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# Ontogeny of the serotonergic projection to rat neocortex: Transient expression of a dense innervation to primary sensory areas

(serotonin/citalopram/development/cerebral cortex/"barrels")

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ABSTRACT **The development of serotonergic innervation to rat cerebral cortex was characterized by immunohistochemical localization of serotonin combined with autoradiographic imaging of serotonin-uptake sites. In neonatal rat, a transient, dense, serotonergic innervation appears in all primary sensory areas of cortex. In somatosensory cortex, dense patches of serotonergic innervation are aligned with specialized cellular aggregates called barrels. The dense patches are not apparent after 3 weeks of age, and the serotonergic innervation becomes more uniform in adult neocortex. This precocious neonatal serotonergic innervation may play a transient physiologic role in sensory areas of cortex or may exert a trophic influence on the development of cortical circuitry and thalamocortical connections.**

Serotonergic neurons of the dorsal and median raphe project extensively throughout the cerebral cortex (1). The behavioral function of serotonin (5-hydroxytryptamine) in the cortex is unknown. Selective effects of antidepressants upon serotonergic neurotransmission implicate this transmitter in affective states (2), while actions of psychedelic drugs via serotonin receptors imply a role in perceptual integration (3).

By use of antibodies to serotonin, the ontogeny of serotonergic neurons and projections in the brain was characterized (4, 5), as was the pattern of cortical innervation (1). Recently, we have labeled neuronal serotonin-uptake sites biochemically and autoradiographically with [$^3$H]citalopram (6), a potent and selective inhibitor of serotonin uptake (7). The present study examines the development of serotonergic innervation in newborn rat cerebral cortex by combined immunohistochemical localization of serotonin and autoradiographic imaging of neuronal serotonin-uptake sites. We demonstrate a transient, dense, serotonergic innervation to primary sensory areas (visual, auditory, and somatosensory areas) of the developing rat cerebral cortex.

## MATERIALS AND METHODS

For immunocytochemical studies, Sprague–Dawley rats at various postnatal ages were administered a monoamine oxidase inhibitor, *trans*-2-phenylcyclopropylamine hydrochloride (10 mg/kg of body weight, i.v.), 3–5 hr before they were killed by perfusion; half of the animals received an additional injection of L-tryptophan (100 mg/kg) 30–60 min prior to perfusion. These pharmacologic agents elevate serotonin levels in axon terminals and augment immunocytochemical labeling but do not change the density of serotonergic axons. Under Metofane (methoxyflurane) anesthesia, rats were transcardially perfused with ice-cold phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde plus 0.1% glutaraldehyde in 0.15 M phosphate buffer (pH 7.4). Brains were postfixed in 4% paraformaldehyde for 12 hr and cryoprotected in 10% dimethyl sulfoxide in PBS. For immunocytochemistry, frozen sections (40-$\mu$m coronal) were incubated for 48–72 hr (4°C; free-floating) in antiserum against serotonin (1), processed by the avidin–biotin peroxidase (ABC) method (8), intensified with osmium (9), dehydrated, cleared, and coverslipped. Adjacent sections were stained with 0.5% cresyl violet acetate (pH 3.7).

Several litters of rats received two s.c. injections of the serotonergic neurotoxin 5,7-dihydroxytryptamine (100 mg/kg; free base in isotonic saline with 1 mg of ascorbic acid per ml) 12 and 36 hr after birth. Thirty minutes before 5,7-dihydroxytryptamine injection, desmethylimipramine (20 mg/kg, s.c.) was injected to block 5,7-dihydroxytryptamine uptake into noradrenergic terminals (10).

In autoradiographic studies, male Sprague–Dawley rats of various ages were anesthetized with sodium pentobarbital and perfused with phosphate buffer/sucrose, and brains were processed for autoradiography as described (6). In brief, tissue sections on slides were incubated with 1 nM [$^3$H]citalopram in 50 mM Tris/HCl buffer (pH 7.4 at 25°C) with 120 mM NaCl and 5 mM KCl for 60 min at room temperature. Nonspecific binding, estimated with 1 $\mu$M paroxetine, was <10% of total binding. Sections were apposed to $^3$H-sensitive Ultrofilm (LKB) at 4°C for 4 weeks to generate autoradiograms.

The regional and laminar distribution of [$^3$H]citalopram binding or of serotonin-immunoreactive axons in rat cortex was determined in matched brain sections from separate animals by use of a microscope equipped with a camera lucida drawing tube. To determine which regions or layers contained [$^3$H]citalopram binding sites or serotonin-immunoreactive axons, a template drawn from adjacent cresyl violet-stained sections was superimposed through the drawing tube upon the image of the autoradiographic or immunocytochemical sections.

## RESULTS

In somatosensory cortex (SI) of the adult rat, serotonin-uptake sites labeled by [$^3$H]citalopram form tangentially continuous bands in layers I and V. Binding sites are more prominent in lateral than in dorsal regions of cortex. A markedly different pattern is apparent in parietal cortex of neonatal rats. A striking feature of neonatal rat is the transient appearance of dense patches of autoradiographic grains in dorsolateral cerebral cortex. At postnatal days (P) 7, 12, and 17, [$^3$H]citalopram binding is substantially more dense than in adult rat and more prominent in dorsal than lateral zones of cortex (Fig. 1). Patches of intense labeling are

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Abbreviations: P*n*, postnatal day *n*; PMBSF, posteriomedial barrel subfield; SI, somatosensory cortex.

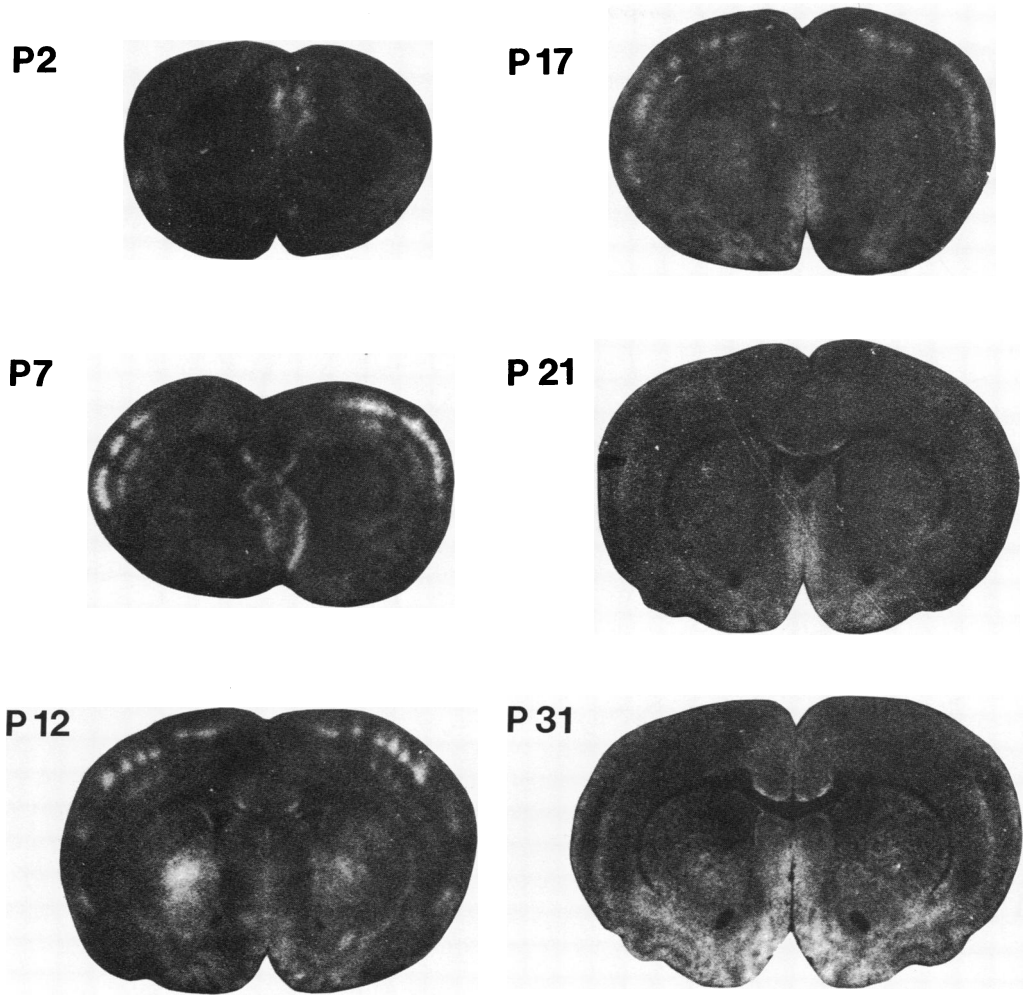


FIG. 1. [$^3$H]Citalopram binding in postnatal rat cerebral cortex. Dense patches of serotonin-uptake sites are observed in cortex from P3 to P17 and are most evident in the second postnatal week. Patches disappear after P21 and are replaced by continuous bands of binding in layers I and V of adult somatosensory cortex (as in P31).

localized in layers IV and VI of neonatal cortex, in contrast to the adult rat where the patches are replaced by continuous bands of less intense labeling in layers I and V. [$^3$H]Citalopram-labeled patches are first seen at P3, the earliest time at which we detect binding sites. Patches are most prominent in the second postnatal week, less pronounced in the third week, and disappear after P21. The disappearance of citalopram-labeled patches is marked first by a progressive decrease in the density of citalopram binding (P17, P21) and later by the appearance of continuous bands of binding sites in other layers.

To characterize the distribution of serotonin-uptake sites, we mapped the dense zones of [$^3$H]citalopram binding in a series of sections extending from the rostral to the caudal poles of the hemisphere (Fig. 2). In the reconstructions, the map of [$^3$H]citalopram binding closely overlaps that of primary sensory areas of cortex, showing that dense [$^3$H]citalopram binding is restricted to these sensory areas. We compared the locations of serotonin-immunoreactive axons and of serotonin-uptake sites in matched sections from littermate rats (Figs. 2 and 3). The distribution of serotonin immunoreactivity closely mirrors [$^3$H]citalopram binding. Zones with a high density of serotonergic axons are confined to primary sensory areas; dense patches of serotonin immunoreactivity are found in SI, while in the visual (area 17) and auditory (area 41) cortex continuous bands of dense axons extend across these areas. In contrast to sensory areas, motor cortex (M) as well as parietal and visual (18a) association areas have a sparse serotonergic innervation and few citalopram-labeled sites. Although the spatial distribution of serotonin-immunoreactive axons closely matches that of citalopram binding sites, the timetable for the two markers differs slightly. Unlike the [$^3$H]citalopram-labeled sites, which persist through P21, the patches of serotonin-immunoreactive axons are not easily seen after P12. [$^3$H]Citalopram autoradiography has higher contrast and greater sensitivity than immunocytochemistry, which may account for the more extended period of detection for patches of [$^3$H]citalopram binding.

In the forelimb (FL) and hindlimb (HL) areas of SI, the distributions of serotonergic innervation and of [$^3$H]citalopram-labeled uptake sites differ from that in lateral SI [whisker area, the posteromedial barrel subfield (PMBSF)]. The limb areas exhibit continuous tangential bands of serotonergic axons and uptake sites. In the barrel field (PMBSF), serotonin-immunoreactive axons and citalopram-labeled uptake sites are clustered in small patches. These patches of dense innervation are separated from each other by zones that have a much lower density of serotonin axons and uptake sites. In the barrel field, the patches have two components, a dense cluster of serotonergic axons in layer IV and another in layer VI (Fig. 4). In every patch, the two axonal clusters are in register vertically and appear to form bilaminar radial modules that impart a strong columnar appearance to the patches. A comparison of the immunocytochemical preparation with the adjacent cresyl violet-

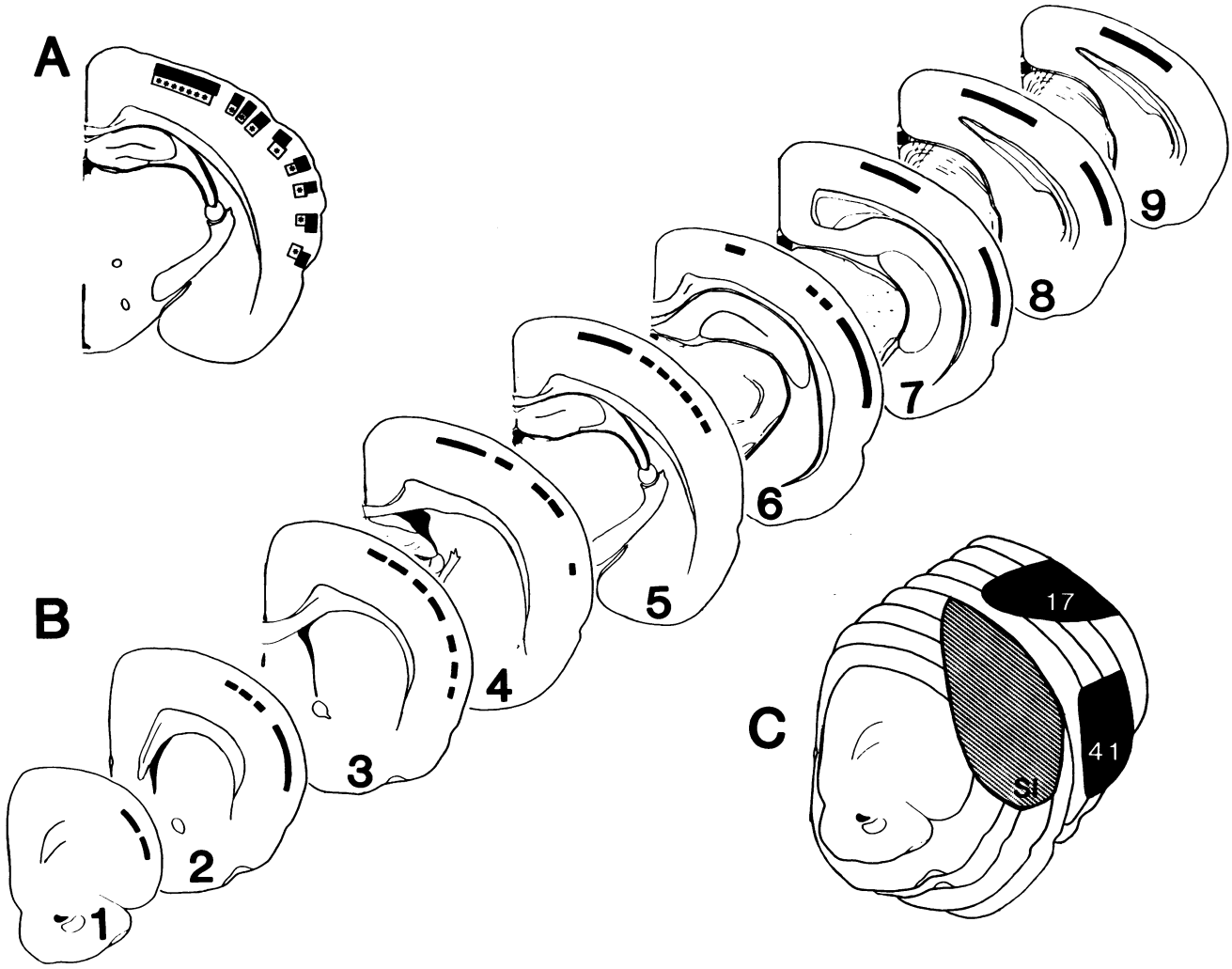


FIG. 2. The distribution of serotonin immunoreactivity (*A*) and of [$^3$H]citalopram binding (*B* and *C*) in sensory areas of cortex at P6. (*A*) Immunocytochemical preparations and cell clusters were mapped in SI. Solid black lines represent the patches of serotonin immunoreactivity; asterisks represent clusters of granule cells in the barrels of the posteromedial barrel subfield (PMBSF). The dense patches of serotonergic axons are in register with the barrels. (*B*) Schematic representation of [$^3$H]citalopram (solid black lines) binding in a series of coronal sections. (*C*) Serial reconstruction of sections illustrated in *B*. Zones of dense [$^3$H]citalopram binding are confined to primary sensory areas of cortex.

stained section shows that the radial modules of serotonergic axons are in register with the barrels of the rat PMBSF (Fig. 2). At higher magnification, each serotonin-immunoreactive patch comprises a dense basket-like network of arborized axon terminals (Fig. 4). Within the patches, serotonergic axons are very fine and convoluted and have numerous varicosities that are intensely stained. The density of serotonergic axons is much less in non-patch regions, where the serotonergic axons have varicosities but are predominantly straight, radially directed, and unbranched.

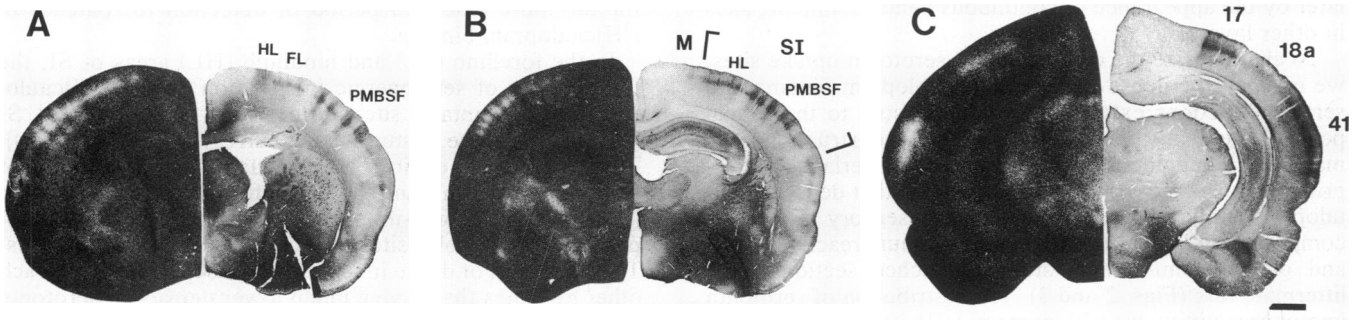


FIG. 3. [$^3$H]Citalopram binding and serotonin immunoreactivity in cortex at P6–P10. The [$^3$H]citalopram-binding pattern matches that of serotonin-immunoreactive axons. Dense zones of serotonin immunoreactivity and of serotonin-uptake sites lie within primary sensory areas of cortex: somatosensory (SI; *A* and *B*), visual cortex (area 17; *C*), and auditory cortex (area 41; *C*). Motor cortex (M), parietal association cortex, and visual association cortex (area 18a) have a lower density of serotonin axons and uptake sites. The dorsal forelimb (FL) and hindlimb (HL) areas exhibit a continuous band of serotonin axons and uptake sites. In the whisker area [posteromedial barrel subfield (PMBSF)], serotonergic innervation forms small dense patches. In contrast to SI, the innervation forms continuous bands within auditory and visual areas (*C*). (Bar = 100 $\mu$m.)

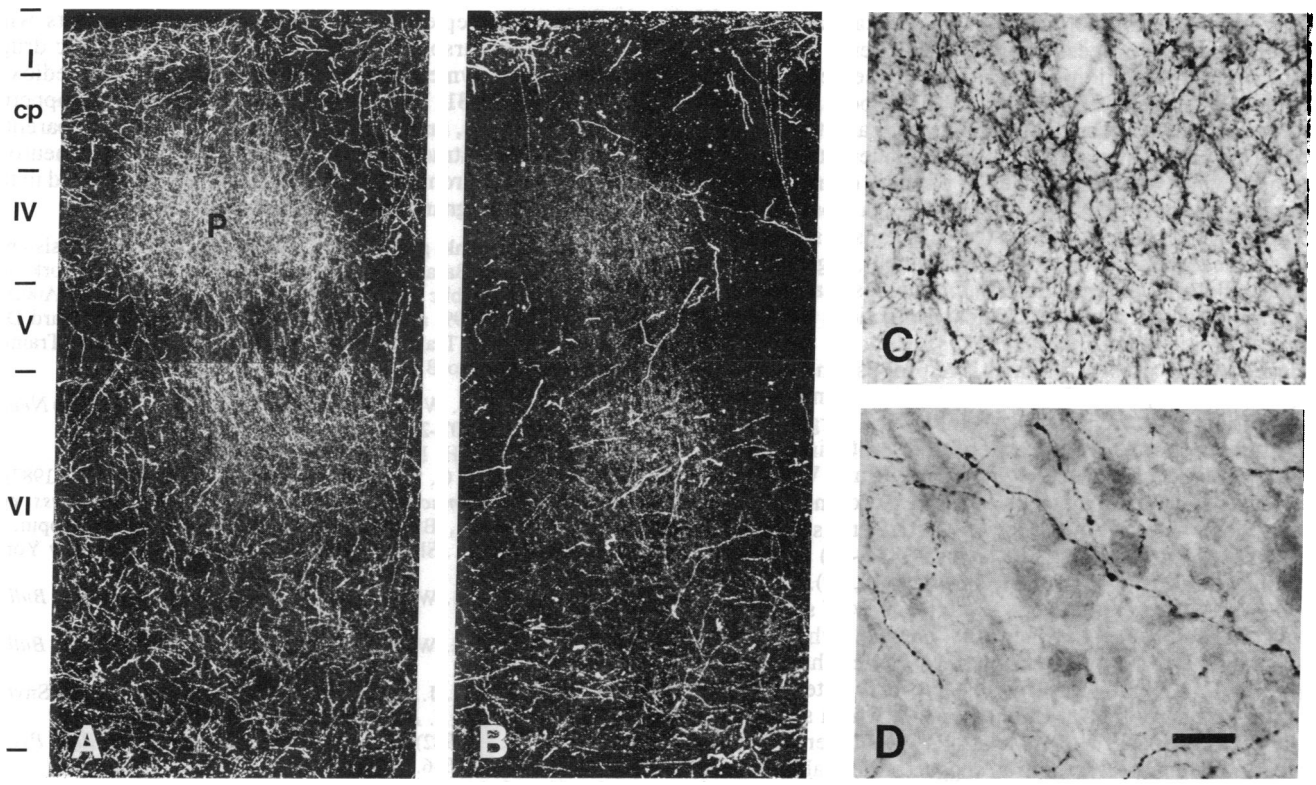


FIG. 4. Serotonergic axons in SI (PMBSF) at P6. (*A*) Laminar distribution of serotonergic axons (serotonin immunocytochemistry, dark-field). Compact patches (P) of dense serotonergic innervation are surrounded by zones of lower axon density. The bilaminar patches, prominent in layers IV and VI, are radially aligned. cp, Cortical plate. (*B*) Following 5,7-dihydroxytryptamine treatment, the density of serotonergic axons decreases in both patch and non-patch (low-density regions). (*C*) Serotonergic axons in patch region showing that the patch of immunoreactivity is made up of highly arborized, varicose axons. (*D*) Serotonergic axons in a non-patch zone where the density is much lower. (Bar = 20 $\mu$m in *C* and *D*.)

After 5,7-dihydroxytryptamine treatment, which produces degeneration of serotonergic axons, there is a substantial decrease in the number and density of axons in both patch and non-patch (low axon density) areas. The density of [$^3$H]citalopram sites is similarly diminished. Although patches are still detected after 5,7-dihydroxytryptamine treatment, the number of axons and binding sites within each patch is significantly reduced.

## DISCUSSION

The major finding of this study is the precocious hyperinnervation of primary sensory areas of cortex by serotonergic axon terminals arising in the raphe nuclei. We have observed three novel features of this projection that are transient and found only during the first postnatal month. (*i*) The density of axons in sensory areas of cortex is extremely high during the first 12 postnatal days and diminishes markedly by the end of the first month. (*ii*) The serotonergic hyperinnervation does not extend uniformly over the cortex but is found only in the primary sensory areas. (*iii*) In SI, serotonergic hyperinnervation takes the form of an array of dense axonal patches each of which is a columnar module with bilaminar, vertically aligned clusters of serotonergic axons. These patches appear coextensive with specialized cellular aggregates in SI known as "barrels." In other sensory areas of cortex, which do not have a patchy organization, the dense serotonergic innervation extends uniformly across the entire cortical field. In adult rats, the patches of serotonergic axons are replaced by a dense plexus of fibers extending through all areas and layers of cortex.

This study, which employed [$^3$H]citalopram binding and transmitter immunocytochemistry, combined multiple presynaptic markers to characterize the maturation of serotonergic axons. The use of these methods for anatomic localization combines the high sensitivity of autoradiography with the fine anatomic detail preserved by immunocytochemistry. The distribution of serotonin-immunoreactive axons matches that of serotonin uptake sites, both of which are patchy in SI but continuous in auditory and visual areas. The nearly identical localization of both markers suggests that they are associated with the same population of fibers. The demonstration of serotonin immunoreactivity in animals that were not preloaded with L-tryptophan indicates that the stained axons contain endogenous serotonin. Moreover, the intense immunoreactivity of varicose serotonergic axons suggests that these fibers have the capacity for vesicular storage of serotonin. The [$^3$H]citalopram binding indicates that young serotonergic axons exhibit binding sites that are associated with the serotonin-uptake carrier. The demonstration of these properties shows that precocious serotonergic axons are functionally mature and capable of synthesis, storage, and uptake of serotonin. These findings further suggest that, in newborn rat, there may be a transient physiologic role for serotonin as a neurotransmitter in sensory areas of cortex.

A dense, patchy distribution of serotonergic axons, similar to that in the rat, has recently been demonstrated in SI of newborn mouse by Fujimiya *et al.* (11). However, the observations in that study were confined to SI, and it was not determined whether serotonergic innervation was present in other cortical areas. In the mouse as in the rat, the dense patches of serotonergic axons are transient and become less evident during the second week of life. At that time we find a progressive increase in the density of serotonergic innervation throughout cerebral cortex. The cortical patches stand out because they contain a compact, dense plexus of serotonergic axons adjacent to a zone of lower axon density. The subsequent loss of the patchy appearance is due in part to the

arborization of serotonergic axons in cortical zones around the patches; this process produces an overall increase in serotonergic innervation density, so that the dense patches become less evident. However, in the newborn, the serotonergic axon density in cortical patches is greater than at older ages. Thus, the overall serotonergic innervation density increases while that in the dense patches decreases. This decrease in axonal density within patches is most likely due to expansive growth of the cortex that causes serotonergic axons to become spread apart. Alternatively, degeneration of axonal collaterals as a mechanism for decreased axon density has been proposed for other cortical projections that initially form excessive axon branches (12, 13).

Based on the present findings, we propose that serotonin may exert a trophic influence on the development of thalamocortical pathways or on their cortical target cells. In immature neocortex, dense patches of serotonin axons and uptake sites occur exclusively in layers IV and VI, the main recipients of thalamic input. In SI, dense serotonergic axonal patches are selectively associated with barrels, which are aggregates of granule cells in layer IV (14–17) and receive thalamic input from individual vibrissae (18, 19). In contrast to the barrel centers, there is a low density of serotonergic axons in the septa between the barrels, which receive association and callosal connections rather than thalamic input (20, 21). Barely discernible barrels are detected for the first time at P3 in the rat (22), the age at which serotonergic patches first appear. The association of serotonergic hyperinnervation with barrel formation suggests that thalamocortical development may be influenced by serotonin. Both raphecortical (4) and thalamocortical (23) serotonergic axons reach the cerebral cortex several days before birth. However, serotonergic axons grow into the cortical gray matter by birth (4), whereas the thalamic projection does not grow into layer IV until 4 days later (24). Thus, the arborization of thalamocortical axons closely follows the period of serotonergic innervation. Thalamic axons have been shown (25) to exhibit a "waiting period" below the cortex before they undergo rapid, intracortical arborization. We speculate that the termination of this waiting period may depend on a trophic effect of serotonin upon cortical target neurons, which in turn signal thalamic axons to grow, or that there may be a direct influence of serotonin on thalamic axons.

The timing of serotonergic hyperinnervation coincides with a period of pronounced growth and synaptogenesis in cerebral cortex (26). This temporal association suggests that serotonin may influence synaptic development. In cultures of neonatal rat visual cortex (27), low concentrations of serotonin (20 $\mu$M) stimulate neuronal differentiation, neuropil formation, and synaptogenesis. Since serotonin accelerates synapse formation *in vitro*, the precocious serotonergic input to SI may have a trophic effect on the formation of thalamocortical connections *in vivo*.

The initial projection of serotonin neurons to restricted areas of cortex indicates that raphe neurons have specific cortical targets rather than a global, diffuse projection upon all cortical areas. The patches of serotonergic innervation in SI provide further evidence for the specificity of this projection to cortical regions that receive sensory inputs from the thalamus. The patchy innervation of SI may reflect an early form of the mosaic organization of raphe projections found in the adult rat (28). The selective association of serotonergic innervation with primary sensory areas of cortex in the neonate may be related to the established role of serotonin in sensory perception in the adult. Thus, psychedelic drugs, such as lysergic acid diethylamide and mescaline, that evoke subjective intensification of sensory perception act via serotonin receptors (3). A role for serotonin in the sleep–wakefulness cycle is supported by behavioral effects of raphe lesions, drug depletions of serotonin, and treatments with serotonin precursors (29). Moreover, the psychedelic drugs methylenedioxymethamphetamine (30) and methylenedioxyamphetamine (31) and fenfluramine, a widely used appetite suppressant (32), markedly deplete brain serotonin, apparently by neuronal destruction. Consequently, serotonergic neurons and sensory neuronal systems may be adversely affected in the offspring of pregnant mothers who ingest these drugs.

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