Pontomedullary Distribution of 5-HT$_{2A}$ Receptor-Like Protein in the Rat

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ABSTRACT

Serotonin (5-HT) exerts excitatory effects in many brainstem regions involved in autonomic, somatic, motor, and sensory functions, and in control of vigilance. To determine the potential role of 5-HT$_{2A}$ receptors in these effects, we immunohistochemically mapped the distribution of 5-HT$_{2A}$ receptor-like protein in the rat pontomedullary brainstem. Areas containing the densest labeling included the trigeminal, facial, hypoglossal, dorsal vagal motor nuclei, medullary linear nucleus, and the inferior olive. In the nucleus ambiguus, labeled cells were located in the areas containing pharyngeal and laryngeal motoneurons. Intensely labeled cells were loosely scattered in the reticular formation adjacent to the raphe magnus and obscurus nuclei, in the gigantocellular region, in the caudal pedunculopontine and laterodorsal tegmental nuclei, dorsomedial pontine reticular formation, and nucleus subcoeruleus. In the nucleus prepositus hypoglossi, all vestibular, abducens, cuneate, and lateral reticular nuclei, labeled neurons commingled with unlabeled ones. Few labeled neurons were located in the rostral and caudal ventrolateral medulla and parvicular reticular formation. In the nucleus of the solitary tract, two patches of diffuse labeling not associated with cellular profiles were present: one in the medial, and the other in the interstitial subnucleus. Similar diffuse labeling was present in the lateral parabrachial region and the lateral rim of the caudal spinal trigeminal sensory nucleus. No labeled cells were found in the locus coeruleus, dorsal raphe, superior olive, or area postrema. The distinct pontomedullary distribution of 5-HT$_{2A}$ receptors, combined with the known arousal-dependent activity of serotonergic neurons, show that these receptors may mediate post- and presynaptic effects in the motor, selected somatic and visceral sensory, oculo-vestibulo-precerebellar, and sleep-related regions. J. Comp. Neurol. 418:323–345, 2000.

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Indexing terms: immunohistochemistry; motor control; serotonin receptors; sleep; vagus; vestibular system

Mammalian serotonin (5-HT)-containing neurons are distributed along the midline of the brainstem and are divided into a rostral and caudal division. The rostral division, comprising the dorsal, caudal linear, and medial raphe nuclei of the midbrain and rostral pons, innervates forebrain targets. Serotonergic cells in the caudal division, including the magnus, obscurus, and pallidus raphe nuclei of the caudal pons and medulla, innervate the lower brainstem and spinal cord (Steinbusch, 1981; see Jacobs and Azmitia, 1992, for a review). Serotonergic neurons play an important role in a variety of physiological functions, including cardiorespiratory, motor, sensory, cognitive, affective, and arousal (Jacobs and Fornal, 1995; Mason and Leung, 1996). These functions are mediated through multiple 5-HT receptor subtypes that use different second messengers, are coupled to distinct ion channels, and produce excitatory or inhibitory effects at many central nervous system (CNS) sites (see Zifa and Filion, 1992, for a review).

Type 2 5-HT receptors, of which at least two subtypes (A and C) are present in the CNS, are coupled to phosphoinositol second messenger systems and generally mediate excitatory effects. In ligand binding studies, they are recognized by their high affinity for agonists such as (+)1-(2,5-Dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (DOI), and antagonists such as mesulergine and ritanserin (Zifa and Filion, 1992). Both ligand binding
and mRNA distribution studies point to a particularly high density of these receptors in suprapontine regions, including the cortex, thalamus, hypothalamus, amygdala, and basal ganglia (Pazos et al., 1985; Pompeiano et al., 1994). The 5-HT2A receptors are, however, also present in the brainstem and spinal cord (Morilak et al., 1993; Pompeiano et al., 1994; Wright et al., 1995), where they have been implicated in motor, viscerosensory, and cardiorespiratory functions, sexual behaviors, the regulation of food intake, and sleep (Zifa and Fillion, 1992). Nevertheless, the majority of studies of 5-HT2 receptors have focused on forebrain regions, whereas the distribution and role of 5-HT2A receptors in the brainstem received relatively less attention.

Electrophysiological studies have extensively documented that 5-HT1A receptors mediate excitatory effects of 5-HT in the brainstem orofacial motor nuclei (Rasmussen and Aghajanian, 1990; Kubin et al., 1992; Larkman and Kelly, 1992; Arita et al., 1995). Excitatory effects of 5-HT having properties compatible with their mediation by 5-HT2 receptors were also observed in other brainstem locations (e.g., Stevens et al., 1992; Bobker, 1994; Sugi-hara et al., 1995; Bayliss et al., 1997). However, the identity of type 2 receptor subtypes mediating these effects is uncertain because of the limited selectivity of the available pharmacological tools. Similarly, the classical receptor autoradiographic methods have limited receptor subtype specificity and, in addition, they cannot clearly relate binding sites to distinct cellular elements.

We have employed the technique of neurotransmitter receptor immunohistochemistry to study the localization of one type of 5-HT2 receptors, the A subtype. This technique has recently been used successfully to study the distribution of other second messenger-coupled receptors (e.g., Talley et al., 1996; Rosin et al., 1996). Immunohistochemistry provides better spatial resolution and selectivity than the previously used autoradiographic methods by allowing one to associate the presence of receptor protein with individual cells. This is particularly relevant for those brain regions where the average receptor density may be low, but where individual cells may express the receptor protein in high abundance. By using receptor immunohistochemistry, we were able to both confirm the presence of the 5-HT2A receptor subtype protein in numerous brainstem locations where the presence of this receptor was suggested by other techniques, and identify sites where 5-HT2A receptors have not been previously reported. A preliminary report has been published (Fay and Kubin, 1997).

**MATERIALS AND METHODS**

Fourteen Sprague-Dawley rats (250–400 g; Charles River, MA) were used in this study. All procedures conformed to the guidelines set forth by the National Research Council (1996), and were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

**Tissue harvesting and immunohistochemical procedures**

The animals were administered an overdose of pentobarbital sodium (150 mg/kg i.p.; Nembutal, Abbott Laboratories, North Chicago, IL) and perfused through the aorta with 1 liter of 10 mM phosphate-buffered saline (PBS; pH 7.4) containing 3,000 units heparin at room temperature followed by 1 liter of ice-cold 4% paraformaldehyde and 5% dimethyl sulfoxide (DMSO) in 10 mM PBS. The brains were removed, postfixed in the same fixative overnight, washed in 10 mM PBS, and cryoprotected in 30% sucrose/PBS, all at 4°C. Coronal (11 animals) or sagittal (3 animals), 35-μm sections of the brainstem were cut on a cryostat and all, or every other, were collected for processing. The sectioning in coronal plane extended from approximately -8.30 to -14.60 mm from bregma (about 90 or 180 sections). Sagittal sections were collected to cover both halves of the brainstem.

All sections were pretreated in 1% sodium borohydride in PBS for 30 minutes followed by washes in PBS and 30 minutes incubation in 70% methanol. Selected sections (see below) were then incubated for 60 minutes in a solution containing 1% normal goat serum and 0.3% Triton X-100 in 2% bovine serum albumin (BSA) at room temperature followed by incubation in rabbit anti-5-HT2A receptor antiserum (Incstar, Stillwater, MI) and further incubation in a goat anti-rabbit antibody (Incstar, Stillwater, MI) and finally incubation in an avidin-biotin complex and diaminobenzidine chromogen (Vector, Burlingame, CA). Sections were counterstained with neutral red.

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>4V</td>
<td>fourth ventricle</td>
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<tr>
<td>5-HT</td>
<td>serotonin</td>
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<tr>
<td>5-HT2A</td>
<td>serotonin 2A receptor subtype</td>
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<tr>
<td>5St</td>
<td>spinal trigeminal nucleus</td>
</tr>
<tr>
<td>7n</td>
<td>facial nerve</td>
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<tr>
<td>12 n</td>
<td>hypoglossal nerve</td>
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<td>Amb</td>
<td>nucleus ambiguus</td>
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<tr>
<td>AP</td>
<td>area postrema</td>
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<tr>
<td>Aq</td>
<td>cerebral aqueduct</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CVLM</td>
<td>caudal ventrolateral medulla</td>
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<tr>
<td>DCM</td>
<td>dorsal cochlear nucleus</td>
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<tr>
<td>DOI</td>
<td>(±)-1-(2,5-Dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride</td>
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<tr>
<td>DTN</td>
<td>dorsal tegmental nucleus</td>
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<td>DVM</td>
<td>dorsal vagal motor nucleus</td>
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<tr>
<td>icp</td>
<td>inferior cerebellar peduncle</td>
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<td>IO</td>
<td>inferior olive</td>
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<td>LC</td>
<td>locus coeruleus</td>
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<tr>
<td>LDN</td>
<td>lateral dorsal tegmental nucleus</td>
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<tr>
<td>LRN</td>
<td>lateral reticular nucleus</td>
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**Key Abbreviations**

- **MVe**: medial vestibular nucleus
- **Mo 5**: trigeminal motor nucleus
- **Mo 7**: facial motor nucleus
- **Mo 12**: hypoglossal motor nucleus
- **NST**: nucleus of the solitary tract
- **PAG**: periaqueductal gray
- **PaC**: pontine nucleus, caudal
- **PaO**: pontine nucleus, oral
- **PaV**: pontine nucleus, ventral
- **PPN**: pedunculopontine tegmental nucleus
- **Pr5**: principal trigeminal sensory nucleus
- **py**: pyramidal tract
- **RTtg**: reticulotegmental nucleus
- **RVLMM**: rostral ventrolateral medulla
- **s5**: spinal trigeminal tract
- **scp**: superior cerebellar peduncle
- **sol**: solitary tract
- **SpVe**: spinal vestibular nucleus
- **SubC**: nucleus subcoeruleus
polyclonal antibody was raised against amino acids 22–41 on the N-terminal of the rat’s 5-HT\(_{2A}\) receptor protein. Subsequently, after washes in PBS, the sections were incubated for 45–60 minutes with an anti-rabbit biotinylated secondary antibody made in goat (Vector Laboratories, Burlingame, CA), washed in PBS, and incubated for 45–60 minutes in an avidin-horseradish peroxidase (HRP) complex (Vector). HRP was visualized by using 0.3% diaminobenzidine (DAB) with nickel intensification (0.5% nickel ammonium sulfate) as the chromogen in the presence of 0.0009% H\(_2\)O\(_2\).

Two series of sections from each brainstem cut in the coronal plane were used for immunohistochemical localization of the 5-HT\(_{2A}\) receptor protein. Of those, one was and the other was not counterstained with Neutral Red (Sigma, St. Louis, MO). In four animals, two additional series of sections were saved and used for control procedures. One control consisted of incubation with the 5-HT\(_{2A}\) antibody with the addition of 5-HT\(_{2A}\) receptor peptide (5 \( \mu \)g/ml; Incstar, Stillwater, MN); as the other control, the primary antibody was omitted from the incubation sequence. Only two series of sections were made from the sagittally sectioned brainstems, one of which was counterstained with Neutral Red and the other was not. All sections were mounted onto gelatin-coated glass slides, dehydrated in alcohol, cleared in xylene, and coverslipped with DPX mountant (Electron Microscopy Sciences, Fort Washington, PA).

Data analysis
The mounted sections were analyzed by using a Leitz Aristoplan microscope. Immunopositive cells and cellular processes were identified by the black granular reaction product characteristic of the nickel-intensified DAB-HRP reaction product that was easily distinguished from the background and the Neutral Red counterstaining. Neurons were classified as immunopositive if the cell’s nucleus and at least one dendrite were present and contained DAB-HRP reaction product as described above. Most of the analysis was done by using the counterstained series of sections. Cell counts were made from coronal sections, whereas sagittal sections were mainly used to relate the rostrocaudal extent of the regions containing labeled cells to the boundaries of the corresponding brainstem nuclei. To derive a semiquantitative assessment of the number of labeled cells in different nuclei, cells were counted in successive coronal sections spaced at an interval of 140 \( \mu \)m. Because the sections adjacent to those analyzed were subjected to control procedures, it was not possible to use the physical disector (e.g., Coggeshall and Lekan, 1996) with this material. To avoid overestimation of cell counts, only those profiles that looked likely to represent a major portion of the cell body and had one or more dendrites attached were counted. Those profiles which, by comparison with other fully recognizable neurons in the same area, could have represented only a fraction of a neuron whose main portion was likely to be located on an adjacent section were disregarded. Labeled processes were classified as dendritic only when the structure was seen to emanate from a cell body. In each analyzed section, individual immunopositive cells were identified according to the above criteria and counted in two optical planes. The distance between the optical planes was adjusted manually and could vary from section to section, which represents a deviation from the strict rules of the optical disec-

tor counting method described by Coggeshall and Lekan (1996).

Within each animal, cell counts per section counted within each neuroanatomical structure analyzed were averaged for the corresponding regions on both sides of the brainstem for each rostrocaudal level in 140-\(\mu\)m increments. These counts were subsequently averaged across three animals from which labeling of particularly good quality was consistently obtained throughout the analyzed rostrocaudal extent of the brainstem. The boundaries of each nuclear region containing labeled neurons were determined with the aid of the Paxinos and Watson (1986) rat brain atlas, and additional observations of sagittal sections from our material. The obex was used as the zero reference point for determination of all coordinates in the anteroposterior direction, and its level was identified at the point immediately below the caudal tip of the area postrema, corresponding to −14.30 mm relative to bregma.

The criteria for counting individual neurons were not suitable for certain regions where the labeling was not associated with distinct cellular profiles, or where the high density of labeling made a precise counting of cells impossible. In such regions, the nature and relative density of labeling of individual cells and/or whole nuclei was described qualitatively and, where appropriate, classified as light or dark relative to other labeled cells or regions within the same section.

RESULTS
Distinct patterns of 5-HT\(_{2A}\) receptor-like immunoreactivity were present in many regions of the brainstem. The labeling occurred either as neuronal somatic or somatodendritic, was associated with fiber tracts, or was of a diffuse nature and not related to distinct cellular profiles. Examples of these three forms of labeling will be presented in more detail as we successively describe labeling present in functionally related groups of distinct nuclei: orofacial motor, cardiorespiratory motor, and viscerosensory nuclei, oculo-vestibulo-precerebellar, and in the pontomedullary reticular formation. Figure 1 provides a gross representation of the extent of the labeling from rostral (Fig. 1A) to caudal (Fig. 1M) levels of the pons and medulla. In particular, it clearly shows those regions where the entire distinct nuclei, or their slected portions, were intensely labeled. Such regions included all orofacial motor nuclei (Fig. 1C, D, F–M), the dorsal tegmental nucleus (Fig. 1C), and the inferior olive (Fig. 1H–L).

The specificity of the labeling was verified by either preadsorption of the primary antibody with the 5-HT\(_{2A}\) receptor control peptide or omitting the anti-5-HT\(_{2A}\) receptor antibody (primary antibody) from the incubation procedures. Both controls resulted in abolition of all immunoreactivity. Figure 2 shows the results of the control experiment with antibody preadsorption for two of the most intensely stained regions: the trigeminal motor nucleus (Fig. 2A) and the inferior olive (Fig. 2C).

Orofacial motor nuclei
When inspected at a low magnification (\( \times 4 \) or \( \times 10 \) objectives), the trigeminal (Mo 5), facial (Mo 7), and hypoglossal (Mo 12) motor nuclei, all were intensely labeled throughout the area of each nucleus (Fig. 3). The intensity of the labeling in these nuclei made it difficult to distin-
Fig. 1. Gross pontomedullary distribution of serotonin 2A receptor subtype (5-HT$_{2A}$) labeling at different rostrocaudal levels. Note the distinct labeling in the orofacial motor nuclei, including trigeminal (Mo 5; D), facial (Mo 7; F,G), and hypoglossal (Mo 12; J–M) motor nuclei; nucleus ambiguus (Amb; H,I), inferior olive (H–L), and dorsal tegmental nucleus (DTN; C). Numbers indicate the rostrocaudal level relative to bregma; sections shown are spaced by approximately 0.55 mm. For other abbreviations, see list. Scale bar = 1 mm.
guish individual labeled neurons and their processes. At a higher magnification (×25 or ×40 objectives; Fig. 3C, F, and G), it was apparent that the somata of all, or almost all, neurons were labeled in these nuclei. The dark, but transparent cell bodies were embedded in a network of fibers that were often stained more intensely than motoneuronal somata. Due to the overall high intensity of staining, it was difficult to determine whether all of the stained fibers represented motoneuronal dendrites. However, based on the pattern of distribution of fibers radiating in characteristic directions beyond the nuclear boundaries (cf., Card et al., 1986 for Mo 5; Watson et al., 1982 for Mo 7; Altschuler et al., 1994; Fay and Norgren, 1997b, for Mo 12) and the observation of those motoneurons that were located near the edges of the nuclei, it was possible to conclude that most of the strongly labeled fibers represented dendrites of orofacial motoneurons. The small and large arrows in the coronal sections of Figure 3B show selected examples of the somatic and dendritic labeling, respectively, in Mo 5. In Mo 12, somata and dendrites of motoneurons located within the main body of the nucleus (Fig. 3F), and those located just ventrolateral to it (Fig. 3G), show intense labeling. The latter cells represent geniohyoid motoneurons characteristically located ventrolateral to the main body of the nucleus (e.g., Altschuler et al., 1994; Fay and Norgren, 1997b).

The labeled dendrites from Mo 5 motoneurons extended mainly dorsally, laterally, and ventrolaterally into the supra-, intermediate, and principal trigeminal regions (Fig. 3B, large arrow). The dendritic labeling originating from Mo 7 was particularly clear ventral to the nucleus where it coursed toward the ventral surface of the brainstem (Fig. 3D, arrowheads). Another, smaller contingent of labeled dendrites emanated from Mo 7 dorsally. Mo 12-immunopositive dendrites extended laterally and ventrally into the reticular formation (Fig. 3E). This pattern of hypoglossal dendritic labeling was particularly evident in sections through rostral and caudal aspects of the nucleus, and less so in the intermediate regions. Labeling in sagittal sections revealed that additional motoneuronal dendrites coursed in the anteroposterior direction within the boundaries of each of these nuclei (not shown). Such longitudinally oriented dendrites were probably the source of intensely labeled small nonsomatic profiles.
Fig. 3. Coronal sections depicting serotonin 2A receptor subtype (5-HT$_{2A}$) receptor immunoreactivity in the trigeminal, facial, and hypoglossal motor nuclei (Mo 5, Mo 7, and Mo 12). A: Schematic sagittal view of the brainstem showing the approximate levels of the sections shown in B–G. B: Labeling in Mo 5. Selected motoneurons located on the edges of the nucleus are pointed out by the small arrows. Dendritic arborizations extend dorsally, into the supratrigeminal region, laterally and ventrolaterally into the principal trigeminal sensory nucleus (large arrow). C: High magnification of Mo 5 depicting labeled cell bodies and dendrites emanating from Mo 5 motoneurons. D: Labeling in the main and accessory portions of Mo 7. Labeled dendrites extend ventrally towards the ventral surface of the medulla (arrowheads) and dorsally into the reticular formation. Note the labeled accessory motoneurons dorsal to Mo 7 proper (arrows). E: Labeling in Mo 12. Labeled dendrites emanate laterally and ventrally. Labeled cells located ventrolateral to the main body of the nucleus, presumably geniohyoid motoneurons (arrow), also show intensely labeled dendrites radiating into the reticular formation. Strongly labeled cells also are present in the lateral portion of the dorsal motor nucleus of the vagus, just dorsal to Mo 12 (arrowheads). No labeling is present in area postrema (AP). F: High magnification of ventromedial region of Mo 12. Although the immunoreactivity in Mo 12 was extremely dense, in some locations, it was possible to differentiate somatic labeling in the main body of the nucleus. G: A higher magnification of geniohyoid motoneurons (arrow) located ventrolateral to the main body of Mo 12. All examples are from sections lightly counterstained with Neutral Red. For abbreviations, see list. Scale bars = 25 µm in C,F; 250 µm in E (also applies to B,D); 50 µm in G.
present in a particularly high abundance in the coronal cross-sections.

The overall intensity of labeling within Mo 5 and Mo 12 was not obviously different in distinct functional compartments of these nuclei, e.g., medial and lateral Mo 5, containing predominantly motoneurons controlling jaw opening and closing muscles, respectively (Lynch, 1985; Fay and Norgren, 1997a), or dorsal and ventral Mo 12, which respectively innervate tongue retracting and protruding muscles (Aldes et al., 1988; Dobbins and Feldman, 1995; Fay and Norgren, 1997b).

In the nucleus ambiguus (Amb), the pattern of labeling was somewhat different from that in Mo 5, 7, and 12. In the rostral pole of the nucleus (approximately between -11.60 and -11.80 mm relative to bregma), intensely labeled individual cell bodies occupied only the ventral portion of the nucleus, whereas its dorsal portion was filled with labeling having a lighter and relatively diffuse appearance (Figs. 4A and B; 5A and B). Based on their cellular morphology and position, the intensely stained cells were likely to represent Amb motoneurons of the semicompact formation (Bieber and Hopkins, 1987). Labeled dendrites from these immunopositive neurons extended in the dorsal, medial, and ventral directions, and were most prominent in the dorsoventral orientation (Fig. 4B and triangles in Fig. 5B).

At intermediate levels of Amb rostrocaudally (−12.80 through −13.24 mm to bregma), the immunoreactive cell bodies formed a densely packed group within the boundaries of the nucleus. The dendritic labeling present at this level was not as extensive as that in the other orofacial motor nuclei or the rostral portion of Amb (Fig. 5C and D). At, and caudal to, the obex, Amb contained relatively few immunopositive cell bodies, whereas labeled profiles that might have represented dendrites of more rostrally located Amb motoneurons were relatively abundant (Fig. 5E and F). This interpretation was supported by sagittal sections in which rostrally located Amb motoneurons often had caudally oriented labeled dendrites that coursed within the outlines of the nucleus (Fig. 4).

**Medullary and pontine regions involved in autonomic regulation**

In the medulla, labeling was present in the dorsal motor nucleus of the vagus, the viscerosensory nucleus of the solitary tract, and caudal spinal trigeminal nucleus im-
important for both somatic and visceral sensory transmission. Relatively few distinctly labeled cells were seen in the area ventral to Amb, at levels corresponding to the

region of the rostral and caudal ventrolateral medulla. In the pons, diffuse labeling was present in the lateral parabrachial region.

Fig. 5. Coronal sections showing serotonin 2A receptor subtype (5-HT_{2A}) labeling in the nucleus ambiguus (Amb), and the adjacent regions. A,B: Rostral medulla. Labeled neurons occupy the ventral part of Amb (large arrows in B), whereas the dorsal part of Amb contains mainly diffuse, nonsomatic labeling. The arrows in A point to smaller labeled cells in the rostral ventrolateral medulla (RVLM). Arrowheads in B show selected labeled dendrites. C,D: Intermediate medullary level. In contrast to rostral Amb, labeled neurons are densely packed and occupy a large portion of the nucleus (large arrows in D). Diffuse labeling is less prominent than in A, but can still be seen dorsal to the cluster of labeled motoneurons. The caudal linear nucleus also contains labeled neurons and dendrites (arrowheads in C). Small arrow in D shows a labeled cell in RVLM. E,F: Obex level. Few labeled Amb neurons (arrows) are present, and staining is less intense than at more rostral levels. Scale bars = 150 µm for A,C,E; 50 µm for B,D,F.
Dorsal motor nucleus of the vagus (DVM). Similar to the orofacial motor nuclei, the DVM also was intensely labeled, with the intensity of somatic labeling often stronger than that seen in the somata of the adjacent Mo 12 (Figs. 3E, triangles; 6C and D). In contrast to the orofacial motor nuclei, however, the immunoreactivity was not evenly distributed throughout the rostrocaudal extent of the nucleus (Figs. 6 A, C, and E; 7A). At levels caudal to

![Fig. 6. Coronal sections showing serotonin 2A receptor subtype (5-HT2A) labeling in the dorsal motor nucleus of the vagus (DVM). Each level (rostral, intermediate, and obex) of the DVM was photographed from the same tissue section as the Amb in Figure 5. A,B: DVM in the rostral medulla. Arrows in A show immunoreactive DVM neurons located ventromedial to the nucleus of the solitary tract (NST). Arrowheads in B point to labeled dendrites of DVM cells that extend into the NST. C,D: Intermediate level. At this level, DVM motoneurons occupy the whole mediolateral extent of the nucleus, but are more densely packed in its medial aspect (arrows show two labelled cells). Some DVM dendrites extend towards the 4th ventricle (4V; arrowheads in D). E,F: Cells in the DVM are not labeled at, and caudal to, this level. Scale bars = 150 μm and 50 μm in left and right panels, respectively.]}
Fig. 7. The rostrocaudal distribution of the number of cell bodies within the dorsal motor nucleus of the vagus (DVM; A), the rostral and caudal ventrolateral medulla (RVLM and CVLM, respectively; B), and the lateral reticular nucleus (LRN; C). Average data from three animals, with the error bars representing the standard error of the mean. The line beneath the abscissa in A marks the entire rostrocaudal span of the DVM; note that labeled DVM neurons were absent from the caudal one-third of the nucleus. The dashed line and solid portions of the line below the abscissa in B mark the rostrocaudal extent of the CVLM and RVLM, respectively. The line beneath the abscissa in C shows that labeled cells in LRN were present at all rostrocaudal levels of the nucleus. Note that the number of labeled cells in CVLM is much lower than in the adjacent and extending further caudally LRN.
the obex, the DVM contained little or no somatic labeling (Fig. 6E and F). Rostral to this level, intensely stained motoneurons first appeared in the lateral aspects of the nucleus, then gradually filled its whole mediolateral extent, and then shifted towards its medial portion (Fig. 6C). This shift occurred over a relatively short rostrocaudal distance, between −14.30 and −13.30 mm to bregma. The rostralmost end of the nucleus contained intensely labeled motoneurons (Fig. 6A and B). This pattern of labeling was consistently present in all experiments. The largest number of labeled DVM cell bodies was found at intermediate levels of the rostral half of the nucleus (Fig. 7A). Labeled dendrites of DVM motoneurons coursed in several directions, some towards the nucleus of the solitary tract (Fig. 6B, triangles), whereas others extended toward the 4th ventricle (Fig. 6D, arrowheads).

Nucleus of the solitary tract (NST). In the NST, the labeling was present in two small regions, one located just medial and the other within and just lateral to the solitary tract (Fig. 8A and B). However, no distinct labeled cell bodies were present in either of these locations (Fig. 8A and B); rather, the labeling was diffuse and uniformly covered these two loci. In spite of their diffuse appearance, these two labeled patches were present consistently in all brains and were abolished by control procedures. The medial labeling (Fig. 8A, small arrow) was located between the solitary tract and the area postrema (AP), within the medial subnucleus of the NST (approximately −13.80 mm relative to bregma). The position of the second labeled locus (Fig 8A, large arrow) corresponded to the interstitial subnucleus (Mrini and Jean, 1995). This labeling could be seen as a longitudinal band coursing in a close association with the solitary tract for approximately 420 μm in the rostrocaudal direction in sagittal sections (not shown). No labeling was present in the area postrema adjacent to NST (Fig. 8A).

Spinal trigeminal sensory nucleus (5St). The lateral rim of the caudal division of the nucleus was encircled
by a diffuse extracellular labeling having similar appearance to that in the NST. This labeling was consistently present only in sections caudal to the obex. The pattern of this labeling is shown in Figure 8C (arrows). Rostral to the obex, labeling having a similar diffuse appearance occurred only in patches scattered along the lateral aspects of the 5St. One such patch extended into the fascicles of the spinal trigeminal tract and into the paratrigeminal zone.

Distinctly labeled cell bodies were not present within the 5St, but a few labeled cells were located along the medial border of 5St within the parvicellular reticular formation (PCRt), between the level of the pyramidal decussation and the rostral pole of the area postrema (not illustrated). Other trigeminal sensory nuclei, such as the principal and mesencephalic nuclei, were devoid of immunoactivity.

**Rostral and caudal ventrolateral medulla (RVLM and CVLM).** This region contained few immunoreactive neurons, of which most were lightly labeled. Many cells were intertwined within the labeled dendrites of Amb neurons, and were distinguished from the labeled cells associated with the ventral border of Amb by their smaller size (Fig. 5A–D). A few labeled neurons also were present in the region corresponding to the CVLM. Overall, however, the counts of cells located within either CVLM or RVLM (~11.80 to -14.60 mm from bregma) were of the order of only 5–10 per section (Fig. 7B), and the labeled cells represented a very small fraction of all cells present in this region. In contrast to the few and lightly labeled neurons in the RVLM and CVLM, the caudal linear nucleus, located dorso- and ventrolateral to the Amb, contained intensely labeled cells intermixed among equally intensely labeled dendrites and superimposed on the background of diffuse label (Fig. 5C, arrowheads).

**Lateral parabrachial and Kölliker-Fuse regions.** A diffuse pattern of labeling, not associated with discernible cell bodies, was present in the lateral parabrachial region. The labeling extended throughout the region and included the internal, central, ventral, dorsal, and external lateral parabrachial subnuclei (Feil and Herbert, 1995). The labeling consisted of thin varicosities superimposed on a larger area of diffuse labeling similar to that seen in the NST (Fig. 8D). Some of those varicosities extended into the fascicles of the brachium. Thus, they may have represented labeling associated with brachial fibers terminating in the parabrachial region. In the Kölliker-Fuse region, labeling was located on fibers, rather than cell bodies (Fig 8D, open arrow). Because of the proximity of Mo 5, immunopositive dendrites emanating from the latter nucleus may have contributed, in part, to the labeling present in the Kölliker-Fuse region.

**Medullary regions related to oculomotor and vestibulocerebellar systems**

Labeled cell bodies and dendrites were present in the abducens nucleus, nucleus prepositus hypoglossi, vestibular complex, lateral reticular, inferior olive, and cuneate nuclei.

**Abducens nucleus.** Large cell bodies and their dendrites were clearly labeled, and extended throughout the rostrocaudal range of the nucleus (not shown). The motoneuronal labeling shared similar qualitative characteristics as those found in the orofacial motor nuclei, but many smaller unlabeled cells were interspersed among larger ones that were immunoreactive, so that the labeled cells were not as densely packed as in the orofacial motor nuclei. This allowed for definite determination that dendrites of many motoneurons extended into all planes, with the most prominently labeled ones coursing ventrally. The labeled abducens motoneurons could be clearly distinguished from the cells of the caudally located interstitial nucleus and the dorsal paragigantocellular regions, as those did not contain labeled neurons.

**Nucleus prepositus hypoglossi.** The labeling in this nucleus was both somatic and dendritic and concentrated in its medial aspects (Fig. 9A, large arrows), although less densely packed and less intensely stained cells also were present in its lateral portion (Fig. 9A, small arrows). Intensely stained cell bodies were interspersed among many unlabeled neurons. The greatest number of labeled cells was located approximately 2.5 mm rostral to the obex, near the pontomedullary junction (Fig. 10A). A portion of the extrasomatic labeling in the medial aspects of the nucleus might have corresponded to the rostrally oriented dendrites of Mo 12 motoneurons. This intense labeling concentrated along the edge of the fourth ventricle and obscured observation of dendritic fields of the medially located prepositus hypoglossi neurons.

**Vestibular complex.** The spinal, medial, lateral, and superior vestibular nuclei contained somatic and dendritic labeling in some cells that were intermixed with unlabeled neurons. The distribution and staining intensity of the somatic labeling differed among these nuclei. The cell bodies in the spinal vestibular nucleus were relatively lightly labeled but were packed rather densely (Fig. 9B). In contrast, the cells of the medial vestibular nucleus were stained more intensely, but were relatively loosely scattered among unlabeled neurons throughout the nucleus (Fig. 9B, white arrows). The majority of somatic labeling for these two vestibular nuclei was found above the pontomedullary junction, about 2.8 mm rostral to the obex (Fig. 10B and C). Labeled dendrites radiated from many spinal vestibular cells in all directions (Fig. 9B, small arrows), whereas few of the intensely stained fibers also present in the medial vestibular nucleus could be unequivocally identified as originating in the local cell bodies. At the level of the caudal pole of the dorsal cochlear nucleus (about -11.45 mm from bregma), a distinct band of heavily immunoreactive fibers coursed between the dorsolateral border of the spinal vestibular nucleus and the dorsal cochlear nucleus (Fig. 9B, arrowheads). We could not identify the origin of these fibers, but they seemed to be related either to the cell group Y, the acoustic stria, or corresponded to a bundle of fibers of the 8th nerve. A portion of these fibers could also originate from the spinal vestibular nucleus.

In the more rostrally located lateral and superior vestibular nuclei, the labeling was quantitatively and qualitatively different from that in the spinal and medial vestibular nuclei. In the transition zones between the spinal and lateral vestibular nuclei, the smaller and consistently labeled spinal vestibular cells were located ventral to the larger and infrequently labeled lateral vestibular neurons. Rostral to this transition zone, when the large neurons typical of the lateral vestibular nucleus became dominant, only a few immunoreactive neurons were found in each section. At these levels, the superior vestibular nucleus also contained some lightly labeled cells.
Lateral reticular nucleus (LRN). Immunoreactive cell bodies were present throughout the rostrocaudal length of the nucleus (Fig. 7C), with the labeling intensity varying from light to very intense (Fig. 9C). The density of labeled cells was not uniform throughout the rostrocaudal extent of the nucleus. More cells were labeled in the rostral than in the caudal half of the nucleus (Fig. 7C). In the caudal half, the parvicellular division of the nucleus contained a relatively uniform density of stained cells, whereas in the magnocellular division labeled cells were preferentially located in its ventral portion. The highest numbers of labeled cells, about 100 cells per section, were counted in sections that were within 0.3 mm rostral and caudal to the obex. Labeled dendrites extended from both intensely and lightly labeled cells (Fig. 9C, small arrows), but in contrast to Mo 7, they remained within the boundaries of the nucleus (cf., Fig. 3D).

Inferior olive (IO). A very intense labeling was present in the IO, but it was not uniform (e.g., Figs. 2C and 12B and C). The most homogenous, dark labeling was present in the caudal one-third of the nucleus (cf., Fig. 12C). Inspection of the IO by using ×25 or higher objectives was needed to see that individual cell bodies in all darkly stained subnuclei were labeled extremely strongly, so that most of them were not transparent (Fig. 9D, arrows). Further rostrally, only the dorsal and principal subnuclei were labeled very intensely, whereas other areas were lighter (Fig. 2C). This intense somatic labeling obscured morphological details of the labeling of fibers that could only occasionally be traced at some length within the nucleus, and rarely extended beyond the zone of intense staining. For the same reason, it was difficult to determine whether the differences in labeling intensity between different regions were due to true variations in the density of cells expressing the receptor protein, or to changes in dendritic orientation within the neuropil. Nevertheless, it appeared that all fibers present in the IO represented...
Fig. 10. Histograms showing the average numbers of labeled cell bodies per section within the nucleus prepositus hypoglossi (A), spinal (B), and medial (C) vestibular nuclei. Labeled cell bodies were found throughout the entire length of each nuclear region. Average data from three animals, with error bars representing the standard error of the mean.
dendrites of IO cells, as no stained fiber bundles were seen entering or exiting the nucleus. The intense labeling present in the IO was specific, as it was abolished by either of our control procedures (Fig. 2C and D).

**Cuneate and external cuneate nuclei.** Immunopositive cells were present in the portion of cuneate nucleus spanning from the rostral pole of the area postrema (−13.30 mm from bregma) to approximately the midpoint of the area postrema. This area lies rostral and is distinct from the area described by Basbaum and Hand (1973) as containing cellular “bricks.” This rostral region contains numerous cell types including spindle-shaped and multipolar cells, whereas the caudal region contains only small round cells (Basbaum and Hand, 1973). Many neurons were moderately stained and some had labeled proximal dendrites. The labeling in the external cuneate nucleus was less prominent, consisting of very lightly stained cells, some with labeled dendrites. It was first evident in the sections just above the obex and continued rostrally to the level of the spinal vestibular nucleus. In contrast, the gracilis nucleus contained no labeling.

**Pontomedullary reticular formation**

Somatic labeling was present in several locations within the pontomedullary reticular regions implicated, among others, in the control of sleep and vigilance. These locations comprised the laterodorsal nuclei (LDN) and pedunculopontine nuclei (PPN), the nucleus subcoeruleus, the medial pontine reticular formation, and the medullary reticular formation adjacent to the raphe nuclei.

**Laterodorsal and pedunculopontine nuclei (LDN and PPN).** The position of immunopositive cell bodies lying medial to the brachium conjunctivum (Fig. 11A and B, arrows) was consistent with the location of acetylcholine and nitric oxide synthase-containing neurons of the PPN (Armstrong et al., 1983; Rye et al., 1987; Ruggiero et al., 1990; Vincent and Kimura, 1992; Dun et al., 1994). These cells were present at high density only in the caudal pole of the PPN (−8.30 mm from bregma) and extended rostrally to about the level of the middle of the inferior colliculus.

Labeled LDN neurons only were located in the caudal portion of the nucleus (between approximately −8.80 mm
to −9.30 mm from bregma; Fig. 11C and D). This location contained a mix of intensely and lightly immunoreactive neurons. Although many of these cells possessed labeled proximal dendrites, a dense plexus of relatively intensely labeled fibers also was present in this region (Fig. 11D). The origin of most of these fibers could not be unequivocally ascertained, but a few could be identified as dendrites of LDN neurons. The adjacent dorsal tegmental nucleus (DTN) also was intensely stained. Observation through ×40 objectives revealed that, in contrast to LDN, this staining was mainly present on diffusely distributed fibers, with relatively sparse intensely labeled neurons (Fig. 11D).

**Subcoeruleus region (SubC).** The cells labeled in the SubC were scattered among other unlabeled neurons of this reticular region delimited by the dorsomedial tip of Mo 5 and the ventral border of the locus coeruleus (LC). The cells were labeled relatively lightly compared to the adjacent Mo 5 (Fig. 11E).

**Medial pontine reticular formation.** Labeled neurons were scattered throughout the caudal (PnC) and oral (PnO) subdivisions of the medial pontine reticular formation (Fig. 11C and E). The cells were present at rostrocaudal levels between −8.00 mm to −10.04 mm relative to bregma. Most were of medium size and relatively lightly labeled when compared to the large, intensely labeled cells in the medullary gigantocellular region (described below). Labeled dendrites often extended from the larger of those pontine cells.

Near the mesopontine junction, beginning at approximately −9.30 mm from bregma and continuing rostrally, intense labeling was seen in the reticulotegmental nucleus (Fig. 1C). This labeling consisted of cell bodies and dendrites and was qualitatively very similar to that seen in the DTN. In contrast, the ventral pontine reticular nucleus (PnV) was marked by the presence of only a few lightly labeled neurons.

The dorsal raphe nucleus and LC were devoid of any labeled profiles, somatic or dendritic, whereas the periaqueductal gray (PAG) contained few labeled neurons located along the outer border of its dorsolateral subdivision (Beitz, 1985). These cells were lightly stained and mingled with a diffuse labeling similar to that seen in the spinal trigeminal nucleus. Unlike the inferior olive, the superior olive was devoid of any somatic or dendritic labeling (Fig. 1C–E).

**Medial medullary reticular formation.** Intensely labeled cell bodies with long labeled dendrites were present in the ventromedial reticular formation of the caudal pons and rostral medulla. Intensely immunoreactive neurons were located along the raphe magnus and obscurus nuclei. At more caudal levels, they extended laterally towards the ventromedial reticular formation, including the pars alpha and ventralis parts of the gigantocellular region (Fig. 12A), parallel to, but clearly away from, the dorsal edge of the IO (Fig. 12B). The morphology of these cells could be fully appreciated, as they were loosely scattered among many other unlabeled neurons. Their labeled dendrites had predominantly a mediolateral orientation, with some crossing the midline.

There was a clear rostrocaudal gradient of the density of labeled cells in the medial medullary reticular formation (Figs. 12A–C and 13). In Figure 12, immunoreactive neurons are most numerous in A, which shows the gigantocellular region, the raphe magnus, and the rostral pole of hypoglossal motor nucleus (Mo 12). Although much less numerous at this level, labeled cell bodies distributed along the midline (small arrows) and within the gigantocellular regions (large arrows) were still present. C: At obex level, few or no labeled cells are present along the midline, and fewer cells are found laterally in the ventral medullary reticular nucleus. IO, inferior olive; py, pyramidal tract. Scale bar = 300 μm.
Fig. 13. Histograms showing the average number of labeled cell bodies in the nucleus reticularis gigantocellularis (A), the gigantocellular pars alpha (B), gigantocellular pars ventralis (C), and along the midline of the medulla in distinct raphe nuclei (D–F) at different rostrocaudal levels. Error bars represent the standard error of the mean.
tral portions of the obscurus, and pallidus nuclei. At midmedullary levels, the number of labeled cells near the midline decreased dramatically, whereas the lateral paragigantocellular region still contained many intensely stained neurons (Fig. 12B). In the caudal medulla, labeled cell bodies in the ventral medullary reticular nucleus were infrequent, and immunopositive cells near the midline almost completely absent (Fig. 12C). In contrast to the region of magnus and obscurus nuclei, the nucleus raphe pallidus region contained very few labeled cells (1–5 per section, as opposed to 10–20 in the magnus and rostral obscurus; Fig. 13).

**DISCUSSION**

This study describes the pontomedullary distribution of neurons expressing the 5-HT₂A receptor-like protein. It reveals a distinct pattern of expression of this protein in the orofacial motor nuclei, DVM, precerebellar nuclei, regions involved in vestibular and oculomotor functions, ventromedial medullary reticular formation, and discrete groups of cells in the dorsal pons. In contrast to the vastness of serotonergic innervation of the brainstem, as determined from the distribution of 5-HT-containing fibers and terminals (Steinbusch, 1981; see Jacobs and Azmitia, 1992, for a review), the distribution of 5-HT₂A-like protein is relatively circumscribed.

There was a good correspondence between previously reported 5-HT terminal density (Steinbusch, 1981) and the pattern of 5-HT₂A labeling found in our study for many, but not all brainstem regions. Consistent with the presence of a dense serotonergic innervation, the Mo 5, 7, and 12, vestibular regions, gigantocellular nuclei, and the DVM contained cells with extremely intense or at least moderate 5-HT₂A receptor labeling. On the other hand, few 5-HT-containing terminals were found in the IO (Steinbusch, 1981), whereas 5-HT₂A labeling within this nucleus was very strong. On the other end of the spectrum, a relatively high density of 5-HT terminal labeling within the NST and the external rim of the caudal spinal trigeminal nucleus (Steinbusch, 1981) contrasts with the absence of somatic labeling for the 2A receptor protein in these locations. Thus, overall, there is a limited agreement between the brainstem distribution of 5-HT-containing terminals and 5-HT₂A receptor labeling. This is not surprising, considering that 5-HT₂A receptors represent only one of at least eleven 5-HT receptor subtypes present in the CNS. Given the predominantly excitatory nature of the effects mediated by 5-HT₂A receptors, this study helps establish the anatomic basis for excitatory influences of 5-HT within the intermediate and caudal brainstem. The specificity of the antibody used was verified by its preadsorption with 5-HT₂A receptor peptide prior to its application to the tissue. This resulted in the abolition of all immunoreactivity throughout the neuropil, including the most intensely stained regions such as the IO and orofacial motor nuclei. Thus, the labeling patterns observed in our study were specific for the 5-HT₂A receptor protein.

Comparison to previous anatomical and functional studies

In one earlier immunohistochemical study of the brainstem distribution of 5-HT₂A receptor-like protein, the antibody used was generated against the same portion of 5-HT₂A receptor protein (Morilak et al., 1993). However, only the pontine labeling described in that study is consistent with our present findings. Similar to Morilak et al. (1993), we found labeled cells in the subcoeruleus area of the pontine reticular formation and in the regions corresponding to the PPN and LDN. In a subsequent study, the same group has demonstrated that many neurons expressing 5-HT₂A-like protein in the latter two nuclei are cholinergic (Morilak and Ciaramello, 1995). The same group observed labeling in other regions of the pons and medulla, but with the exception of three small sites within the nucleus of the solitary tract and spinal trigeminal nucleus, dismissed it as nonspecific on the basis of the low labeling intensity relative to the background and lack of dendritic labeling (Morilak et al., 1993). In particular, all labeling in the orofacial motor nuclei, DVM, and the IO was disregarded. Furthermore, neither the strongly labeled cells scattered along the midline and in the paragigantocellular region of the rostral medulla nor the very consistent, albeit diffuse and clearly nonsomatic labeling associated with the solitary tract were reported. Thus, significant discrepancies exist between our present and that earlier study.

These discrepancies may be due, at least in part, to the particularly high background reported for the brainstem sections by Morilak et al. (1993). This may have obscured their ability to discern specific labeling. The high background may have resulted from the high concentration of the anti-5-HT₂A antibody used in their experiments (1:50, compared to 1:750 in our study). Importantly, the high antibody concentration used in those experiments also may have prevented specific labeling in many regions. It is possible that, due to excessive concentrations of the primary antibody, the avidin–biotin complexes made the subsequent enzyme-substrate reaction ineffective (Hoffman, 1998). Given the high intensity of labeling in the orofacial motor nuclei, IO, LRN, and DVM in our experiments, it is conceivable that appreciable “crowding” occurred in the study of Morilak et al. (1993), which prevented adequate labeling.

Very recently, another study of the distribution of 5-HT₂A receptor-like protein throughout the CNS, including the pons and the medulla, was published (Cornea-Hébert et al., 1999). The study illustrates and discusses the finding from pontomedullary regions to a very limited extent, but it is clear that the nature of labeling is very different from ours, and it appears that the level of background labeling in the brainstem is again relatively high (e.g., plate 6). The major differences include: peculiar somatic labeling whose density decreases dramatically over the nucleus, high intensity of labeling in fiber tracts, and the absence of dendritic labeling in other than primary dendrites. These observations have lead the authors to suggest that most of the labeling corresponds to intracellularly located receptors that either solely play a role in intracellular communication between the soma, dendrites, and axons, or became internalized following their activation by endogenous ligands. In addition, although there is a good agreement between the work by Cornea-Hébert et al. (1999) and our study regarding the presence of somatic labeling for many brainstem structures (e.g., most motor and vestibular nuclei), there are also major discrepancies. The latter include strong or moderate labeling in the parvicellular reticular field, superior olive, spinal and principal sensory trigeminal nuclei, where we found minimal or
Pontomedullary 5-HT$_{2A}$ receptors in the rat

no labeling, and moderate or no labeling in the dorsal motor nucleus and the inferior olivary, where we found extremely intensely stained cells. Because the histochemical methods used in that study are similar to ours, it appears that the use of a different antibody is the main reason for the differences.

In contrast to significant differences between our immunohistochemical results and those of Morilak et al. (1993) and Cornea-Hébert et al. (1999), there is a high degree of consistency between our observations and earlier autoradiographic receptor binding and in situ hybridization investigations describing the distribution of 5-HT$_2$ receptors and the mRNA expression, respectively (Pazos and Pala- cios, 1985; Pompeiano et al., 1994; Wright et al., 1995). It has to be recognized, however, that the inherent limitations of autoradiographic techniques did not allow those studies to visualize the morphological details of the cells containing the receptor protein or its mRNA, and that pharmacological distinction between at least two closely related 5-HT$_2$ receptor subtypes (2A and 2C) present in the central nervous system could not be fully ascertained in studies that relied on pharmacological ligands. Nevertheless, those earlier studies reported a high or moderate density of 5-HT$_{2A}$ receptor binding sites (Pazos et al., 1985), and expression of the corresponding mRNA (Pompeiano et al., 1994; Wright et al., 1995), in the orofacial motor nuclei, DVM, IO, LRN, and several other regions where we found 5-HT$_{2A}$ receptor-like protein. On the other hand, relatively low and/or only nonspecific binding was reported in those areas where we observed intensely labeled cells that were loosely scattered among other unlabeled neurons (the medial medullary reticular formation adjacent to the raphe nuclei and the paragigantocellular region), and 5-HT$_{2A}$ receptor mRNA was not observed in those regions where we found labeling not clearly associated with distinct cell bodies and dendrites (parts of the NST, linear nucleus, lateral edge of the caudal 5St, and portions of the parabrachial region). These latter differences may be explained if we assume that the sites containing diffuse labeling in our study corresponded to the locations where cells synthesizing 5-HT$_{2A}$ receptor protein were absent, whereas the protein was present because it was delivered by intra-axonal transport in axons of cells located elsewhere in the central or peripheral nervous system. Thus, our results are supported by earlier anatomical studies, and extend them by providing an improved morphological identification of those cellular elements that possess 5-HT$_{2A}$ receptors.

The distribution of 5-HT$_{2A}$ receptor-like protein revealed by this study is also consistent with numerous functional pharmacological studies demonstrating excitatory effects of 5-HT, or 5-HT-like compounds having a strong affinity to 5-HT$_2$ receptors. Thus, pressure or iontophoretic applications of 5-HT or 5-HT$_2$ receptor agonists had excitatory effects on motoneurons of the Amb and other orofacial motor nuclei and the DVM (Katakura and Chandler, 1990; Ribeiro-do-Valle et al., 1991; Kubin et al., 1992; Arita et al., 1995; Albert et al., 1996; Hsiao et al., 1997). Furthermore, the 5-HT$_{2A}$ agonists o-methyl-5-HT and DOI, excited IO cells (Llinas and Yarom, 1986; Sugihara et al., 1995), a subpopulation of nucleus prepositus hypoglossi neurons (Bobker, 1994), selected cells of the vestibular nuclei (see Darlington et al., 1995 for a review), and selected cells in the medial pontine reticular formation (Stevens et al., 1992).

In agreement with our finding of large cells with intensely labeled cell bodies and dendrites distributed along the midline of the medulla and in the paragigantocellular region, other studies reported cells excited by 5-HT in the raphe magnus (Wessendorf and Anderson, 1983; Hentall et al., 1993), and in the more caudally located raphe obscurus and pallidus nuclei (Fenik et al., 1996; Bayliss et al., 1997). Those cells were often intermingled with 5-HT-containing neurons, but by numerous criteria were determined to represent a distinct, nonserotonergic population. Those criteria included the absence of tryptophan hydroxylase, the precursor enzyme for serotonin (Bayliss et al., 1997), relatively fast axonal conduction velocities, weak or absent inhibitory effects of 5-HT (Wessendorf and Anderson, 1983; Hentall et al., 1993; Fenik et al., 1996), and the presence in at least some cells of this type of a strong excitatory tactile somatosensory input uncharacteristic of 5-HT-containing neurons (Wessendorf and Anderson, 1983; Fenik et al., 1996). Consistent with those findings, in preliminary double-labeling experiments, we have determined that cells expressing 5-HT$_{2A}$ receptor-like protein are distinct from those containing tryptophan hydroxylase, even though the two cell types are often present next to each other and have a similar general distribution in the rostral medulla (Fay and Kubin, unpublished observations). Based on this proximity of 5-HT$_{2A}$-immunopositive processes, Jansson et al. (1998) suggested that excitatory effects of 5-HT may be mediated in the raphe region by means of volume neurotransmission.

Excitatory effects mediated by 5-HT receptors were reported for cells in the RVLM (Wang and Lovick, 1992; McCall and Clement, 1994; Hwang and Dun, 1998). This is in agreement with our observation of the presence in this region of cells whose cell bodies and at least proximal dendrites express 5-HT$_{2A}$ receptor-like protein. Although direct pharmacological evidence for 5-HT$_{2A}$ receptor-mediated excitation has not been reported for the LRN, the high density of 5-HT$_{2A}$ receptor mRNA (Pompeiano et al., 1994; Wright et al., 1995) and the prominent immunoreactivity described in our experiments strongly implicated 5-HT$_{2A}$ receptors in the activation of LRN neurons. Recently, Murphy and Behbehani (1995) reported that stimulation of the nucleus raphe magnus produced excitatory responses in some LRN neurons. Thus, the consistency of our results with those from earlier neuroanatomical and pharmacological experiments confirms that the pontomedullary cells and regions in which 5-HT$_{2A}$ receptor-like protein was detected in the present study represent those where 5-HT exerts excitatory effects.

**Additional morphological observations not revealed by previous studies**

In contrast to most other locations where 5-HT$_2$ receptor-like protein was clearly associated with distinct cellular profiles, the labeling observed in the nucleus of the solitary tract was diffuse in appearance, and could not be related to distinct cellular profiles (Fig. 8A and B). This pattern of labeling suggests that the antibodies were bound to fibers, rather than cell bodies, and, therefore, a presynaptic location of the protein. A predominantly pre-synaptic location of 5-HT$_{2A}$ receptors in the NST is consistent with the study of Okabe et al. (1997), who reported that 5-HT$_{2A}$ receptor mRNA was absent from this nucleus, and is indirectly supported by an electrophysiological study which cast doubts on the occasional reports suggesting that postsynaptic excitatory effects of 5-HT are...
mediated in the NST by 5-HT\(_{2A}\) receptors (Feldman, 1995). Although we could not determine the identity of those cellular elements that expressed the protein in the NST, our observation of a diffuse labeling associated with the interstitial subnucleus, a small region largely embedded in the solitary tract, suggests that 5-HT\(_{2A}\) receptors may be present on selected vagal afferent endings. Interestingly, ultrastructural observations point to a particularly high incidence of axo-axonic synapses in the intestinal subnucleus (Mrini and Jean, 1995). Many of those synapses involve vagal afferents from the larynx, which may suggest that 5-HT\(_{2A}\) receptors modulate transmission in this afferent pathway important for breathing, deglutition, and phonation.

The appearance of the labeling in the Mo 5, Mo 7, and Mo 12 nuclei was relatively diffuse, as reported (Morilik et al., 1993). By observation through \(\times 25\) or higher objectives, however, we could determine that it was confined to somata and dendrites of individual motoneurons. In addition, in all these nuclei we observed labeled dendrites extending beyond the nuclear boundaries. Individual labeled somata and long dendrites also could be observed in those motoneurons that were located near the boundaries of each nucleus. Thus, excitatory effects mediated by 5-HT\(_{2A}\) receptors onto orofacial motoneurons are likely to be exerted over both their somata and dendrites. Indeed, in the Mo 12, the dendritic labeling was more intense than that overlying the motoneuronal cell bodies. Thus, although these observations still leave open the possibility that a portion of the labeling seen in the soma of orofacial motoneurons represents a pool of internalized receptors (cf., Cornea-\'Herbert et al., 1999), the intense and sharp labeling of dendrites is more compatible with a close association of the labeled protein with dendritic membrane than with a relatively diffuse appearance of immunohistochemically labeled internalized receptors (e.g., studies of \(\alpha\)\(_{2A}\)-adrenergic receptors by Talley et al., 1996 and Guyenet et al., 1994).

The distribution of labeled cells in the Amb region differed from that in the other orofacial motor nuclei in that the distribution of 5-HT\(_{2A}\) receptor-like protein was much less uniform. This may be related to the relative homogeneity of this region (Bieger and Hopkins, 1987; Ellenberger and Feldman, 1990; Nu\'ez-Abades et al., 1992; Lee et al., 1992). Upper airway motoneurons are intermixed here with motoneurons innervating the alimentary tract (Bieger and Hopkins, 1987; Altschuler et al., 1994). In our material, groupings of large and intensely stained cells were primarily found in the ventral aspect of the rostral nucleus ambiguous, corresponding to the semicompact and loose formations. These neurons possessed typical motoneuronal morphology and were present within the region containing pharyngeal and laryngeal motoneurons (Bieger and Hopkins, 1987; Nu\'ez-Abades et al., 1992). Esophageal motoneurons are located more dorsally, within the compact formation where the labeling was less intense. Thus, the latter motoneurons are likely to receive a weak excitatory input mediated by 5-HT\(_{2A}\) receptors. The ventral parts of Amb also contain cardiac vagal motoneurons that are intensely innervated by 5-HT-containing terminals (Lee et al., 1992; Izzo et al., 1993; Hopkins et al., 1996). Application of 5-HT into the Amb excites these cells and results in vagally mediated bradycardia (Izzo et al., 1988). In addition, a longitudinal column of bulbospinal respiratory premotor neurons, the ventral respiratory group, runs parallel to the Amb (Ellenberger and Feldman, 1990). Some of these cells are excited by \(\alpha\)-methyl-5-HT and, therefore, may express 5-HT\(_{2A}\) receptors (Lalley et al., 1995). However, at least in the cat, inhibition is the dominant effect of 5-HT on respiratory-modulated cells other than respiratory motoneurons present in this region (Arita and Ochiishi, 1991). Thus, the amount of 5-HT\(_{2A}\) protein expressed by bulbospinal respiratory neurons may be low, consistent with the greatly reduced number of labeled cells in the Amb at levels caudal to the obex. In addition, the generally less intense labeling in Amb than in other orofacial motor nuclei may explain the less prominent excitatory effects of 5-HT on laryngeal than on Mo 12 motoneurons (Fenik et al., 1997). Overall, however, appropriate double labeling studies are necessary to determine the functional identity of those neurons located in the Amb region that express 5-HT\(_{2A}\) receptor-like protein.

**Physiologic significance of pontomedullary 5-HT\(_{2A}\) receptors**

One condition when brainstem serotonergic neurons are strongly activated is during states of arousal accompanied by centrally generated motor activity (Jacobs and Fornal, 1995). In contrast, serotonergic cells are silenced, or nearly silenced, during the rapid eye movement (REM) stage of sleep (Jacobs and Azmitia, 1992). These extreme conditions provide a perspective with which one can consider the significance of the distribution within the pontomedullary brainstem of 5-HT\(_{2A}\) receptor-like protein.

The potential role of 5-HT\(_{2A}\) receptors present in the orofacial motor nuclei has been discussed earlier in relation to the decrements in upper airway motoneuronal activity that occur during REM sleep (Kubin et al., 1998). It has been suggested that, in predisposed individuals, the excitatory effects mediated by 5-HT in upper airway motoneurons play an important role in the maintenance of upper airway motor tone during wakefulness. During sleep, and REM sleep in particular, a withdrawal of serotonergic excitation contributes to a loss of upper airway motor tone that, in turn, may lead to upper airway obstructions. Our finding that 5-HT\(_{2A}\) receptor-like protein in all orofacial motor nuclei is consistent with this receptor subtype mediating a part of the wakefulness-dependent excitatory effect. In addition, serotonergic excitatory effects in orofacial motoneurons may facilitate engagement of these motoneurons in various stereotyped motor behaviors necessary for chewing, breathing, swallowing, and phonation (Jacobs and Fornal, 1995; Hsiao et al., 1997).

The presence of 5-HT\(_{2A}\) receptor-like protein in the IO, vestibular, and cuneate nuclei, and the LRN implicates 5-HT\(_{2A}\) receptors in control of the afferent input to the cerebellum. One may speculate that 5-HT\(_{2A}\) receptors in these nuclei alter the transmission in spino-olivo-, spino-cuneo-, spino-reticulo-cerebellar, and vestibulo-cerebellar pathways in a sleep state-dependent manner. Of importance here is to note that many of these nuclei also project to the ventrolateral posterior thalamus. This, in turn, may provide the basis for the behavioral state-dependence of the transmission and processing of the information reaching the thalamus. It is, however, unclear why 5-HT\(_{2A}\) receptor labeling in the cuneate nucleus receiving afferents from upper parts of the body is stronger than in the gracilis nucleus receiving afferent information from caudal regions. Pontomedullary 5-HT\(_{2A}\) receptors located in
the RVLM, only selected neurons expressed 5-HT2A receptor agonists. Alterations in the activity of neurons in these nuclei produced by lysergic acid and DOI may facilitate the occurrence, or modulate the expression, of states in which the mind operates in disconnection from the actual external and internal sensory context. On the other hand, the relatively weak labeling of lateral vestibular neurons, which are important for the maintenance of postural tone (see Pompeiano, 1980 for refs.), suggests that a withdrawal from lateral vestibular neurons of serotonergic excitatory drive mediated by 5-HT2A receptors is not the major mechanism leading to decrements in vestibulospinal reflexes during the atonia of REM sleep; rather, state-dependent inhibition at motoneuronal level plays a major role (Pompeiano et al., 1983).

It is interesting to note that, with the exception of the IO, in all the nuclei involved with precerebellar and/or oculomotor functions (abducens, vestibular, and prepositus hypoglossi), labeled cells were intermingled with unlabeled ones. This, in agreement with earlier iontophoretic studies (e.g., Bobker, 1994; Darlington et al., 1995), suggests that only certain subpopulations of neurons in these nuclei can be excited by 5-HT2A receptors. The heterogeneous labeling pattern in different subnuclei of the IO also may indicate that different IO regions are not equally sensitive to the excitatory influence of 5-HT. The prominence of 5-HT2A receptor protein expression in the dorsal IO suggests that these receptors may serve to enhance the integration of somatic inputs, as this region receives afferent input from the portion of cuneate nucleus (McCurdy et al., 1998), which we also found expressing 5-HT2A receptors. Therefore, studies combining identification of 5-HT receptors with further neuroanatomical (e.g., projection sites) and functional classification of neurons are needed in order to better elucidate the role of 5-HT2A receptors in these nuclei. It appears conceivable that 5-HT2A receptors are activated in precerebellar and oculomotor regions in association with motor activation that requires an enhanced sensorimotor coordination.

Another interesting coincidence is that two nuclei showing intense or moderate 5-HT2A receptor labeling, the prepositus hypoglossi and the RVLM, respectively, represent two major sources of afferent innervation of the LC (Aston-Jones et al., 1991). This again relates neurons possessing 5-HT2A receptors in these two regions to those brainstem systems that are relevant for control of sleep states and behavioral arousal. Because the prepositus hypoglossi mediates inhibitory effects onto noradrenergic LC neurons (Aston-Jones et al., 1991), our data support the earlier work suggesting that serotonergic excitation of the prepositus hypoglossi neurons leads to the suppression of LC activity (Gorea et al., 1991; Haddjeri et al., 1997). In the RVLM, only selected neurons expressed 5-HT2A receptor-like protein. Therefore, it remains to be determined whether those are the ones that project to the LC and whether they mediate excitatory or inhibitory effects on LC activity. It is possible that serotonergic effects exerted in the prepositus hypoglossi and RVLM may play a role in the maintenance of a relative balance between activation of the brainstem noradrenergic and serotonergic neurons. This, in turn, may have important implications for neuropsychiatric disorders. Other pontine regions related to sleep and arousal also contained 5-HT2A receptors (PPN, LDN, SubC). This has been reported previously (Morilak et al., 1993; Morilak and Ciaranello, 1993), or suggested by neuropharmacological data (Stevens et al., 1992), but so far there is no comprehensive explanation of the role of 5-HT2A receptors in pontine regions controlling the sleep-wake cycle.

One common feature of the diffusely distributed labeling seen in association with the fibers of the solitary tract, along the lateral rim of the caudal 5St, and in the Kölliker-Fuse and parabrachial regions of the pons, is that all these sites receive, directly or indirectly, afferent information from visceral afferents (Kalia and Sullivan, 1982; Mrini and Jean, 1995; Feil and Herbert, 1995). This suggests that presynaptic or diffuse nonsynaptic effects exerted by 5-HT2A receptors play an important role in the transmission of signals relevant for various aspects of autonomic regulation. So far, there is no specific evidence for presynaptic effects exerted by 5-HT2A receptors within the nucleus of the solitary tract, but presynaptic effects mediated by 5-HT2A have been demonstrated in other systems (Maura et al., 1991).

Thus, in summary, numerous sites and neuronal groups in the pontomedullary brainstem express 5-HT2A receptors. The pattern of distribution of these receptors implies that control of sleep and arousal, motor activation, somatic and visceral sensory transmission, and sensorimotor coordination.

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PONTOMEDULLARY 5-HT₂A RECEPTORS IN THE RAT


