*Neuroscience Letters*, 118 (1990) 141–146
Elsevier Scientific Publishers Ireland Ltd.



NSL 07202

# Anatomical evidence for multiple pathways leading from the rostral ventrolateral medulla (nucleus paragigantocellularis) to the locus coeruleus in rat

Bernadette Astier[2], Elisabeth J. Van Bockstaele[1,3], Gary Aston-Jones[1] and Vincent A. Pieribone[4]

*[1]Department of Mental Health Sciences, Hahnemann University, Philadelphia, PA 19102-1192 (U.S.A.), [2]Laboratoire de Neuropharmacologie, CNRS UMR 105, Faculté de Pharmacie, Université Claude Bernard, Lyon (France), [3]Department of Biology, New York University, New York, NY 10003 (U.S.A.) and [4]Department of Histology, Karolinska Institutet, Stockholm (Sweden)*

(Received 16 April 1990; Revised version received 4 June 1990; Accepted 19 June 1990)

*Key words:* Adrenergic; Afferent pathway; C1 group; Electrolytic lesion; Fluoro-Gold; Medullary bundle; Phenylethanolamine-*N*-methyl-transferase; Retrograde transport

Using retrograde transport of Fluoro-Gold (FG) combined with immunofluorescence for phenylethanolamine-*N*-methyltransferase (PNMT), we have examined afferents to the nucleus locus coeruleus (LC) from the rostral ventrolateral medulla (nucleus paragigantocellularis; PGi) in rats sustaining lesions of the medullary adrenergic bundle (MB). In lesioned rats, very few adrenergic LC-projecting neurons persist in the PGi ipsilateral to the lesion, representing a 90% decrease in comparison to non-lesioned animals. These results indicate that almost all adrenergic input to the LC from C1 neurons in PGi is conveyed by the MB. In contrast, the number of non-adrenergic LC afferent neurons in the PGi ipsilateral to the lesion only decreased by 48% after such lesions. Thus, this pathway also provides non-adrenergic projections to LC from PGi, but many of these are conveyed by other route(s) as well.

Retrograde tracing experiments revealed that the paragigantocellularis (PGi) located in the rostral ventrolateral medulla [1] is a major afferent to the rat nucleus locus coeruleus (LC) [4, 13]. While the location of these LC afferent neurons has been described in some detail [5, 14], the projection pathway(s) from the PGi to the LC has not been fully examined. Immunohistochemistry combined with retrograde labeling [13] has revealed that many (approximately 21%) of LC afferents from the PGi contain phenylethanolamine-*N*-methyltransferase (PNMT), a marker for putative adrenergic neurons. PNMT-immunoreactive (PNMT-IR) fiber projections from the PGi course rostrally and dorsally, entering the catecholamine medullary bundle (MB) at the level of rostral PGi, and exit at the level of the LC to innervate this structure, suggesting a pathway whereby C1 cells innervate LC [3, 8, 14]. This proposed pathway is consistent with studies [2] which demonstrate that the bulk of PNMT-IR fiber innervation of the LC is eliminated by lesion of the MB.

In contrast to these findings, *Phaseolus vulgaris*-leucoagglutinin (PHA-L) injections into subregions of PGi identified a more ventrolateral pathway to the LC, with fibers passing dorsolateral to the superior olive and proceeding rostrally to course dorsally at the level of the parabrachial complex, which they traverse as they move medially to innervate the LC [6]. These findings have been confirmed by our own recent studies using PHA-L [16].

Together these results indicate that there may be at least two distinct projection pathways from the PGi to the LC, with adrenergic fibers predominantly restricted to the more medial MB route. In the present study, we tested this hypothesis by examining retrograde labeling of PNMT-IR neurons in the PGi with injection of the tracer Fluoro-Gold (FG) in the LC of animals sustaining lesions of the MB. Our results confirm the possibility that the adrenergic inputs to the LC are almost entirely provided by the MB while non-adrenergic projections from PGi to LC project through the MB as well as by other route(s) [16].

Experiments were performed on 12 male Sprague–Dawley rats weighing 250–300 g. Animals were anesthetized with 400 mg/kg chloral hydrate, i.p., and placed in a stereotaxic instrument with the incisor bar lowered to place the skull approximately 30° from the horizontal plane. In 9 animals, a unilateral lesion of the MB was performed by electrolytic coagulation (4 mA DC current

*Correspondence:* G. Aston-Jones, Department of Mental Health Sciences, Hahnemann University, MS 403, Broad and Vine, Philadelphia, PA 19102-1192, U.S.A.

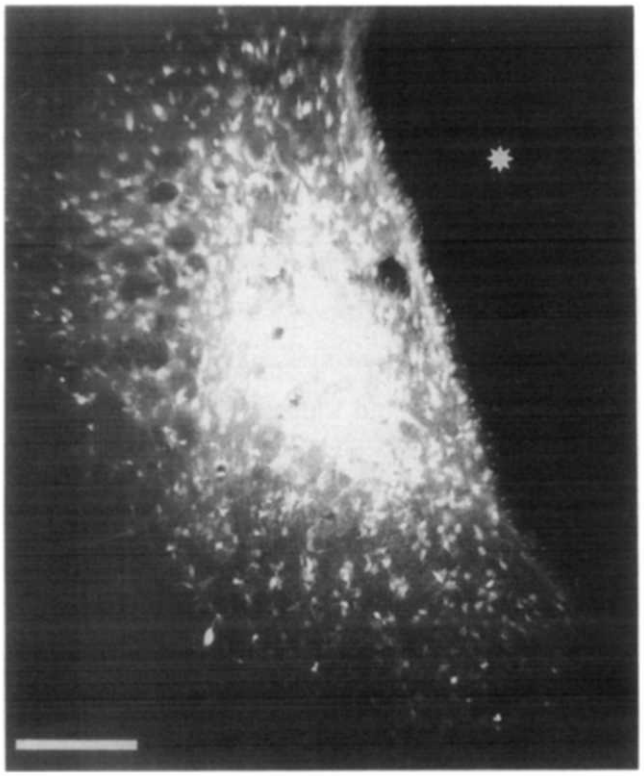

Fig. 1. An epifluorescent (UV illumination) photomicrograph of a coronal section showing a typical iontophoretic deposit of FG in the LC. Asterisk indicates the IVth ventricle. Bar = 245 $\mu$m.

for 5 s) with a monopolar stainless-steel electrode (250 $\mu$m diameter with an exposed tip length of 200 $\mu$m). The electrode was stereotaxically placed at the following coordinates: 2150 $\mu$m rostrally, 1600 $\mu$m ventrally and 1000 $\mu$m laterally with the calamus scriptorius serving as the reference point.

Two weeks after receiving lesions, animals were reanesthetized with chloral hydrate for FG injection into the LC ipsilateral to the lesion. As previously described [12], precleaned micropipettes with 10 $\mu$m tips were filled with a 1% solution of FG in 0.1 M sodium acetate buffer (pH 3.3). Extracellular unit recordings were obtained from the injection pipette and the LC was tentatively identified by its characteristic discharge properties. Iontophoretic deposits of FG were made using +1.0 $\mu$A of pulsed current (50% duty cycle) for 10 min. FG was similarly injected into the LC of non-lesioned rats ($n = 3$). Animals were allowed to survive 5 days and were then deeply anesthetized and perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed and immersed in the same fixative for 90 min at 4°C.

Frozen, 30-$\mu$m thick serial sections of the medulla and pons were taken coronally and processed for detection of PNMT by using a highly specific primary antiserum produced in a rabbit against bovine adrenal PNMT according to Joh and Goldstein [9], previously character-

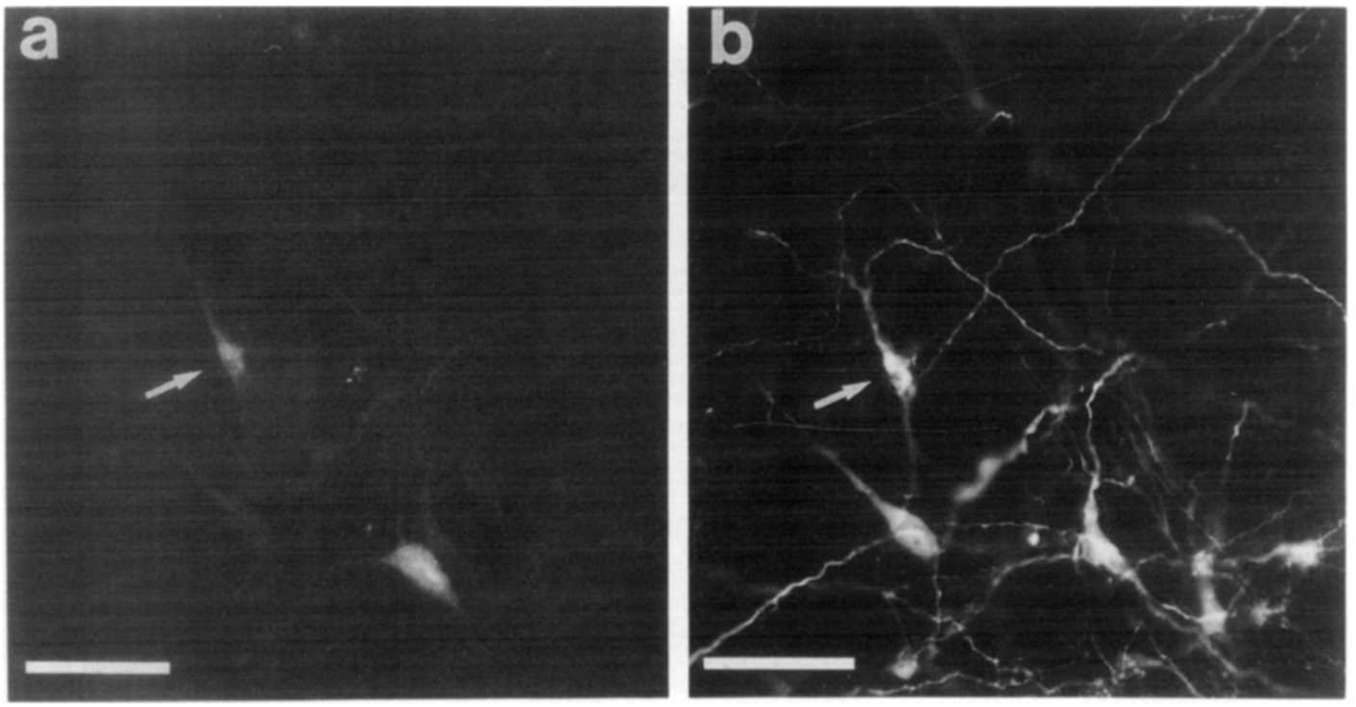


Fig. 2. a: an epifluorescent photomicrograph (UV illumination) showing neurons retrogradely labeled with FG from LC. Bar = 60 $\mu$m. b: a photomicrograph of the same field as in (a) showing PNMT-IR neurons in the PGi with RITC fluorescence. The neuron denoted by the arrows in (a) and (b) contains both retrogradely transported FG and PNMT immunofluorescence.

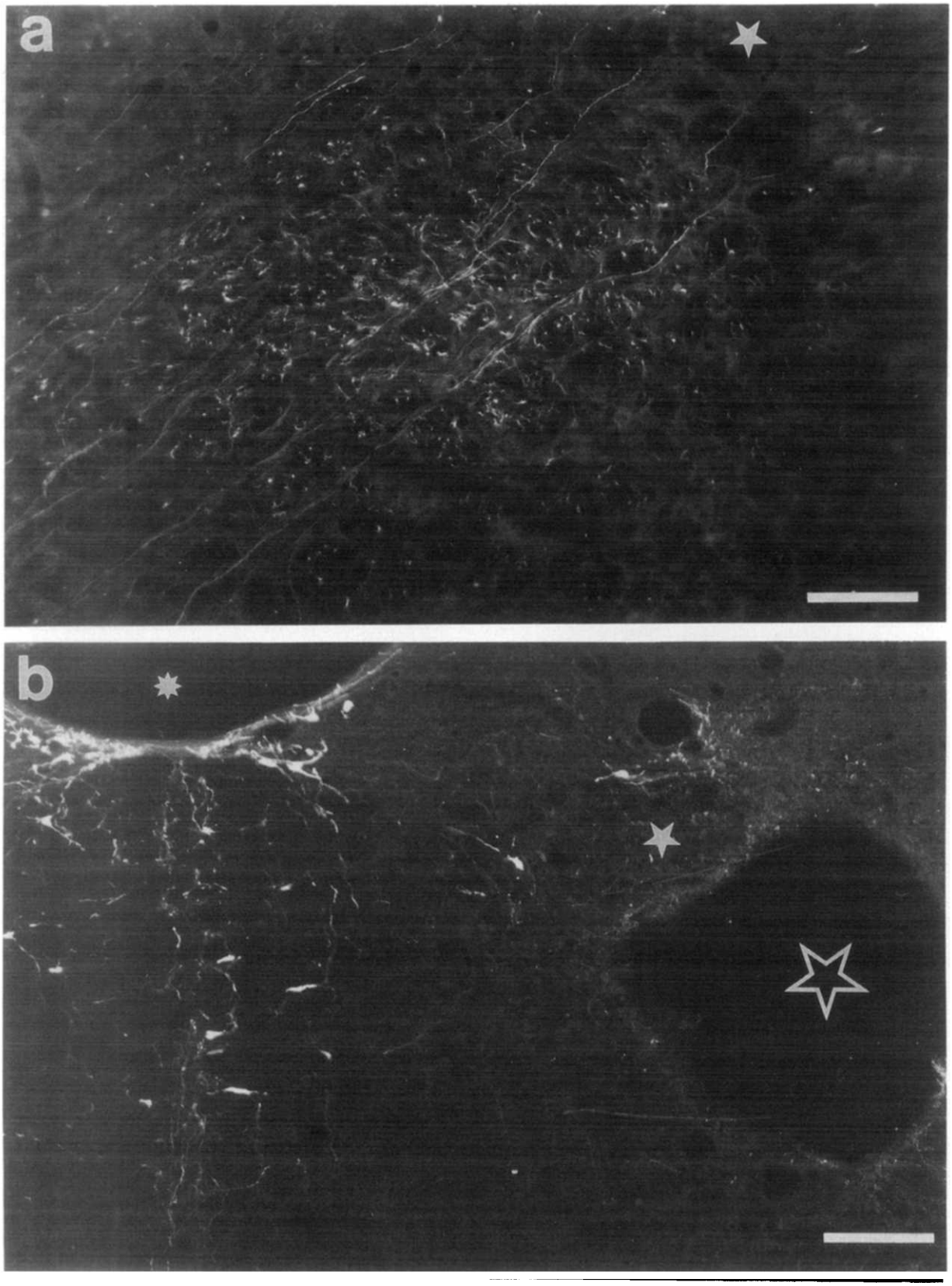


Fig. 3. Epifluorescent photomicrographs of coronal sections at the level of MB lesions, illuminated to reveal RITC labeled PNMT immunoreactivity. a: a field contralateral to a lesion of MB, showing fine PNMT-IR fibers cut in cross section in the MB ventrolateral to the intermediate reticular nucleus (at closed star). Bar = 120 $\mu$m. b: a field from the same brain as in (a) but through the lesion of MB (lesion at open star). Note PNMT-IR neurons of the CA3 group near midline on the floor of the IVth ventricle. Closed star indicates location of intermediate reticular nucleus on this side. Asterisk is on the midline in the IVth ventricle. Bar = 240 $\mu$m.

ized by Kitahama et al. [10]. In tissue sections, the PNMT antiserum did not stain dopaminergic (A9, A10) or noradrenergic neurons (A1–A7). Most sections were processed for rhodamine isothiocyanate (RITC)-immunofluorescent detection of PNMT; some sections of the pons were processed for PNMT using an improved peroxidase-anti-peroxidase technique previously described [10]. The primary antiserum was localized with affinity-purified, goat anti-rabbit antiserum conjugated to RITC (Boehringer Mannheim, Indianapolis, IN) according to Pieribone and Aston-Jones [12]. Sections were then dehydrated in alcohols and coverslipped in DPX mountant (DBH Limited, Poole, U.K.). FG was visualized under UV epi-illumination (330 nm excitation peak, low-end emission cut off at 450 nm), and RITC under green illumination (546 ± 4 nm peak). By alternating illumination wavelengths, neurons containing FG, RITC immunofluorescence, or both were discriminated and counted. All sections of the medulla were collected and examined for singly- or doubly-labeled neurons. Black and white photographs were made with Kodak Plus-X film (ASA 125) for fluorescent images and Panatomic-X (ASA 32) for brightfield images on a Leitz Aristoplan microscope.

Injections of FG using the above procedures were centered in the LC in all animals (Fig. 1). A significant advantage of our method was that the iontophoretic deposit of FG produced no damage at the site of injection, as previously described [12–14]. Animals with injection sites that apparently spread beyond the LC proper were not analyzed.

Examination of the lesion sites in PNMT-reacted material (Fig. 3a) revealed that the bulk of PNMT-immunofluorescent fibers in the MB were destroyed in 6 cases. In these cases, the great majority of PNMT-IR terminal fibers was eliminated in the ipsilateral LC, compared with sections of non-lesioned rats or to the contralateral LC of the lesioned rat (Fig. 4a, b). Animals with improperly placed lesions were not further analyzed.

In non-lesioned animals, cell counts indicated that 21% (mean ± S.E.M.: 154 ± 50 of 739 ± 79 FG-labeled cells) of the neurons of the ipsilateral PGi that contained retrogradely transported FG also exhibited PNMT-IR

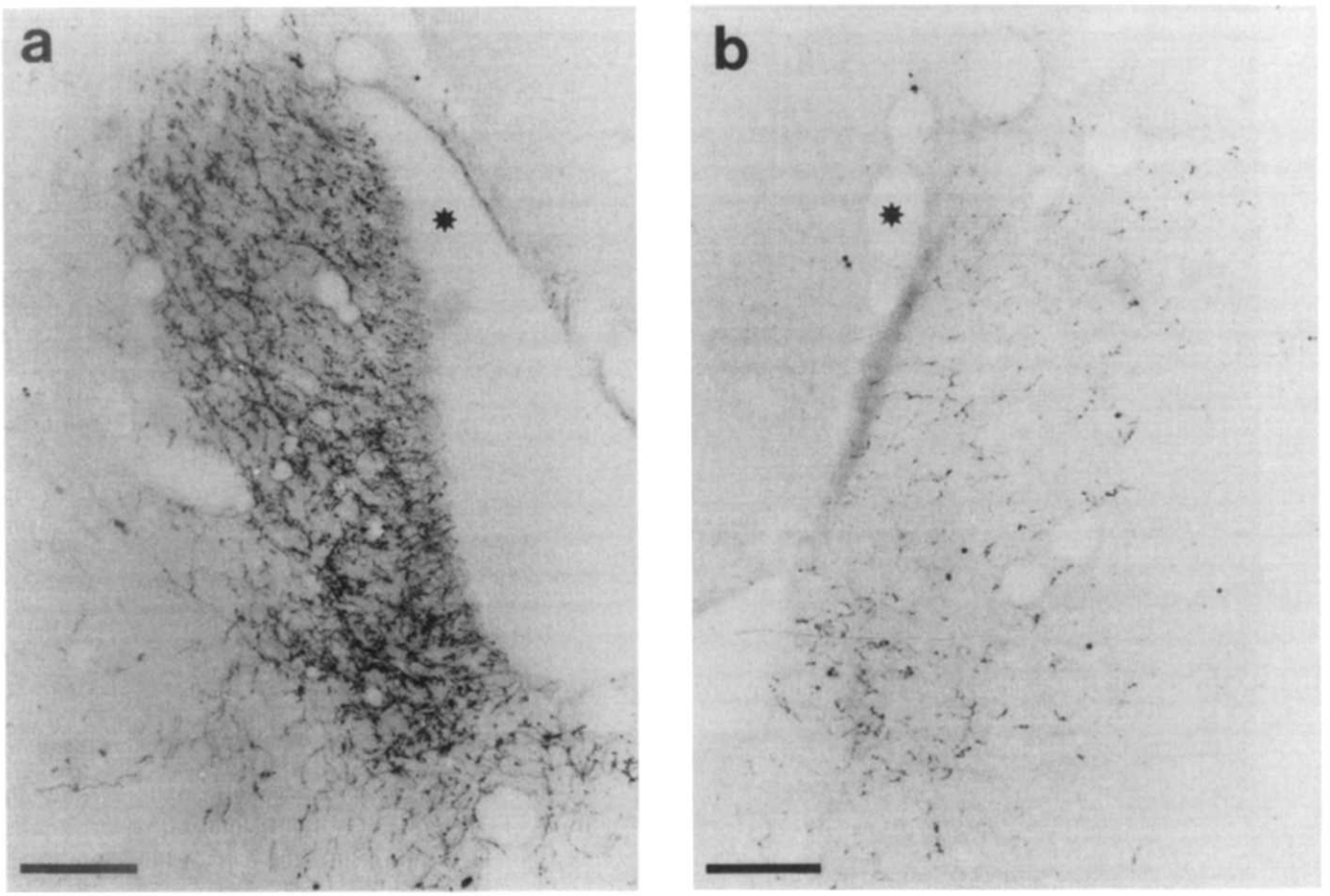


Fig. 4. a: bright-field photomicrograph of a coronal section showing dense PNMT-IR fiber innervation in the LC contralateral to an MB lesion. Asterisk denotes the IVth ventricle. b: a similar photomicrograph as in (a) but ipsilateral to the MB lesion showing a marked decrease of PNMT-IR fibers in LC (same animal as in Fig. 3). Asterisk denotes the IVth ventricle. Bars = 245 μm.

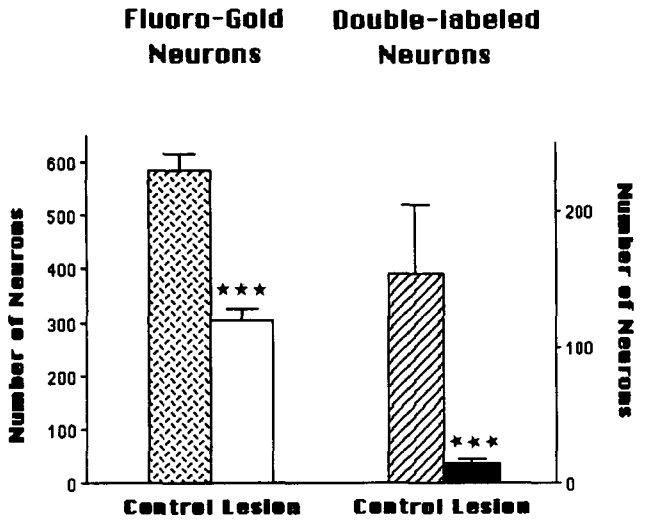


Fig. 5. Graph summarizing the results of cell counts obtained for control and lesioned animals. Fluoro-Gold neurons: data were collected for neurons labeled only with FG in the PGi. The mean number of FG-only neurons in ipsilateral PGi of control animals was $585 \pm 30$, and in lesioned animals was $303 \pm 23$, representing a decrease of 48%. Double-labeled neurons: data were collected for double-labeled FG/PNMT neurons. The mean number of doubly-labeled neurons in the ipsilateral PGi in control animals was $154 \pm 50$, and in lesioned animals was $15 \pm 3$, reflecting a decrease of 90%. $^{***}P < 0.001$ ($t$-test).

(Fig. 5). In the 6 animals containing MB lesions, there was a 90% decrease in the number of doubly labeled neurons in the ipsilateral PGi, and only 5% of LC afferents were PNMT-IR (mean $\pm$ S.E.M.: $15 \pm 3$ of $318 \pm 23$ FG-labeled cells). In contrast to the nearly total elimination of doubly labeled cells, these lesions reduced the number of FG-only labeled cells in the ipsilateral PGi by only 48% (mean $\pm$ S.E.M.: $303 \pm 23$ cells and $585 \pm 30$ cells in lesioned and non-lesioned animals, respectively).

In the present study, we found that 21% of LC afferent neurons in the PGi of intact rats co-localized with PNMT. This percentage is similar to previous results obtained with a different antibody raised against rat adrenal medulla PNMT [13]. However, these results differ from those of Guyenet and Young [6] who reported that 72% of PGi neurons retrogradely labeled with rhodamine-conjugated latex microspheres from the LC area are also PNMT-IR (using an antibody raised against bovine PNMT). However, in a more recent abstract [7], this same laboratory reported that only 36% of LC afferents in the PGi are also PNMT-IR. The discrepancy between the higher percentage reported [6] and our findings may be due to the fact that their injections were not restricted to LC; areas neighboring LC receive substantial adrenergic input [3, 8, 14] which may derive from C1 neurons [4, 6, 11, 16]. In addition, microbead injections such as theirs can create lesions so that some labeling could be due to uptake by non-terminal fibers of passage. This was not the case for our iontophoretic deposits of FG, which do not damage injection sites and do not label neurons by uptake into passing fibers [12].

The prominent decrease of PNMT-IR fibers in the LC, and of adrenergic LC afferents in the ipsilateral PGi of lesioned rats, demonstrates that the adrenergic input to the LC is mainly provided by ipsilateral C1 neurons projecting through the MB, as previously proposed [2, 13]. The residual adrenergic innervation in the LC following such lesions may originate (i) from PNMT-fibers joining the MB rostrally to the genu of the facial nerve [3] (i.e., rostral to the lesions) or (ii) from the contralateral C1 group [14].

It is interesting that the MB provides both the major adrenergic innervation to the LC as well as to the intermediolateral cell column (IML) in the spinal cord. It has been suggested that the C1 projections to the IML play an important role in cardiovascular regulation [15]. However, it is uncertain if such a function is also conveyed by the C1-LC projection, as it is not known whether the same C1 neurons project both to the LC and to the IML.

Furthermore, our results indicate that a large number of non-adrenergic PGi afferents to the LC also utilize this pathway. Nonetheless, the persistence of a substantial number of FB labeled cells in the lesioned rat indicates the existence of pathway(s) other than the MB from PGi to LC, consistent with results obtained with anterograde transport of PHA-L [6, 16].

In any case, the smaller percentage decrease in non-adrenergic LC afferent cells compared to adrenergic afferent cells in lesioned animals indicates that many non-PNMT LC-projecting neurons utilize pathway(s) other than the MB, while nearly all PNMT afferents to the LC from the PGi project via the MB. These different pathways may convey functionally different inputs to the LC, and are being further investigated.

We are particularly grateful to Dr. L. Denoroy (INSERM-U52, Lyon, France) for providing us antiserum to PNMT. We wish to thank Yan Zhu for excellent technical assistance and S. Aston-Jones for secretarial assistance. We are also grateful to the Philippe Foundation. This study was supported by PHS Grants NS 24698 and DA 06214.

1 Andrezik, J.A., Chan-Palay, V. and Palay, S.L., The nucleus paragigantocellularis lateralis in the rat. Conformation and cytology, Anat. Embryol., 161 (1981) 335–371.

2 Astier, B., Kitahama, K., Denoroy, L., Jouvet, M. and Renaud, B., Immunohistochemical evidence for the adrenergic medullary longitudinal bundle as a major ascending pathway to the locus coeruleus, Neurosci. Lett., 74 (1987) 132–138.

3 Astier, B., Kitahama, K., Denoroy, L., Jouvet, M. and Renaud, B., Additional adrenergic projection pathways in the rat brain: an immunohistochemical study using a phenylethanolamine-*N*-methyl-transferase antibody, 13th Annual meeting of the European Neuroscience Association, Stockholm, in press.

4 Aston-Jones, G., Ennis, M., Pieribone, V.A., Nickell, W.T. and Shipley, M.T., The brain nucleus locus coeruleus: restricted afferent control of a broad efferent network. Science, 234 (1986) 734–737.

5 Aston-Jones, G., Shipley, M.T., Ennis, M., Williams, J.T. and Pieribone, V.A., Restricted afferent control of locus coeruleus neurons revealed by anatomic, physiologic and pharmacologic studies. In C.A. Marsden and D.J. Heal (Eds.), The Pharmacology of Noradrenaline in the Central Nervous System, Oxford University press, Oxford, in press.

6 Guyenet, P.G. and Young, B.S., Projections of nucleus paragigantocellularis to locus coeruleus and other structures in the rat, Brain Res., 406 (1987) 171–184.

7 Haselton, J.R. and Guyenet, P.G., PNMT neurons of the C1 group with projections to both locus coeruleus and spinal cord, Soc. Neurosci. Abstr., 13 (1987) 809.

8 Hökfelt, T., Johansson, O. and Goldstein, M., Central catecholamine neurons as revealed by immunohistochemistry with special reference to adrenaline neurons. In A. Björklund and T. Hökfelt (Eds.), Handbook of Chemical Neuroanatomy, Vol. 2, Classical Transmitters in the CNS, Elsevier, Amsterdam, 1984, pp. 157–276.

9 Joh, T.H. and Goldstein, M., Isolation and characterization of multiple forms of phenylethanolamine-*N*-methyltransferase, Mol. Pharmacol., 9 (1973) 117–129.

10 Kitahama, K., Pearson, J., Denoroy, L., Kopp, N., Ulrich, J., Maeda, T. and Jouvet, M., Adrenergic neurons in human brain demonstrated by immunocytochemistry with antibodies to phenylethanolamine-*N*-methyl-transferase (PNMT). Discovery of a new group in the nucleus tractus solitarius, Neurosci. Lett., 53 (1985) 303–308.

11 Loewy, A.D., Wallach, J.H. and Mckellar, S., Efferents projections of the ventral medulla oblongata in the rat, Brain Res. Rev., 3 (1981) 63–80.

12 Pieribone, V.A. and Aston-Jones, G., The iontophoretic application of Fluoro-Gold for the study of afferents to deep brain nuclei, Brain Res., 475 (1988) 259–271.

13 Pieribone, V.A., Bohn, M.C. and Aston-Jones, G., Adrenergic and non-adrenergic afferents to the nucleus locus coeruleus from the C1 and C3 adrenergic cell groups, Neurosci. Lett. 85 (1988) 297–303.

14 Pieribone, V.A. and Aston-Jones, G., Adrenergic innervation of the rat nucleus coeruleus arises predominantly from the C1 adrenergic cell group in the rostral medulla, Neuroscience, in press.

15 Ross, C.A., Ruggiero, D.A., Park, D.H., Joh, T.H., Sved, A.F., Fernandez-Pardal, J., Saavedra, J.M. and Reis, D.J., Tonic vasomotor control by the rostral ventrolateral medulla: effect of electrical or chemical stimulation of the area containing C1 adrenaline neurons on arterial pressure, heart rate and plasma catecholamine and vasopressin, J. Neurosci., 4 (1984) 479–494.

16 Van Bockstaele, E.J., Astier, B., Pieribone, V.A., Aston-Jones, G. and Shipley, M.T., Multiple projection pathways from the ventrolateral medulla to locus coeruleus in rat, Soc. Neurosci. Abst., 15 (1989) 1013.