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Invited Review

Chemocytoarchitecture of the rat locus ceruleus

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Summary. I shall elaborate on the cytochemical classifications of the rat locus ceruleus (LC) and state how each of these could be identified. In the LC, 80% (1,309/1,642) of the noradrenergic (NA) neurons are also GABAergic. This is found by demonstrating that two immunoreactivities coexist in adjacent sections alternately immunostained by anti-TH or anti-GABA antibody. Pharmacological manipulations with pargyline (75 mg/kg, i.p., 2 h prior to perfusion) and L-tryptophan (150 mg/kg, 1.5 h prior to perfusion) revealed 5-HT-like immunoreactivity (5-HT-LI) in most LC cells (masked 5-HT cells) that produce 5-HT but not other indoleamines. If 5-HTP is applied instead of tryptophan, 5-HT-LI is shown by the raphe nuclei and a few LC cells (masked indoleamine cells) in the marginal zone. Anti-GAD antibody reveals GAD-LI in 32% of GABA neurons predominantly in the dorsal division. *In situ* hybridization studies detected tryptophan hydroxylase mRNA and GAD mRNA in many small- and medium-sized neurons. It is concluded that the LC consists mostly of an NA population that is possibly synthesizing multiple transmitters, such as GABA, GAD and 5-HT in single neurons so that the system enables the LC simultaneously to innervate the entire CNS.

Key words: Locus ceruleus, Immunocytochemistry, *In situ* hybridization, Coexistence, Multiple neurotransmitters

1. Neuronal morphology identified by classical methods

Cytoarchitectonic studies using the Golgi and Nissl methods (Shimizu and Imamoto, 1970; Ramon-Moliner, 1974; Swanson, 1976; Shimizu et al., 1978) have clearly shown the presence of small- and medium-sized neurons in the LC of rats and cats. In rats, Shimizu et al. (1978) subclassified the small neurons into at least three types of small oval cells (10 x 15 μm in perikaryal size) with axons terminating in their vicinity after moderate branching, and the medium-sized neurons into fusiform and multipolar shapes (20 x 30 μm in perikaryal size), according to their morphology. These medium-sized neurons probably correspond to the projection neurons which monosynaptically innervate the entire CNS (Cedarbaum and Aghajanian, 1978; Mugnaini and Oertel, 1985).

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2. Cytochemical classification of LC neurons

In addition to the major transmitter, noradrenaline (NA) (Dalhström anf Fuxe, 1964), LC neurons have been shown to contain other «classical» transmitters, such as serotonin (5-HT) (Steinbusch, 1984; Iijima, 1989; Iijima and Sato, 1991), GABA (Bérod et al., 1984; Iijima and Ohtomo, 1988; Steindler and Troske, 1989; Iijima et al., 1992), and glutamic acid decarboxylase (GAD) (Kosaka et al., 1985, 1987; Jones, 1991; Iijima et al., 1992, 1993), as well as peptides, including neuropeptide Y (Everitt et al., 1984), substance P (Chan-Palay et al., 1978), and vasopressin (Caffé et al., 1985).

Burnstock (1976) suggests that each neuron has the potential to synthesize all transmitter substances, but that expression of one gene required for the synthesis of a transmitter may supress expression of genes for alternate transmitters. However, because of the recent findings in the rat LC (Iijima et al., 1992, 1993), the latter part of this opinion requires modification to explain the mechanism of the coexistence of two transmitters, similarly to that needed in the case of the coexistence of dopamine and GABA in A12-A15 cell groups of the rat diencephalon (Kosaka et al., 1987).

3. Classification of 5-HT cells in the LC

In order to avoid confusion in the LC region it is necessary to classify the 5-HT cells into three groups; A, B and C. «Group A, 5-HT cells» show 5-HT-like

immunoreactivity (5-HT- LI) against a specific anti-5-HT antiserum in untreated primate, cats and rats (Figs. 1, 2). In total, about 20 LC neurons per rat may belong to this category and these are located mainly in a peripheral part of the LC proper (Sladek and Walker, 1977; Léger et al., 1979; Steinbusch, 1984; Iijima, 1989).

«Group B, masked 5-HT cells» (e.g., LC) reveal 5-HT-LI after treatment with pargyline (75 mg/kg, i.p., 2h; Aghajanian and Wang, 1978) (Figs. 3, 4) and L-tryptophan (150 mg/kg, i.p.) load prior to transcardial perfusion with paraformaldehyde-picric acid-glutaraldehyde fixative (PPG, Kosaka et al., 1985, 1987). The LC consists mostly of masked 5-HT cells, which do not show 5-HT-LI to administration of p-Chrorophenylalanin (PCPA, Koe and Weissman, 1966) prior to pargyline and L-tryptophan (Fig. 5), but reveal 5-HT-LI if PCPA is given prior to pargyline and 5-HTP load (Fig. 6) (Iijima, 1989). This difference exhibited by different combinations of PCPA and either 5-HT precursor has been interpreted by the report that PCPA inhibits 5-HT synthesis at the step of L-tryptophan (Koe and Weissman, 1966).

Group C, «masked indoleamine cells», produce not only 5-HT but also other indoleamines in the cytoplasm (Figs. 5, 6). These cells have been found in certain nuclei such as the dorsomedial nucleus of the diencephalon (Frankfurt and Azimitia, 1983), the posterior hypothalamus (Sakumoto et al., 1984; Maeda et al., 1984), and the area postrema (Nishida et al., 1985). Nishida et al. (1985) summarized the cells comprising these nuclei and a few ventromedially-located cells to the LC as «masked indoleamine cells». They particularly noted a high MAO activity in the cytoplasm which degrades 5-HT so quickly that it becomes difficult to detect 5-HT without use of pargyline in order to inhibit MAO activity. Although Shimizu (1961) suggested that the LC may belong totally to the group C «masked indoleamine cells», it has recently become clear that major LC cells should be classed in group B, «masked 5-HT cells» (Iijima, 1989) (Figs. 4, 5).

Several neurons, which are ventro-medial to the LC, may be composed of 5-HT cells (A) and masked indoleamine cells (C) (Figs. 5, 6). Their 5-HT-LI is significantly higher in intensity than that of masked 5-HT cells (B) (Fig. 6). Because of their limited number, their unequivocal identification needs observation of serial sections including these cells (Iijima, 1989).

4. How to demonstrate the coexistence of two «classical» neutrotransmitters in a single cell

In order to demonstrate the coexistence of two classical neurotransmitters in single LC neurons, thin (6 µm-thick) serial cryostat sections of the LC were alternately immunostained for either tryrosine hydroxylase (TH) (Figs. 9, 11, 13, 17), or any other neurotransmitter under consideration (Figs. 10, 12, 14, 15, 18) according to the PAP (Sternberger, 1986) or ABC (Hsu et al., 1981) methods, because TH is a major transmitter in the LC (Lewis and Schon, 1975).

In order to determine whether two immuno-reactivities coexist in the same neurons, stet pairs of adjacent sections of the series alternately immunostained by the two different antisera were studied (Hökfelt et al., 1982; Kosaka et al., 1985; Kubota et al., 1986).

5. Various preparations are needed for analysis of LC

5.1. Karyometry is useful for classifying LC neurons into small- and medium-sized groups (cf. Iijima and

1-6. Localization of serotonin-like immunoreactivity (5-HT-LI) in the locus ceruleus (LC). PAP method. PPG fixation.

Fig. 1. Low-power photomicrograph showing localization of 5-HT-LI in the frontal section of the LC area. Two medium-sized neurons are mildly immunostained without treatment (group A, 5-HT cell). V: the 4th ventricle. x 150

Fig. 2. Higher magnification of the two 5-HT-LI neurons immunostained in Fig. 1. One of the proximal dendrites, as well as a positive-beaded neuropil revealstet 5-HT-LI. x 600

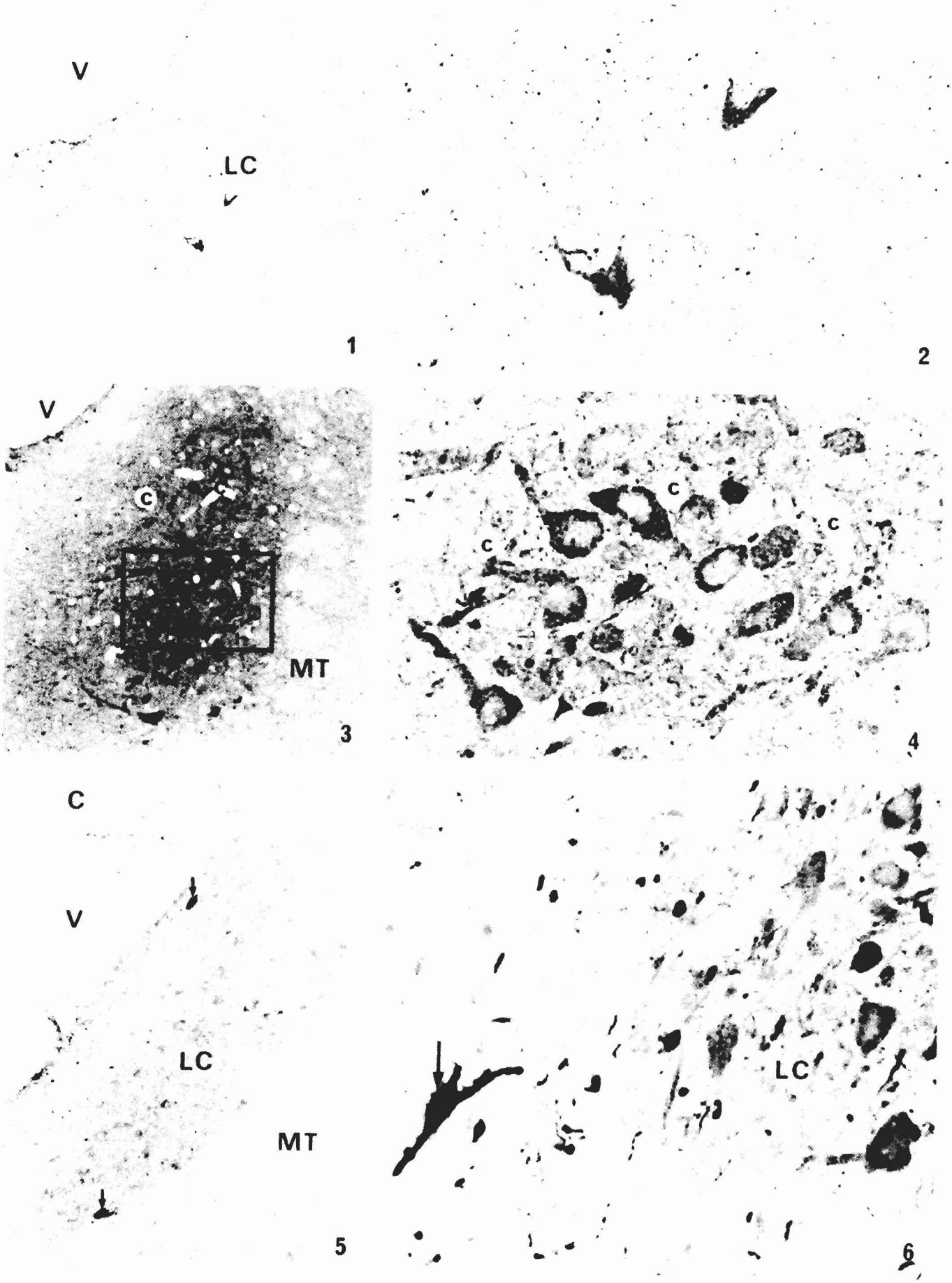
Fig. 3. Low-power photomicrograph of the LC showing a much higher 5-HT-LI than seen in Fig. 1, because of treatment with pargyline (75 mg/kg, i.p., 2h) prior to transcardial perfusion. Many capillary lumina (c) appear as wide and tortuous spaces. The MT does not show 5-HT-LI. MT: Mesencephalic tract nucleus of the trigeminal nerve; V: the 4th ventricle. x 150

Fig. 4. Higher magnification of the boxed area shown in Fig. 3. No nuclear staining is recognizable, whereas neuronal perikarya exhibit moderate to strongly positive 5-HT-LI. Many capillary lumina (c) are also noted; which correlate Figs. 3 and 4. x 600

Fig. 5. Low-power photomicrograph showing the absence of 5-HT-LI in the LC region (LC) after treatment with PCPA prior to both pargyline and L-tryptophan. This figure illustrates the lack of immunoreactive cells (masked 5-HT cells, group B) within the LC and surrounding structures, such as the cerebellum (C), the 4th ventricle (V) and the mesencephalic tract nucleus of the trigeminal nerve (MT). At the border of the LC, the two neurons (short arrows) showing 5-HT-LI may correspond to masked indoleamine cells. x 150

Fig. 6. High-power photomicrograph showing a ventro-medial margin of the LC indicates the difference between a masked indoleamine cell that is strongly positive (arrow, group C) and masked 5-HT cells corresponding to the majority of LC cells (LC, group B), x 500

Figs. 3-5. Iijima 1989, Acta Histochem. vol. 87. VEB-Gustav Fischer, Jena.


V
LC
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2
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Ohtomo, 1988).

Similar applications of karyometry to the analysis of the LC have been performed in our studies on 5-HT (Iijima et al., 1990, 1992).

5.2. Zamboni's fixation is the key for the optimal detection of GABA-LI.

After PPG fixation, Kosaka et al. (1987) demonstrated the coexistence of dopamine and GABA-LI in some neurons of A12 to A15 cell groups of the rat diencephalon, but they did not provide positive evidence for the coexistence of TH and GABA-LI in the rat LC.

However, it is now clear that glutaraldehyde should be omitted for the fixation of the brain, especially when the detection of GABA-Li is scheduled (see Kosaka et al., 1987; Iijima et al., 1992).

5.3. It seems important that masked 5-HT cells are greatly enhanced in revealing 5-HT-LI by pharmacological manipulations such as pargyline and 5-HTP treatment prior to transcardial perfusion with PPG (Aghajanian and Wang, 1978; Iijima, 1989). These cells prefer 5-hydroxytryptophan (5-HTP, 200 mg/kg, i.p.) to L-tryptophan (Aghajanian and Asher, 1971; Graham-Smith, 1971) as a 5-HT precursor (Bogdansky et al., 1958; Aghajanian et al., 1970). Intraperitoneal injection of pargyline followed by that of 5-HTP (200 mg/kg, i.p.) (e.g., posterior hypothalamus, Sakumoto et al., 1984), clearly increased 5-HT-IR in the neuronal perikarya of most LC neurons (72% immunopositive for 5-HT after, 5, 7-DHT treatment) (Iijima, 1989).

5.4. *In situ* hybridization detected signals of tryptophan hydroxylase mRNA and GAD mRNA (Fig. 16) in both small and medium-sized neurons of the LC. This method (Larsson and Hougaard, 1990) has been applied to demonstrate the ability to synthesize 5-HT or GABA in the rat LC. The specificity of hybridization was established by Northern blot analysis.

5.5. A polaroid camera enables us to analyse all neurons in a short period. Counting should be limited only to the neurons whose nucleoli were cut through.

6. Purification of Antibody

In order to demonstrate the existence of either three combinations of NA + 5-HT, NA + GABA, or TH + GAD in the LC, we examined whether the two immunoreactivities coexisted in alternately immunostained sections of the series. For this purpose, each antibody was obtained and purified according to the precautions of the original authors. Anti-GABA antibody (Iijima et al., 1986) was obtained from a rabbit and purified according to the methods of Storm-Mathisen et al. (1983), and Ottersen and Strom-Mathisen (1984). The production of anti-5-HT antibody was performed according to the method of Grota and Brown (1974). Polyclonal antisera and monoclonal antibodies against tyrosine hydroxylase (TH) were raised in rabbits and mice (Okuno and Fujisawa, 1982, 1985; Oomori et al., 1989). Polyclonal antiserum against GAD was provided by Chemicon International Inc (1992). The antigen was derived from expression of a cloned cDNA.

7. Multiple neurotransmitters synthesized in single cells

7-1 NA cell population

A 6-hydroxytryptophan (6-OHDA) study (Lewis and Schon, 1975) suggested that the rat LC consists mostly of a single NA-ergic cell population, and hence the LC has been called A 6 cell group by histofluorescence (Dahlström and Fuxe, 1964).

This concept has been substantiated by many studies, since the central part of the LC consists exclusively of NA (Lewis and Schon, 1975; Garver and Sladek, 1975; Swanson, 1976; Amaral and Sinnamon, 1977). However, since Steinbusch (1984) reported the presence of a few 5-HT cells in a ventral part of the LC, a question has remained to be clarified as to whether the rat LC consists of a single population of NA

7-10. Localization of serotonin-like immunoreactivity (5-HT-LI) in the locus ceruleus (LC). PAP method. PPG fixation.

Figs. 7 and 8. Both photomicrographs show 5-HT-LI of rats treated with 5,7-DHT (i.v.t.) prior to administration of pargyline and L-tryptophan (i.p.), and 5-HT-LI in the dorsal pons and a part of the cerebellum (C). The frontal section of Fig. 7 is located 300 µm anterior to that from which Fig. 8 is taken. CG: central grey; V: 4th ventricle; MT: Mesencephalic tract nucleus of the trigeminal nerve. Both x 180

Fig. 7. The LC consists only of the dorsal division at this proximal level of the brain. The majority of LC neurons (most of them, B group) reveal 5-HT-LI, and many of these cells are moderately to strongly positive, and are randomly scattered throughout the whole LC. However, the immunoreaction is less intense in the dorsal edge (line dE) of the LC than in its other parts. Capillary lumina (c).

Fig. 8. The LC consists of both dorsal and ventral divisions at this brain level. The solid line suggests the possible demarcation of such divisions.

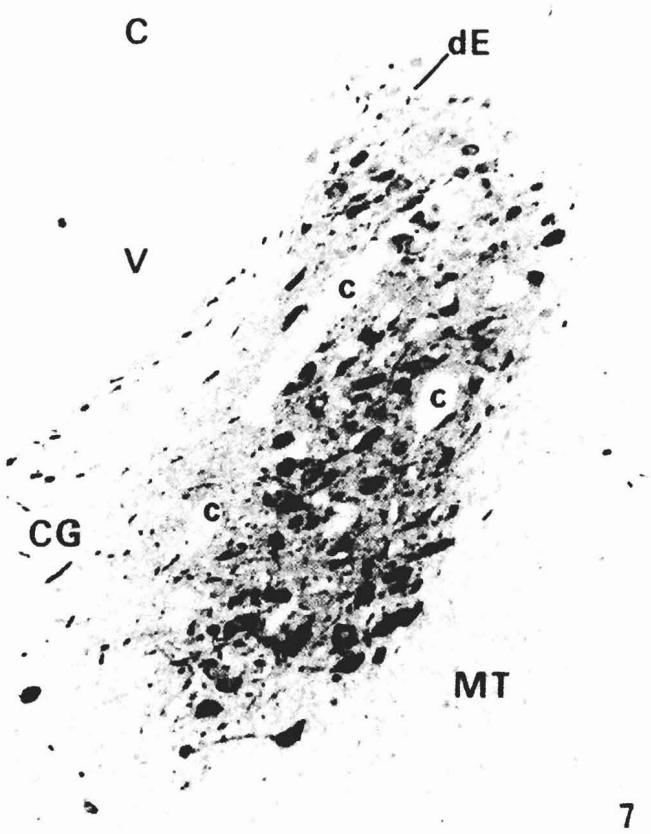
Figs. 9 and 10. Paired medium-power photomicrographs showing the localization of TH-LI (Fig. 9) and 5-HT-LI (Fig. 10) in adjacent sections. Animals were treated with pargyline (75 mg/kg, i.p., 2h) and 5-HTP (5-hydroxytryptophan 100 mg/kg, i.p., 1.5 h) prior to perfusion (Zamboni's). Cells 1-8 show TH-LI in Fig. 9, and 5-HT-LI in Fig. 10. Comparison of the two figures demonstrates the co-existence of TH and 5-HT in single LC neurons. CG: central grey; MT: mesencephalic tract nucleus of the trigeminal nerve. Capillary lumina (c) correlates Figs. 9 and 10. x 380

Figs. 7-10. Iijima and Sato 1991. Acta. Histochem. vol. 90. VEB-Gustav-Fischer, Jena.

or more than two populations with different transmitters.

7-2. GABAergic neurons in the LC

The possibility that the NA cell population of the LC might contain GABA has remained to be elucidated even after the immunocytochemical study of Storm-Mathisen et al. (1983), and Ottersen and Storm-Mathisen (1984). In addition, Bérod et al. (1984) reported the absence of GADergic neuronal cell bodies within the LC. As a



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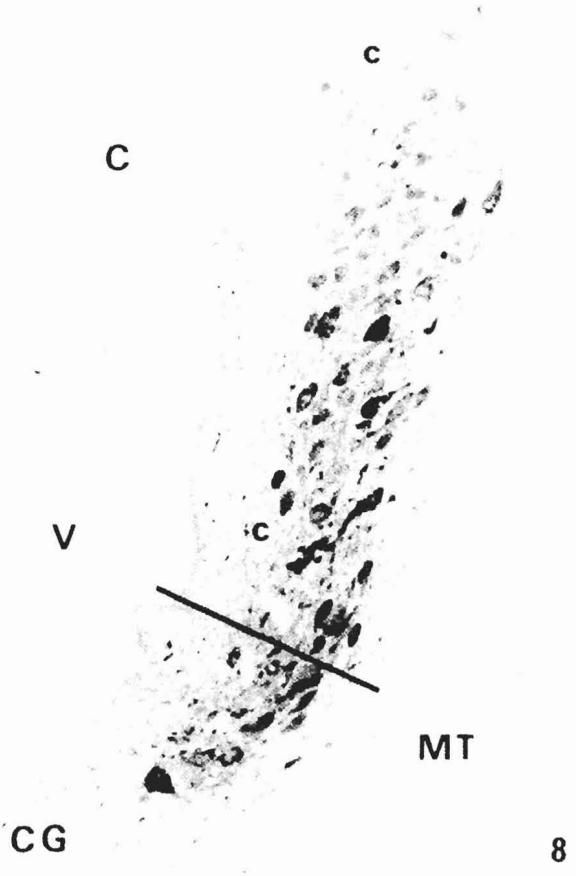

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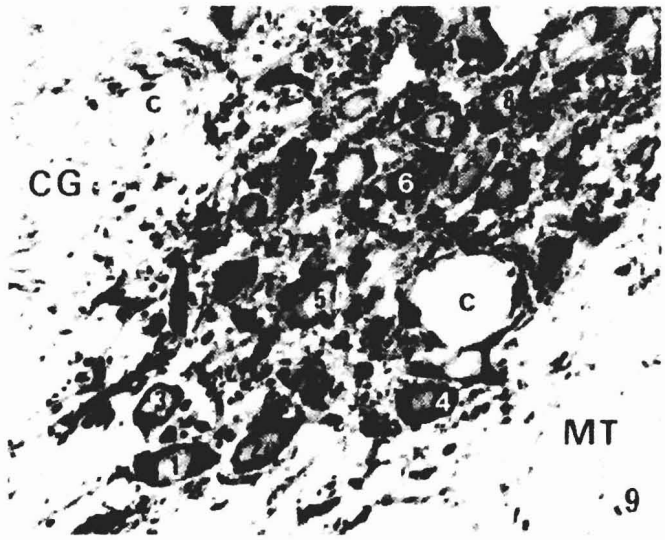

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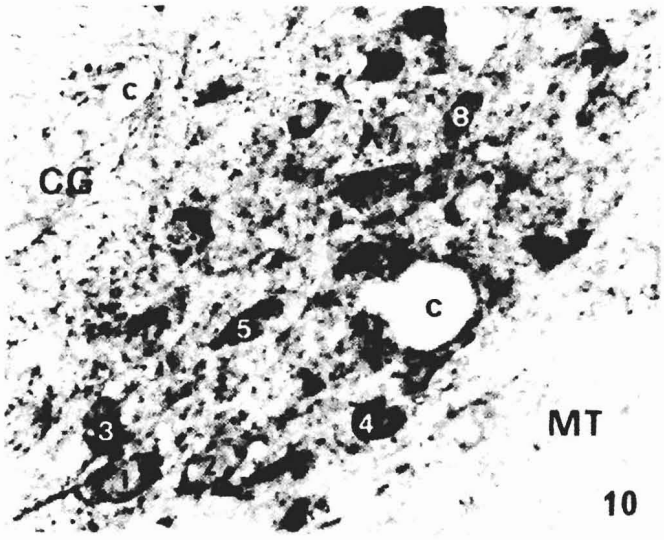

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result, it is generally considered that the LC is rich in GABAergic neuropil, but that it possibly lacks GABAergic neurons (Mugnaini and Oertel, 1985).

This conventional concept that the LC of rats is composed of a single NAergic population, however, soon required modification (Steindler and Troske, 1989) when a few small GABAergic neurons were identified throughout sets of thin serial frontal sections of the rat LC after PPG fixation (Iijima et al., 1987). Thereafter, Iijima and Ohtomo (1988) found that GABA-LI was more widely distributed in small and medium-sized neurons of the rat LC at both light and electron microscopic levels, using the PAP method with the same PPG fixation. In support of these findings, Steindler and Troske (1989) reported, in their developmental study, that two types of LC neurons appeared on different embryonic days in the mouse. Although Jones (1991) regarded these intrinsic GABAergic neurons as inhibitory interneurons, they appear to be different from proper, medium-sized LC neurons. In 1989, Iijima (1989) still considered that GABAergic neurons in general belong to another group apart from the NA group.

7-3. Convincing evidence for the coexistence of TH and GABA in single LC neurons

The coexistence of GABA-LI and TH-LI in single neurons of the rat LC has been demonstrated (Iijima et al., 1992) (Figs. 11-14). The profiles of these cells were labelled by alternately immunostaining adjacent sections for GABA-LI or TH-LI by the ABC method (Hsu et al., 1981) or the PAP method (Sternberger, 1986) after perfusion (either Zamboni's fixative or PPG), and observations at light and electron microscopic levels. For light microscopy, pairs of more than 590 (Zamboni's) (Figs. 11-14) and 260 (PPG) adjacent sections, and for electron microscopy, 40 ultrathin sections, cut from adjacent semithin plastic sections (Zamboni's), were examined. GABA-LI was found in 80% (1,309/1,642 in total) of small and medium-sized neurons, uniformly scattered throughout the LC (Figs. 11-14). These observations unequivocally demonstrated that the majority of GABAergic (Figs. 13, 14) neurons are also noradrenergic (Figs. 13, 14). Several neurons were neither noradrenergic nor GABAergic (Figs. 13, 14), while other noradrenergic neurons did not show GABA-LI (cells 6-7 in Fig. 14). It was also shown that astrocytes (cell 9 in Fig. 14), but not oligodendrocytes (cell 10 in Fig. 14), contained GABA. *In situ* hybridization, using a fragment of the glutamic acid decarboxylase (GAD) cDNA, amplified by the polymerase chain reaction, as probe, showed GAD mRNA signals in many neurons throughout the LC (Fig. 16), supporting the presence of a GAD/GABA system in the LC. Hence, multiple «classical» neurotransmitters, including NA, GABA, and GAD, coexist in many LC neurons and possibly contribute to its widely diverging projections throughout the entire CNS (cf. Iijima et al., 1992).

In the LC, TH-LI can be considered to indicate NA neurons exclusively, since dopamine- or adrenaline-containing cell bodies have not yet been reported at any significant level (Amaral and Sinnamon, 1977).

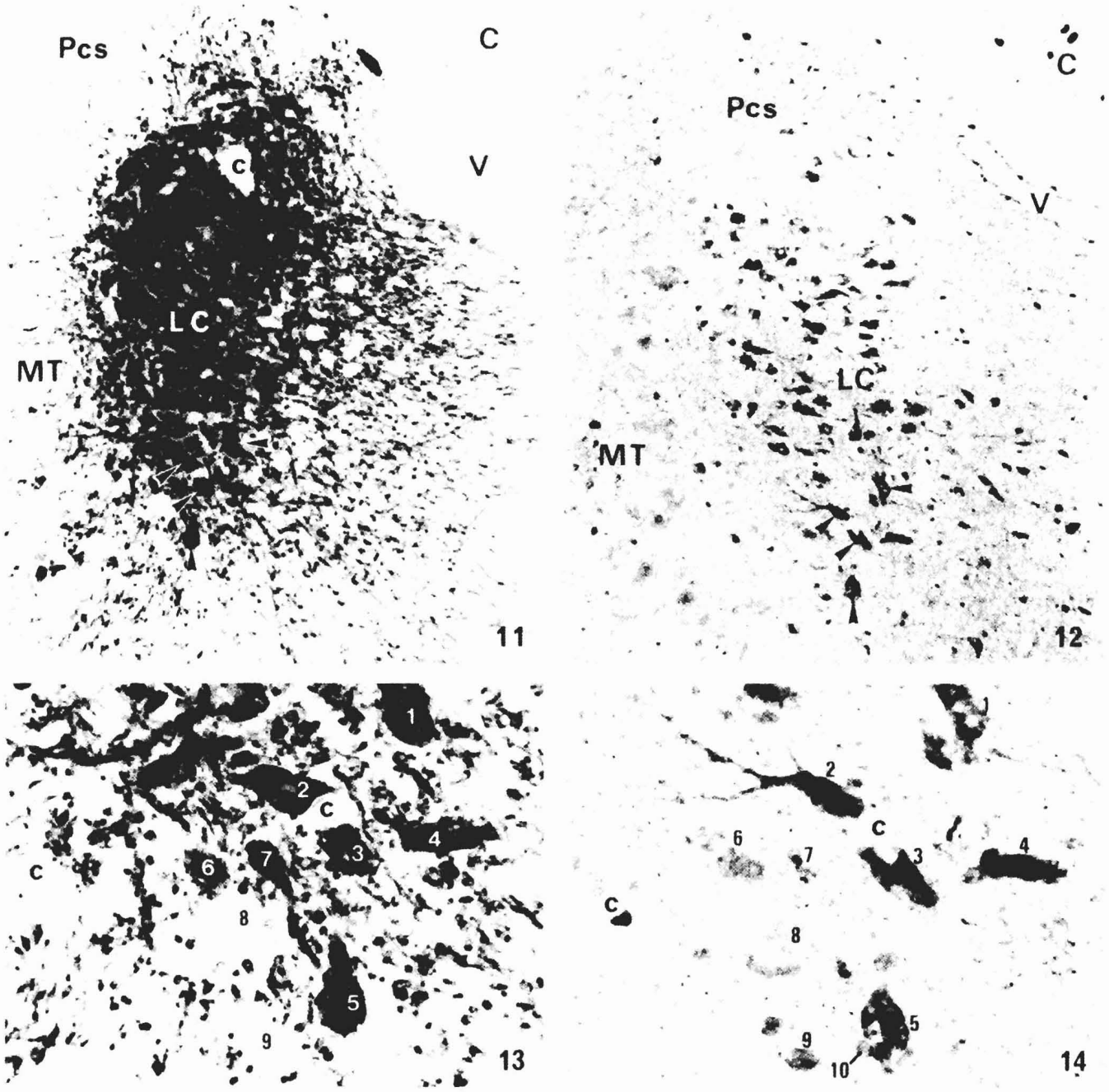
In considering the significant overlaps between two populations containing different transmitters (GABA 80%, 5-HT 72%, GAD 33%, in total LC neurons), the conventional concept, that considers the LC to be A6 cell group (Dahlström and Fuxe, 1964), would become more acceptable if changed into the modified hypothesis that the LC consists exclusively of the NA group while it is simultaneously synthesizing other multiple classical transmitters such as GABA, GAD and 5-HT (Iijima et al., 1992, 1993).

7-4. GAD-LI also coexists with NA and GABA in single LC neurons

Recently, Jones (1991) described the presence of a few small GAD neurons inside the rat LC. However, previous authors had not reported GAD-LI on the perikarya of most neurons of the LC in contrast to the abundant GABA and GAD neuropil and pericellular nerve terminals (Bérod et al., 1984; Kosaka et al., 1987). We have obtained positive evidence for the coexistence of NA, GABA, and GAD (for demonstration of the co-existence of TH and GAD, see Figs. 17, 18) in the rat LC by immunocytochemistry and *in situ* hybridization, due to successful Zamboni fixation (Iijima et al., 1993).

Four sets of serial cryostat sections, alternately immunostained for TH-LI or GABA-LI (240 pairs, 6 µm-thick, Zamboni's fixation), were examined. Statistical analysis, performed by Student's t-tests, revealed that the difference between the number of total TH-ergic neurons and total GABA-ergic neurons was not significant, indicating that a single population contains both NA and GABA. The ABC method for GAD-LI was applied to rats treated with colchicine (100 µg), 48 h prior to Zamboni's perfusion. GAD-LI was found in 32% (433/1,367) of TH cells in alternate sections stained for TH-LI or GAD-LI. The low rate of GAD to GABA (433/1,321) appears to be due to technical limitations. GAD cells of similar shape tended to assemble and form clusters, predominantly in the dorsal division.

In situ hybridization, using fluorescin isothiocyanate (FITC) for GAD mRNA (Fig. 16), revealed the signals in a greater number of neurons than GAD-LI cells, but in a significantly fewer number of cells than those showing TH mRNA signals (Iijima et al., 1993). This study therefore proves the presence of GAD in many LC neurons of rats, and provides further evidence for the coexistence of TH and GABA, supporting the recent notion that the LC consists of a single population that uses multiple neurotransmitters (Iijima et al., 1992) as mentioned above.



Figs. 11 and 12. Medium-power photomicrographs showing localization (arrowheads) of TH-LI (Fig. 11) and GABA-LI (Fig. 12) in adjacent sections of the LC. The LC is clearly delineated from the surrounding tissue by TH-LI (Fig. 11). Many neurons showing GABA-LI are uniformly scattered throughout the LC (Fig. 12). Comparison of the two figures demonstrates the co-existence of TH and GABA in the LC neurons. Parts of these neurons are indicated by arrowheads. C: cerebellum; Pcs: superior cerebellar peduncle; MT: mesencephalic tract nucleus of the trigeminal nerve; V: the 4th ventricle. Both x 180

Figs. 13 and 14. Higher magnifications of the ventral parts of the LC showing TH-LI in Fig. 11 (Fig. 13), and GABA-LI in Fig. 12. (Fig. 14). Cells 1-5 show both TH-LI and GABA-LI; cells 6-7 show TH-LI but not GABA-LI; cell 9 shows GABA-LI (Fig. 14), but not TH-LI; whereas cell 8 shows neither TH-LI nor GABA-LI. Cell 10, seen as a negative image in Fig. 14, and missing in Fig. 13, is an oligodendrocyte. Capillary lumina (c) correlate Figs. 13 and 14. Both preparations used Zamboni's fixative. ABC method. x 560

Figs. 11-14. Iijima et al., 1992. Anat. Rec. vol. 234. Wiley and Liss INC., New York.

7-5. More than 70% of LC neurons reveal 5-HT-LI after neurotoxin treatment

Earlier studies had already detected 5-HT cells around, but rarely at the limit of, the LC in the primate, cat, and rat by histofluorescence, autoradiography, and immunohistochemistry (Sladek and Walker, 1977; Léger et al., 1979; Steinbusch, 1984). Some authors had already succeeded in clearly revealing 5-HT-LI in certain brain regions with high MAO activity, such as the dorsomedial nucleus of the hypothalamus, the posterior hypothalamus and the area postrema (Nishida et al., 1985), by pharmacological manipulations with pargyline and 5-HTP prior to vascular perfusion. As expected, similar treatments to these prior to perfusion with PPG have also been effective in drastically revealing 5-HT-LI in most of the LC neurons (Iijima, 1989). Furthermore, injection of PCPA almost completely abolished 5-HT-LI of the LC if the rats were loaded with pargyline and L-tryptophan (Koe and Weissman, 1975) instead of pargyline and 5-HTP (Iijima, 1989). This study, using PCPA (Iijima et al., 1990), raises the high possibility that the NA cell population of the LC is also simultaneously synthesizing 5-HT under normal conditions.

7-6. Demonstration of the ability of 5-HT synthesis in an LC neuron

7-6-1. Application of neurotoxins for preventing leakage from rapheal 5-HT terminals in the LC.

A horseradish peroxidase (HRP) study (Loughlin et al., 1986) classified the HRP-labelled neurons of the LC into core cells, fusiform cells, small oval cells, and large pyramidal cells according to their morphology. Chemical lesioning of the 5-HT terminals, arising from the nuclei raphes and terminating in the LC (Conrad et al., 1974; Cedarbaum and Aghajanian, 1978), should provide a useful method for preventing leakage from such rapheal axon terminals to the LC cells (Pickel et al., 1977).

The localization of 5-HT-LI was studied by the PAP method using a purified antibody (Grota and Brown, 1974) obtained from a rabbit (Figs. 1-8, 10). The antibody was applied to serial cryostat sections with alternate counterstaining by cresyl violet, after intraventricular injections of 5,6-dihydroxytryptamine (5,6-DHT), or 5,7-dihydroxytryptamine (5,7-DHT) (Figs. 7, 8) prior to treatment with pargyline and a precursor of 5-HT (Baumgarten et al., 1971; Baumgarten and Lachenmayer, 1972). The majority of LC neurons were immunopositive (Figs. 3, 4), and more than half of all LC neurons clearly showed 5-HT-LI (60% positive cells for 5,6-DHT; 74% for 5,7-DHT in serial sections (Figs. 7, 8); Iijima et al., 1990). Although core cells were the most predominant, all types of neurons were immunopositive, and randomly scattered throughout the LC.

7-6-2. Immunocytochemical and *in situ* hybridization evidence for the coexistence of NA, 5-HT and TPH mRNA

In situ hybridization (Larsson and Hougaard, 1990), using an immunocomplex of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP), shows that many small and medium-sized neurons with signals of tryptophan hydroxylase (TPH) mRNA are uniformly scattered throughout the LC of rats (Iijima and Sato, 1991), and that a few extrinsic neurons, just ventromedial to it, also show the hybridization signals. The specificity of the technique has been established by Northern blot analysis (Iijima and Sato, 1991).

Synthesis of 5-HT in intrinsic neurons is indicated not only by the results of *in situ* hybridization (Larsson and Hougaard, 1990), but also by the fact that the distribution of masked 5-HT cells (Fig. 16), immunocytochemically revealed after pargyline and 5-HTP loading (Iijima, 1989), corresponds to neurons showing the mRNA hybridization signals. Identification of the same neurons in adjacent cryostat sections (Kosaka et al., 1985, 1987), immunostained alternately for tyrosine hydroxylase or 5-HT after loading (Figs. 9, 10), provides evidence for the coexistence of NA and 5-HT in a single neuron of the LC (for the method see Kubota et al., 1986; Kosaka et al., 1987). A few extrinsic, non-specific indoleamine cells located ventromedial to the LC (Fig. 6) may arise from the lateralization of the raphe neurons. Expression of tryptophan hydroxylase (TPH) in the CNS appears to be restricted to specific regions, such as the LC and raphe nuclei (Iijima and Sato, 1991).

7-7. Evidence for the coexistence of NA, 5-HT, and GAD in the LC

The LC consists of a single cell population that most probably uses multiple neurotransmitters, including NA, GABA, GAD and 5-HT simultaneously in single neurons (Iijima and Sato, 1991; Iijima et al., 1993). Identifying the same neurons in adjacent sections, alternately immunostained by TH-LI and GABA-LI in some series, and by TH-LI and GAD-LI in other series, demonstrated the coexistence of NA, GABA and GAD in single LC cells (Iijima et al., 1993). The coexistence of NA and 5-HT was already proven by observation of adjacent sections (Figs. 9, 10) and *in situ* hybridization to detect tryptophan hydroxylase (TPH) mRNA (Iijima and Sato, 1991). However, the low ratio of GAD to GABA at 32%, in accordance with Kosaka et al. (1987), who have already demonstrated the coexistence of dopamine and GABA in some neurons of A12-A15 cell groups in the rat diencephalon, appears to be due to technical limitations. Further study is needed to settle this problem.

In considering the anatomically unique situation of the LC, which has monosynaptically diverging

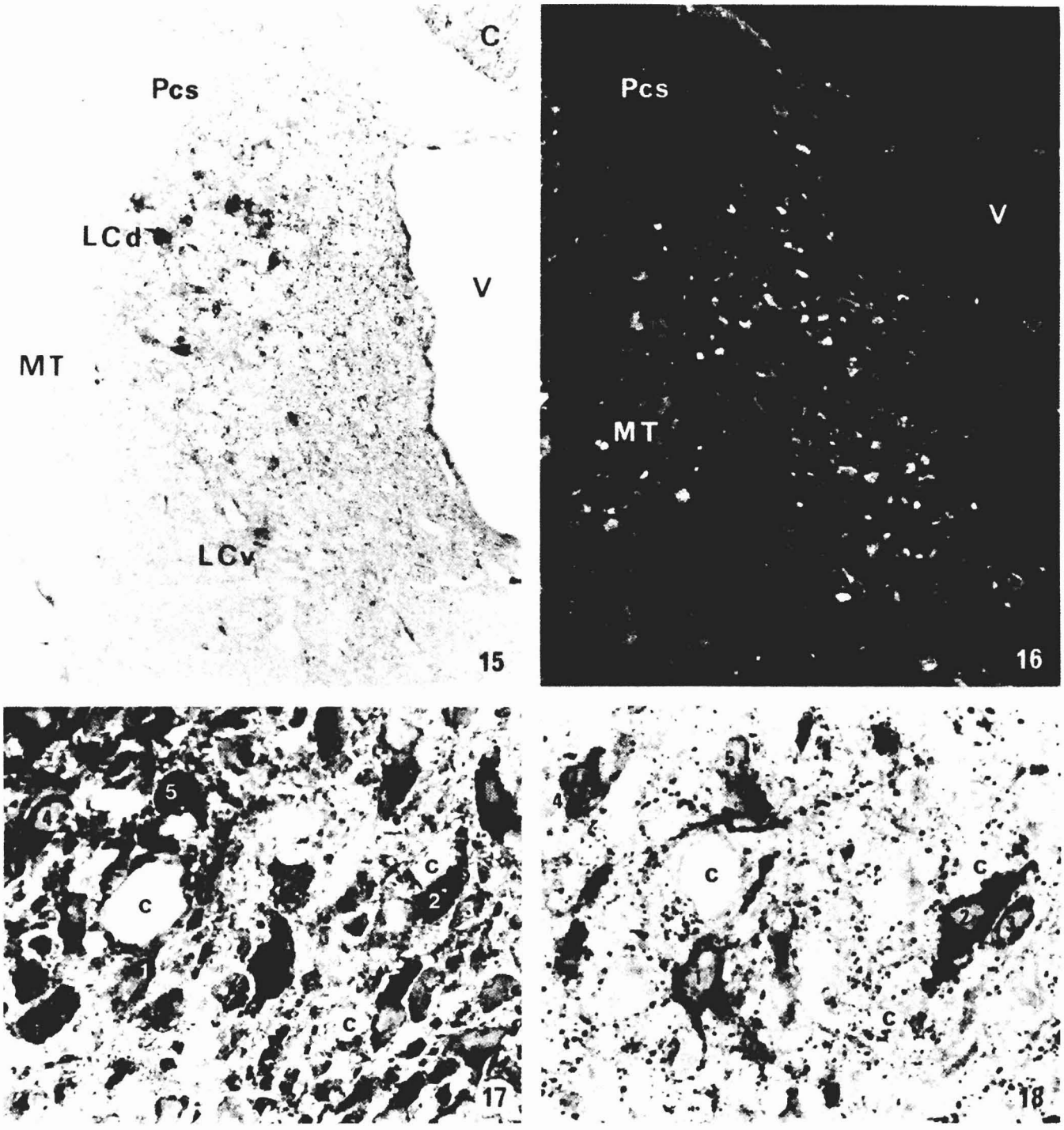


Fig. 15. Low-power photomicrograph showing the localization of glutamic acid decarboxylase-like immunoreactivity (GAD-LI) in the LC area. GAD-LI was scattered in similarly shaped cell bodies which were predominantly located in the dorsal (LCd) rather than the ventral (LCv) division of the LC. C: cerebellum; Pcs: superior cerebellar peduncle; MT: Mesencephalic tract nculeus of the trigeminal nerve; LCd: dorsal division of the LC; LC: locus ceruleus; LCv: ventral division of the LC, respectively. ABC method. x 180

Fig. 16. *In situ* hybridization, using fluorescein isothiocyanate (FITC) to detect GAD mRNA in the LC area, revealed signals in a greater number of neurons than by labelling with the ABC method (Zamboni's). The signals were more densely distributed in the dorsal division. In the MT area, the weak yellow-brown fluorescence is not significant. x 180

Figs. 17 and 18. High power photomicrographs showing TH-LI (Fig. 17) and GAD-LI (Fig. 18) in adjacent sections (colchicine treated i.v.t.). Cells 1-5 show both TH-LI and GAD-LI, and thus demonstrate the co-existence of TH and GAD in single neurons. Capillary lumina (c) correlate the two figures. Both use Zamboni's fixative. ABC method. x 600

Figs. 15-18. Iijima et al. 1993. Acta Histochem. Cytochem. Vol. 26, Nakanishi Print. Co., Kyoto.

projections into the entire CNS (Cedarbaum and Aghajanian, 1978), it is possible that this particular structure with multiple transmitters in single LC neurons, would enable each axon to simultaneously innervate a variety of cells with different receptors (Iijima et al., 1993).

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