate-pentobarbital anesthesia and aided by a dissecting microscope, the right (or anterior) vagal trunk was cut between two 3-0 silk ligatures as high as possible on the esophagus below the diaphragm and above the hepatic and accessory celiac branches. Sham

Received August 30, 2000

Accepted January 13, 2001

*This work was supported by NIH DC03198 (TAH).

vagotomy consisted of the same procedure without touching either the esophagus or nerves ($n = 2$).

Four days after the vagotomy, the rats were overdosed with sodium pentobarbital and transcardially perfused, first with 100 ml heparinized isotonic saline containing 0.5% NaNO, then with 400 ml 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB). The brains were dissected, blocked, post-fixed for 2 hr, and transferred into 30% sucrose solution for cryoprotection. Forty micron coronal sections were cut on a freezing, sliding microtome through the rostral-caudal extent of the DMN. The right side of each brainstem was marked by a needle puncture. Sections were cut from the caudal DMN, caudal to the area postrema (bregma -14.6 mm), to the rostral extent of the DMN (bregma -12.8 mm). All coordinates were based on Paxinos and Watson.¹⁵ Free-floating tissue sections were permeabilized for 15 min in 0.1 M Tris buffer, 0.1% Triton X-100, at 37°C, followed by a 15 min reaction in 0.1 M Tris buffer, 0.1% Triton X-100, 0.05% β -NADPH, 0.0125% nitroblue tetrazolium at 37°C. The reaction was terminated with ice-cold 0.1 M Tris buffer. Sections were mounted in anatomical order from 0.05 M sodium phosphate buffer onto the glass slides, then dehydrated and cover-slipped.

Cells in the DMN expressing NADPH-d were hand counted after digitizing the 720×540 micron images of all consecutive sections using an Olympus Provis microscope, a MTI-CCD 72 camera, and a Macintosh computer. Counts per section were aligned by using the most caudal aspect of the area postrema as a landmarker. For statistical analysis, the total unilateral cell counts were summed across three regions of the DMN (caudal to the area postrema, subpostremal, and rostral to the area postrema). The cell counts from the axotomized and intact sides were compared by paired t-tests.

RESULTS

As previously described by others,¹⁶⁻¹⁹ small numbers of NADPH-d positive cells were seen in the sham-operated DMN (Fig. 1A, 1B, and 2A).

Some cells were detected in the most caudal DMN, almost no cells were found in the subpostremal DMN, and a larger number of cells were seen in the rostral DMN.

Four days after the right subdiaphragmatic vagotomy, NADPH-d positive cells were observed on almost every section of the axotomized (left) DMN from the most caudal to most rostral extent (Fig. 1C, 1E, and 2B). The number of NADPH-d positive cells in the axotomized DMN appeared increased at all levels compared to the DMN of the sham-operated rats. NADPH-d cells were distributed throughout the subpostremal DMN, and in the lateral DMN concentrated laterally immediately below the centralis subnucleus of the nucleus tractus of solitarius.

Compared to the intact (right) DMN, significantly greater number of cells were induced in the axotomized DMN in the subpostremal and rostral DMN, but not in the caudal DMN (Fig. 1, D and F). Also, more NADPH-d cells appeared to be induced in the intact, rostral DMN of all unilateral vagotomized rats compared to the DMN of the sham-operated rats (Fig. 2B).

Very few NADPH-d positive cells were observed in the NA of any rats, and no difference was observed between the intact and axotomized sides (data not shown).

DISCUSSION

We found that subdiaphragmatic vagotomy induced NADPH-d in the vagal motor neurons of the DMN but little or no NADPH-d in the motor neurons of the NA. This is consistent with the known topography of the vagal motor neurons. Because the NA motor neurons do not project below the stomach, only the most caudal innervation of the esophagus would have been damaged by subdiaphragmatic vagotomy.²⁰ Conversely, the left DMN neurons project to the stomach, liver, pancreas, intestines, etc., and would be axotomized by right subdiaphragmatic vagotomy.²¹

The cell counts across consecutive serial sections of the DMN revealed increases in the number of NADPH-d positive cells in the axotomized subpostremal and rostral DMN. Surpris-

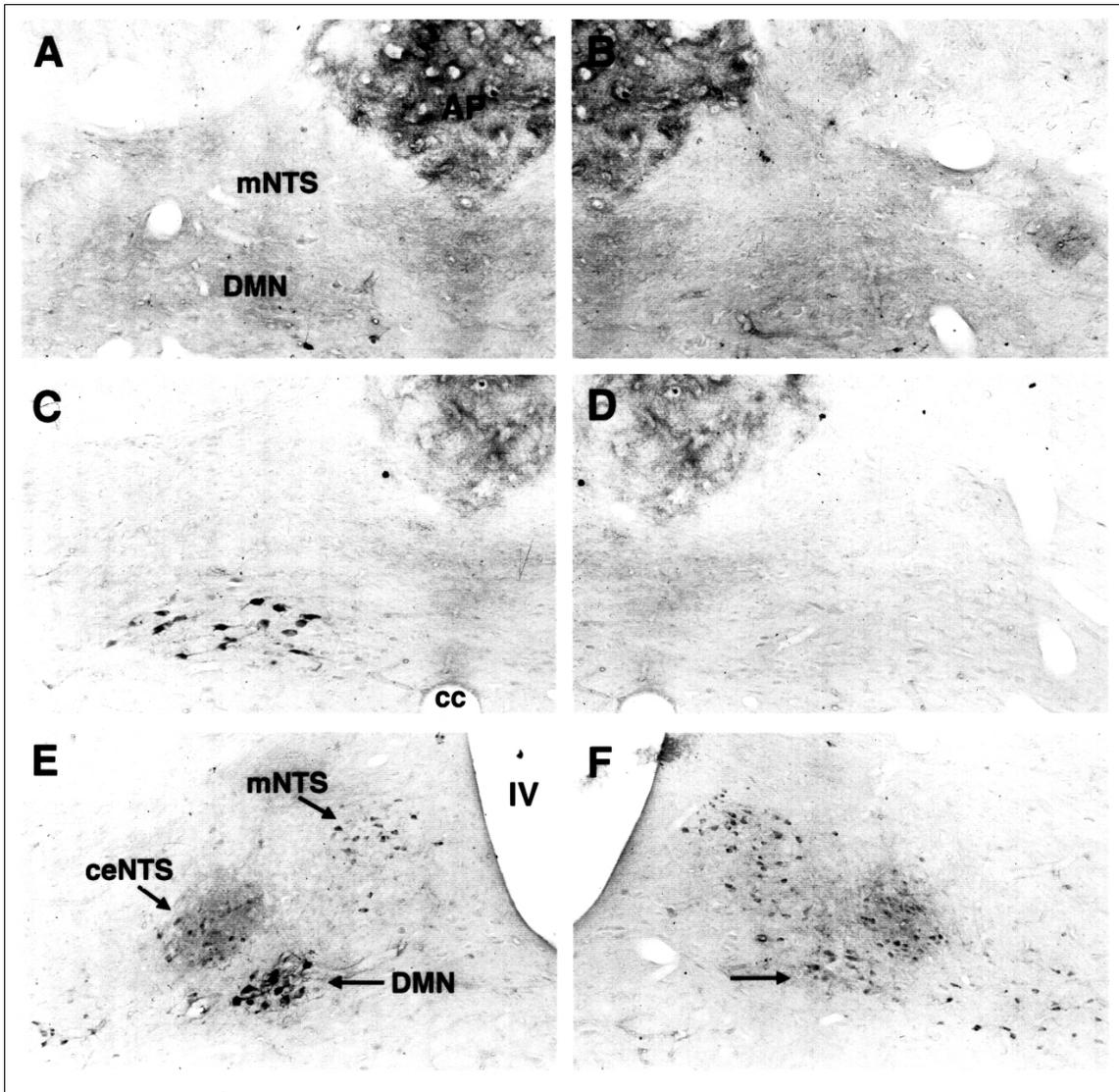


Fig. 1. Photomicrographs of NADPH-d in the dorsal vagal complex of a sham-operated rat (A,B) and an unilaterally vagotomized rat (C-F). Few or no NADPH-d-positive cells were observed in the DMN of the sham-operated rats (A, B). Four days after the right subdiaphragmatic vagotomy, NADPH-d-positive cells were detected in the left (axotomized) DMN beneath the AP (C) and rostral to the AP (E, arrow). No NADPH-d positive cells were seen in the right (intact) DMN subpostremally (D), but some NADPH-d positive cells were seen in the right DMN rostral to the AP (F, arrow). Stained cells were also detected in the ceNTS and mNTS rostral to the AP of both axotomized (E) and intact (F) sides of the vagotomized rats, and sham-operated rats (not shown). AP, area postrema; mNTS, medial nucleus of the solitary tract; DMN, dorsal motor nucleus of the vagus; cc, central canal; IV, fourth ventricle; ceNTS, centralis subnucleus of the NTS.

ingly, while subdiaphragmatic vagotomy induced more NADPH-d in the rostral left DMN, it also induced NADPH-d in the non-axotomized neurons of the right DMN rostral to the area postrema, although significantly less than on the axotomized side. This bilateral induction of NADPH-d after unilateral subdiaphragmatic

vagotomy in the intact DMN has occasionally been reported in the intact DMN after unilateral cervical vagotomy.²

The number of cells expressing NADPH-d after vagotomy in the subpostremal and intermediate DMN also appeared to be less than the total number of motor neurons that project to the abdo

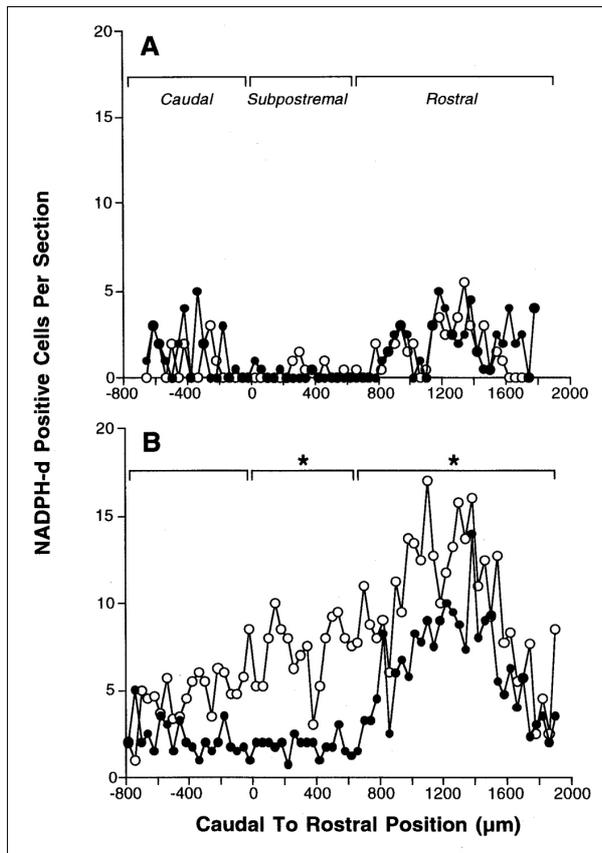


Fig. 2. Mean number of NADPH-d-positive cells across the left (white circles) or right (black circles) DMN of the sham-operated (A) and vagotomized rats (B). Cell counts of the consecutive serial sections were aligned by the most caudal aspect of the area postrema ($0\mu\text{m}$ on the x-axis). The sham operated rats ($n = 2$) had only a small number of NADPH-d-positive cells in the DMN caudal and rostral to the subpostremal DMN. The subdiaphragmatic vagotomy ($n = 4$) significantly increased the number of NADPH-d-positive cells in the left (axotomized) vs. right DMN beneath and rostral to the area postrema. * $p < 0.05$.

men as retrogradely labeled by intraperitoneal Fluoro-gold.^{22,23} This could be due to selective induction of NADPH-d in a subset of DMN cells. Alternatively, it may be that only a small number of axotomized DMN cells express NADPH-d at 4 days after the vagotomy, and greater numbers would express NADPH-d at later time points.

Our results show that subdiaphragmatic vagotomy is sufficient to induce NADPH-d in the axotomized DMN. By quantifying the NADPH-d positive cells, we have shown that there is a

differential response to axotomy along the rostral caudal extent of the DMN, such that the subpostremal and rostral DMN are more sensitive to axotomy. Furthermore, subdiaphragmatic vagotomy induces some bilateral NADPH-d in the rostral DMN.

Because the right vagus was axotomized ~6 cm below the level of the cervical vagus, we conclude that the proximity to the cell body is not absolutely necessary for the axotomy to induce NADPH-d in the vagal motor neurons. The proximity of the axotomy to the cell body has been shown to correlate with the induction of cell death both centrally²⁴ and peripherally,²⁵ such that axotomy more than 4 mm from the cell body results in minimal cell death. Despite the distance of the nerve cut from the cell bodies of the DMN, however, NOS expression was induced. This result stands in contrast to the response of the spinal motoneurons, which do not express NOS after distal axotomy.²⁵ Because the subdiaphragmatic vagal motor neurons and afferents can regenerate to the target organs after axotomy,¹² the response of the dorsal vagal complex after subdiaphragmatic vagotomy may be a useful model for studying the role of NOS in neuronal injury and regrowth.

Jeong Won Jahng, Ph.D.

Department of Pharmacology,

Yonsei University College of Medicine,

C.P.O. Box 8044, Seoul 120-752, Korea.

Tel: 82-2-361-5233, Fax: 82-2-313-1894,

E-mail: jwjahng@yumc.yonsei.ac.kr

REFERENCES

1. Lin LH, Sandra A, Boutelle S, Talman WT. Up-regulation of nitric oxide synthase and its mRNA in vagal motor nuclei following axotomy in rat. *Neurosci Lett* 1997;221:97-100.
2. Yu WH. Regulation of nitric oxide synthase expression in motoneurons following nerve injury. *Dev Neurosci* 1997;19:247-54.
3. Yeo JF, Tang FR, Leong SK. Expression of glutamate receptor subunit 1 and nitric oxide synthase in the hypoglossal nucleus and dorsal vagal nucleus in the rat after neurectomy. *Int J Neurosci* 1997;90:9-20.
4. Kristensson K, Aldskogius M, Peng Z-C, Olsson T, Aldskogius H, Bentivoglio M. Co-induction of neuronal

- interferon-gamma and nitric oxide synthase in rat motor neurons after axotomy: a role in nerve repair or death? *J Neurocytol* 1994;23:453-9.
5. Zhao X-L, Yanai K, Hashimoto Y, Steinbusch HWM, Watanabe T. Effects of unilateral vagotomy on nitric oxide synthase and histamine H3 receptors in the rat dorsal vagal complex. *J Chem Neuroanat* 1996;11:221-9.
 6. Yu W-HA. Nitric oxide synthase in motor neurons after axotomy. *J Histochem Cytochem* 1994;42:451-7.
 7. Gonzalez MF, Sharp FR, Sagar SM. Axotomy increases NADPH-diaphorase staining in rat vagal motor neurons. *Brain Res Bull* 1987;18:417-27.
 8. Jia Y, Wang X, Ju G. Nitric oxide synthase expression in vagal complex following vagotomy in the rat. *Neuroreport* 1994;5:793-6.
 9. Ruggiero DA, Mtui EP, Otake K, Anwar M. Central and primary visceral afferents to nucleus tractus solitarii may generate nitric oxide as a membrane-permeant neuronal messenger. *J Comp Neurol* 1996;364:51-67.
 10. Ling EA, Shieh JY, Wen CY, Yick TY, Wong WC. The dorsal motor nucleus of the vagus nerve of the hamster: ultrastructure of vagal neurons and their responses to vagotomy. *J Anat* 1987;152:161-72.
 11. Laiwand R, Weman R, Yarom Y. Electrophysiology of degenerating neurones in the vagal motor nucleus of the guinea-pig following axotomy. *J Physiol* 1988;404: 749-66.
 12. Powley TL, Prechtel JC, Fox EA, Berthoud HR. Anatomical considerations for surgery of the rat abdominal vagus: distribution, paraganglia and regeneration. *J Auton Nerv Syst* 1983;9:79-97.
 13. Rinaman L, Levitt P. Access to gastric tissue promotes the survival of axotomized neurons in the dorsal motor nucleus of the vagus in neonatal rats. *J Comp Neurol* 1991;313:213-26.
 14. Smith GP, Jerome C. Effects of total and selective abdominal vagotomies on water intake in rats. In: Kral JG, Powley TL, Brooks C, editors. *Vagal Nerve Function: Behavioral and Methodological Considerations*. Amsterdam: Elsevier Science Publ.; 1983. p.259-71.
 15. Paxinos G, Watson C. *The rat brain in stereotaxic coordinates*. 2nd ed. San Diego: Academic Press; 1986.
 16. Lawrence AJ. Nitric oxide as a modulator of medullary pathways. *Clin Exp Pharmacol Physiol* 1997;24:760-3.
 17. Krowicki ZK, Sharkey KA, Serron SC, Nathan NA, Hornby PJ. Distribution of nitric oxide synthase in rat dorsal vagal complex and effects of microinjection of nitric oxide compounds upon gastric motor function. *J Comp Neurol* 1997;377:49-69.
 18. Dun NJ, Dun SL, Forstermann U. Nitric oxide synthase immunoreactivity in rat pontine medullary neurons. *Neuroscience* 1994;59:429-45.
 19. Rodrigo J, Springall DR, Uttenthal O, Bentura ML, Abadia-Molina F, Riveros-Moreno V, et al. Localization of nitric oxide synthase in the adult rat brain. *Philos Trans R Soc Lond B Biol Sci* 1994;345:175-221.
 20. Bieger D, Hopkins DA. Viscerotopic representation of the upper alimentary tract in the medulla oblongata in the rat: the nucleus ambiguus. *J Comp Neurol* 1987;262: 546-62.
 21. Berthoud H-R, Powley TL. Topography of efferent vagal innervation of the rat gastrointestinal tract. *Am J Physiol* 1991;260:R200-R7.
 22. Powley T, Fox EA, Berthoud HR. Retrograde tracer technique for assessment of selective and total subdiaphragmatic vagotomies. *Am J Physiol* 1987;253:R361-R70.
 23. Houpt TA, Frankmann SP, Berlin RA. Unilateral vagotomy attenuates c-Fos induction by cholecystokinin and bombesin in the contralateral nucleus of the solitary tract. *Soc Neurosci Abs* 1996;22:1413.
 24. Sofroniew MV, Isacson O. Distribution of degeneration of cholinergic neurons in the septum following axotomy in different portions of the fimbria-fornix: a correlation between degree of cell loss and proximity of neuronal somata to the lesion. *J Chem Neuroanat* 1988;1:327-37.
 25. Gu Y, Spasic Z, Wu W. The effects of remaining axons on motoneuron survival and NOS expression following axotomy in the adult rat. *Dev Neurosci* 1997;19:255-9.