

Serotonergic Neuronal Systems: What Their Anatomic Organization Tells Us about Function

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A review of the anatomic organization of ascending serotonin projections is followed by recent findings showing that these axonal projections are not diffuse but have an intricate and orderly pattern. The dorsal and median raphe nuclei and the B9 cell group have overlapping but differential projections to all parts of the forebrain. While most raphe projections extensively overlap, the dorsal raphe projects most heavily to frontal cortex and striatum, while the median raphe predominantly innervates hippocampus and septum. Small clusters of raphe cells project in a mosaic pattern to multiple, widely distributed islands of cortex. Yet, a coarse topographic order is preserved in the ascending dorsal raphe projections. Recent studies demonstrate two classes of serotonin axon terminals that differ in axon morphology, cells of origin, regional distribution, and response to psychotropic drugs. Dorsal raphe axons are extremely fine and highly vulnerable to certain neurotoxic amphetamines, e.g., 3,4-methylenedioxymethamphetamine; median raphe axons have large varicosities and are resistant to these mood-elevating drugs. We propose that there are two anatomically and functionally distinct serotonergic projections to cortex and that neurons in the dorsal raphe nucleus appear to play a major role in the control of affective state.

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THIS paper provides a brief overview of the neuroanatomic organization of serotonergic [5-hydroxytryptamine (5-HT)] projections to the forebrain. Selected anatomic features of serotonergic neuronal projections are first summarized, followed by a description of recent findings supporting the view that serotonin projections comprise multiple neuronal sub-

systems that have a high degree of specificity and precision in their organization. Anatomic studies of serotonergic neuronal systems have depicted the locations of serotonin-containing neuronal cell bodies, the axonal pathways by which these cells project to distant parts of the brain, and the patterns of axonal arborization in regions where the axons terminate upon target neurons. Over the past 20 years, numerous techniques have been employed to delineate the neuronal circuitry of serotonin cells with progressively increasing sensitivity, precision, and resolution. The recent acquisition of more detailed and comprehensive anatomic data has provided new insights into the functional role of serotonin in the brain and strongly supports early speculation that serotonin neurons play an important role in mental function, especially in the control of affective state and emotional expression.

Serotonin is one of several biogenic amines that are widely distributed within the central nervous system. These monoaminergic compounds, which also include norepinephrine, dopamine, and epinephrine (acetylcholine is often included), have been extensively studied with regard to their neurotransmitter role in the central and peripheral nervous system. The presence of serotonin was first demonstrated in the brain using bioassay techniques based on detection of its vasoconstrictive effects.^{5, 106} However, the sensitivity of bioassays is quite low, and the next advances were made following the development of spectrophotofluorometry at the National Institutes of Health. Employing this new chemical method, Bogdanski and coworkers¹³ reported in 1957 substantial concentrations of serotonin in the dog and cat brain (1 to 2 µg/g), with marked regional differences in levels in different areas of the brain. These authors found especially high 5-HT levels in limbic areas of the forebrain, leading to their speculation that serotonin may be involved in emotional expression. The greatest single breakthrough in this field came in 1962 from the University of Gothenburg, Sweden, where Falck, Hillarp, and their colleagues³⁰ developed a histo-

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chemical method for the visualization of biogenic amines in tissue sections. Fascinating historical accounts of the development of this method have recently been published by Carlsson¹⁵ and by Dahlstrom and Carlsson.²⁵ This powerful new method was then systematically employed by a series of investigators in Sweden who proceeded to map the locations of biogenic-amine-containing neurons and their axonal projections in the brain. The Falck-Hillarp method employed freeze-dried brain tissue that was subsequently exposed to humid formaldehyde vapor, which reacted with the biogenic amines, converting them to fluorescent derivatives, and provided morphologic fixation of the tissue. The serotonin molecule was converted to a beta-carboline, which emits yellow fluorescence when excited by ultraviolet light. The localization and intensity of the fluorescence was sufficient to be visualized in the light microscope, making it possible for the first time to detect serotonin (as well as catecholamines) within neuronal cell bodies and axonal processes.

Localization of Serotonergic Cell Groups

While serotonin-containing processes were detected throughout the nervous system, serotonergic cell bodies were restricted to the brainstem where they were localized in specific nuclei. The first attempt to map serotonergic cell bodies found them localized in discrete clusters that were situated along the midline of the brainstem. The majority of serotonin-containing neurons were observed²⁶ within neuronal groups that were previously designated as the raphe nuclei based upon cytoarchitectural criteria.¹⁰⁴ The histofluorescence method showed that serotonergic cell groups closely, but not exactly, match the raphe nuclei; that some serotonin cells lie outside the boundaries of the raphe nuclei; and that not all raphe cells are serotonergic. Consequently, in 1964 Dahlstrom and Fuxe²⁶ described nine groups of serotonergic cell bodies, which they designated B1-B9 and which correspond largely to the raphe nuclei defined in Nissl-stained preparations. For example, the rostral-most 5-HT cell groups found in the midbrain and rostral pons are classified as groups B6 to B9. Group B7 is the largest group of serotonergic neurons and corresponds to the dorsal raphe nucleus, which lies within the periaqueductal gray matter of the midbrain at a level just behind the oculomotor nucleus and extends caudally down to the rostral end of the fourth ventricle. This B7 group is continuous caudally with a smaller number of serotonergic cells (B6) that lie in the central gray close to the midline along the floor of the fourth ventricle, at levels through the rostral part of the locus coeruleus. Cell groups B6 and B7 are now often lumped together as comprising the dorsal raphe nucleus, and many investigators include B6 as the caudal extension of the dorsal raphe (Figs. 1 and 2).

The B8 cell group corresponds to the median raphe nucleus and has also been designated the nucleus central superior. The median raphe nucleus lies within the core of the reticular formation at the junction between the pons and midbrain. The caudal border of the median raphe lies at a level just rostral to the facial colliculus, and the nucleus extends rostrally to the decussation of the superior cerebellar peduncle, which forms its anterior border. The median raphe has been subdivided into a dorsal midline cluster of densely packed medium sized cells and a more ventral and lateral zone of smaller and more widely spaced cells.¹⁰⁴ Laterally, in the vicinity of the tectospinal tract, the median raphe merges with the giant cell portion of the pontine reticular formation. The position of the median raphe is best appreciated in parasagittal sections (cf. Fig. 31 of Lidov and Molliver⁶⁹). In this longitudinal plane, the median raphe is tilted forward and ventrally such that its caudal-most portion merges dorsally with the dorsal raphe nucleus and the most rostral portion lies in the ventral midbrain where it is continuous with the interpeduncular nucleus (Fig. 3). In addition to serotonergic neurons, the median raphe also contains a substantial proportion of neurons that do not contain serotonin.^{81, 100, 114}

Another prominent collection of serotonergic neurons in the rostral brainstem was designated cell group B9 by Dahlstrom and Fuxe²⁶ and corresponds in part to the tegmental reticular nucleus of the pons.^{96, 104} The B9 group lies in the ventrolateral tegmentum of the pons and midbrain and is therefore not one of the midline raphe nuclei. The serotonin cells of B9 appear to form a lateral extension of the median raphe and lie along the dorsal surface of the medial lemniscus (Fig. 4; refer to Fig. 2 of Dahlstrom and Fuxe²⁶ and Figs. 25 to 27 of Lidov and Molliver⁶⁹).

The three raphe nuclei described above, dorsal raphe, median raphe, and B9, are of particular significance because they give rise to most of the ascending serotonergic projections to the forebrain. The other raphe nuclei, B1 to B5, contain many fewer serotonergic cells and are located along the midline raphe extending from the mid-pons to the caudal medulla. These more caudally situated serotonergic cell groups give rise primarily to intrinsic brainstem connections and some project to the spinal cord. Further discussion of these cell groups is beyond the scope of this review; they have been described elsewhere in greater detail^{21, 26, 69, 73, 99, 100, 102} and are shown in Figure 3B.

Visualization of Ascending Serotonergic Projections

Following the initial discovery of serotonin-containing cells in the rostral brainstem,²⁶ the histofluorescence method of Falck and Hillarp revealed the presence of serotonergic axon terminals in widespread areas

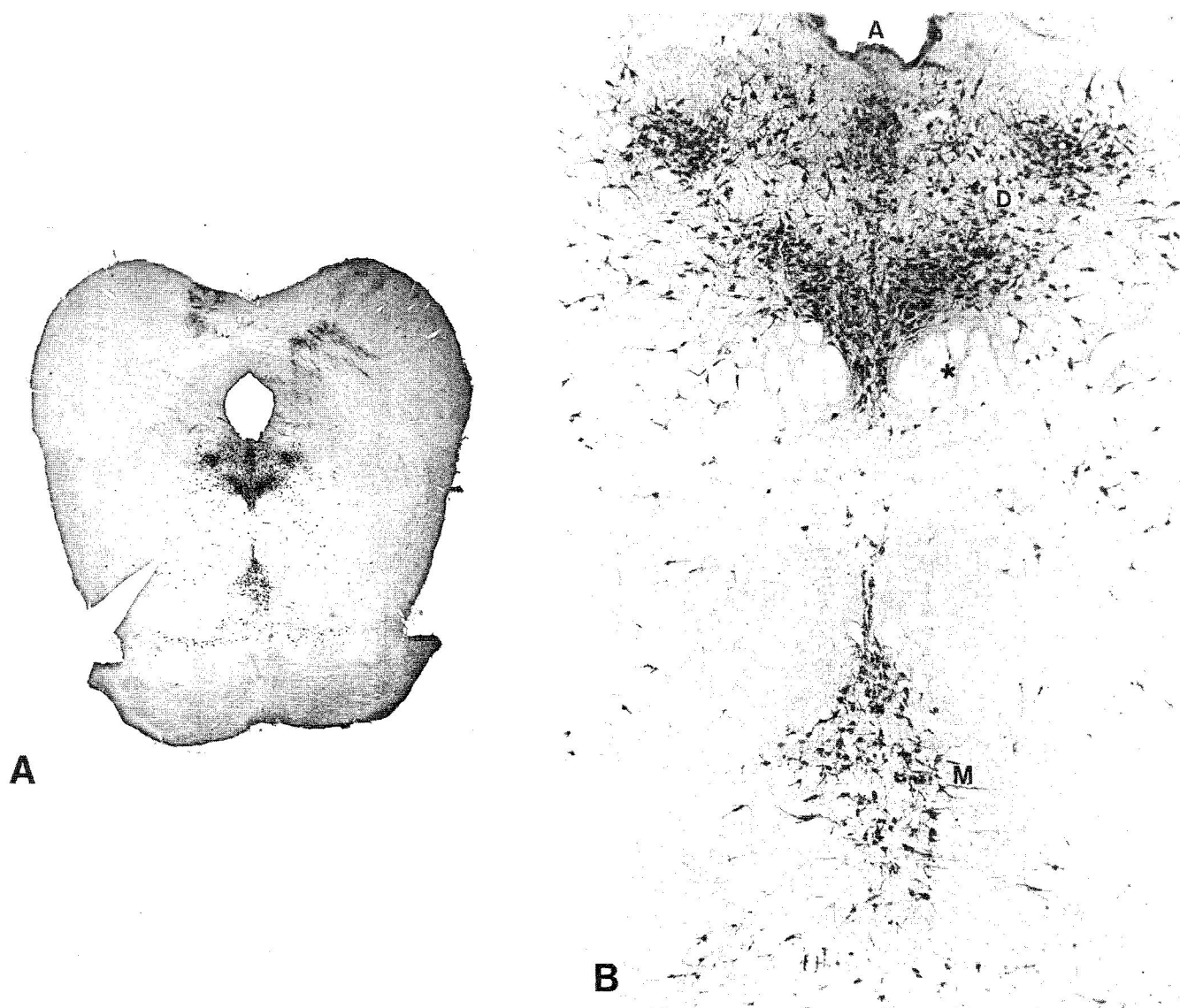


FIG. 1. Serotonergic cell bodies in the midbrain raphe nuclei demonstrated by 5-HT immunocytochemistry. *A*, Low magnification survey view of transverse section through the rat midbrain. The three raphe cell groups shown here in the midbrain give rise to widespread serotonergic projections to cerebral cortex and other parts of the forebrain. *B*, Higher magnification micrograph showing 5-HT neurons in dorsal and median raphe nuclei. The dorsal raphe nucleus (B7) lies in the central gray matter just beneath the cerebral aqueduct. In the transverse plane, the dorsal raphe can be further subdivided into a ventromedial cell cluster between and just above the MLFs, a smaller dorsomedial group just below the aqueduct, and large bilateral cell groups, the lateral wings. The principal cortical serotonin innervation arises from the ventromedial zone of the dorsal raphe. The median raphe nucleus (B7) lies in the central core of the midbrain, below the MLF. In addition to a modest projection to neocortex, the median raphe projects heavily to hippocampus, septum, and hypothalamus. The B9 cell group lies in the ventrolateral midbrain along the fibers of the medial lemniscus. Figure courtesy of Laura Mamounas, Johns Hopkins University. (Abbreviations: D = dorsal raphe, M = median raphe, A = aqueduct, * = MLF [medial longitudinal fasciculus].)

of the forebrain including cerebral cortex, septum, amygdala, and diencephalon.³⁵ However, no serotonergic cell bodies were observed in any part of the forebrain. To determine the origin of the diverse 5-HT-containing axon terminals, this group of investigators in Sweden proceeded to combine the histofluorescence technique with attempts to interrupt the serotonergic axons by surgical lesions. Employing this experimental approach, Anden and colleagues⁶ demonstrated a loss of

fluorescent 5-HT axons in forebrain following lesions of the anterior raphe nuclei or of the hypothalamus. These experimental results, combined with the inability to find serotonin-containing cell bodies in the forebrain, led to the proposition that most of the ascending serotonergic projections arise from cell bodies situated in the raphe nuclei of the midbrain and that axons ascend mainly in the medial forebrain bundle (MFB), which lies in the lateral hypothalamus.^{6, 107} Thus, this discov-

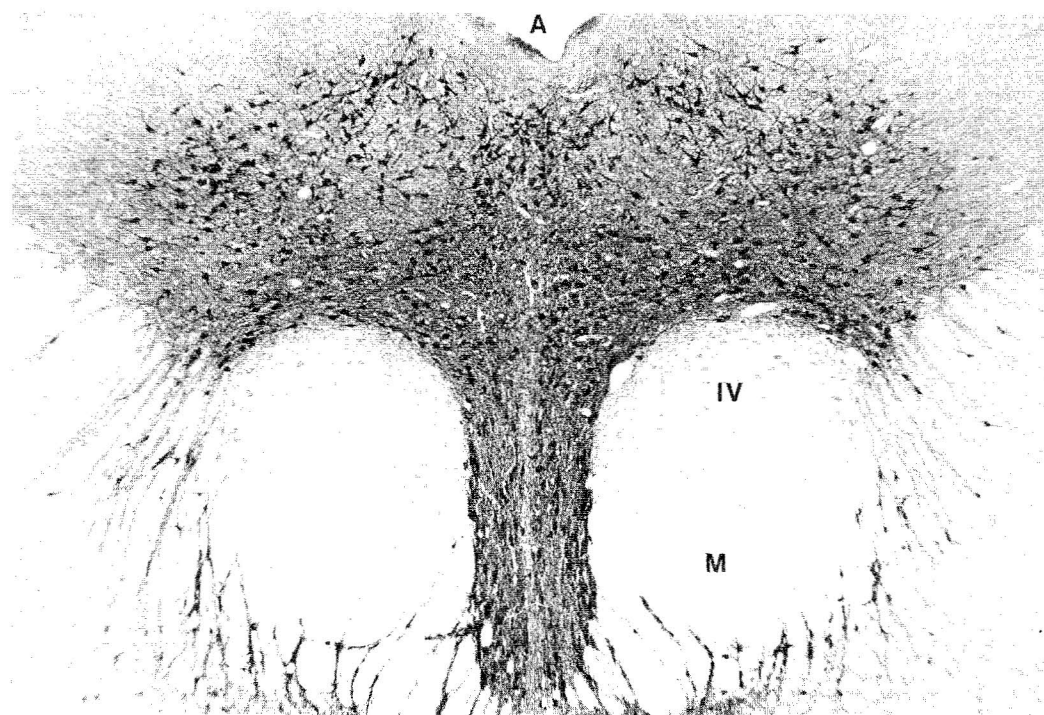


FIG. 2. Serotonergic neurons in the primate midbrain demonstrated by immunocytochemistry. Note the highly differentiated arrangement of 5-HT neurons in the dorsal raphe nucleus just below the cerebral aqueduct. Compared with the rat, the primate dorsal raphe shows a more intricate anatomic organization and the cells are more widely spaced. The dorsal and median raphe nuclei give rise to a highly differentiated and topographically organized projection to cerebral cortex in the primate. Micrograph courtesy of Mary Ann Wilson, Johns Hopkins University. (Abbreviations: A = aqueduct, M = MLF, IV = trochlear nucleus.)

ery in the 1960s that a distinct group of serotonin-containing cells in the brainstem projects to widespread areas of the cerebral cortex and other parts of the forebrain had a revolutionary impact on neuroscience. The previous notion that all projections to cerebral cortex were relayed through the thalamus was no longer tenable. This new histochemical approach provided the impetus to redefine the organization of neuronal connections in terms of sets of neurons that use particular neurotransmitters. The seemingly divergent projections of the monoaminergic neurons suggested that they might be involved in regulation of neuronal excitability across functionally diverse and widely separated areas of the forebrain. The demonstration of direct projections from the central core of the brainstem reticular formation to multiple areas of cerebral cortex led to speculation that the biogenic amine-containing neurons in the brainstem core may be involved in brain functions such as attention, vigilance, mood, or motivation.

In the two decades since the initial discovery of serotonergic projections with the Falck-Hillarp method, a multitude of new methods have been developed to obtain a more detailed and accurate depiction of serotonergic and other monoaminergic projections to forebrain.

The original histofluorescence method was limited by relatively weak fluorescence and rapid fading due to photodecomposition of the fluorescent molecules. There was an apparent discrepancy in regard to innervation density since the number of axon terminals detected by fluorescence was too low in view of the transmitter levels.^{6, 36} In retrospect, it was found that limited sensitivity of histofluorescence did not permit detection of many fine axon terminals in the forebrain, so that the density of serotonergic innervation was initially underestimated. Newer anatomic methods that have been of particular value in obtaining a more accurate delineation of serotonergic projections include the use of anterograde axonal transport of tritiated amino acids,^{23, 75} localization of tritiated serotonin accumulated directly through the high affinity uptake pump of axon terminals,²⁹ and immunocytochemistry employing an antibody directed against serotonin developed by Steinbusch and colleagues at the University of Nijmegen.^{99, 101, 102} Several problems that were initially addressed with these methods included identifying the precise origin of serotonergic projections to forebrain, conducting a more detailed analysis of the pathway by which axons reached various forebrain regions, and ob-

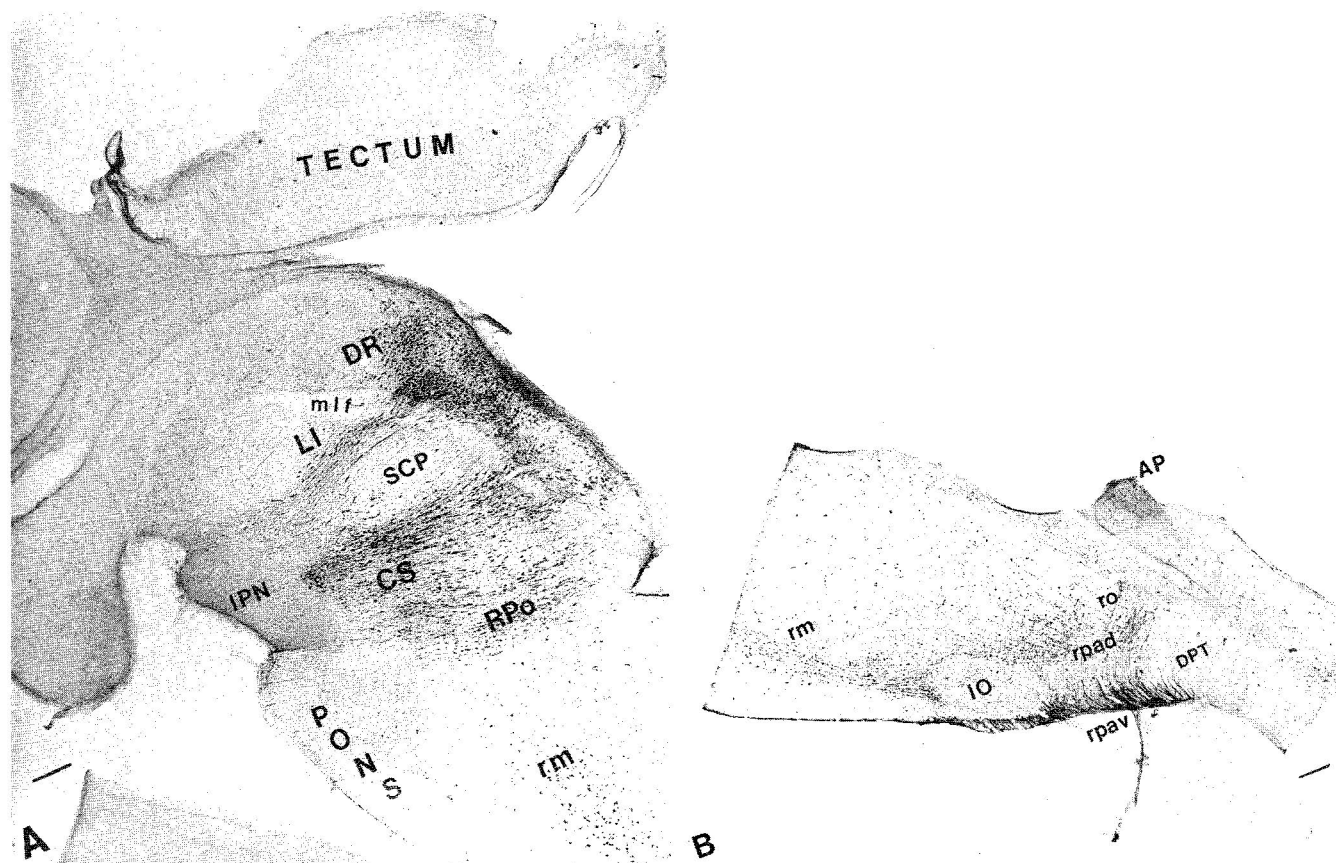


FIG. 3. Parasagittal section through rat brainstem showing the longitudinal arrangement of serotonergic cell bodies in the raphe nuclei. A, Note the elongated dorsal raphe nucleus extending through the central gray beneath the aqueduct forming a continuous cell group from the rostral midbrain to the anterior pons where it lies in the floor of the fourth ventricle. The median raphe (CS = central superior) lies more ventrally where it angles forward and ventrally to the interpeduncular nucleus (IPN). B, Parasagittal caudal view of serotonin neurons in pons and medulla. (Abbreviations: rm = raphe magnus, ro = raphe obscurus, rpad = raphe pallidus.) Other landmarks are: DPT = decussation of pyramidal tract and inferior olive (IO). Figure A above also shows raphe pontis (RPO), the decussation of superior cerebellar peduncle (SCP), and a small anterior group of serotonin cells, the nucleus linearis intermedius (LI). See text for further details. Figure from Lidov and Molliver.⁶⁹ (Reprinted from *Brain Research Bulletin* with permission of Pergamon Press.)

taining a more complete and accurate depiction of patterns of terminal innervation. An important organizational question addressed by many investigators was to determine whether serotonin projections are diffusely organized or whether individual raphe cell groups have restricted projections to particular forebrain areas. The issue of whether there are specific, differential projections is of particular importance in understanding the function of serotonin neurons in that these cell groups may either exert a single global effect or they may have multiple independent functions dependent upon particular sets of 5-HT neurons and their pathways. Moreover, if there are separate, independent functions of serotonin based on differential neuronal projections, it would be important to determine whether specific pharmacologic agents can be found to act selectively on different sets of serotonin neurons.

Raphe Projections to Forebrain

The initial proposal that serotonergic neurons in the midbrain projected directly to cerebral cortex^{6, 26, 35} was a radical departure from conventional thinking about brain organization. Several laboratories proceeded to test this hypothesis using independent methods and to provide further details about the route of ascending monoaminergic pathways. Initial attempts to examine the hypothesis of direct serotonergic projections to cortex were conducted by Moore and Heller at the University of Chicago. These investigators showed that lesions in the lateral hypothalamus interrupting the MFB led to marked reductions in forebrain levels of norepinephrine and serotonin.⁴⁴ Investigators at Yale University provided further evidence for raphe-cortical projections by showing that lesions of the raphe nuclei produce a

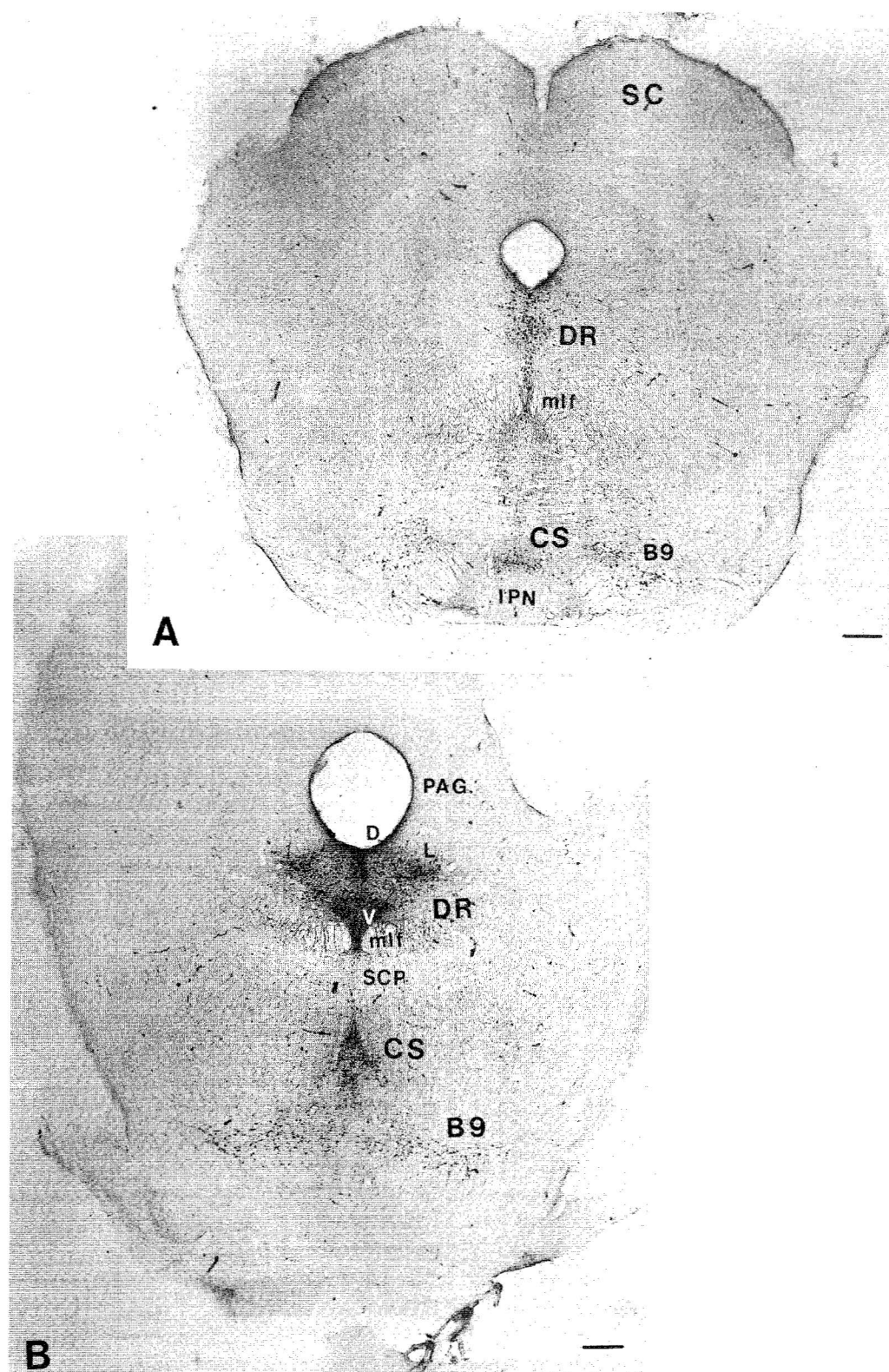


FIG. 4. Serotonergic cell bodies at two transverse levels of the midbrain showing their orientation with respect to other brain structures. Note the change in cell number and configuration of the dorsal raphe from a more rostral section (A) to a more caudal level (B). These figures show the location of the B9 cell group that lies in a ventrolateral position in the midbrain. The B9 cell group has a small projection to neocortex, but its connections have not been fully characterized. Figure reproduced from Lidov and Molliver.⁶⁹ (Reprinted from *Brain Research Bulletin* with permission of Pergamon Press.)

large reduction in cortical levels of serotonin, in serotonin uptake, and in tryptophan hydroxylase activity.^{61, 62} In a study using bilateral MFB lesions that produced large reductions in cortical serotonin levels, direct anatomic evidence for raphe-cortical projections could not be established using the Nauta method, a silver stain for degenerating axons.⁷⁶ However, using the Fink-Heimer method, a more sensitive modification of the Nauta technique, Hedreen⁴³ observed a small number of degenerating axons in cingulate cortex, hippocampus, and entorhinal cortex following large median raphe lesions; this result was verified by Conrad and associates²⁰ in a more detailed analysis of raphe projections, thus providing anatomic evidence for the existence of direct raphe-cortical projections.

Further evidence for direct projections from the raphe nuclei of the midbrain to cerebral cortex was obtained by the autoradiographic demonstration of anterograde axonal transport after raphe injections of tritiated amino acids.^{7, 12, 20, 74, 75} The anterograde axonal transport-autoradiographic method showed axonal pathways reaching parts of the cerebral cortex; however, the sensitivity of this technique was not sufficient to detect most axon terminals presumably due to dilution of the label in this long, highly collateralized pathway.⁷⁵ The pattern of terminal innervation in cortex was not adequately described until a method for the intracellular detection of 5-HT by immunocytochemistry became available¹⁰² (see below). The preceding autoradiographic axonal transport studies confirmed the main pathway of ascending serotonin axons through the MFB from which axons fan out to reach neocortex, septum, hippocampus, and amygdala. A more detailed depiction of the trajectory of ascending 5-HT axons was later obtained by Parent and coworkers⁸³ by means of autoradiographic detection of tritiated serotonin that was taken up directly into axons following intraventricular infusion of labeled serotonin. The autoradiographic uptake and transport studies were of particular value in demonstrating preterminal axonal pathways; they clearly showed the trajectory of ascending raphe axons in the lateral hypothalamus, with branches from the MFB to hippocampus, septum, olfactory bulb, cingulum, as well as fibers moving laterally from the MFB (in the ansa peduncularis) to reach amygdala and ventrolateral cortical areas^{7, 20, 75, 83} (Fig. 5).

Topographic Organization of Raphe Projections to Forebrain

While the early studies cited above showed that the serotonergic innervation of forebrain arises from the raphe nuclei in the rostral brainstem, they did not address the further issue of whether individual clusters of

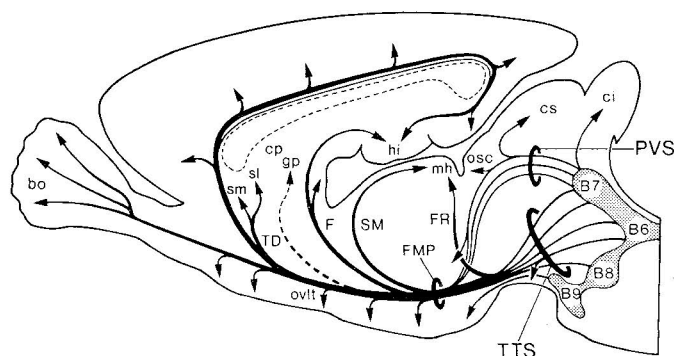


FIG. 5. A diagrammatic representation of ascending serotonergic pathways from midbrain raphe nuclei to various parts of the forebrain. Two main pathways emerge from the midbrain raphe nuclei, a dorsal periventricular path (PVS) and the more ventral tegmental radiations (TTS). Both pathways merge in the caudal hypothalamus where they enter the medial forebrain bundle. At various levels, fibers branch out from this main ascending bundle to innervate specific regions of forebrain. Most notable are fiber bundles to the habenular nuclei, thalamus, septum, striatum, olfactory bulb (bo), and the cingulum bundle and fornix (F). The principal ascending pathway of serotonergic axons is via the medial forebrain bundle through lateral hypothalamus and basal forebrain. For further details see text and references 83 and 75. This figure reproduced courtesy of Parent and coworkers.⁸³ (Reprinted from *Neuroscience* with permission of Pergamon Press.)

raphe neurons project to different areas of the forebrain. This issue is of importance in determining whether 5-HT neurons always have global effects on brain function or whether they may have more specific, independent actions. In order to understand the functional organization of 5-HT neurons and to evaluate the behavioral effects of drugs that affect 5-HT, it is necessary to establish whether particular raphe nuclei project to different parts of the brain, and whether topographically organized projections arise from adjacent but separate zones within individual raphe nuclei. The widespread distribution of raphe axons initially led to the interpretation that serotonergic projections were highly collateralized with little evidence for topography or anatomic subdivisions.⁷⁵ Additional support for the existence of divergent projections came from retrograde axonal transport studies showing that some individual raphe neurons send axon collaterals to multiple forebrain areas.^{28, 31, 108} However, several lines of evidence have subsequently accumulated showing that different serotonergic nuclei project to specific regions of the forebrain with a complex and intricate pattern of topographic organization. Although the serotonergic projections are considerably more divergent than the myelinated pathways that subserve sensory and motor systems directly, the raphe nuclei exhibit a pattern of restricted projections that appears to be functionally significant. Azmitia and Segal⁷ showed that the dorsal and median raphe nuclei give

rise to multiple, anatomically distinct sets of axons that form separate pathways to different brain structures. Moreover, there is both anatomic and biochemical evidence that the dorsal and median raphe nuclei have different patterns of forebrain projections; the two nuclei project to different sets of forebrain areas, and in regions where the projections overlap, the relative contribution from each of these nuclei is different.

The first clear demonstration that the dorsal and median raphe nuclei give rise to different patterns of forebrain projections was based on finding differential regional decreases in serotonin levels following selective lesions of the dorsal and median raphe. Experiments that were independently verified in separate laboratories demonstrated that 5-HT levels in the hippocampus are markedly reduced following median raphe lesions, whereas striatal and neocortical serotonin appears to arise largely in the dorsal raphe.^{52, 71} Further confirmation for this differential pattern was obtained by measuring serotonin uptake and tryptophan hydroxylase activity after selective raphe lesions.^{38, 53} Anatomic confirmation of differential median and dorsal raphe projections was obtained by anterograde transport of amino acids in a comprehensive analysis of ascending 5-HT axons.⁷ The preceding studies provided further evidence that the median raphe projects predominantly to hippocampus and the dorsal raphe to striatum, while both nuclei have different but overlapping projections to neocortex. Evidence for differential projections arising from cells within different parts of the dorsal raphe nucleus was subsequently obtained using the method of retrograde axonal transport of horseradish peroxidase. Jacobs and colleagues⁵⁰ showed that the rostral portion of the dorsal raphe projects to both striatum and neocortex, while the caudal portion of the dorsal raphe, along with cells in the median raphe, projects to the hippocampus. In another study employing anterograde transport of horseradish peroxidase, Tohyama and co-workers¹⁰⁵ found that rostral versus caudal cells in the dorsal raphe project to cortex via separate pathways; rostral cells project to ventrolateral areas of cortex; and caudal dorsal raphe cells send axons into the cingulum bundle. The apparent discrepancy between the seemingly widespread nature of raphe projections and the demonstrated topographic order was explained by evidence that individual zones of the raphe nuclei project to functionally related parts of the brain, such as striatum and substantia nigra⁴⁸ (Fig. 6) and to multiple but related areas of neocortex.⁵⁸

Differential Cortical Projections from the Midbrain Raphe Nuclei

The question of whether cells in different regions of the raphe nuclei project topographically to separate

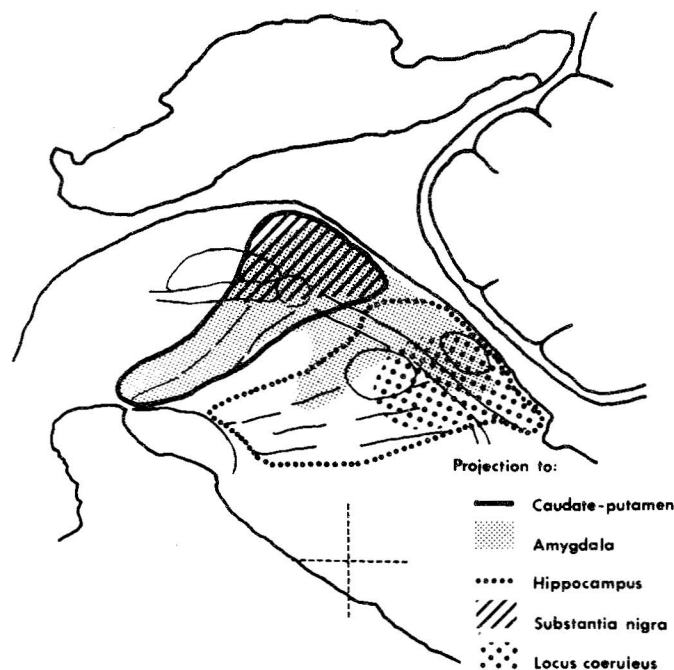


FIG. 6. A schematic parasagittal view of raphe nuclei showing that 5-HT projections are topographically organized. Moreover, particular zones of the dorsal raphe and median raphe project to functionally related parts of the brain. For example, rostral dorsal raphe projects to the striatum and substantia nigra, while caudal regions project to hippocampus and locus coeruleus. The amygdala receives projections from both divisions. This figure reproduced courtesy of Imai and colleagues.⁴⁸ (Reprinted from *Journal of Comparative Neurology* with permission of Alan R. Liss, Inc.)

areas of cortex was then addressed in studies that employed retrograde transport of fluorescent dyes that were injected into different cortical fields. In studies of the serotonergic innervation of hippocampus and entorhinal cortex, Kohler and Steinbusch^{54, 57} found a topographic pattern of dorsal and median raphe projections from cells in restricted parts of these nuclei and also reported that some raphe neurons have divergent axon collaterals that project to both of these areas. In a quantitative study from this laboratory using retrograde transport of fluorescent dyes, the dorsal raphe, median raphe, and B9 cell group were found to have different patterns of projections to the neocortex.⁸¹ In this study, O'Hearn observed that dorsal raphe cells do not project diffusely to cortex but are topographically organized with a rostrocaudal gradient of dorsal raphe cells projecting from frontal to occipital areas of cortex.⁸¹ Three times as many dorsal raphe cells project to frontal cortex than elsewhere, while median raphe cells project more uniformly to all neocortical areas. A similar rostrocaudal gradient of dorsal raphe cortical projections has been confirmed independently by Waterhouse and associates.¹¹³ The rostrocaudal topography within the dorsal raphe suggests that this nucleus is heteroge-

neously organized and that particular zones within the dorsal raphe project to different cortical areas. Thus, the raphe nuclei are not diffusely organized but have dissimilar patterns of organization, and each nucleus may have different actions upon different cortical areas.⁸¹

The organization of ascending raphe projections has been examined in further detail by Kosofsky, using anterograde axonal transport of the lectin, PHA-L. This new, highly sensitive method developed by Gerfen and Sawchenko³⁷ has the advantage of visualizing the fine, distal axonal projections from small clusters of cells within the raphe nuclei. The principal new findings of this study (Kosofsky and Molliver, 1987, manuscript in preparation) are that small clusters of raphe cells form discrete patches or islands of cortical innervation and that these islands have a multifocal pattern of distribution.⁵⁸ Each cluster of dorsal raphe cells projects to multiple patches of cortex, typically frontal cortex plus one or more additional areas, with preservation of a coarse topographic order. This organizational pattern indicates that the raphe nuclei form a distributed system of efferent connections that systematically influences widely separated cortical areas. The multifocal pattern of raphe-cortical organization appears similar to that reported by Pearson, Powell, and colleagues⁸⁶ for the cholinergic projections of the basal nucleus to neocortex in the monkey; zones of the basal nucleus were shown to project upon areas of neocortex that are interconnected by cortico-cortical association connections. Thus, it may be a general principle that separate cortical areas that are interconnected and functionally related may receive projections from small groups of amine-containing subcortical neurons. Consequently, small groups of cells within the raphe nuclei (and in the basal nucleus) may influence the excitability of multiple, functionally related cortical areas.⁵⁸

Serotonergic Innervation Patterns in Neocortex

The differential projection of raphe nuclei to different areas of cortex is reflected in regional specializations of the cortical serotonergic innervation pattern. Due to technical limitations of histofluorescence and anterograde transport methods (e.g., low sensitivity and incomplete detection of axons, as described above) it was not feasible to visualize the pattern and full extent of cortical innervation by axon terminals from the raphe nuclei. With the advent of immunocytochemistry for serotonin developed by Steinbusch and coworkers,¹⁰² it became possible to visualize the full extent of fine serotonergic axon terminals with a high degree of sensitivity and specificity. An antibody to serotonin produced in this laboratory by H. Lidov was used to analyze

the serotonergic innervation pattern of cerebral cortex,⁶⁷ and a series of additional studies in rodents and primates have clarified further details of the innervation pattern in papers to be published elsewhere (B. E. Kosofsky, M. A. Wilson, M. E. Blue). In the initial immunocytochemical study of 5-HT cortical innervation, Lidov and colleagues⁶⁷ demonstrated a markedly high density of 5-HT-containing axons throughout the cerebral cortex of the rat. There are marked regional differences observed in the density of axons and in the laminar pattern of innervation (Fig. 7). 5-HT innervation density is extremely high in frontal cortex and falls off in more caudal areas. Within different cortical areas, laminar differences are seen in the morphology, density, and orientation of 5-HT axons. In posterior cingulate cortex and in hippocampus there is a distinct lamination of 5-HT fibers, with coarse, beaded axons forming bands of high density at the bottom of layer I in posterior cingulate cortex and along the hilus of the dentate gyrus.⁶⁷

In the primate brain, an extremely dense plexus of serotonergic axons was found in visual cortex with a distinctive pattern of lamination.^{60, 77} A striking finding in the latter study is a localized decrease of serotonin axon density in layer IVc-beta of area 17 in the Macaque. This innervation pattern suggests that 5-HT neurons have little effect on the input of visual information to area 17 but a potent effect on subsequent processing of visual information. Wilson and Molliver¹¹⁵ have observed a high 5-HT innervation density in somatosensory cortex of the monkey compared with a low density in motor cortex, in addition to striking regional differences in the pattern of serotonin innervation among other cortical areas. A detailed, anatomic description of serotonin innervation patterns in both rat and monkey is beyond the scope of this review and will be published separately. The main point to be emphasized in this survey is that there are characteristic regional differences in innervation in several cortical areas. Such differences in the density and pattern of innervation may reflect different functional influences of serotonin neurons upon separate cortical areas.

Development of Cortical Innervation

Serotonin neurons appear in the fetus at an early stage of development and form one of the earliest neuronal systems to have projections that ascend from the brainstem. The early development of 5-HT neurons and their ascending projections has been described in a number of reports.^{63-65, 69, 82, 95, 112} In a study from this laboratory, immunocytochemistry was used by Lidov and Molliver⁶⁸ to analyze the development of serotonergic projections to cerebral cortex in the rat. Raphe

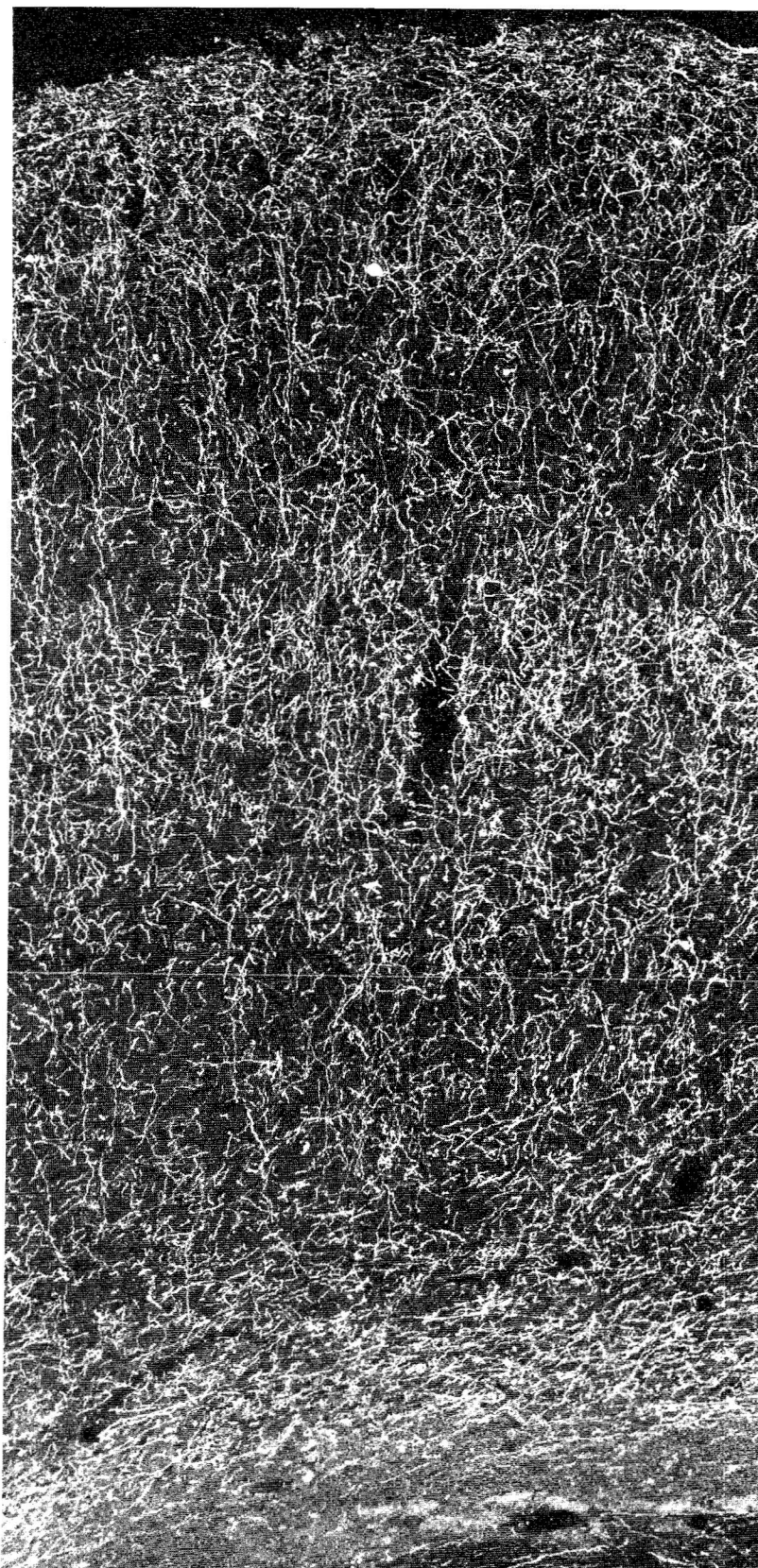


FIG. 7. Serotonergic innervation of cerebral neocortex demonstrated by 5-HT immunocytochemistry. Darkfield microscopy reveals the extremely dense plexus of highly arborized serotonergic axons that extend through all layers of parietal cortex and are in a position to contact nearly all cortical neurons. A dense plexus of serotonin axon terminals is seen in all regions, but there are subtle variations among different cortical areas in the density and laminar distribution of fibers. Original photomicrograph courtesy of Mary E. Blue, Johns Hopkins University.

axons were observed to reach cerebral cortex before birth and to provide extensive innervation in the early neonatal period. Serotonergic axons initially have a bilaminar pattern arborizing in layer I and in the deepest zone of cortex; through further growth, axons arborize into the middle cortical layers. In a subsequent study, the serotonin innervation of somatosensory cortex was analyzed in postnatal rat by D'Amato and associates²⁴ using immunocytochemistry for 5-HT axons and autoradiography for the identification of serotonin-uptake sites labeled with [³H]citalopram, a selective inhibitor of serotonin uptake. A precocious hyperinnervation of primary sensory areas of cortex by serotonergic axon terminals was present transiently. The density of axons was greatest during the first 2 weeks of life, and then rapidly diminished.²⁴ The hyperinnervation was found only in primary sensory areas of cortex, and serotonin axon terminals form dense patches in layer IV of somatosensory cortex; these patches are coextensive with cellular aggregates known as "barrels," the specialized zones that receive inputs from vibrissae on the face, a highly important sensory organ for the rodent. The distribution of serotonin axons matches that of 5-HT uptake sites labeled with citalopram, showing that these precocious axons are relatively mature and are capable of both storage and uptake of serotonin. The early formation of this specialized innervation of sensory cortex indicates that raphe neurons have specific cortical targets in early ontogenesis, rather than initially forming a diffuse projection to cortex. The patchy innervation of SI provides further evidence for the specificity of 5-HT projections in the neonate and may reflect early development of the mosaic organization⁵⁸ of raphe projections described in the adult rat (Kosofsky and Molliver, 1987, manuscript in preparation). The neonatal formation of this dense serotonin innervation just precedes the arborization of thalamocortical axons leading to the suggestion that serotonin may have a trophic effect upon the growth of thalamic inputs to cortex.²⁴

Two Classes of 5-HT Axon Terminals

Serotonin axon terminals in several parts of the brain typically exhibit heterogeneous morphology. Chan-Palay^{17, 18} studied the ultrastructural features of serotonin axon terminals labeled by uptake of tritiated serotonin. She described multiple, distinctive types of serotonin axon terminals, and raised the question of whether these axons arise from different raphe cell groups.¹⁷ Immunocytochemical studies also revealed morphologically different axon types in cerebral cortex. Lidov and colleagues⁶⁷ reported that extremely fine 5-HT axons were distributed throughout the cerebral cortex, while a special group of coarse, varicose fibers

were observed in restricted areas, notably in posterior cingulate cortex and in the hilus of the dentate gyrus. In entorhinal cortex, Kohler and coworkers^{55, 56} found that, in addition to a somewhat diffuse terminal plexus of fine 5-HT fibers, there is a small, distinct zone in layer III of lateral entorhinal cortex that contains a dense plexus of coarse varicose axons. During the analysis of anterograde axonal transport from the dorsal raphe and median raphe, Kosofsky and Molliver⁵⁹ made the unexpected discovery that there are consistent morphologic differences between cortical axon terminals that arise from these two nuclei. Axon terminals from the median raphe nucleus appear relatively coarse and are beaded with large, spherical varicosities.⁵⁹ In contrast, axons from the dorsal raphe are very fine and typically have minute, pleomorphic varicosities that are granular or fusiform in shape (Figs. 8 and 9). The fine axon terminals are the type most abundant in cortex, but the coarse beaded fibers have a distinctive appearance and a characteristic regional distribution. The beaded median raphe type axons are especially numerous in the outer layers of parietal cortex, in layer II of posterior cingulate cortex, in the lateral entorhinal area, and in hippocampus. Thus, the regional distribution of beaded axons^{59, 78} agrees with previous reports (reviewed above) of median raphe projections to particular cortical areas. The dissimilarities in serotonergic axon morphology described here suggest that individual raphe nuclei may form different types of synaptic contacts. Moreover, further studies from this laboratory (see below) have shown that the two axon types have different pharmacologic properties and are therefore likely to be functionally distinct (Fig. 10).

5-HT Receptors in Cortex

Serotonin binding sites are present in high density throughout the brain. These presumed serotonin receptors have been the subject of active investigation and their pharmacologic properties and regional distribution are summarized in recent reviews by Altar and coworkers³ and by Leysen.⁶⁶ The physiologic actions at serotonin receptors and their effects on neuronal activity have also been subject to extensive investigation, and this topic has been reviewed by Aghajanian and colleagues² and de Montigny and Blier.²⁷ One of the major discoveries in serotonin neuropharmacology during the past decade has been the identification of multiple serotonin binding sites.⁸⁸ The two main classes of serotonin receptors were originally classified by Peroutka and Snyder⁸⁷⁻⁸⁹ into 5-HT₁ and 5-HT₂ receptors. Recent studies subdivide the 5-HT₁ receptors into at least three subtypes, while 5-HT₂ receptors appear to form a homogeneous population of binding sites. Similar numbers of both 5-HT₁ and 5-HT₂ receptors are found in

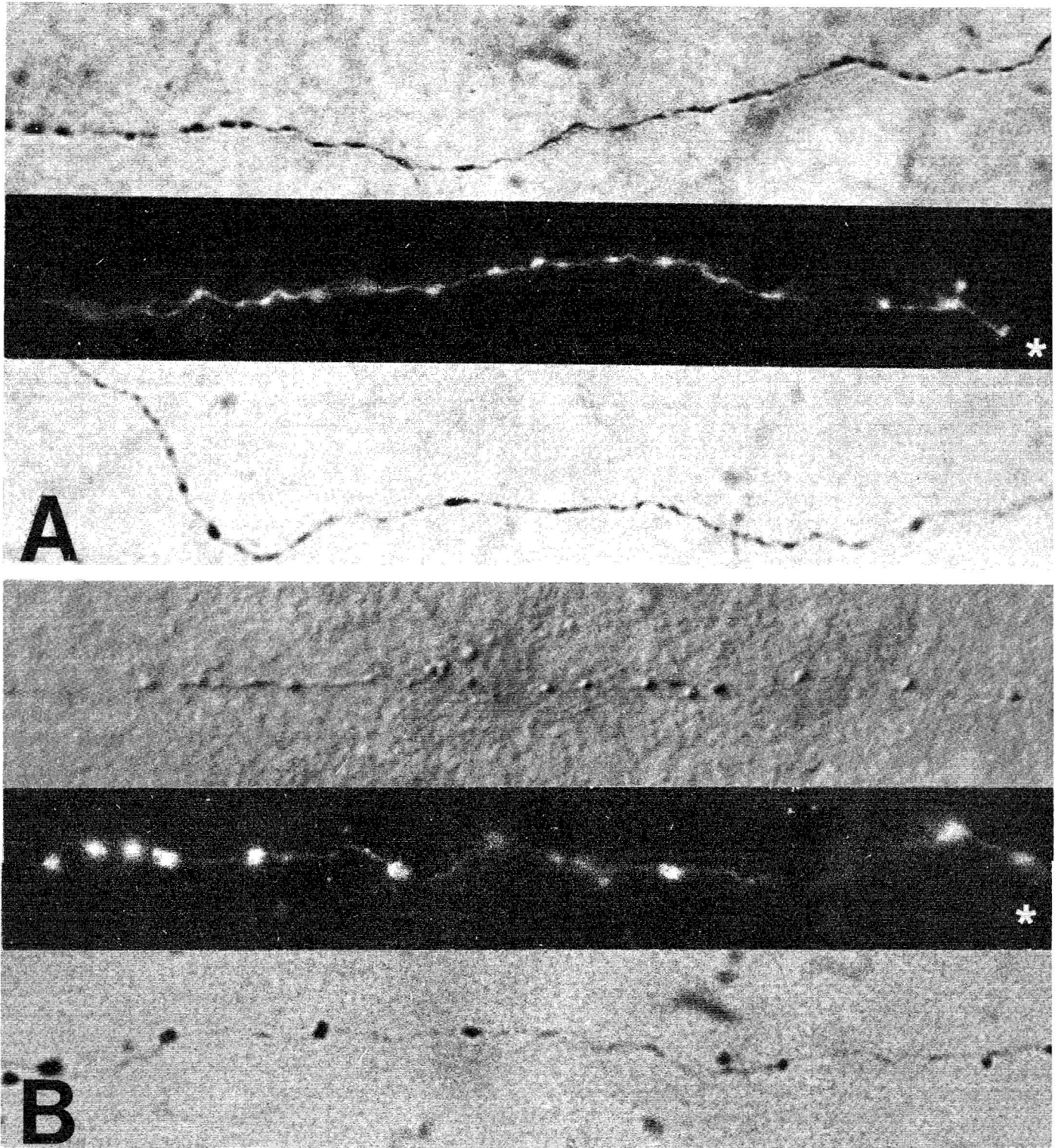


FIG. 8. Raphe cortical axons in the rat labeled by anterograde transport from the raphe nuclei of the lectin PHA-L (phaseolus vulgaris leucoagglutinin). A, fine, dorsal raphe type fibers visualized in frontal neocortex after PHA injection in the dorsal raphe nucleus. B, median raphe type fibers with large varicosities visualized in parietal neocortex after PHA injection in the median raphe. Figure from Kosofsky and Molliver.⁵⁹ (Reprinted from *Synapse* with permission of Alan R. Liss, Inc.)

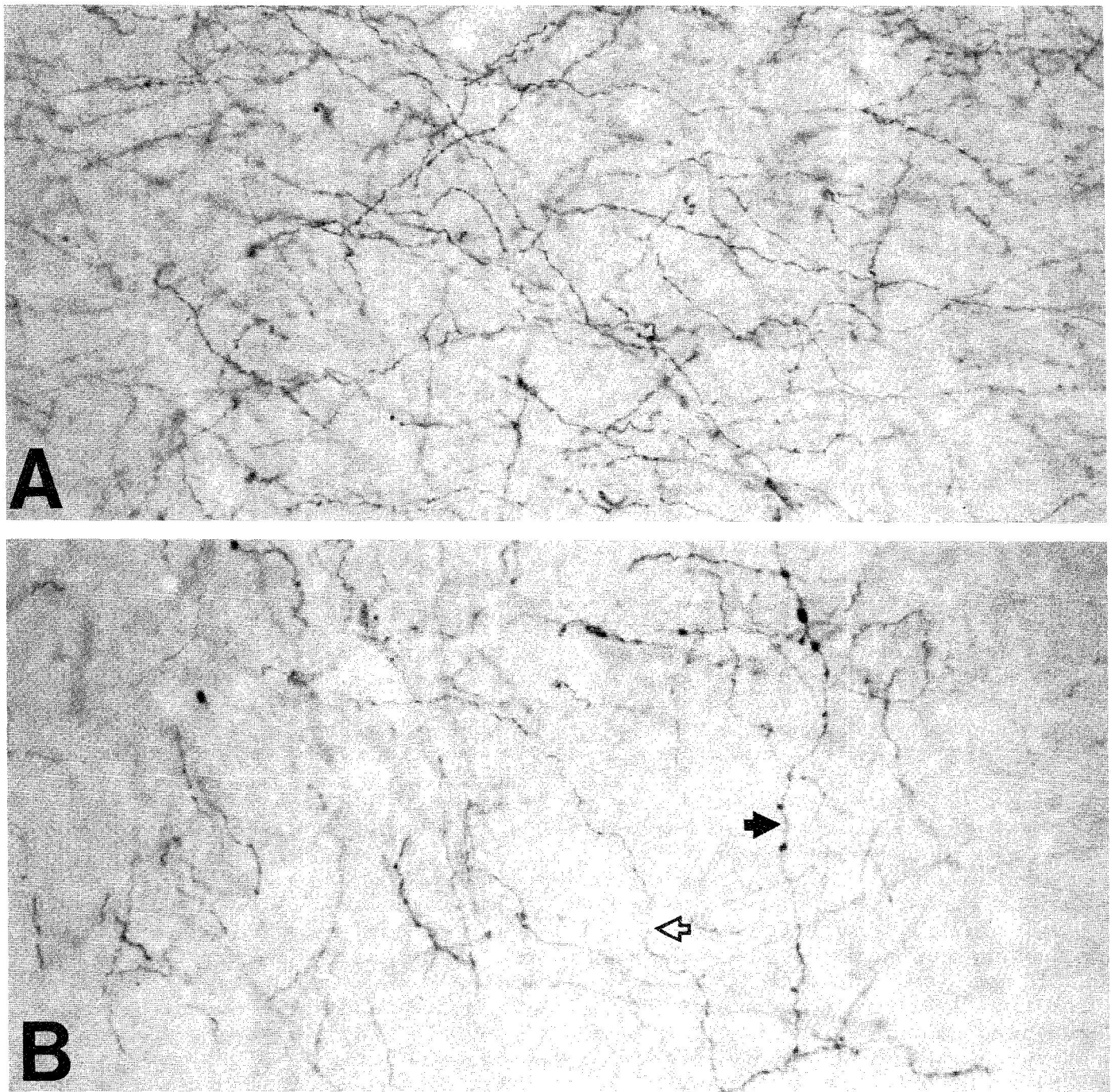


FIG. 9. Serotonin immunoreactive axons in cerebral cortex of rat. A, dorsofrontal cortex. Note the high density of fine 5-HT axons with small, granular or fusiform varicosities of the type arising from the dorsal raphe. B, posterior parietal cortex. Note heterogeneity of axon morphology. One prominent fiber of the median raphe type (*solid arrow*) has large varicosities. This is adjacent to a fine axon with minute varicosities of the dorsal raphe type (*open arrow*). Figure from Kosofsky and Molliver.⁵⁹ (Reprinted from *Synapse* with permission of Alan R. Liss, Inc.)

cerebral cortex,⁸⁹ yet each type differs in its anatomic distribution within the cortex.^{4, 84, 85} A new ligand (¹²⁵I-MIL) has been developed for detecting 5-HT₂ receptors with a high degree of specificity and sensitivity,^{46, 47} and this ligand has greatly improved the capability for

localizing this receptor type with increased precision. This laboratory has recently employed the ligand ¹²⁵I-MIL in collaboration with Hartig in order to compare directly the distribution of 5-HT immunoreactive axons in cortex with the distribution of 5-HT₂ receptors. In

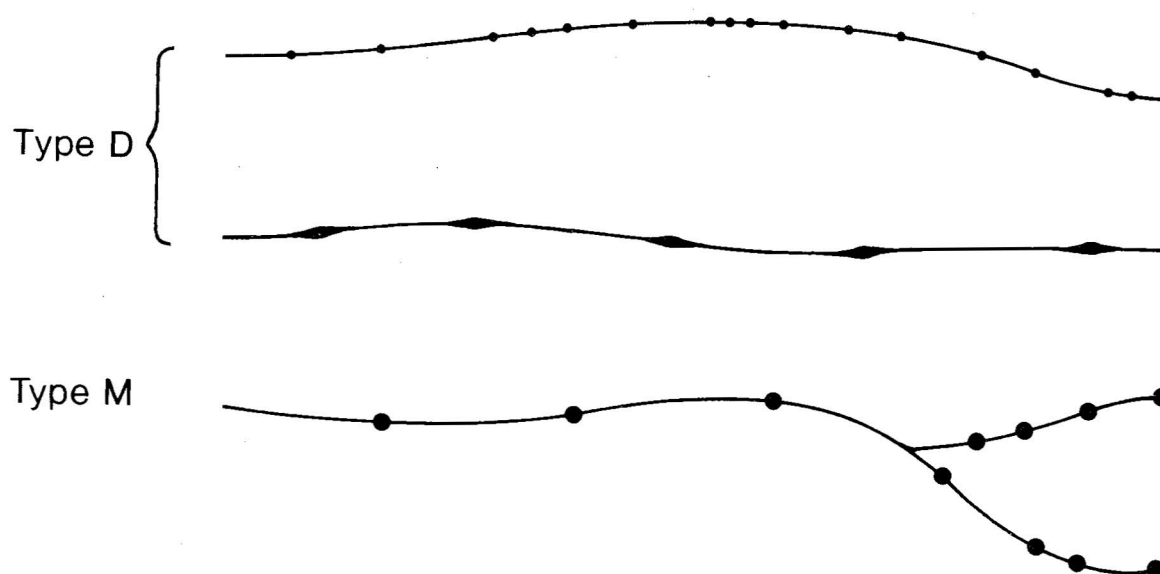


FIG. 10. Schematic representation of the two classes of raphe-cortical axon terminals. Axons that arise in the dorsal raphe nucleus (type D) have heterogeneous varicosities that range from small granular dilatations (above) to fusiform varicosities (1 to 3 μm in diameter). Axons from the median raphe nucleus (type M) have large spherical varicosities (3 to 5 μm in diameter). Figure taken from Kosofsky and Molliver.⁵⁹ (Reprinted from *Synapse* with permission of Alan R. Liss, Inc.)

this study, Blue and associates^{10, 11} observed that the density of 5-HT₂ receptors corresponds closely to the distribution of 5-HT axons in most areas of the forebrain. In particular, it was noted that the fine type of 5-HT axon terminals was most closely associated with 5-HT₂ receptors, a relationship that is especially evident in somatosensory cortex where both of these elements are quite dense in the upper portion of layer V. These results lead to speculation that the 5-HT₂ receptors throughout the forebrain may be selectively associated with the fine axon terminals that arise from the dorsal raphe nucleus.^{11, 59} This proposed relationship raises the possibility that separate serotonin projections arising from different raphe nuclei may terminate in relation to specific 5-HT receptor subtypes and therefore form multiple and distinct functional systems of serotonin projections. It may therefore become important to explore the suggestion^{32, 39} that separate serotonin projections may subserve different behavioral functions and therefore be amenable to selective pharmacologic modification.

Neurotoxicity of Psychotropic Drugs

Recent studies of the effects of hallucinogenic amphetamines have provided new information concerning the function and organization of serotonergic projections to forebrain. There is considerable, previous evidence suggesting that hallucinogenic, mood-altering drugs act primarily on serotonin neurons or synapses in the central nervous system (see review by Jacobs and Trulson⁵¹). For example, the prototypic indoleamine

hallucinogen, LSD, produces small increases of 5-HT levels in the brain³³ presumably due to inhibition of dorsal raphe neurons.^{1, 2} Other mood-altering drugs such as the phenethylamine hallucinogens (e.g., mescaline or 2,5-dimethoxy-4-methylamphetamine [DOM]) have little direct effect on the activity of raphe cell bodies yet have a high affinity for 5-HT₂ receptors,⁴⁰ and their effects are blocked by ketanserin, a selective 5-HT₂ receptor antagonist⁴⁵; this group of drugs may act postsynaptically at 5-HT synapses.⁴⁹ In recent years, a number of hallucinogenic amphetamine derivatives that are chemically related to mescaline have become widely self-administered for recreational use because of their potent mood-elevating effects. Two of these compounds, which are known as "designer drugs," have attracted particular public interest: these are 3,4-methylenedioxymphetamine (MDA) and 3,4-methylenedioxymethamphetamine (MDMA). The latter drug, known by the street name of "Ecstasy," has been proposed for medical use as an adjunct to psychotherapy because it produces augmented mood and enhanced interpersonal communication.^{41, 42} In a seminal study from the University of Chicago, Ricaurte and coworkers⁹⁰ demonstrated that repeated, large doses of MDA cause profoundly decreased serotonin levels in the forebrain of rats. Additional studies confirmed that both MDA and MDMA cause depletion of biochemical markers for forebrain 5-HT.^{8, 19, 93, 94, 103} These results were interpreted as indicating that the hallucinogenic amphetamine derivatives may cause degeneration of serotonin projections to forebrain. Based on the evidence that mood-altering

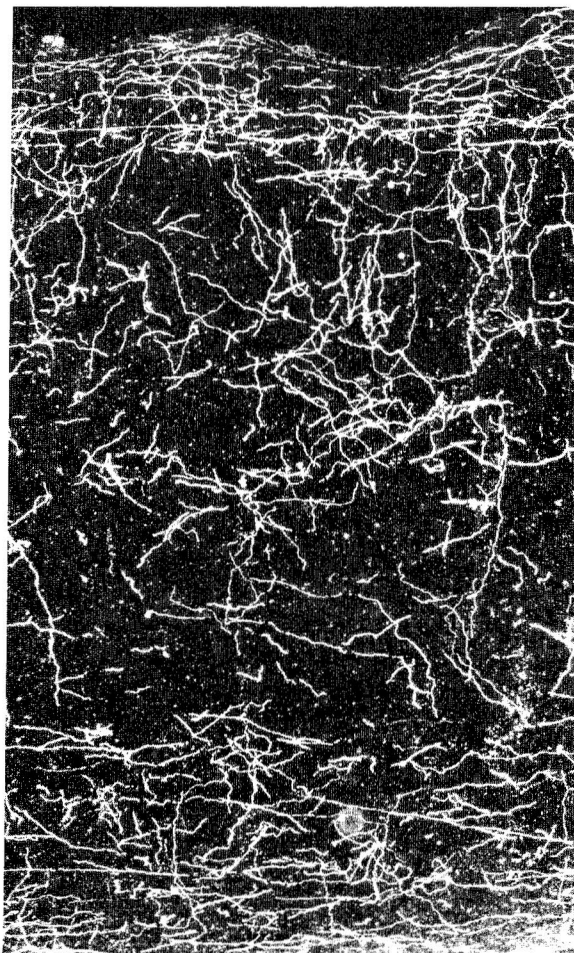
drugs act upon serotonergic neurotransmission and may be neurotoxic, it was of interest to examine the anatomic effects of MDA and MDMA upon serotonin neurons in order to identify the specific neuronal structures that are affected. Therefore, an immunocytochemical study of the neurotoxic effects of MDA and MDMA was conducted in this laboratory by E. O'Hearn in collaboration with Battaglia, De Souza, and Kuhar at the National Institute on Drug Abuse.^{79, 80} Serotonin immunocytochemistry showed that repeated doses of MDMA and MDA in rats cause a profound loss of serotonergic axons throughout the forebrain, especially in neocortex, striatum, and thalamus (Fig. 11). Regional differences in toxicity are exemplified by partial sparing of axons in hippocampus (Fig. 12), hypothalamus, basal forebrain, and brainstem. At short survival times, positive evidence for acute degeneration of 5-HT axons was observed in the form of greatly swollen, fragmented 5-HT nerve terminals; at longer survival times, the persistent loss of axons reflects lasting denervation. It was possible to specify the structural compartment of 5-HT neurons that is affected since there was a selective loss of axon terminals, while preterminal axons of passage and raphe cell bodies are spared. The corresponding loss of 5-HT uptake sites provides further support for a direct effect of these drugs on 5-HT axon terminals.⁸ Preterminal fibers exhibit increased immunoreactive staining and are thickened presumably due to damming-up of neurotransmitter secondary to terminal axotomy. MDA produced more severe cytotoxicity than MDMA, and there appeared to be selective vulnerability of fine axon terminals with relative sparing of beaded fibers that have large varicosities. These findings indicate that MDA and MDMA act at serotonin axon terminals where they ultimately produce a cytotoxic effect leading to degeneration after large repeated doses. This selective action of mood-altering drugs at serotonin terminals supports the idea that serotonergic neurons play a major role in the regulation of affective state.

Dual Serotonergic Projections to Forebrain

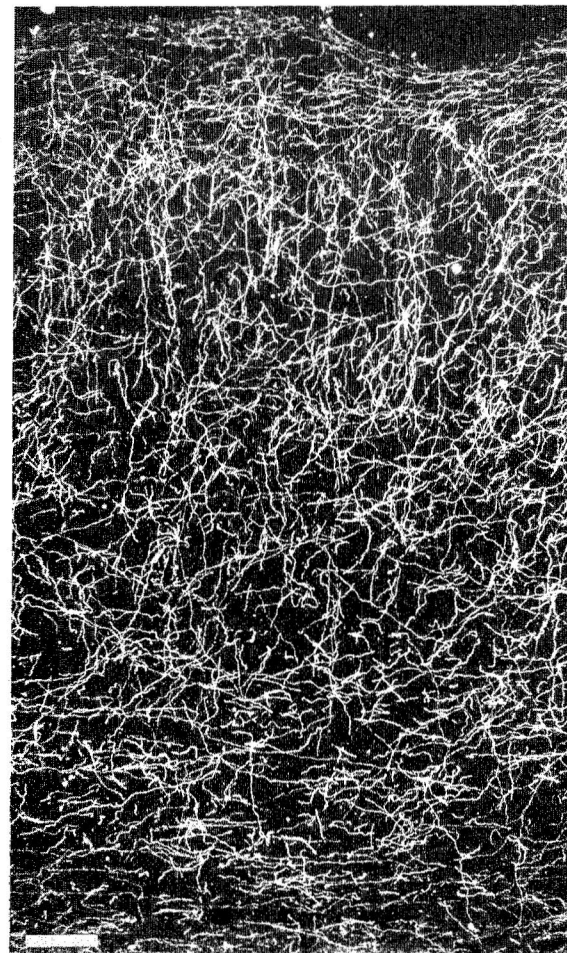
In order to better characterize the cytotoxicity of hallucinogenic drugs upon 5-HT neurons, the effects of several psychotropic amphetamine derivatives have been compared and their effects on serotonin axon terminals have been examined in greater detail. The effects of MDA were compared with *p*-chloroamphetamine (PCA), a neurotoxic amphetamine derivative with biochemical effects on 5-HT markers that have previously been extensively studied.^{91, 92} If the effects of PCA are similar to those of MDA, it would be an excellent model drug to study the selectivity and mechanisms of amphetamine neurotoxicity on 5-HT neurons. Initial results indicate

that both drugs cause a marked reduction in the density of 5-HT axon terminals in forebrain.⁷⁸ A nearly identical anatomic profile of neurotoxicity was seen with both compounds. At all survival times studied, fine 5-HT terminals are almost completely ablated; nearly all spared 5-HT terminals in cerebral cortex are of the beaded type with large varicosities, and they are morphologically identical to the beaded axons that were shown to originate in the median raphe nucleus.⁵⁹ Mullen and coworkers⁷⁸ found that the regional distribution of spared axons after PCA or MDA matches the distribution of beaded median raphe axons in the normal animal. These results demonstrate that the beaded 5-HT axons are resistant to the neurotoxic effects of MDA and PCA, while the fine axon terminals are selectively vulnerable. Therefore, this series of studies distinguishes two classes of serotonin axons based on their morphology and their differential vulnerability to psychotropic drugs. The mechanism for the selective neurotoxicity has not yet been elucidated. Concomitant administration of MDMA or PCA with the serotonin uptake inhibitor, fluoxetine, provides complete protection of serotonin axons against the neurotoxic effects of these psychotropic amphetamines.^{9, 34, 93} Since neurotoxicity of both MDA and PCA has been shown to depend on binding to the serotonin uptake carrier,^{34, 93} these results lead to the suggestion that there may be different 5-HT uptake receptors associated with the two axon types.

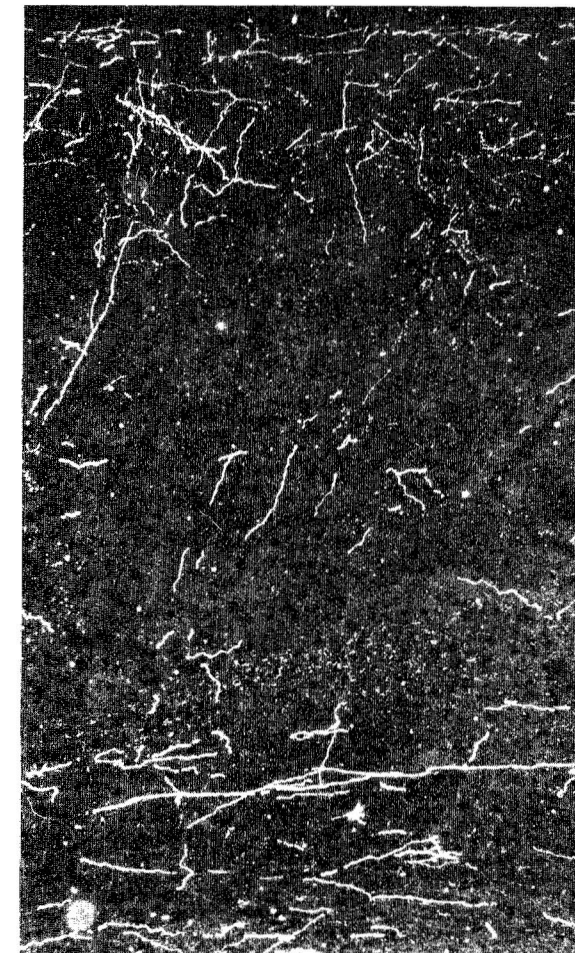
Based on previous evidence for the differential origin of the two morphologically distinct serotonin axon types,⁵⁹ we postulated that dorsal raphe axon terminals are selectively vulnerable to the neurotoxic amphetamines, while median raphe (beaded) axons are drug resistant.^{78, 80} In order to establish whether the two axon types originate from separate raphe nuclei, Marmounas conducted a retrograde axonal transport study to determine which 5-HT projections remain intact following PCA treatment. Employing fluorescent dye injections (fluorogold) in parietal cortex after the animals had been treated with PCA, the number of labeled neurons in the dorsal raphe nucleus is markedly reduced (80% decrease), but that of labeled neurons in the median raphe does not differ from controls.⁷² These results confirm that the dorsal raphe projection to cortex is selectively vulnerable to the neurotoxic effects of PCA, while the median raphe projection is resistant to this drug. Moreover, fine and beaded axon terminals, which have different pharmacologic properties, arise from different sets of cells located in separate raphe nuclei. The serotonergic innervation of cortex thus comprises two separate, functionally dissimilar projections that differ in cells of origin, axon morphology, regional distribution, and response to psychotropic drugs.⁷²



MDMA



CONTROL



MDA

FIG. 11. Neurotoxic effects of hallucinogenic amphetamines upon serotonin axon terminals in rat neocortex. This figure demonstrates the effects of MDA and MDMA upon the serotonin innervation of visual cortex. The normal serotonin innervation shown in the control panel demonstrates a high density of arborized 5-HT axon terminals. Following repeated systemic administration of MDA or MDMA (20 mg/kg) there is a marked reduction in the density of fine serotonin axon terminals. This reduction reflects selective degeneration of 5-HT axons. Fine axon terminals of dorsal raphe origin are selectively vulnerable, while the smaller population of varicose terminals from the median raphe are resistant to this class of drugs. These findings support the view that psychotropic amphetamines act directly upon the fine serotonin axon terminals and may eventually cause their destruction. This original photomicrograph is based on data from O'Hearn and associates.⁸⁰

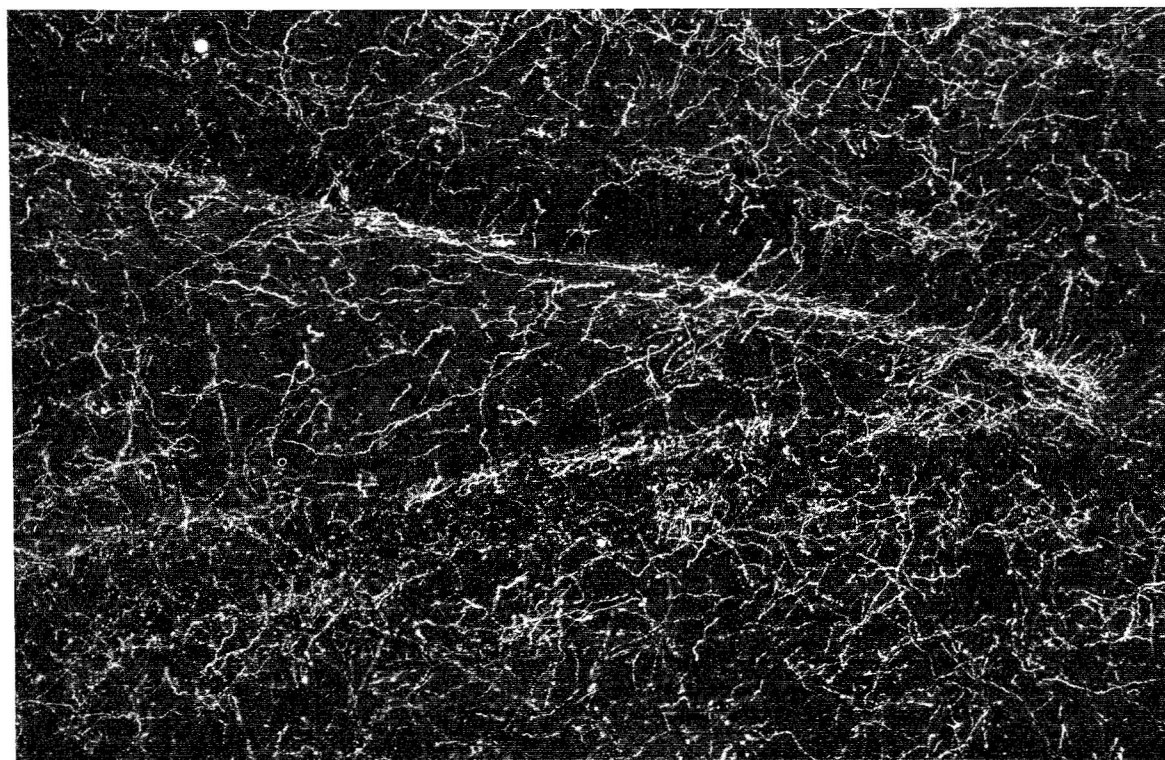
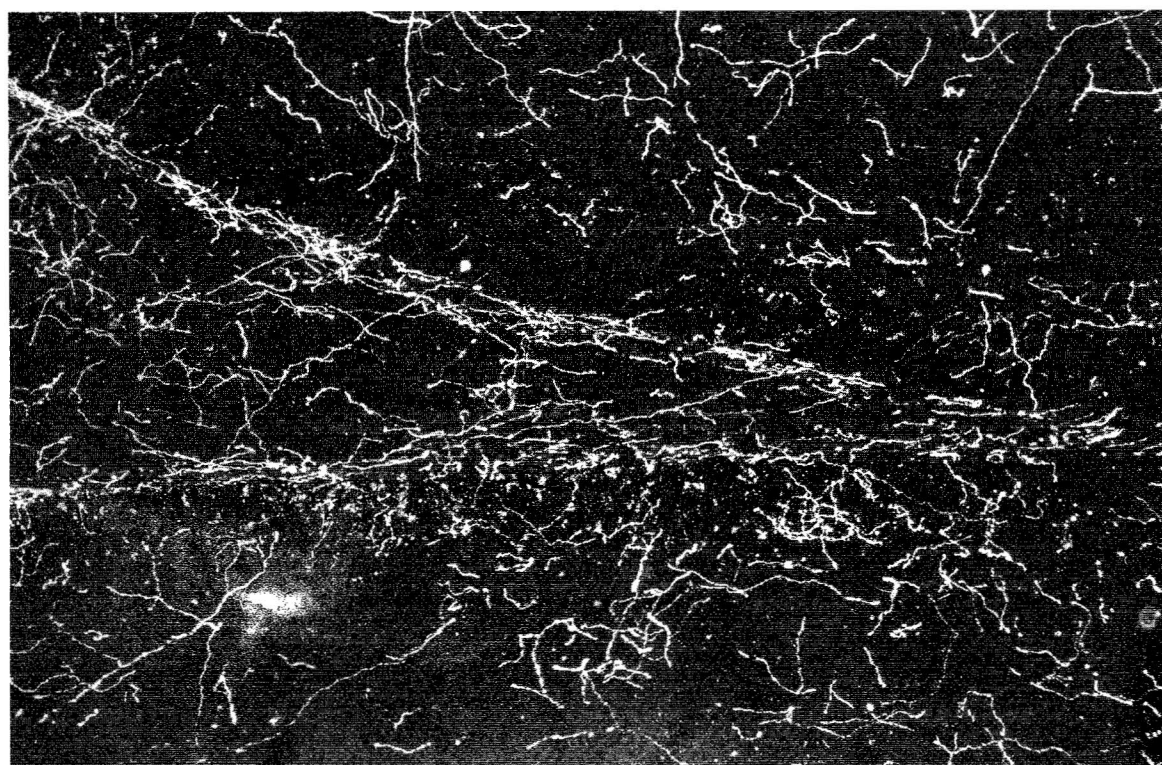
**CTRL****MDA**

FIG. 12. Serotonergic innervation of the dentate gyrus in the rat hippocampal formation. The control panel above shows a high density of 5-HT axons in the hilus and molecular layer of the dentate gyrus. This region is innervated by fine terminals from the dorsal raphe and a dense plexus of coarse, varicose axon terminals that are especially prominent along the V-shaped margin of the dentate granule cell layer. The lower panel (MDA) shows a marked loss of fine axon terminals following treatment with the psychotropic drug MDA (see text). The 5-HT innervation of hippocampus is relatively spared by MDA due to the survival of the coarse, varicose axons from the median raphe that densely innervate this region. This figure demonstrates the differential vulnerability of the two serotonin axon types and shows that limbic areas of forebrain receive an especially dense 5-HT innervation from the median raphe nucleus. Original photomicrograph courtesy of E. O'Hearn based on data from O'Hearn and associates.⁸⁰

The neurotoxicity of PCA and MDA appears to be associated with release of neurotransmitter. At short time intervals (4 hours) after administration of MDA or PCA, there is nearly total depletion of serotonin from fine axon terminals, but no apparent effect on the beaded fibers is demonstrated by immunocytochemistry.⁹ These results confirm that PCA and MDA act initially by causing the release of serotonin from axon terminals and demonstrate that the neurotoxicity of these drugs appears related to their ability to cause massive release of neurotransmitter. Recent studies in collaboration with Ricaurte and colleagues at Stanford Medical Center indicate that neurotoxic effects of MDMA on serotonin axons are found in primate cortex as in the rat. Moreover, Wilson and colleagues¹¹⁶ have observed that in the primate there also appear to be two separate, functionally distinct 5-HT projections with relative sparing of coarse, beaded axons that have a highly characteristic regional distribution.

Conclusion

The preceding studies showing the toxic effects of psychotropic amphetamines on particular serotonin axons, combined with previous evidence for differential serotonin projections to forebrain, demonstrate that there are two classes of 5-HT axons that differ in origin and pharmacologic properties. These results indicate that there are separate and parallel serotonergic projections from the raphe nuclei to the forebrain. These projections arise in the dorsal and median raphe nuclei respectively and give rise to widely distributed but multifocal patches of innervation, each system having a different pattern of termination. The selective vulnerability of dorsal raphe axons to mood-elevating drugs leads us to speculate that the 5-HT projection from the dorsal raphe plays an important role in the control of affective state and may also be involved in perceptual integration. There are several lines of evidence that support the role of 5-HT in regulation of mental state and in disorders of mood. The initial observation that reserpine causes behavioral depression and was found to deplete 5-HT and other monoamines was the basis for postulating a role for the biogenic amines in depression. The observations that LSD produces a psychedelic state with hallucinations and was also found to act upon serotonin neurons and receptors led to early proposals of a relationship between serotonin and mental state. The widespread projections of serotonin axons from raphe neurons in the limbic midbrain area provided an anatomic substrate for a functional system that may be influenced by emotional state and affect extensive areas of the brain. Clinical evidence that antidepressant drugs block serotonin uptake and prolong its activity¹⁶

provided support for the view that a serotonin deficiency was involved in the pathogenesis of depression.^{22, 109-111} Decreased levels of 5-hydroxyindoleacetic acid were found in depressed patients and suicide victims.⁷⁰ Serotonin uptake sites labeled by imipramine were greatly reduced in platelets of depressed patients¹⁴ and in brain samples of frontal cortex from suicide victims,⁹⁸ who also showed marked increases in levels of 5-HT₂ receptors.⁹⁷ Thus, depressive disorders appear to be associated with a loss of markers for serotonin innervation in forebrain and a consequential up-regulation of 5-HT₂ receptors. The additional evidence that psychotropic amphetamines act either at 5-HT₂ receptors or on 5-HT axon terminals provides a basis to postulate that a principal function of a group of serotonin neurons in the midbrain is to regulate affective state along the continuum from depression to euphoria.

Much more needs to be learned about the functional organization of the serotonin projections and about their possible role in disorders of affect and aggression. Recent information about their intricate anatomic organization provides a springboard for more detailed analyses of the functional and chemical properties of these neuronal systems. The duality of 5-HT projections and the postulated role of dorsal raphe neurons in mood fosters the hope for development of new pharmacologic agents with a high degree of specificity for modifying affective state without influencing other neuronal systems or other 5-HT projections.

Acknowledgments

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