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The Brain Nucleus Locus Coeruleus: Restricted Afferent Control of a Broad Efferent Network

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Dense, focal injections of wheat germ agglutinin conjugated–horseradish peroxidase in the locus coeruleus of rats labeled afferent neurons in unexpectedly few brain regions. Major inputs emanate from only two nuclei—the paragigantocellularis and the prepositus hypoglossi, both in the rostral medulla. The dorsal cap of the paraventricular hypothalamic nucleus and the spinal intermediate gray are possible minor afferents to locus coeruleus. Other areas reported to project to locus coeruleus (for example, amygdala, nucleus tractus solitarius, and spinal dorsal horn) did not exhibit consistent retrograde labeling. Anterograde tracing and electrophysiologic experiments confirmed the absence of input to locus coeruleus from these areas, which instead terminate in targets adjacent to locus coeruleus. These findings redefine the anatomic organization of the locus coeruleus, and have implications for hypotheses concerning the functions of this noradrenergic brain nucleus.

THE LOCUS COERULEUS (LC) HAS attracted intense interest in the last two decades, largely because of its pervasive noradrenergic fiber projections throughout the central nervous system (CNS). A considerable body of evidence implicates this nucleus in global brain functions such as emotion and vigilance, as well as in the etiology of mental disorders such as depression and dementia of the Alzheimer type (*1, 2*). At the cellular level, perhaps more is known about the anatomical projections and postsynaptic effects of the LC than any other system in brain (*3*). Recent studies have also revealed a homogenous set of discharge properties for these cells and shown that specific behavioral and sensory events evoke concerted activity of LC neurons (*4, 5*; reviewed in *6*).

In contrast to this knowledge of the physiology, pharmacology, and efferent anatomy of the LC system, little is known about the neural inputs that regulate activity in this nucleus. Specification of the afferents that control LC discharge is critical to understanding the neural circuitry in which this system functions and, therefore, to generating hypotheses concerning its role in brain and behavioral processes.

In previous attempts to define inputs to rodent LC, injections of horseradish peroxidase (HRP) were used to label afferent neurons by retrograde transport. In the few studies of this type (*7*), many CNS structures were found to contain retrogradely labeled cells, but no functional pattern of afferent innervation emerged. Since these initial neuroanatomic studies were conducted, retrograde tracing techniques have improved substantially. In particular, recent methods employing the tracer wheat germ agglutinin–conjugated HRP (WGA-HRP), combined with the histochemical substrate tetramethylbenzidine (TMB), are many times more sensitive than techniques utilizing nonconjugated HRP as the tracer and diaminobenzidine as the substrate (as in the above studies) (*8*). Thus, we reexamined inputs to LC, taking advantage of the more sensitive techniques presently available; we anticipated that additional afferents to LC might be found. However, we found that the LC is innervated by only a few brain regions, with the bulk of afferents arising from two rostral medullary nuclei. These results substantially redefine the circuit relationships of this pervasive noradrenergic brain system and, therefore, have a direct bearing on hypotheses of LC function.

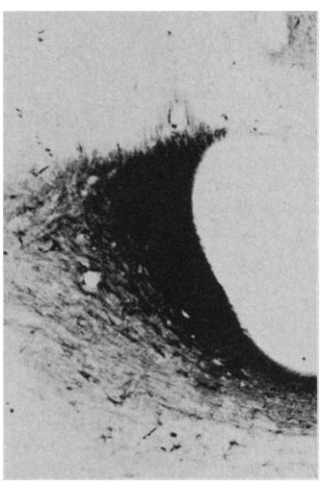

Fig. 1. LC injection site. Bright-field photomicrograph of a coronal section through LC of an experimental rat brain. WGA-HRP was iontophoresed into LC and tissue processed with TMB after a 24-hour survival period (*9, 11*). Note the dense deposit of tracer (black area) confined to the LC. Medial (fourth ventricle) is to the right and dorsal is at the top.

Dense, focal injections of WGA-HRP were made by iontophoresis from glass micropipettes (*9*) in adult, male Sprague-Dawley rats. Single cell recordings through the injection pipette were used to accurately place injections [for example, LC neurons were recognized by their distinctive waveform and responsiveness to sensory stimuli (*3, 10*)]. Eighteen to thirty-six hours after injection, brains were prepared for HRP histochemistry with TMB as the substrate (*11*).

Our conclusions are based on an analysis of injection sites centered unilaterally in the LC (33 cases) (Fig. 1). Neurons were considered retrogradely labeled if at least ten TMB reaction granules were visible within the Nissl-stained cell. Injections confined to

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the LC labeled numerous neurons consistently and strongly in only two areas—the nucleus prepositus hypoglossi (PrH) and the nucleus paragigantocellularis (PGi), both in the medulla. Labeled neurons in the PrH, located in rostral dorsomedial medulla, are densely aggregated along the dorsolateral border of the medial longitudinal fasiculus (mlf) where it meets the fourth ventricle; some cells were seen also scattered along the lateral aspects of, and occasionally within, the mlf (Fig. 2, A and B). Retrograde labeling in PrH is bilateral, but slightly greater contralaterally. The second major source of afferents to LC, and the most prominent (in terms of number of labeled neurons), is PGi as defined by Andrezik *et al.* (*12*), located in rostral ventrolateral medulla. Retrogradely labeled PGi neurons are centered slightly caudal to labeled PrH cells and are predominantly, though not exclusively, ipsilateral to the injection site. Labeled cells are scattered between the rostral lateral reticular nucleus and the caudal facial nucleus and between the inferior olive and the spinal trigeminal nucleus (Fig. 2, C and D). Some labeled neurons are present just dorsal to the lateral aspect of the inferior olive, and others reside medial to the facial nucleus, near its caudal limit.

The dorsal cap of the paraventricular hypothalamic nucleus (PVH) and the intermediate gray of the spinal cord (SpC) also exhibited weak but consistent retrograde labeling. For the dorsal cap cells, labeling was bilateral but slightly greater ipsilaterally. Neurons in more central portions of the PVH were only labeled when injections substantially exceeded the boundaries of LC (see below). Labeled SpC neurons were scattered in the intermediate gray of the cord near the central canal, predominantly contralaterally. Many of these cells were so weakly labeled as to be on the threshold of detection.

The ventral tegmental area (VTA), dorsal and median raphe nuclei, and lateral as well as rostral hypothalamus contained a few weakly labeled cells in some animals, but were unlabeled in many others.

Our results for retrograde labeling, therefore, indicate that the LC receives inputs from only two major (PrH and PGi) and possibly two minor (PVH and SpC) sources. However, previous tracing studies (*7, 13*) describe afferents to LC from many areas that lack consistent retrograde labeling in our material (for example, amygdala, spinal dorsal horn, nucleus tractus solitarius, and VTA). To investigate the cause of these discrepancies, we performed several additional experiments.

First, to assess the possibility that our small iontophoretic injections failed to label all afferents, we made injections in the occipital cortex (border between areas 17 and 18) using the same procedures as for our LC injections. Retrogradely labeled cells were found in all sites that are known to project to rat occipital cortex (*14*). This result indicates that our injection method labeled all known afferents in another, well-characterized system.

Second, examination of cases with LC injections that impinged on neighboring parabrachial, vestibular, or central gray nuclei revealed substantial retrograde labeling in areas previously reported to project to LC but that remain unlabeled after restricted LC injections (for example, amygdala, spinal dorsal horn, nucleus tractus solitarius, and insular cortex). Thus, with injections exceeding the boundaries of the LC, our results are similar to previous reports (*7, 13*). This suggests that tracer uptake in areas nearby but outside the LC may have substantially contributed to previously reported labeling. This possibility is consistent with our physiologic studies revealing robust antidromic activation in PGi and PrH, but not in rostral nucleus tractus solitarius (NTS) and other areas of discrepancy, after focal electrical stimulation of LC (*15*).

Third, large injections of WGA-HRP were made into areas reported to project to LC (*7*) and the resulting patterns of antero-

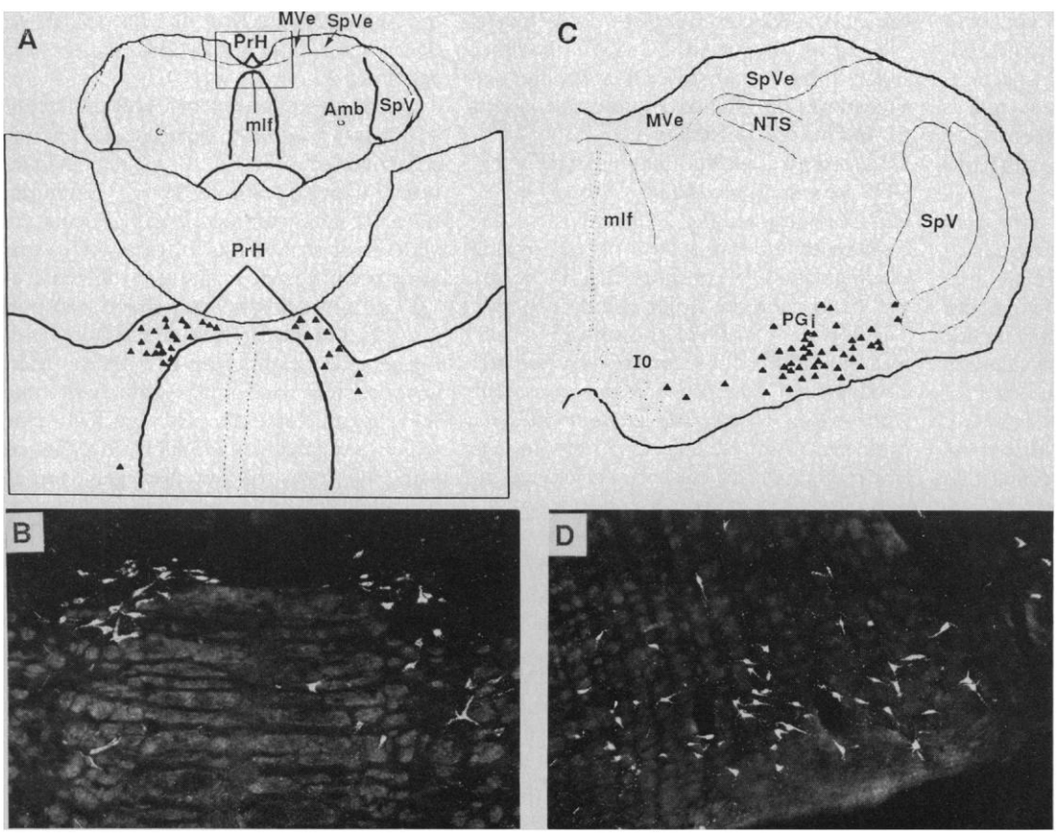

Fig. 2. Major retrogradely labeled LC afferents, in PrH (A and B) and in PGi (C and D). (A) Video computer-aided plot (Nikon/Joyce-Loebl Magiscan) of retrogradely labeled neurons in a coronal section through rostral medulla after an injection of WGA-HRP into LC. Low- (upper) and high-power (lower) views of the same section are given for orientation. Amb, nucleus ambiguous; mlf, medial longitudinal fasiculus; MVe, medial vestibular nucleus; PrH, prepositus hypoglossal nuclei; SpV, spinal trigeminal nucleus; SpVe, superior vestibular nucleus. (B) High-power, dark-field, polarized-light photomicrograph of retrogradely labeled neurons in PrH. Same orientation as in (A). (C) Computer-aided plot of retrogradely labeled neurons in a coronal section through PGi [slightly caudal to section in (A)] after an injection of WGA-HRP into LC ipsilaterally. Midline is at left. IO, inferior olive; NTS, nucleus tractus solitarius; PGi, paragigantocellularis lateralis; other abbreviations are as in (A). (D) High-power, dark-field, polarized-light photomicrograph of retrogradely labeled neurons in PGi. Same orientation as in (C).

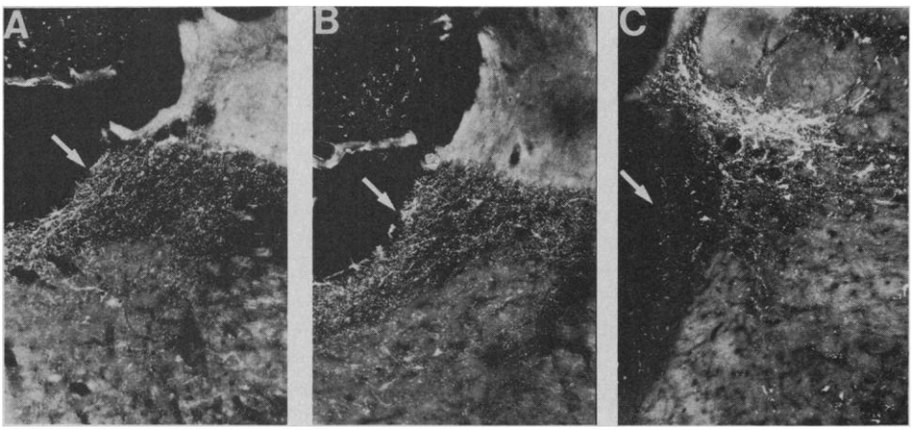

Fig. 3. Anterogradely transported fiber labeling. (A) Dark-field, polarized-light photomicrograph of coronal section showing dense innervation of LC (at arrow) by WGA-HRP–labeled fibers after an injection into PrH. (B) Photomicrograph as in (A), except following injection of WGA-HRP into PGi. Compare dense labeling within LC in (A) and (B) to contrasting labeling seen in (C). (C) Photomicrograph as in (A), except after injection of WGA-HRP into the central nucleus of the amygdala. Note dense innervation of parabrachial area (immediately lateral and dorsal to LC) by labeled fibers, but lack of fiber labeling within LC (at arrow).

grade labeling were assessed. Injections into the central nucleus of the amygdala, previously reported to be the major afferent to LC (*7*), revealed no afferent fiber labeling in LC, but instead produced dense anterograde and retrograde labeling in the nearby ventromedial parabrachial nucleus (Fig. 3C). Injections in the dorsal horn of the thoracic spinal cord, rostral NTS, and VTA yielded similar results—anterograde labeling was absent in LC but present in neighboring parabrachial and central gray nuclei. Sparse, scattered fibers were observed in LC from caudal NTS injections; however, since retrogradely labeled neurons are consistently absent in this area after our dense injections confined to LC, we conclude that such fiber labeling reflects transit axons projecting to the adjacent, heavily labeled parabrachial nucleus.

Finally, to confirm our own data from retrograde transport studies, anterograde labeling was examined after WGA-HRP injections into PGi and PrH, the two major afferents to LC identified in our retrograde analysis. Anterograde labeling was dense in LC after injection into either PGi or PrH (Fig. 3, A and B).

These results are consistent with our recent physiologic experiments revealing that electrical stimulation of PGi at low intensities reliably yields short-latency robust excitation in LC neurons (Fig. 4) (*16*). In contrast, stimulation of the central nucleus of the amygdala apparently yields no direct synaptic response in LC but markedly excites nearby parabrachial neurons (*16*). Therefore, at least for this major input to areas adjacent to the LC, possible innervation of distal, extranuclear LC dendrites (*17*) does not appear to be a significant afferent mechanism.

Other possible sources of afferents to LC are more difficult to examine with tracing methods because of their proximity to the LC. In particular, parabrachial and vestibular nuclei often contained labeled cells located within the relatively faint "halo" of the injection site centered in LC. We further examined these structures in eight rats, using very small injections of WGA-HRP in LC (<0.5 μA for 5 minutes) and short survival times (2 to 8 hours). In three cases there was no halo in regions adjacent to LC and no retrograde labeling in parabrachial or vestibular nuclei, even though positive neurons were present in PGi. We therefore suggest that these adjacent areas are not afferent to LC; additional studies are needed to confirm this possibility.

In summary, we find that remarkably few CNS areas are directly afferent to LC in rat. Only two nuclei, the PGi and the PrH, consistently exhibit substantial retrograde labeling, and we conclude that these cell groups provide the major afferents to LC. The dorsal cap of PVH and spinal intermediate gray also exhibit consistent retrograde labeling after confined LC injections, but with sparse, weakly labeled neurons. Because areas near LC injection sites (for example, parabrachial nuclei) may receive inputs from these structures, conclusions concerning these weakly labeled nuclei must remain tentative until confirmed by anterograde transport studies. We therefore consider these to be possible minor LC afferents.

The PGi area is a crossroads for circuitry pertaining to autonomic neuron integration as well as polymodal environmental and painful stimuli (*12, 18*). Each of these activities may influence LC through PGi projections; our results, in light of the heavy PGi projection to the intermediolateral cell column of the spinal cord (*19*) and the observations by Svennson and colleagues that LC discharge parallels sympathetic nerve activity (*20*), raise the possibility that a set of PGi cells may coregulate LC and sympathetic preganglionic neurons. In addition, we and others (*2–5*) have found that LC neurons exhibit pronounced excitation following painful or polymodal nonnoxious stimuli (in waking animals), suggesting that the LC is strongly regulated by afferents that integrate sensory information across modalities, such as PGi. This latter possibility is consistent with our recent finding that LC is strongly activated by PGi stimulation (*16*).

The physiology and anatomy of the PrH have led many investigators to conclude that this nucleus is involved in the control of gaze (*21*). However, it is only recently that PrH has received close experimental investigation, and such a characterization may be incomplete. As McCrea *et al.* (*22*) point out, "When one considers the diversity of afferents as well as efferents from the prepositus, it is apparent that this nucleus is more than a simple site for oculomotor or precerebellar activity." Possible nonoculomotor functions of the PrH may be performed by adrenergic neurons located in this region (*23*), which may innervate LC. Additional studies are under way to determine the circuitry and physiologic functions of the subsets of PGi and PrH neurons that are afferent to LC to better characterize the functional influences of these projections on the LC system.

Our study indicates that a large number of structures previously reported to provide substantial input to LC (*7, 13*) remain unlabeled following restricted WGA-HRP injections in LC. Additional experiments revealed that at least four of these areas (that have been regarded as strong afferents to LC) do not support anterograde transport into LC, but instead yield robust fiber labeling in areas neighboring LC. These results are consistent with other recent studies that demonstrate that the parabrachial area, which is adjacent to LC, is strongly innervated by many of the discrepant nuclei, including the insular cortex, central nucleus of the amygdala, spinal dorsal horn, VTA, and NTS (*24*). Thus, it may be that many structures containing retrogradely labeled cells in previous studies are attributable to spread of injected tracer, and consequent uptake by terminals in areas adjacent to LC. This conclusion is supported by results of our recent physiologic experiments, described above. In retrospect, it is not surprising that previous HRP studies attributed numerous afferents to LC inasmuch as the

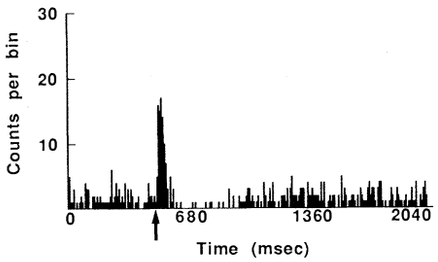

Fig. 4. Time histogram of discharge from an LC neuron before and after stimulus, accumulated during 50 trials of PGi stimulation (0.5 stimuli per second, 400 μA intensity, presented at arrow). Note short-latency (10-msec onset), robust excitation. Inhibition after the activation may be due to intracoerulear feedback mechanisms (*3, 28*).

extensive inputs to the adjacent parabrachial area were not known at that time.

Several studies have found that a number of different neurotransmitter systems appear to innervate LC (*3*), and this has generally fostered the notion that LC receives afferents from a large number of sources. It is noteworthy, however, that the few nuclei afferent to LC identified in our study contain neurons that stain for markers of most of these same neurotransmitters. For example, neurons in the PGi and PrH areas have been reported to stain positively for markers of serotonin, enkephalins, substance P, acetylcholine, neurotensin, corticotropin-releasing factor, and somatostatin (*25*), and Hokfelt *et al.* and others (*23*) have provided evidence for major adrenergic cell groups within both the PrH and PGi. Studies are in progress to determine the neurotransmitters used by the presently identified afferents to LC as it is possible that multiple transmitter systems impinge on LC from only a few nuclei.

There are several implications of this study for LC function. (i) Because so few cell groups project directly to LC, the diverse sensory and behavioral events that influence LC discharge probably act on this nucleus by common pathways. (ii) Because signals reaching the LC are thus likely to be highly preprocessed, and LC neurons exhibit uniform, concerted activity (*2–5, 26*), it is unlikely that this nucleus is engaged in complex processing of several types of information. Rather, the LC may weigh activity in inputs from two major sources, and widely distribute a uniform message over its divergent efferents (*5, 26*). (iii) By far, the preponderance of afferent control over the LC emanates from the medulla; there is virtually no forebrain control of this nucleus, which itself pervasively innervates most of the forebrain. Therefore, processes associated primarily with forebrain areas (such as, memory, emotion, learning, or fear and anxiety) (*27*) probably do not require the LC for their primary functioning, although such functions may be gated or modulated as part of a more global function of LC [for example, in behavioral orientation and the level of vigilance (*5, 26*)]. Thus, the proposed role of LC in such forebrain processes may require reexamination. (iv) Our results emphasize the need to learn more about the anatomy and physiology of PGi and PrH. The integrative functions of these two medullary nuclei hold the key to understanding the functional role of LC in brain and behavioral processes.

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