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Descending Projections from the Prefrontal Cortex to the Locus Coeruleus of the Rat

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The fiber projection from the prefrontal cortex to the locus coeruleus (LC) in the periventricular region was analyzed in rat using anterograde and retrograde tracing methods. Following injection of an anterograde tracer, Phaseolus vulgaris leucoagglutinin (PHA-L), into prelimbic and infralimbic regions of the medial prefrontal cortex, labeled axonal fibers with varicosities were observed bilaterally within the LC, with *ipsilateral predominance*. *Terminal labeling was also observed* in the region medial to the nucleus at rostral to middle levels of the LC, whereas axonal labeling in the caudal LC was minimal. Anterogradely-labeled axonal fibers were not found in the subcoerulear region. A retrograde tracer, gold-conjugated and inactivated wheatgerm-agglutinin horseradish-peroxidase (WGA-apo-HRP-gold), was injected into several rostro-caudal levels of the LC. Majority of retrogradely-labeled cells were observed in the prelimbic or infralimbic regions of the medial prefrontal cortex when the injections were made into rostral to middle levels of the LC. Only a few cells were observed in cingulate, dorsal peduncular, orbital, or insular cortices. The present findings suggest that the nucleus LC receives restricted, excitatory inputs from cognitive, emotional, and autonomic centers of the cerebral cortex and might secondarily have influences on widespread brain regions via its diversified monoaminergic innervation.

The nucleus locus coeruleus (LC) in the periventricular region is a compact nucleus that projects fibers into various areas of the cerebral cortex, diencephalon, brainstem, cerebellum, and spinal cord (Grzanna and Molliver, 1980; Felten and Sladek, 1983). The LC and its efferent projections have been suggested to be involved in brain functions such as paradoxical sleep, facilitation and inhibition of sensory neurons, and wakefulness of the animal via cortical activation. Although the broad influence of this brainstem monoaminergic cell group on the cerebral cortex is well-established, evidence for descending cortical projection to the LC is much less investigated.

Descending cerebro-cortical projections to the LC have been analyzed using anterograde labeling techniques and functional significance of this pathway has been implicated in monkey (Arnsten and Goldman-Rakic, 1984; Chiba et al., 2001). Previous studies reported that the dorsomedial and dorsolateral parts of the prefrontal cortex might be the major cortical input to the LC which might modulate emotional and autonomic parameters of the animal (Arnsten and Goldman-Rakic, 1984). The role of the ventromedial prefrontal lobe in autonomic and limbic functions was recently suggested based on the fact that individuals with lesions of this area are unable to generate autonomic responses to emotional stimuli (Neafsey, 1990; Hurley et al., 1991; Bacon and Smith, 1993; Buchanan and Powell, 1993; Neafsey et al., 1993; Bechara et al., 1994). Physiological characteristics of subregions of the ventromedial prefrontal lobe were further specified in that the viscerosensory inputs reach specific areas within the agranular insular cortex in primates as in rodents, whereas infralimbic and prelimbic regions are involved in autonomic motor functions (Carmichael and Price, 1994; Chiba et al., 2001).

The present study was designed to further investigate anatomical organization of the projection from the prefrontal cortex into the LC in the periventricular region in rat. An anterograde tracing study employing Phaseolus vulgaris leucoagglutinin (PHA-L) was performed to examine the projection from medial prefrontal cortex to the LC. In the second series of experiments, a retrograde tracer, gold-conjugated and inactivated wheatgerm-agglutinin horseradish-peroxidase (WGA-apo-HRP-gold or WG), was injected into several rostro-caudal levels of the LC and distribution of retrogradely-labeled neurons at various regions of the prefrontal cortex was investigated. The result of the anterograde and retrograde labeling studies

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on the projection from the prefrontal cortex into the monoaminergic cell group would provide the basis for functional implications of this descending pathway and its subsequent effect on widespread efferent targets of the LC in rat.

Materials and Methods

A total of twenty Sprague-Dawley rats including both sexes and ranging in weight from 300 to 350 g were used in this study. Prior to surgery, each rat was anesthetized with intraperitoneal injection of chloral hydrate (3.6% in distilled water, 1 ml/100 g body weight).

PHA-L injection and immunocytochemistry

An anterograde tracer, PHA-L, was injected within prelimbic or infralimbic regions of the medial prefrontal cortex and labeled axonal fibers and terminal arborizations were investigated at the LC. The location of the cortical injection site was determined from the atlas of Paxinos and Watson (1998). A glass micropipette (tip diameter, 10-15 μm) containing 2.5% PHA-L (Vector, L-1110) was stereotaxically lowered over the medial prefrontal cortex. The tracer was then ejected iontophoretically with 5 μA pulsed current on a 5-sec duty cycle for 20-30 min. Post-injection survival time ranged from 7 to 10 d.

The animals were perfused using 150 ml of saline followed by 300 ml of 4% paraformaldehyde in 0.1 M acetate buffer (pH 6.5) and 300 ml of 4% paraformaldehyde in 0.05 M borate buffer (pH 9.5). The brain was removed and stored in the borate fixative containing 30% sucrose overnight. Tissue blocks containing the prefrontal cortex or the LC were sectioned at the thickness of 50 μm using a cryostat. A series of every 5th section was collected in tissue-culture wells. The sections were washed with 0.02 M potassium-phosphate buffered saline (KPBS, pH 7.4) followed by 1 h incubation in 2% normal horse serum (NHS, Vector, S-2000) with 0.3% Triton X-100. The sections were then incubated for 24-48 h at 4°C in goat anti-PHA-L (Vector, AS-2224) diluted 1:1000 in KPBS with Triton and 2% NHS. After rinses, sections were incubated for 90 min in biotinylated horse anti-goat IgG (Vector, BA-9500). They were then incubated in the avidin-biotin complex (ABC, Vector, PK-6100) for 90 min. Sections were reacted with the peroxidase substrate 3, 3'-diaminobenzidine (DAB, Vector, SK-4100) at 4°C for 2-3 min. The nickel solution in this kit was often added into the DAB solution to make terminal fibers and varicosities dark-brown.

Dopamine-beta-hydroxylase (DBH) immunocytochemistry

Following detection of PHA-L labeled fibers, the LC sections were subsequently processed for DBH immunocytochemistry to localize the PHA-L labeled axonal fibers and varicosities contacting the norepinephrine-containing LC cells. Sections were washed in 0.1 M tris-buffered saline (TBS, pH 7.4) and incubated in 0.5% H_2O_2 in TBS for 20 min to inhibit endogenous peroxidase activity. After rinses, sections were incubated in 10% normal rabbit serum (NRS, Vector, S-5000) for 1 h to block nonspecific binding. After the buffer rinse, free-floating sections were incubated in 1:1000 dilution of mouse monoclonal anti-bodies against DBH (Chemicon, MAB308) for 24-48 h (4°C). Sections were then incubated in 1:100 dilution of rabbit anti-mouse IgG (Chemicon, AP160). After rinses, they were incubated in mouse peroxidase-antiperoxidase (PAP, Chemicon, PAP14) at a dilution of 1:500. Sections were stained with DAB (Vector, SK-4100) at 4°C for 1-2 min. Positive tissue controls were performed using sections from other noradrenergic brainstem regions including the solitary nucleus and the dorsal motor nucleus of the vagus nerve. Negative control experiments involved omission of the primary antibody and substituting it with equally diluted normal serum or reacting a series of sections with increasing dilutions of the primary antibody until all staining was lost. Similarly, omission of the secondary antibody or the PAP solution was also performed to test whether either the reagents or the procedures would give rise to non-specific staining.

WGA-apo-HRP-gold (WG) injection

In the second series of experiments, WG was injected into the LC and retrogradely-labeled cells were investigated at the prefrontal cortex. The location of the LC injection site was determined from the atlas of Paxinos and Watson (1998). The WG was synthesized using inactivated wheatgerm-agglutinin horseradish-peroxidase (Sigma, L-0390) and 10 nm colloidal gold (Sigma, G1527), as described in Basbaum and Menetrey (1987). The injection apparatus consisted of a glass micropipette (tip diameter, 20-30 μm) hydraulically linked to a 2.0 μl Hamilton syringe. A total volume of 0.2-0.3 μl of WG was pressure-injected into a single site within the LC over a 30-min period. Post-injection survival time ranged from 3 to 5 d.

Silver enhancement reaction

The animals were perfused using 150 ml of saline followed by a fixative containing 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS, pH 7.4). The brain was removed and stored in PBS containing 30% sucrose overnight. Tissue blocks containing the prefrontal cortex or the LC were sectioned at the thickness of 50 μm using a cryostat. A series of every 5th section was collected in tissue-culture wells. An injection site at the LC as well as retrogradely-labeled neurons at the prefrontal cortex was detected using a commercial silver intensification kit (Sigma, SE-100), as described in L-Smith et al. (1992). Prefronto-cortical sections were mounted, dried, and counter-stained with 1% neutral red to examine the location of retrogradely-labeled cells within

each cortical region. The sections with LC injection sites were further processed for DBH immunocytochemistry to determine the confinement of the tracer within the nucleus.

Results

In the first series of experiments, descending projection from the medial prefrontal cortex to the LC was investigated using an anterograde tracing method. A representative injection site of PHA-L within the prelimbic and infralimbic regions of the medial prefrontal cortex is depicted in Fig. 1A. The uptake of PHA-L by the somata (Fig. 1A, arrowheads) was prominent at layers 5 and 6 of the cerebral cortex. The medio-lateral dimension of the injection site was in the range of 150-200 μm. In upper cortical layers (layers 1 to 4) close to the pial surface, the anterogradely-transported tracer material was densely deposited within axonal fibers (Fig. 1A, open arrows). Following the injection, PHA-L immunoreactive axons and terminal arborizations were observed at rostral (Fig. 1B) and caudal (Fig. 1D) levels of the LC. Terminal fibers were extremely thin, as evidenced in other autonomic and limbic brain regions (Chiba et al., 2001). Higher magnification views, however, exhibited PHA-L labeled

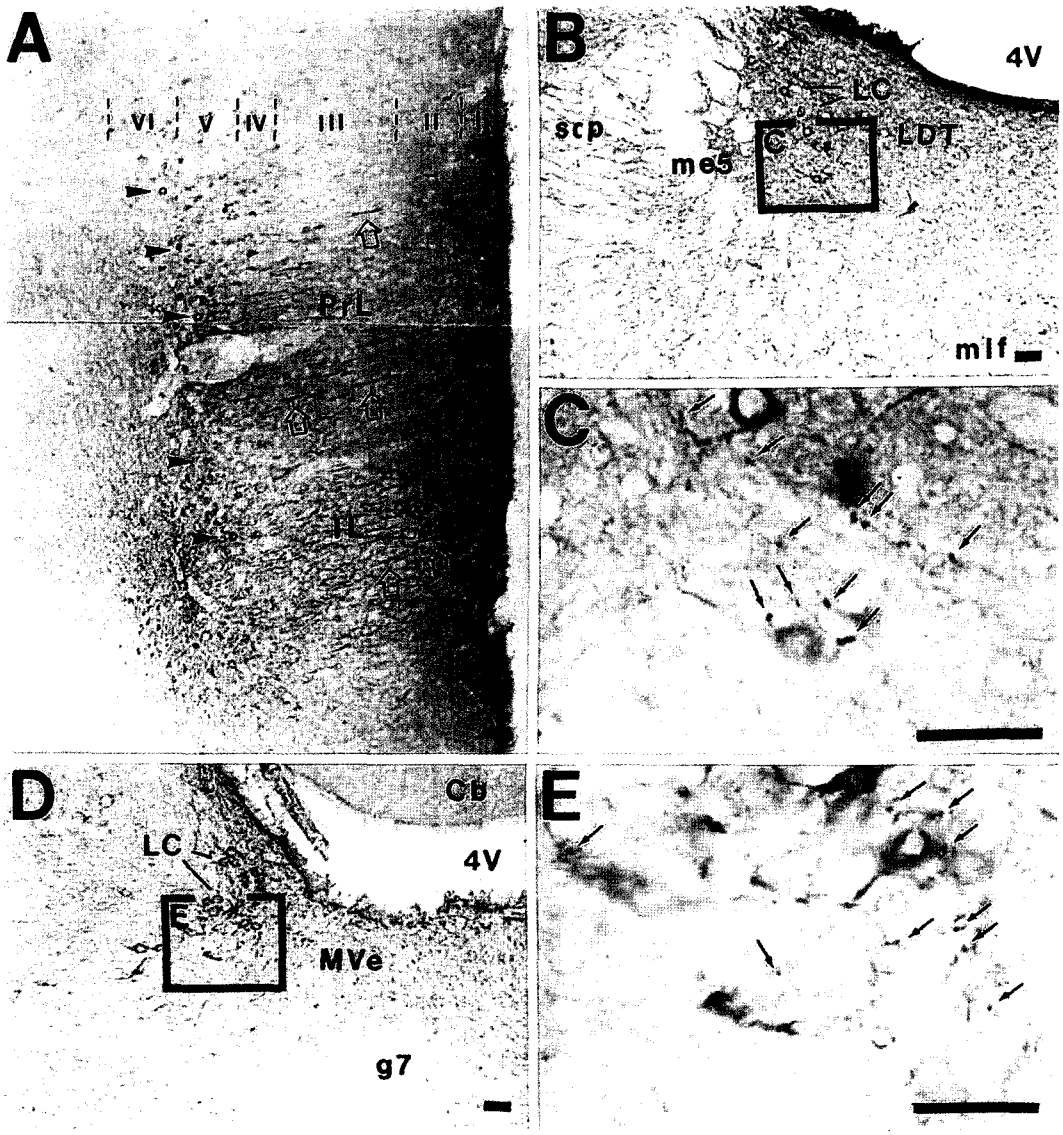

Fig. 1. Iontophoretic injection (rat no. 40) of PHA-L into prelimbic (PrL) and infralimbic (IL) regions of the medial prefrontal cortex produced labeling in the somata (arrowheads) at deep cortical layers as well as axonal fibers (open arrows) at superficial layers (A). I-VI represent cortical layers based on Nissl-stained sections. Anterogradely transported tracer was detected along axonal fibers and varicosities (arrows) close to DBH-immunolabeled somata in rostral (B and C) and caudal (D and E) levels of the locus coeruleus (LC). Cb, cerebellar lobule; g7, genu of facial nerve; LDT, laterodorsal tegmental nucleus; me5, mesencephalic trigeminal tract; mlf, medial longitudinal fasciculus; MVe; medial vestibular nucleus; scp, superior cerebellar peduncle; 4V, the fourth ventricle. Scale bars=50 μm.

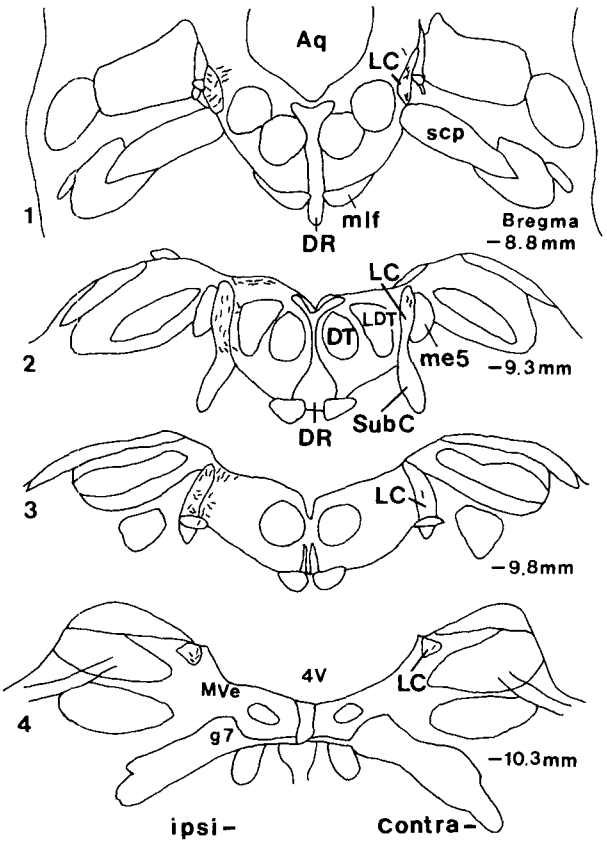

Fig. 2. Following injection (rat no. 40) of PHA-L into the medial prefrontal cortex (Fig. 1A), axonal fibers were observed within the locus coeruleus (LC) at a rostro-caudal series of transverse sections (1-4). Aq, cerebral aqueduct; DR, dorsal raphe nucleus; DT, dorsal tegmental nucleus; g7, genu of the facial nerve; LDT, laterodorsal tegmental nucleus; me5, mesencephalic trigeminal tract; mlf, medial longitudinal fasciculus; MVe, medial vestibular nucleus; scp, superior cerebellar peduncle; SubC, subcoeruleus nucleus; 4V, the fourth ventricle.

axonal fibers and terminal arborizations with varicosities (arrows) close to DBH-immunolabeled LC dendrites and perikarya (Fig. 1C and E).

The distribution of axonal fibers with varicosities is shown at a rostro-caudal series of LC transverse sections (Fig. 2). Following unilateral injection of PHA-L into the prelimbic and infralimbic regions of the medial prefrontal cortex (Fig. 1A), anterogradely labeled axonal fibers were observed bilaterally within the LC, with ipsilateral predominance (Fig. 2). Within the LC, the axonal fibers were relatively homogeneously distributed (Fig. 2, sections 1-4). It should be noted that PHA-L labeled axonal fibers were also observed at the region medial to the LC, which is located within the area between the LC and the laterodorsal tegmental nucleus (Fig. 2, sections 1-3). The axonal fibers with varicosities, however, were not observed at the subcoerulear region.

In the second series of experiments, WG was pressure-injected into several rostro-caudal levels of the LC and retrogradely-labeled cells were examined at the prefrontal cortex (Table 1). Labeled neurons were observed bilaterally within regions of the prefrontal cortex, with ipsilateral predominance. The largest number of double-labeled cells were observed in cases where injections were made into rostral LC (rat numbers 44 and 48). A number of cells were also observed in middle LC injection cases (rat numbers 51 and 53), whereas only a few cells were observed in caudal injection cases (rat numbers 42 and 58). The distribution of retrogradely-labeled cells was most pronounced in prelimbic and infralimbic regions of the medial prefrontal cortex in each case (Table 1).

The confinement of the tracer within the LC at the injection site was confirmed by DBH immunostaining (Fig. 3A, asterisks) following silver enhancement reaction. At the injection site, the amount (0.2-0.3 μl) of injected tracer produced an injection site whose medio-lateral dimension was in the range of 50-100 μm. Examples of retrogradely labeled neurons at the prelimbic and infralimbic regions of the medial prefrontal cortex are depicted after counterstaining with neutral red (Fig. 3B-D). WG-labeled cells were observed at various stages along the thickness of sections. Cells located close to the slide glass were well-counterstained with neutral red (Fig. 3B), whereas those located close to the cover glass were outlined only by the

Table 1. Distribution of retrogradely labeled cells at regions of the prefrontal cortex following the injection of WGA-apo-HRP-gold into rostro-caudal levels of the locus coeruleus (LC) in the rat

Injection site	Rostral LC				Middle LC				Caudal LC			
Cases	Rat # 44		Rat # 48		Rat # 51		Rat # 53		Rat # 42		Rat # 58	
	ipsi	contra	ipsi	contra	ipsi	contra	ipsi	contra	ipsi	contra	ipsi	contra
Cingulate	1	1	1	0	1	1	3	1	0	0	1	0
Prelimbic	14	6	12	7	7	2	5	2	2	1	3	1
Infralimbic	11	5	8	5	4	1	7	5	1	1	0	1
Dorsal peduncular	1	0	1	1	1	0	1	1	1	0	1	0
Medial orbital	0	1	1	0	0	0	2	0	0	0	0	0
Ventral orbital	0	0	0	0	0	0	0	1	0	0	0	0
Lateral orbital	1	0	0	0	0	0	0	0	0	0	0	0
Insular	0	0	0	1	0	0	1	0	0	0	0	0

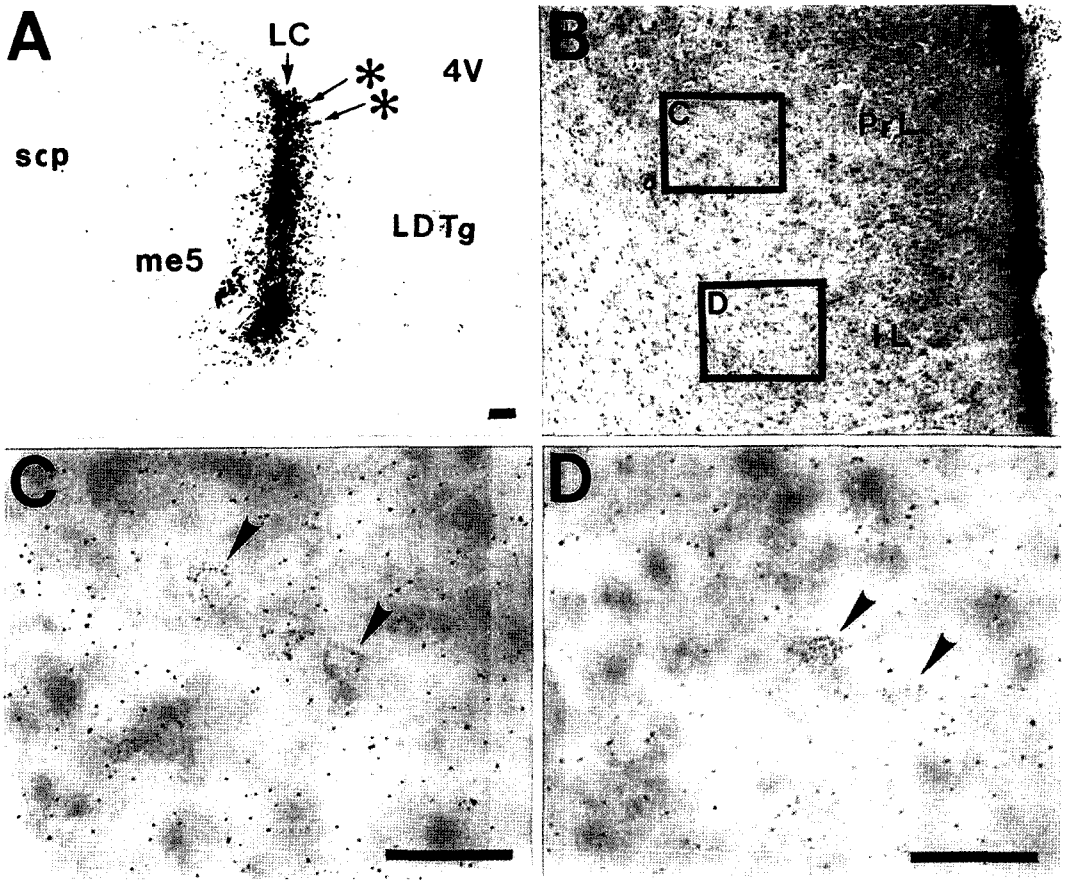

Fig. 3. Following injection (A) of WG into the locus coeruleus (LC), retrogradely-labeled cells (arrowheads) were observed at prelimbic (PrL) and infralimbic (IL) regions of the medial prefrontal cortex (rat no. 44). The confinement of the tracer within the LC was insured by the DBH immunostaining (asterisks). LDT, laterodorsal tegmental nucleus; me5, mesencephalic trigeminal tract; scp, superior cerebellar peduncle; 4V, the fourth ventricle. Scale bars=50 μm.

black WG granules (Fig. 3C and D, arrowheads). The majority of retrogradely labeled neurons were cortical, pyramidal cells located in layers 5 and 6 and the largest diameter of the labeled cells was in the range of 10-15 μm (Fig. 3C and D).

The distribution of retrogradely labeled cells is depicted in a rostro-caudal series of prefronto-cortical sections (Fig. 4). Labeled neurons were located bilaterally within the sections, with ipsilateral predominance. The majority of retrogradely-labeled neurons were located at the prelimbic and infralimbic regions of the medial prefrontal cortex. A few cells were also observed in the medial orbital (Fig. 4, section 1), cingulate (Fig. 4, section 2), insular (Fig. 4, section 3), and dorsal peduncular (Fig. 4, sections 3 and 4) cortices. Cells were observed neither in the ventral, lateral, and dorsolateral orbital cortices, nor at the primary and secondary motor and somatosensory cortices.

Discussion

The present anterograde labeling study indicated that the prelimbic and infralimbic regions of the medial prefrontal cortex projected to the LC bilaterally, with ipsilateral predominance (Figs. 1 and 2). Although axonal fibers with varicosities originating from the medial prefrontal cortex existed close to DBH-immunoreactive neurons within the LC region (Fig. 1C and E, arrows), the labeled fibers were not confined within the boundaries of the LC but were found just medial to the nucleus at rostral and middle levels (Fig. 2, sections 1-3). This observation correlates well with the previous report in the monkey that LC dendrites extend medially for several hundred microns beyond the nucleus proper (Arnsten and Goldman-Rakic, 1984; Chiba et al., 2001). It has also been postulated in the monkey that neurons-containing peptides including enkephalin, substance P, and beta-endorphin which have excitatory or inhibitory actions over the adjacent LC cells exist in medial border of the LC, (Grzanna and Molliver, 1980; Watson et al., 1980; Haber and Elde, 1982). Thus it seems that the prefrontal cortex could have either direct influence on dendrites within the LC nucleus as well as the area just medial to the LC or have indirect influence on LC cells via opioid-containing interneurons located in this area.

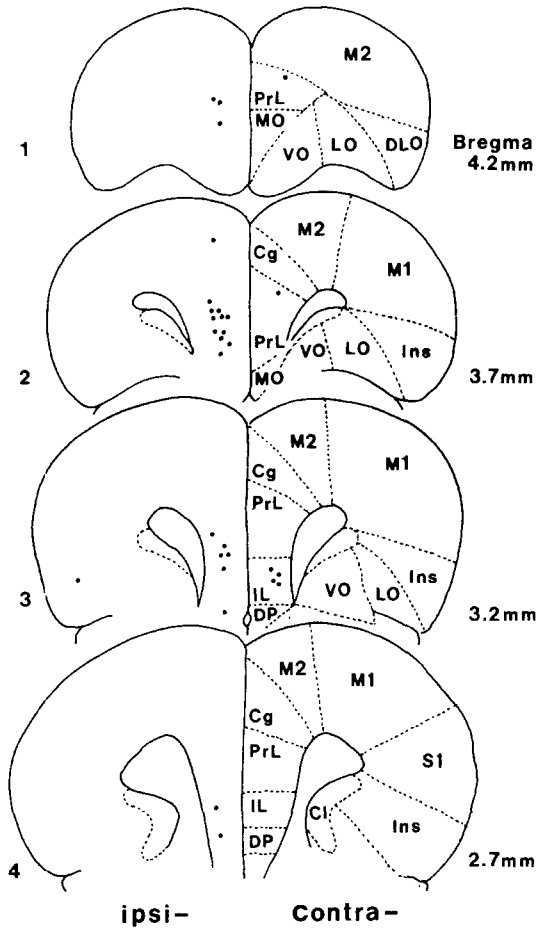

Fig. 4. The distribution of retrogradely-labeled cells (dots) at a rostro-caudal series of prefronto-cortical sections (1-4), following injection of WG into the locus coeruleus (rat no. 44). Cg, cingulate; Cl, claustrum; DLO, dorsolateral orbital; DP, dorsal peduncular; IL, infralimbic; Ins, insular; LO, lateral orbital; MO, medial orbital; M1 and M2, primary and secondary motor; PrL, prelimbic; S1, primary sensory cortices.

In the second series of experiments employing WG injection into several rostro-caudal levels of the LC, a moderate number of retrogradely labeled neurons were observed (Table 1). Only a few cells existed in the medial prefrontal cortex when injected into caudal LC. It was also reported in monkey that the dorsomedial and dorsolateral prefrontal cortex projected mainly to the rostral LC (Arnsten and Goldman-Rakic, 1984; Chiba et al., 2001). In contrast, it was suggested that the compact, caudal LC receives afferent fibers mainly from several brainstem structures including prepositus hypoglossal and paragigantocellular nuclei (Cedarbaum and Aghajanian, 1978; Aston-Jones et al., 1986).

Among various prefronto-cortical regions, the majority of retrogradely-labeled cells were consistently observed within the prelimbic and infralimbic regions of the medial prefrontal cortex (Table 1). In addition, only a small number of descending projection to the LC originating from cingulate, dorsal peduncular, orbital, and insular cortices was observed. This is consistent with the observation in the monkey that cortical projections to this monoaminergic brainstem nucleus originated from the dorsomedial and dorsolateral prefrontal cortex, but few derived from the orbital or insular cortices (Dalsass et al., 1981; Arnsten and Goldman-Rakic, 1984).

It was reported that the prelimbic and infralimbic regions of the medial prefrontal cortex might be the major prefronto-cortical projection to serotonergic cells in the the dorsal raphe (DR) nucleus of the rat (Lee and Kim, 2002). Although direct comparison of the number of retrogradely labeled cells in various regions of the medial prefrontal cortex was not made between DR and LC injections, the major afferent cortical areas including prelimbic and infralimbic regions provide heavier efferent projections to the DR than to the LC when equal amount of tracer was injected into each nucleus (Table 1 in Lee and Kim, 2002). It is well established that the LC is a compact nucleus that projects fibers to portions of the telencephalon, diencephalon, midbrain, cerebellum, pons, medulla, and spinal cord. With the exception of the 5-hydroxytryptamine (5-HT) projection system of the DR, no other brain regions have been demonstrated to have such widespread efferent projections. These norepinephrine or 5-HT containing brainstem regions seem to be in good contrast with dopamine-containing cell groups in the midbrain, which send relatively restricted efferent fibers to the cerebral cortex, but receive rather extensive afferents from widespread regions of the cortical mantle (Bunney and Aghajanian, 1976).

It has been well-established that LC cells fire at the presentation of relevant sensory stimuli and that the baseline firing rate of these cells appears to be related to relevance of ongoing situations in awake, behaving animal (Foote et al., 1980; Aston-Jones and Bloom, 1981). Since the LC receives restricted descending inputs mainly from the prelimbic and infralimbic regions of the medial prefrontal cortex in the rat, this descending projection system might be able to convey highly processed cortical information into the LC and thus secondarily have influence on diversified monoaminergic efferent targets of the nucleus, which might be involved in evaluating the relevance of complex sensory events and situations occuring at specific moments.

Recent anatomical studies indicated that the infralimbic cortex, but not the prelimbic region, projected uniquely to the LC in the Japanese monkey (Chiba et al., 2001). Functional differentiation between the two areas of the medial prefrontal cortex has been suggested. Previous studies indicated that the prelimbic region receives a direct input from the hippocampus, a connection that is essential for spatial memory and production of long-term potentiation (Vickery et al., 1997). In contrast, the infralimbic area is responsible for control of autonomic motor activities of the animal (Neafsey, 1990; Hurley et al.,

1991; Bacon and Smith, 1993; Buchanan and Powell, 1993; Neafsey et al., 1993; Takagishi and Chiba, 1993; Bechara et al., 1994; Chiba et al., 2001). The orbital gyrus in the medial prefrontal cortex is also involved in autonomic functions of the animal in that electrical stimulation of specific regions produced inhibition of respiration, a rise of blood pressure and a decrease in tonus of the gastric musculature of the monkey (Buchanan and Powell, 1993; Neafsey et al., 1993). Thus it might be implicated that the cortical differentiation permits more detailed assessment of specificity of the descending projection in primate as well as rat and that somatosensory, cognitive, autonomic and limbic information received by the LC might have influences on various brain regions via its extensive efferent projection system (Grzanna and Molliver, 1980; Felten and Sladek, 1983, Chiba et al., 2001).

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