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# Serotonin neurons on the ventral brain surface

**(ventral medulla oblongata/light and electron microscopic immunocytochemistry/silver intensification procedure/rat/autoradiography)**

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ABSTRACT **Serotonin neurons and fibers on the subpial surface of the ventral medulla oblongata in the rat are described by immunohistochemistry and autoradiography. The neurons are concentrated in the area encompassed by the origins of the abducens, hypoglossal, glossopharyngeal, and vagus nerves. The highest number of serotonin surface neurons appears along the median medullary fissure or basilar sulcus, where they may represent the most ventral extensions of the raphe pallidus group. As these cells lie on the surface of the brain, they could be directly affected by alterations in the chemical composition of the cerebrospinal fluid and, depending on their connections, could influence important medullary functions.**

The serotonin (5-HT; 5-hydroxytryptamine) system of the rat consists of a relatively small number of neurons in well-defined locations in the brain (1–3) that supply nerve fibers to extensive regions of the brain parenchyma. This innervation includes not only the brain itself but also the supraependymal plexus of 5-HT fibers lining the ventricular surfaces (4–7), the leptomeninges of the spinal cord (6, 8), and the pial vessels (9). There has been growing evidence that the raphe 5-HT neurons within the medulla provide some of these fibers (5, 6, 9); however, it is likely that the raphe neurons are not the only sources of 5-HT innervation to the leptomeninges. In the present paper we provide evidence for a system of 5-HT neurons not described previously. Subpial or superficial 5-HT cells of the medulla are demonstrated by two chemically specific methods: (*i*) autoradiography after *in vivo* infusions of [$^3$H]5-HT for specific uptake by 5-HT cells and fibers and (*ii*) immunocytochemistry with polyclonal antibodies against 5-HT revealed by the diaminobenzidine chromogen and subsequent silver intensification (10).

## MATERIALS AND METHODS

Adult male rats of the Wistar or Charles River strain of 200–300 g of body weight were used. For immunocytochemistry the animals were anesthetized with chloral hydrate and perfused transcardially at 37°C with a prefixative wash of phosphate-buffered saline and then with 4% paraformaldehyde in 0.12 M phosphate buffer at pH 7.4. Brains were postfixed overnight in the same fixative and sliced at 3-mm intervals or cryoprotected with 30% sucrose and sectioned on a cryostat at 150-$\mu$m thickness in the horizontal plane. The peroxidase-antiperoxidase (PAP) method (11) was applied for light microscopy. For electron microscopy, tissue was pretreated with 0.15% Triton X-100 in phosphate-buffered saline for 15 min. Incubations were carried out in the following sequence: 5-HT antibody (1:5000 dilution) up to 72 hr (electron microscopy, 24 hr), goat anti-rabbit IgG (1:100), rabbit PAP complex (1:100), and diaminobenzidine (DAB) reaction. For slice preparations the washes between steps were prolonged for 6 hr. Tissue for electron microscopy was postfixed with 2% aqueous osmium tetroxide for 2 hr and embedded in Durcupan. Thin sections were examined in a JEOL electron microscope. A modified silver intensification process or DAB/silver/gold chromogen reaction (10) was applied to many of the immunochemical sections to enhance the signal-to-noise ratio of the stained material.

The 5-HT antiserum was generated in rabbits. The antigen used was a conjugate of 5-HT and bovine serum albumin, prepared as described (12). Specificity was determined by the ELISA and immunohistochemistry. Addition of 50 $\mu$M human serum albumin–5-HT conjugate, 100 $\mu$M 5-HT, 1 mM tryptamine, or 5 mM 5-methoxytryptamine to the antiserum abolished immunoreactivity; addition of 500 $\mu$M dopamine reduced immunostaining. Addition of 1000 $\mu$M of other monoamines, noradrenalin, adrenalin, histamine, melatonin, L-tryptophan, 5-hydroxytryptophan, and 5-hydroxyindole acetic acid did not decrease immunoreactivity.

Light microscope autoradiograms were prepared as described (2, 13) from the ventral medulla of brains of anesthetized rats infused intraventricularly with [$^3$H]5-HT [creatinine sulfate, New England Nuclear; specific activity, 24 Ci/mmol (1 Ci = 37 GBq); concentration, 0.01 mM] either alone or simultaneously with unlabeled D,L-norepinephrine (concentration, 0.1 mM). The infusions were terminated at 3 hr and the animals were perfused with 1% glutaraldehyde/1% formaldehyde in 0.12 M phosphate buffer (pH 7.4). Horizontal and sagittal sections, 2–4-$\mu$m thick, of Epon-embedded material were prepared for autoradiography with Kodak NTB-2 emulsion and exposed for 3–4 weeks prior to development. Throughout this study special care was taken to prepare tissue through the ventral medulla in ways that would allow visualization of the largest area of the surface and subpial region. The greatest success was attained with horizontal sections taken parallel with the ventral brain surface while maintaining landmarks for the orientation of nerve exits, pyramids, and olivary protrusions. In addition, complete sets of autoradiograms were prepared from serial sections through the ventral medullas of four animals to estimate the numbers of superficial cells marked by [$^3$H]5-HT. Counts were made only of cells in a truly superficial or subpial position, and every cell counted was measured with an eyepiece reticule along its long axis and at right angles to that.

## RESULTS

In the rat brain an extensive system of 5-HT-immunoreactive cells with [$^3$H]5-HT uptake capability is present on the

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Abbreviations: DAB, diaminobenzidine; 5-HT, serotonin (5-hydroxytryptamine); 5-HT-i, 5-HT-like immunoreactive.

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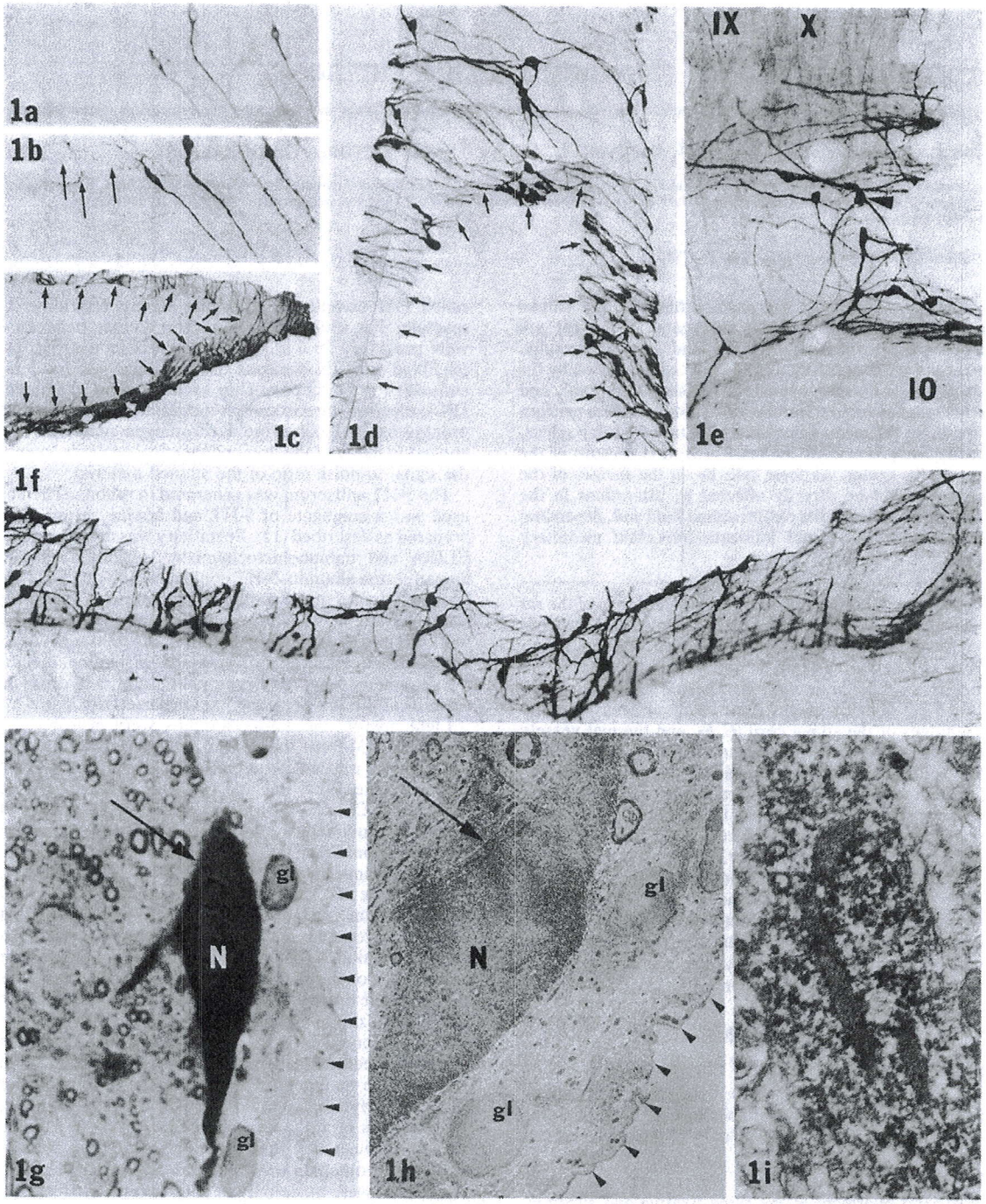


FIG. 1. (*a–f*) Light microscopic demonstration of 5-HT-like immunoreactive (5-HT-i) neurons upon the surface of the ventral medulla by immunoreaction with antibodies against 5-HT; (*b–f*) after the silver intensification procedure. (*a* and *b*) Three 5-HT-i neurons and several fibers demonstrated by the DAB reaction before silver intensification (*a*) and after the procedure (*b*). Intensification by the silver chromogen process improves visibility of neural elements. (×100.) (*c* and *d*) Low and higher magnification of a horizontal section from the ventral medullary surface with 5-HT-i neurons and fibers. The series of arrows points to the surface of the pyramid. (×30, ×100.) (*e*) 5-HT-i cells and fibers at the origins of the glossopharyngeal (IX) and vagus (X) nerve roots, near the inferior olive (IO). (×100.) (*f*) 5-HT-i cells and fibers on the lateral surface of the ventral midsagittal fissure of the medulla. (×100.) (*g* and *h*) A pair of micrographs of serial sections through a 5-HT-i neuron (N, arrow) to demonstrate its subpial position in the ventral surface of the medulla oblongata. The electron micrograph indicates DAB deposits only in the neuron (N) and not in the overlying glial cells (gl). Arrowheads indicate the basal lamina of the external glial limiting membrane. (×1350, ×2500.) (*i*) Higher magnification of a dendrite from the 5-HT-i neuron in *g* and *h*. (×25,000.)

subpial surface of the ventral medulla. These cells are concentrated on the medullary surface, caudal to the exit of the abducens (VI) nerve and rostral to the rootlets of the hypoglossal (XII) nerve. The cells extend laterally over the

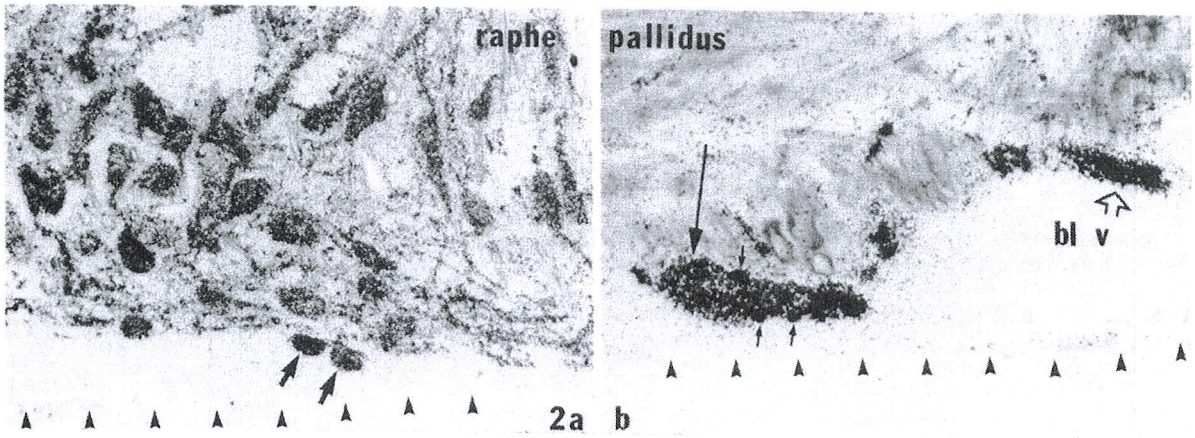


FIG. 2. (*a* and *b*) A pair of autoradiograms after selective uptake of [$^3$H]5-HT by 5-HT neurons and their fibers in the raphe pallidus. Arrowheads indicate the external glial limiting membrane at the brain surface. The subpial 5-HT neurons are indicated by heavy arrows in *a*. (×324, sagittal section.) (*b*) A single 5-HT subpial or surface neuron (large arrow) has upon its somatic and dendritic surface several heavily labeled 5-HT terminals (small arrows). Processes from a 5-HT cell encircle a subpial blood vessel (arrow, bl v), as is typical in this region. (×1428, sagittal section.)

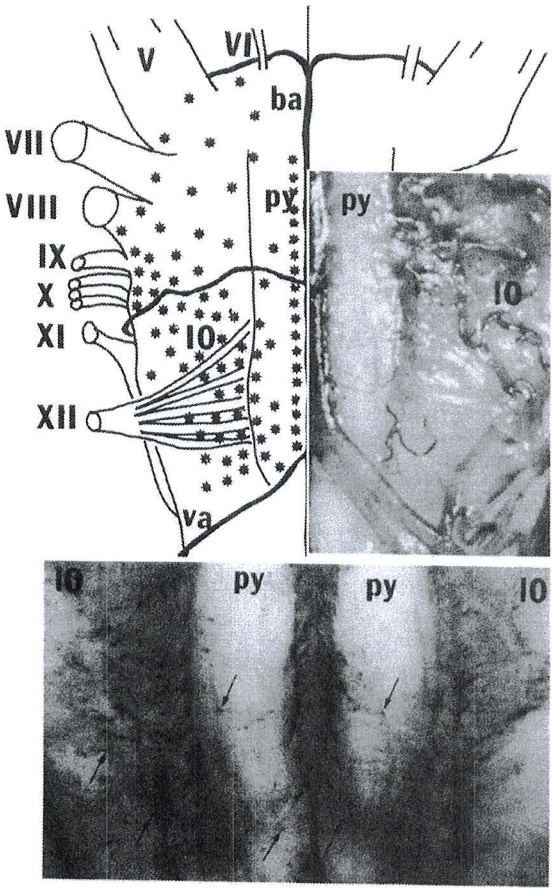


FIG. 3. This composite illustration shows a schematic diagram (upper left) and a photomicrograph (upper right) of the ventral medullary surface in the brain of a rat. Nerve rootlets and blood vessels are identified, and the locations of 5-HT neurons on the brain surface are indicated by asterisks. The photomicrograph (below) shows the same view of the entire ventral medullary surface after incubation with anti-5-HT antibodies. The black structures (some indicated by arrows) are 5-HT neurons. V–XII, nerve rootlets of cranial nerves; va, vertebral artery; py, pyramids; ba, basilar artery; IO, inferior olive; asterisks or arrows, 5-HT neurons. (×90.)

origins of the glossopharyngeal (IX) and vagus (X) nerve roots (schematic diagram in Fig. 3), with a particularly dense area between the Xth and the XIIth rootlets. By comparison, the ventral surface of the pyramids has a sparser population of 5-HT cells. Other 5-HT cells cluster close to the vessels branching off the vertebral and basilar arteries on the ventral medullary surface (see Fig. 1 *a–f* and Fig. 3). The 5-HT neurons were observed in all animals used in these experiments. Silver intensification after the DAB procedure greatly enhanced the visibility of cell perikarya and rendered fine-caliber fibers readily visible where they were not previously. The background was not increased.

These findings are corroborated by parallel studies with autoradiography. 5-HT neurons are heavily labeled with silver grains after uptake of [$^3$H]5-HT. In addition to the usual disposition of raphe and other 5-HT neurons in the parenchyma of the medulla, the subpial system is readily identified (Fig. 2 *a* and *b*). The 5-HT cells are clearly not within the brain parenchyma proper but are situated more superficially, immediately under the external glial limiting membrane. Other characteristics of these cells include the predilection for surrounding blood vessels and the presence of [$^3$H]5-HT-labeled axonal terminals upon the surfaces of their somata and dendrites (Fig. 2*b*). Electron microscopic examination of these immunoreactive 5-HT cells and the autoradiographically labeled material indicated that these cells have the usual characteristics of neurons. The position underneath the external limiting membrane but outside of the true brain parenchyma was also confirmed. In addition, synapses were observed upon the somatic and dendritic surfaces of these 5-HT neurons.

Counts of [$^3$H]5-HT-labeled neurons on the ventral subpial surface of the medulla from four brains, as described in *Materials and Methods*, indicate that a total of between 575 and 625 labeled cells could be found in the ventral surface of each brain. This is an underestimate of the total number of superficial 5-HT cells, as it is likely that this system of neurons is not confined to the ventral medulla. The majority of the cells are fusiform, ranging from 25 to 30 $\mu$m in length and 10 to 15 $\mu$m in width, with some slightly larger. A few cells are multipolar with perikarya in the same size range. The densest clusters are found along the median fissure and beneath the subpial surface of the nerve roots of the IXth, Xth, and especially XIIth cranial nerves. This observation is not surprising, as the median fissure marks the most ventral extent of the raphe pallidus, an important cluster of neurons,

many of which contain 5-HT. Moreover, the existence of 5-HT neurons within the nerve rootlets of the XIIth cranial nerve has been documented (14). Fig. 3 is a schematic representation of these surface 5-HT neurons.

## DISCUSSION

The existence of 5-HT neurons in a superficial position just beneath the external glial limiting membrane of the ventral medulla has, to our knowledge, not been reported previously. The large number of such cells and their proximity to the blood vessels and the subarachnoid space at the base of the brain suggest that their location is not an artifact but is intimately related to their function. Although at present we have no information bearing on their function, it is evident that these cells are in a position to respond to substances and movements in the cerebrospinal fluid bathing the ventral surface of the brain. In addition, the neurons, by virtue of their preferred position underlying blood vessels, are subject to the pulsations of blood vessels and gross movements of the cerebrospinal fluid. Alterations in neurohormones, neuroactive substances, or ions could all have immediate effects upon these cells with widespread results, depending upon their connections. Therefore, it is important to determine by experimental studies the afferent connections and the projections of these cells as well as the extent of their distribution over the surface of the rest of the brain. Further investigations of the 5-HT neurons on the surface of the brain should include experimental determination of possible connections of these neurons, possible coexistence of 5-HT with other neuroactive substances such as substance P, γ-aminobutyric acid, and somatostatin, and a more precise definition of the total extent of this superficial neuronal system elsewhere in the brain.

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