Convergence and Plasticity of Monoaminergic Systems in the Medial Prefrontal Cortex during the Postnatal Period: Implications for the Development of Psychopathology

Francine M. Benes, Jill Bolte Taylor and Miles C. Cunningham

Laboratory for Structural Neuroscience, McLean Hospital, Belmont, MA: Program in Neuroscience and Department of Psychiatry, Harvard Medical School, Boston, MA, USA

A variety of observations have suggested that the dopamine and serotonin systems may play a role in the pathophysiology and treatment of major mental disorders of childhood, adolescence and early adulthood. A recent triple immunofluorescence study has demonstrated a convergence of serotonin and dopamine fibers onto both pyramidal cells and GABAergic interneurons in the rat medial prefrontal cortex (mPFCx). These findings are consistent with the results of an electrophysiological study conducted in another laboratory that suggested such a relationship exists in the pyriform cortex of the rodent brain. During postnatal development, the dopamine system shows a progressive ingrowth of fibers into this region that continues until the early adult period. In contrast, GABAergic neurons appear to complete their postnatal maturation by the fourth postnatal week (the early post-weanling period). As dopamine fibers infiltrate the rat mPFCx, they progressively increase their interaction with neural elements within the neuropil and with the cell bodies of both pyramidal cells and GABAergic interneurons. This process appears to be influenced by the serotonin system, since lesioning of the nucleus raphe dorsalis during the neonatal period results in a significant increase of dopamine fibers. This finding suggests that lesions of the serotonin system induce plasticity of the cortical dopamine system; however, it is not known whether this inferred suppressive effect of serotonin fibers occurs at brainstem levels or within the mPFCx itself. Taken together, these various studies suggest that the convergence of dopamine and serotonin fiber systems on intrinsic cortical neurons shows considerable plasticity during postnatal life that could theoretically contribute to the development of 'miswired' circuits in individuals with neuropsychiatric disorders.

Introduction

The past decade has been characterized by a significant change in how we conceptualize the etiology of mental illness during childhood, adolescence and adulthood (Benes, 1995). Among these disorders, schizophrenia has received the most attention, with recent post-mortem studies having provided compelling evidence for a defect of GABAergic neurotransmission playing a role in its pathophysiology (Bird *et al.*, 1979; Hanada *et al.*, 1987; Simpson *et al.*, 1989; Reynolds *et al.*, 1990; Benes *et al.*, 1991, 1992, 1996a,b, 1997b; Akbarian *et al.*, 1995; Beasley and Reynolds, 1996; Woo *et al.*, 1997; Todtenkopf and Benes, 1998). Taken together, these various neurochemical and microscopic findings reported to date are consistent with the idea that there may be a decrease of GABAergic cells and/or activity in this disorder. Since the mechanism of action of antipsychotic medication involves blockade of both dopamine and serotonin receptors (Meltzer, 1994), a key question is how GABA cells interact with these monoaminergic systems in corticolimbic regions of schizophrenic brain. Thus far, studies of the dopamine (Mackay *et al.*, 1978; Owen *et al.*, 1978; Lee and Seeman, 1980; Cross *et al.*, 1981; Mackay *et al.*, 1982; Cross *et al.*, 1983; Joyce *et al.*, 1988; Kornhuber *et al.*, 1989a,b; Seeman and Niznik, 1990; Ohara *et al.*, 1993; Seeman *et al.*, 1993a,b; Akil and Lewis, 1997; Benes *et al.*, 1997a,b; Joyce and Meador-Woodruff, 1997; Meador-Woodruff *et al.*, 1997) and serotonin (Bennett *et al.*, 1979; Whitaker *et al.*, 1981; Mita *et al.*, 1986; Hashimoto *et al.*, 1991; Joyce *et al.*, 1993) systems have failed to demonstrate consistent abnormalities that can be convincingly distinguished from neuroleptic effects. A recent report, however, has suggested that a subtle 'miswiring' of the dopamine system with respect to pyramidal neurons and GABA cells may be present in the anterior cingulate cortex of subjects with schizophrenia (Benes, 1997a,b; Benes *et al.*, 1997a,b). Such an abnormality could be present without there being any associated changes in the levels of biochemical and molecular markers for the dopamine system. If this latter hypothesis is correct, it will be important to gain some insight into how aberrant connections between monoaminergic fibers and intrinsic cortical neurons may arise and how such changes may influence corticolimbic function.

It is now broadly believed that schizophrenia is a neurodevelopmental disorder (Jakob and Beckmann, 1986; Weinberger, 1987; Benes, 1988), one in which normal maturational changes in the corticolimbic system during late adolescence may 'trigger' its onset in susceptible individuals (Benes, 1988, 1989; Benes *et al.*, 1994). In order to understand further the implications of this hypothesis, not only for schizophrenia but also for other neuropsychiatric disorders that present during childhood and adolescence, the following discussion will examine the anatomic relationship of the monoaminergic systems to intrinsic cortical neurons, particularly GABAergic cells, and will consider how the development of these interactions could potentially go awry during the postnatal period.

Interactions of Dopamine and Serotonin Fibers with Cortical Neurons

It is now well-established that the activity of cortical neurons is probably modulated by both the dopamine and serotonin systems. In the rat medial prefrontal cortex (mPFCx), a homologue of the anterior cingulate cortex of human brain, serotoninergic fibers are abundantly present in both superficial and deep laminae (Lidov *et al.*, 1980; Reader, 1981), while dopamine fibers are most densely distributed in layers V and VI (Emson and Koob, 1978; Lindvall and Bjorklund, 1984). Recent studies have demonstrated that both pyramidal (Seguela *et al.*, 1988; Goldman-Rakic *et al.*, 1989; Verney *et al.*, 1991) and non-pyramidal (Verney *et al.*, 1991; Benes *et al.*, 1993a) neurons of this region receive inputs from dopamine fibers. In some studies, both of these neuronal subtypes showed D1 and D2 receptor binding activity (Vincent *et al.*, 1993, 1995a) and their respective messenger RNAs (Huntley *et al.*, 1992) localized to their cell bodies. Rodent studies in which *in situ* hybridization has been used to localize mRNA for the two subtypes have demonstrated that projection cells of various laminae may express one or the other subtype (Gaspar *et al.*, 1995), although

the D2 receptor seemed to be principally associated with those in layer V. More recent work has demonstrated that mRNA for the D1 and D2 subtypes are also expressed by cortical interneurons subtype (LeMoine and Gaspar, 1998); but the cells showing mRNA for both receptors were principally those containing parvalbumin, while those showing calbindin-immunoreactivity seemed to preferentially express the D1. It is important to emphasize, however, that the ability to localize mRNA for a particular protein is limited by the degree to which this nucleic acid is being expressed at any given time by a subpopulation of cells. Thus, the absence of a particular mRNA in subpopulations of neurons *in situ* hybridization studies does not exclude the possibility that the receptor in question is actually being synthesized and utilized by these cells. Some immunocytochemical studies in primates have preferentially localized the D1 receptor to pyramidal neurons (Smiley *et al.*, 1994; Bergson *et al.*, 1995), while others have found it in interneurons (Muly *et al.*, 1998). It appears likely that different antibody preparations may yield different localization patterns. Using a high resolution Scatchard analysis of the distribution of D1 receptor binding activity, this receptor was found to be expressed by both projection cells and interneurons in rodent mPFCx (Davidoff and Benes, 1998). This latter technique, like the ones employing fluorescently tagged ligands for the D1 and D2 receptors (Vincent *et al.*, 1993, 1995a), have the advantage of localizing the high affinity binding activity and are much less likely to be distorted by indeterminate losses of mRNA or immunoreactivity that are inherent to *in situ* hybridization and immunocytochemistry, respectively. In any case, it seems likely that D1 and D2 receptors are employed by many different neuronal populations to mediate the effects of dopamine in the cortex.

In the pyriform cortex, the activity of both pyramidal and nonpyramidal neurons can also be manipulated with either agonists or antagonists of serotonin receptors (Sheldon and Aghajanian, 1990; Gellman and Aghajanian, 1993). Both pyramidal cells (Jakab and Goldman-Rakic, 1998; Wu *et al.*, 1998) and GABA neurons (Gellman and Aghajanian, 1993; Morilak *et al.*, 1993) in the prefrontal cortex express the serotonin $(5\text{-HT})_{2A}$, whereas pyramidal neurons in the hippocampus (Chalmers *et al.*, 1993) and pyriform cortex (Sheldon and Aghajanian, 1991) have been associated with the $5HT_{1A}$ and $5HT_{1C}$ subtypes, respectively. Thus, the pattern of expression for various receptor subtypes may vary from one region to another.

The above observations suggest that both projection cells and local circuit cells may be *potentially* influenced by the dopaminergic and serotoninergic projections to the mPFCx (Benes, 1995a,b). It is noteworthy that a convergence of these fiber systems onto intrinsic cortical neurons could play an important role in the cortical stress response. Exposure to stress has not only been associated with changes in dopamine (Thierry *et al.*, 1976; Roth *et al.*, 1988) and serotonin in both the prefrontal cortex (Thierry *et al.*, 1986) and hippocampus (Kalen *et al.*, 1989) [for a review see Stanford (Stanford, 1993)] systems, but has also been implicated in the regulation of pyramidal cells (Chalmers *et al.*, 1994) and GABAergic interneurons (Corda and Biggio, 1986; Schwartz *et al.*, 1987).

To understand how the dopamine and serotonin systems may be interacting with intrinsic cortical neurons, it is important to know whether the respective fiber systems project to mutually exclusive neuronal subpopulations or whether perhaps there is a significant degree of overlap in the neurons receiving inputs from these two systems. In order to investigate this question, these two transmitter systems have been localized using a combination of single, double and triple immunocytochemical approaches (Taylor and Benes, 1996). Using a single immunoperoxidase technique, the proportion of 5-HT-IR varicosities that are in apposition with neuronal cell somata in layers II and VI is ~35 and 18%, respectively, while the majority (65 and 82%, respectively) are found in the neuropil of these two laminae (Table 1). For tyrosine hydroxylase (TH)-IR varicosities, a similar pattern is observed, although the percentages associated with cell bodies (23 and 13%, respectively) is somewhat lower for 5-HT-IR varicosities (Table 1). In order to assess whether these interactions might be random in nature, a 'predicted' number of varicosities in neuropil versus on cell bodies was computed by calculating the number of varicosities that would be found in each compartment according to their respective areal percentages. Using this so-called Poisson analysis (Benes *et al.*, 1993), the 'observed' frequencies for 5-HT-IR varicosities in apposition with cell bodies in layers II and VI is 2–3 times higher than the 'predicted' (χ^2 = 93.7, P = 0.0001 and χ^2 = 19.5, P = 0.0001, respectively). The 'observed' frequency with which TH-IR varicosities form appositions with cell bodies shows a similar pattern. In the rat mPFCx, somata <100 μm^2 are believed to be primarily nonpyramidal in nature, while those >100 μm^2 are probably pyramidal cells (Vincent *et al.*, 1993). Using these criteria, both 'nonpyramidal' and 'pyramidal' neurons in layers II and VI show an approximately equal distribution of 5-HT-IR and TH-IR in apposition with their somata. Based on these findings,

Table 1
χ^2 analysis of the randomness of 5HT and TH varicosity interactions with cell bodies and in neuropil

Cortical layer	5HT		TH	
	Neuropil	Cell body	Neuropil	Cell body
Layer II				
Observed	344 (65%)	189 (35%)	429 (77%)	131 (23%)
Predicted	477 (89%)	56 (11%)	505 (90%)	55 (10%)
Layer VI				
Observed	429 (82%)	92 (18%)	1177 (87%)	177 (13%)
Predicted	477 (92%)	44 (8%)	1245 (92%)	108 (8%)

5HT: layer II, $\chi^2 = 93.7$, $P = 0.0001$; layer VI, $\chi^2 = 19.5$, $P = 0.0001$. TH: layer II, $\chi^2 = 37.2$, $P = 0.0001$; layer VI, $\chi^2 = 18.7$, $P = 0.0001$.

The total number of varicosities in the neuropil or on cell bodies of layers II and VI are indicated for both 5HT- and TH-IR fibers. The numbers in parentheses represent the percent of the total (i.e. Neuropil + Cell body). A Poisson analysis was performed by determining the area of the entire field, the area of each neuron's somata in the field, the area of the surrounding neuropil and the areal percent for the two respectively compartments. The 'predicted' number of varicosities was determined by multiplying the areal percent for cell bodies and neuropil by the total number of varicosities present in the sample field. The significance of differences between the 'observed' and 'predicted' number of varicosities was assessed using an $R \times C$ (2×2) contingency table analysis. For both 5HT-IR and TH-IR, the proportion of varicosities in apposition with cell bodies was 2–3 times higher than would be predicted if a random distribution were present.

Table 2
Percent of large (≥100 μm^2) and small (<100 μm^2) neurons with varicosities plus probability of convergence

	Cortical layer	5HT	TH	Probability of convergence (5HT × TH)
Large neurons with varicosities	layer II	54	43	23
	layer VI	38	67	25
Small neurons with varicosities	layer II	53	68	36
	layer VI	44	58	26

The percentage of 5HT-IR and TH-IR cells in apposition with neuron somata in layers II and VI were multiplied by one another to generate a predicted probablility of convergence.

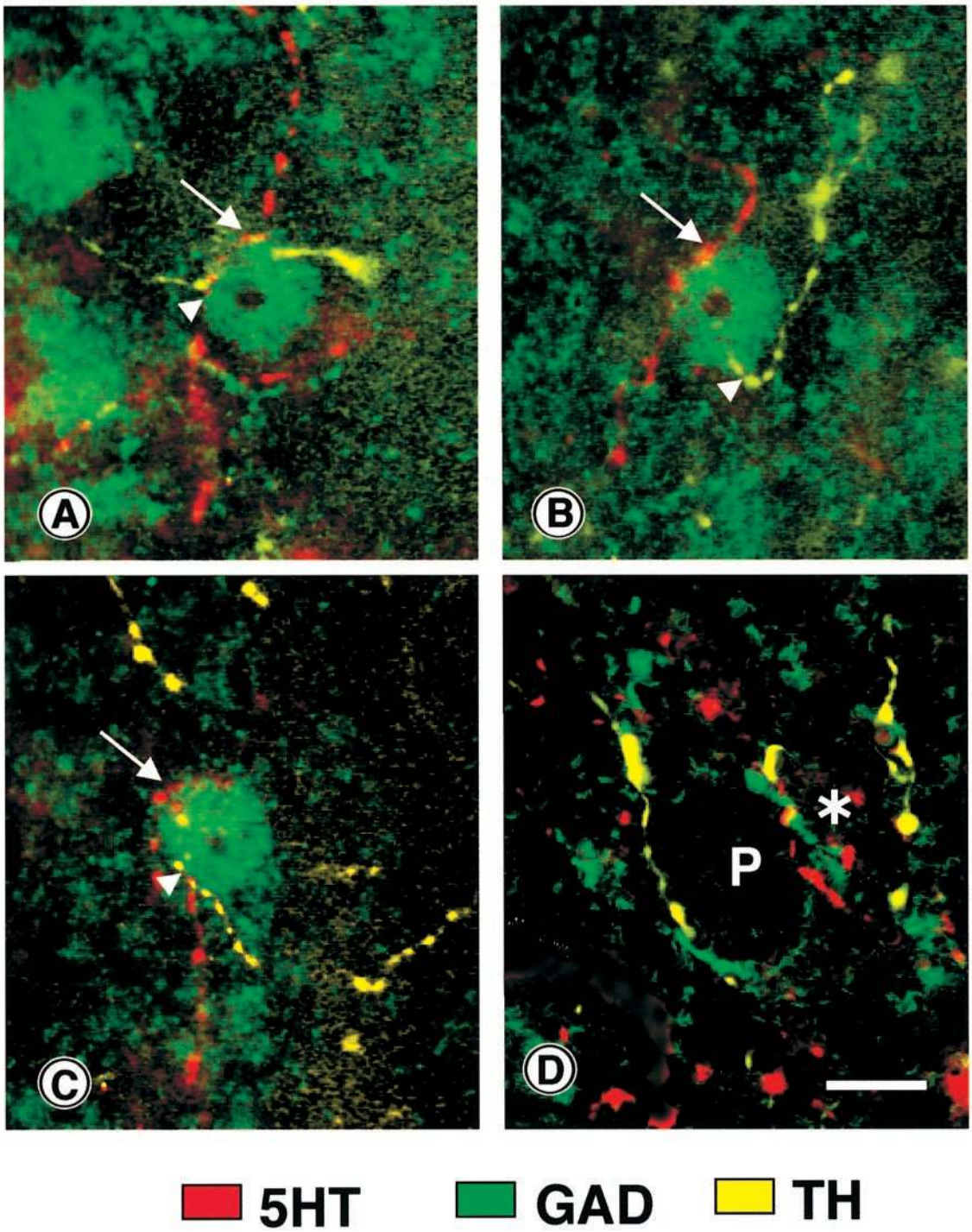


Figure 1. A set of co-registered digital confocal images of 5-HT-, GAD- and TH-IR staining. (*A–C*) Three deconvolved images showing a convergence of monoaminergic inputs (arrows = 5-HT-IR; arrrowheads = TH-IR) to GAD-IR cell bodies in the rat mPFCx. (*D*) 5-HT- and TH-IR varicose fibers converge on a pyramidal neuron (P) with GAD-IR terminals forming 'classic' axosomatic contacts. There also appear to be appositions between GAD-IR terminals and 5-HT-IR varicosities (*). Similar interactions also seem to be occur between GAD-IR terminals and TH-IR varicosities. Bar = 10 μm. Reproduced with permission (Taylor and Benes, 1996).

the probability that both 5-HT- and TH-IR fibers would be found simultaneously on individual somata was found to be 23 and 25% for neurons in layers II and VI, respectively, that were >100 μm^2 in size and 36 and 26%, respectively, for neurons that were <100 μm^2 in size (Table 2). Accordingly, these latter data had suggested that a convergence of 5-HT and dopamine fibers might be a relatively frequent occurrence in the rat mPFCx and that such events probably occur to an equal degree for both pyramidal neurons and interneurons.

When sections are processed simultaneously with antibodies against 5-HT, TH and the 67 kDa isoform of glutamate decarboxylase [GAD_{67}], two patterns of interaction have been

observed (Figure 1*A–D*). On the one hand, both 5-HT-IR and TH-IR varicosities are found in apposition with the same GAD_{67}-IR somata (Figure 1*A–C*), with ~25% of GAD_{67}-IR somata showing a convergence of these two fiber systems. This latter frequency agrees remarkably well with the 'predicted' frequency that was computed using the number of contacts observed for each fiber system in single immunocytochemical preparations (Table 2). The second pattern observed with the triple localization involved appositions of 5-HT-, TH- and GAD_{67}-IR fibers with 'ghosts' of pyramidal neuron cell bodies visualized by the absence of cytoplasmic or nuclear fluorescence. This so-called 'trivergence' of 5TH-IR and TH-IR varicose fibers, together with GAD_{67}-IR puncta forming 'classical' rings of axosomatic synapses (Figure 1*D*), suggests that pyramidal neurons may receive not only traditional synaptic inputs from GABAergic terminals, but also modulatory ones from the DA and 5-HT systems. Taken together, it appears that both the dopamine and serotonin systems may interact extensively with both pyramidal cells and interneurons. Although some of these interactions may be present at the level of the cell body, it is likely that the majority occur within the neuropil area where the dendritic branches of both cell types are localized (see Table 1).

It is important to point out that the double and triple localizations discussed above do not have the spatial resolution needed to determine whether the contacts observed are synaptic in nature. Studies from other laboratories, however, have suggested that both dopamine and serotonin fibers primarily form synaptic contacts with dendrites. For example, at the ultrastructural level, DA-containing varicosities have been shown to form synapses with shafts and spines of distal dendritic branches; although consistent with the findings discussed above and elsewhere (Benes *et al.*, 1993), some also form contacts with neuron somata having a morphological appearance similar to that of inhibitory basket cells (Goldman-Rakic *et al.*, 1989). Based on primate studies, serotoninergic fibers form appositions predominantly on interneurons and, like dopamine fibers, most do not show synaptic profiles (Smiley and Goldman-Rakic, 1993, 1996). The appositions formed by DA varicosities on neuron somata are also typically nonsynaptic in nature and do not show glial processes interposed between them (Verney *et al.*, 1990). Approximately 90% of all varicosities show a small area specialized for synaptic transmission (Seguela *et al.*, 1988), although it is not clear whether this is also true for varicosities in apposition with cell bodies. Similar data are lacking for serotoninergic contacts on neuronal cell bodies. The responses associated with activation of the dopamine system are typically modulatory in nature and show a much longer duration than is typically observed with 'classic' synaptic inputs (Reader *et al.*, 1979; Bunney and Chiodo, 1984; Mantz *et al.*, 1988; Williams and Goldman-Rakic, 1995; Yang and Seamans, 1996; Gulledge and Jaffe, 1998). In contrast, those responses associated with serotoninergic inputs to the pyriform cortex in the rodent brain have the properties of synaptic inputs that primarily influence GABAergic cells and, in turn, exert a secondary influence on pyramidal neurons (Sheldon and Aghajanian, 1991). In the medial prefrontal cortex, the action of serotonin appears to be a presynaptic one on a subpopulation of glutamatergic terminals (Marek and Aghajanian, 1998). Clearly, the action of the monoaminergic systems on intrinsic cortical neurons varies on a region-by-region basis. Further study is needed to identify how the somal contacts made by dopamine and serotonin fibers influence the activity of these intrinsic neurons.

Postnatal Development of Monoaminergic Fibers and Intrinsic Cortical Neurons

It has long been suspected that the development of the corticolimbic regions of the human brain may continue well beyond birth (Flechsig, 1920; Yakovlev and Lecours, 1967; Benes, 1989; Benes *et al.*, 1994). Recently, this idea has received increased attention with the growing realization that normal maturational changes probably play an important role in the appearance of various neuropsychiatric diseases at specific stages of postnatal life (Weinberger, 1987; Benes, 1988). In other words, a normal ontogenetic change at a critical stage of development could potentially act as a 'trigger' for the onset of a given disorder at that stage. Consistent with this concept, many studies in the rodent brain have demonstrated that there are significant changes in several key neurotransmitter systems at key stages of the postnatal period [for comprehensive reviews see Johnston and Parnavelas *et al.* (Johnston, 1988; Parnavelas *et al.*, 1988)].

The Dopamine System

Dopaminergic projections to the rat mPFCx have been found to increase progressively beyond the weanling stage until to the early adult period (Verney *et al.*, 1982; Kalsbeek *et al.*, 1988). During the first 2 weeks of postnatal life (Figure 2, P11) the relative distribution of DA-IR varicose fibers in the rat mPFCx is quite low, but shows the highest density in deeper laminae (Benes *et al.*, 1993a). During the third postnatal week, however, the density of such fibers shows discernible increases (Figure 2, P20), and this pattern continues until adulthood (Figure 2, P45, P52 and Adult). As described by others (Lindvall *et al.*, 1978), the density of DA-containing fibers in the rat mPFCx is greatest in layer VI and shows a progressive decrease toward layer I. Unlike noradrenergic fibers in the mPFCx (Lindvall and Bjorklund, 1984), the dopamine system does not show long, vertical fibers travelling either vertically toward layer I or horizontally within this lamina. Together with the fact that the densest distribution of fibers observed is in deeper layers, it seems unlikely that noradrenergic axons have been included in these analyses, particularly since the antibody preparation employed in one of these studies (Benes *et al.*, 1993a) is 50 times less selective for norepinephrine–glutaraldehyde–protein conjugates than for those made with dopamine (Geffard *et al.*, 1984). Thus, it seems unlikely that a significant number of noradrenergic fibers were included in the count of DA-IR varicosities.

A similar pattern of fiber staining can be distinguished at all postnatal stages examined. The increase of fiber density occurs to a proportionate degree across layers VI–II and does not progress in a distinct 'inside-out' manner. The size of the DA-IR varicosities also increases from ~1.2 μm at P20 to ~2.4 μm by P60; however, it is not likely that this change in size accounts for the increase in the density of fibers during the postnatal period, because the dimensions of the largest varicosities are still quite small relative to the thickness of the sections (40 μm). The size of varicosities reported in this study is larger than that previously described in an ultrastructural analysis (Seguela *et al.*, 1988); however, this apparent discrepancy is probably related to shrinkage incurred during the fixation, dehydration and imbedding that is typically required for electron microscopic studies. Moreover, using a correction for particle size in which the numerical density, N_a, is divided by section thickness, t, plus the average diameter of the particles, D (Abercrombie, 1946; Weibel, 1979), the density of fibers in post-weanling mPFCx is ~3.5-fold higher than in that of pre-weanling rats. For sections in which single immunoperoxidase-processing is combined with cresyl violet

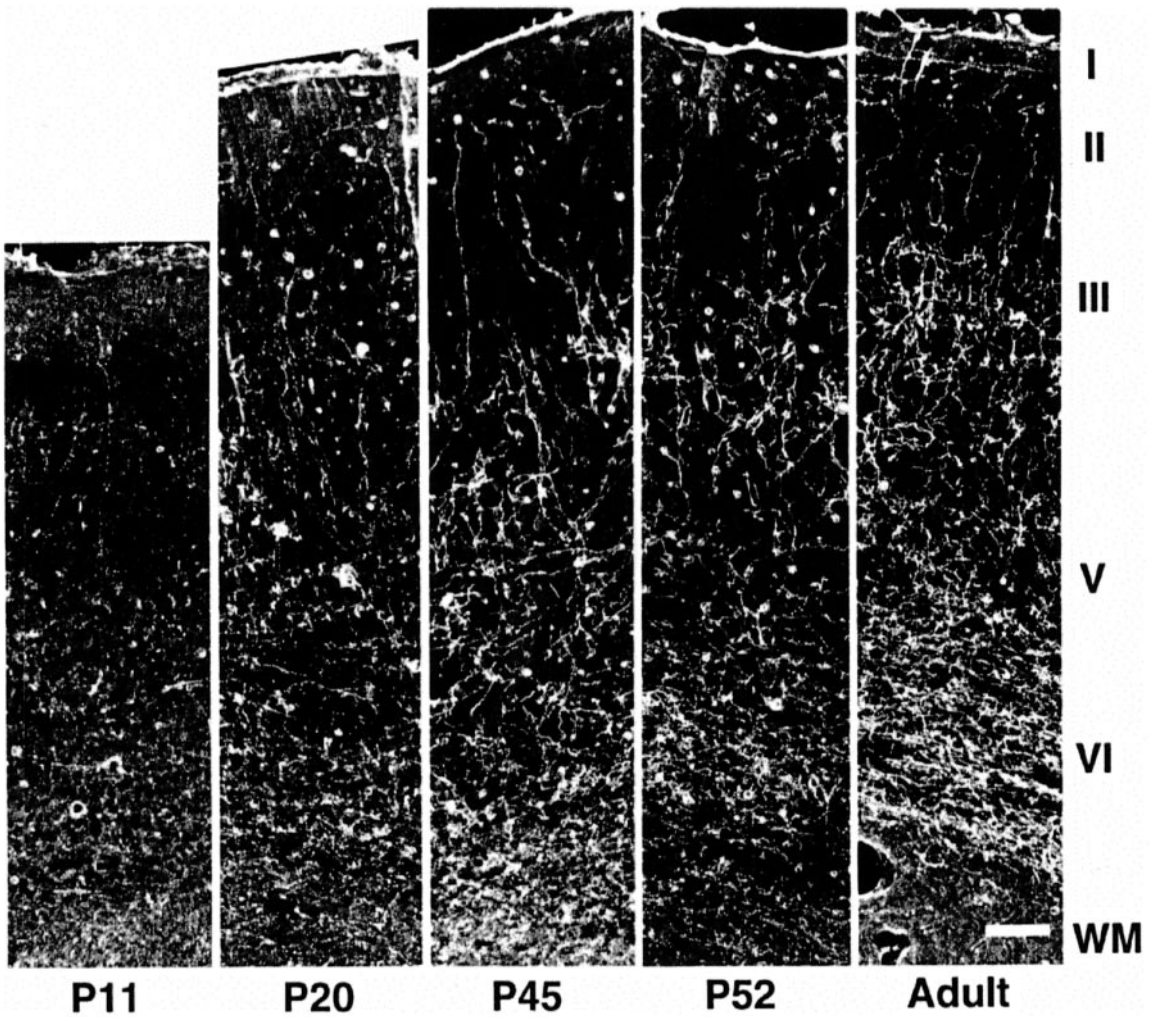


Figure 2. A set of low power, darkfield photomicrographs of dopamine-IR varicose fibers in the rat mPFCx at five different postnatal stages (P11, P20, P45, P52 and early adult). At P11, there is a paucity of dopamine-containing fibers in the cortical mantle, but by P20, these fibers have become much more common. At P45 and P52, dopamine-IR fibers show a typical laminar distribution, with the highest density occurring in deeper laminae V and VI and a progressive decrease in density occurring in a gradient fashion toward layer I. In adult rats, this unique laminar distribution is still present, even though there is a marked increase in the density of fibers in each of the layers. Bar = 100 μm. Reproduced with permission (Benes *et al.*, 1996).

staining, DA-containing varicose fibers can be observed throughout the neuropil, but very commonly, such fibers course toward neuronal cell bodies (Benes *et al.*, 1993). Many cell bodies have one or more varicosities in close apposition, and such contacts are observed in both superficial and deep laminae at all stages of postnatal life examined, although the density of varicosities in layers II and III is characteristically quite low.

Postnatal increases in the density of dopaminergic projections to the rat mPFCx (Verney *et al.*, 1982; Kalsbeek *et al.*, 1988) are paralleled by an increase of D_2 receptor binding activity, which begins prenatally (Bruinink *et al.*, 1983) and continues until the fourth postnatal week (Deskin *et al.*, 1981). Interestingly, administration of 6-OH-dopamine prevents this latter increase of D_2 receptor binding (Deskin *et al.*, 1981), an effect that is associated with dystrophic changes in the basal dendrites of pyramidal neurons (Kalsbeek *et al.*, 1988). Lesions induced in the prefrontal cortex of adult monkeys using 6-OH-dopamine result in an impaired performance of the spatial delayed alternation task (Brozoski *et al.*, 1979) and it seems likely that this functional deficit would be associated with changes in the D2 receptor on pyramidal neurons.

The GABA System

GABA has long been considered the most important inhibitory neurotransmitter in the mammalian brain and extensive neurochemical studies of its development in rodent brain suggest that its maturation continues well into the postnatal period [for a review see Johnston (Johnston, 1988)]. For example, GABA-accumulating cells show a progressive increase in numerical density until P11 (Chronwall and Wolff, 1980). In contrast, the concentration of GABA and the specific activity of glutamate decarboxylase (GAD) (Coyle and Enna, 1976), as well as GABA receptor binding activity (Coyle and Enna, 1976; Palacios *et al.*, 1979) and the messenger [m]RNA for this receptor (Gambarana *et al.*, 1990), all increase until the third postnatal week.

At birth, the numerical density of GAD-IR somata in the anterior cingulate cortex reaches a peak at approximately postnatal day 5 (PN5) then diminishes until PN20, when the thickness of the cortical mantle is maximal (Vincent *et al.*, 1995b). During this same period, the relative amount of neuropil surrounding all cell bodies in this region is expanding as dendritic and axonal fibers are increasing. As shown in Figure 3, not only do GABAergic cell bodies show a gradual increase in

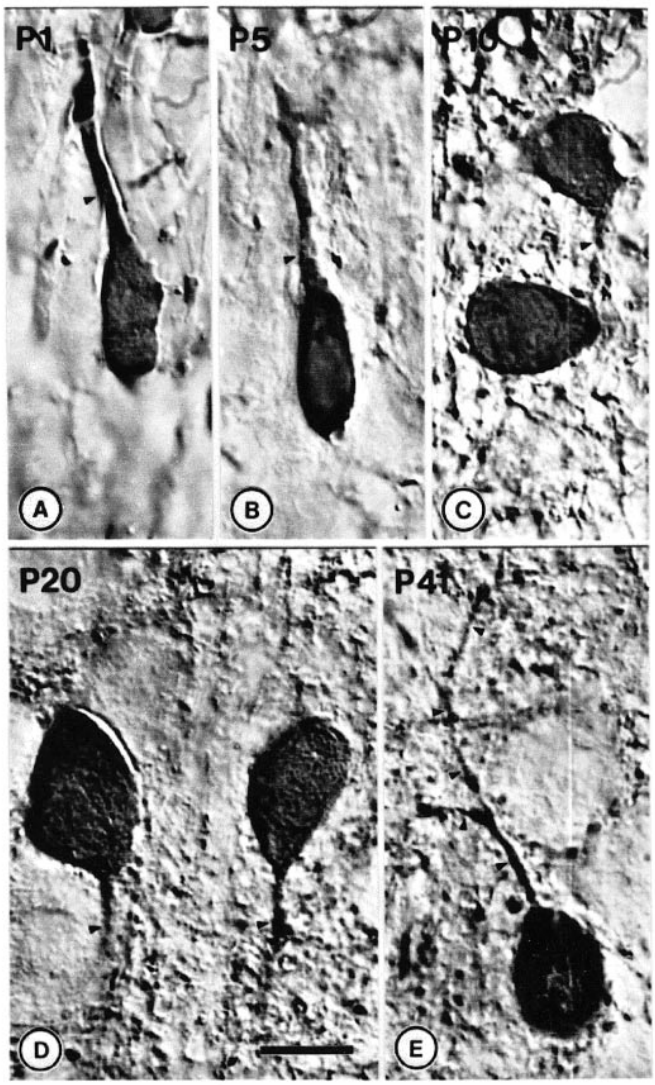


Figure 3. A set of Nomarsky photomicrographs showing GABA-IR cell bodies in the rat mPFCx at various postnatal ages (P1, P5, P10, P20 and P41). (*A*,*B*) At early postnatal stages, GABA-IR cells have an elongated shape and a vertical orientation with respect to the pial surface. These cells also show thick apical dendrites and thinner basal processes (not in plane of focus). (*C*,*D*) At intermediate stages, some neuron somata become round or oval in shape and their dendritic processes show a reduction in caliber. (*E*) At later stages of the postnatal period, most GABA-IR somata show a round or oval shape and the dendrites exhibit secondary and tertiary branching (arrowheads). Scale bar = 10 μm. Reproduced with permission (Vincent *et al.*, 1995).

their size but, by P20–40, primary, secondary and tertiary branches of their dendritic tree can also be visualized. The expansion of neuropil in the rat mPFCx probably involves an increase of both dendritic branches and terminals of GABAergic neurons, a process that continues in the superficial layers until P25 (Vincent *et al.*, 1995b), when the efficacy of GABAergic synaptic transmission also becomes optimal (Luhmann and Prince, 1991). In general terms, the maturation of the GABA system continues for ~2–3 weeks postnatally. As such, its full maturation within the mPFCx is probably complete *before* the dopamine system attains its full postnatal profile. Presumably, then, the dendritic branches of GABAergic interneurons lay in waiting for ingrowing dopamine fibers to target them for the formation of functional interactions.

Postnatal Development of Dopamine–GABA Interactions

As previously reported (Benes *et al.*, 1996), specimens of the rat mPFCx processed with a double-immunostaining technique that localizes both DA-immunofluorescent (-IF) varicosities and GABA-IF cell bodies show a progressive increase in the interaction of these two neuronal elements between the pre-weanling period (Figure 4, P20) and the early stages of the post-weanling period (Figure 4, P25). An increasing number of such varicosities form contacts with GABA-IF neurons as the post-weanling period progresses, and this becomes most apparent at the beginning of the adult period (Figure 4, P60). When the latter double-IF preparations are subjected to blind, semiquantitative analysis, a progressive linear increase in the percentage of GABA-IF cell bodies (Figure 5, upper panel) with apposed DA-IR varicosities occurs between P0 and P60, and these data best fit a first-order polynomial equation (r = 0.75, P = 0.0005). During the pre-weanling period, any given GABA-IR cell body, on average, can show approximately one apposed varicosity. During the post-weanling period, however, the number of DA-IR varicosities (Figure 5, lower panel) in contact with GABA-IR cell bodies shows a curvilinear rise through P60 (r = 0.81, P = 0.0005). Some neurons have no varicosities forming appositions with their somata, while other have more than one, making the average number per cell >1.0. For these latter data, a second-order polynomial equation provides the best fit. When an *index of interaction* similar to that described in Table 4 is computed by multiplying the percentage of GABA cell somata having apposed dopamine varicosities and the number of such varicosities in contact with any given GABA cell body, post-weanling rats have an index that is 1.5 times higher than that seen in pre-weanling animals. By adulthood, this index increases 1.8 times with respect to post-weanling rats and 2.5 times when compared with pre-weanling animals (Benes *et al.*, 1996).

In primates, dopaminergic inputs to neuron somata appear to be minimal (Goldman-Rakic *et al.*, 1989), while in the human cortex, TH-IR varicosities have been shown to form appositions with the cell bodies of both pyramidal and nonpyramidal neurons with a remarkable degree of consistency across many cases (Todtenkopf and Benes, 1998). Using a Poisson analysis (Benes *et al.*, 1993) of a large number of varicosities (>10 000) counted in 15 normal human cases, neurons in layers II (P = 0.0001), III (P = 0.004), V (P = 0.0001) and VI (P = 0.0001) of the anterior cingulate cortex were found to have non-random contacts with TH-IR varicosities (unpublished observations). The data obtained in a parallel analysis of a schizophrenic cohort (n = 10) were remarkably similar. Overall, the 'observed' percentage of varicosities in contact with cell bodies ranged from ~3 to 7% and is much lower than that seen for DA-IR (Benes *et al.*, 1993) and TH-IR (Taylor and Benes, 1996) in the rat mPFCx. Although the majority are clearly associated with the neuropil, the proportion on cell somata is nevertheless quite significant in a Poisson sense. It is not clear, however, why the primate and human brain show such a lower density, although even a small number of varicosities could potentially exert a significant modulatory influence at the level of the cell body.

Taken together, the somata of GABAergic neurons probably act as a site with which sprouting dopaminergic fibers may form appositions. In this process, GABA cells may be a 'passive' target for the formation of interactions, or they may exert an 'active' neurotrophic influence on fiber sprouting and/or contact formation (Spoerri, 1988). Either way, it seems likely that dopaminergic fibers are capable of exerting an increasing modulatory influence on the activity of inhibitory interneurons

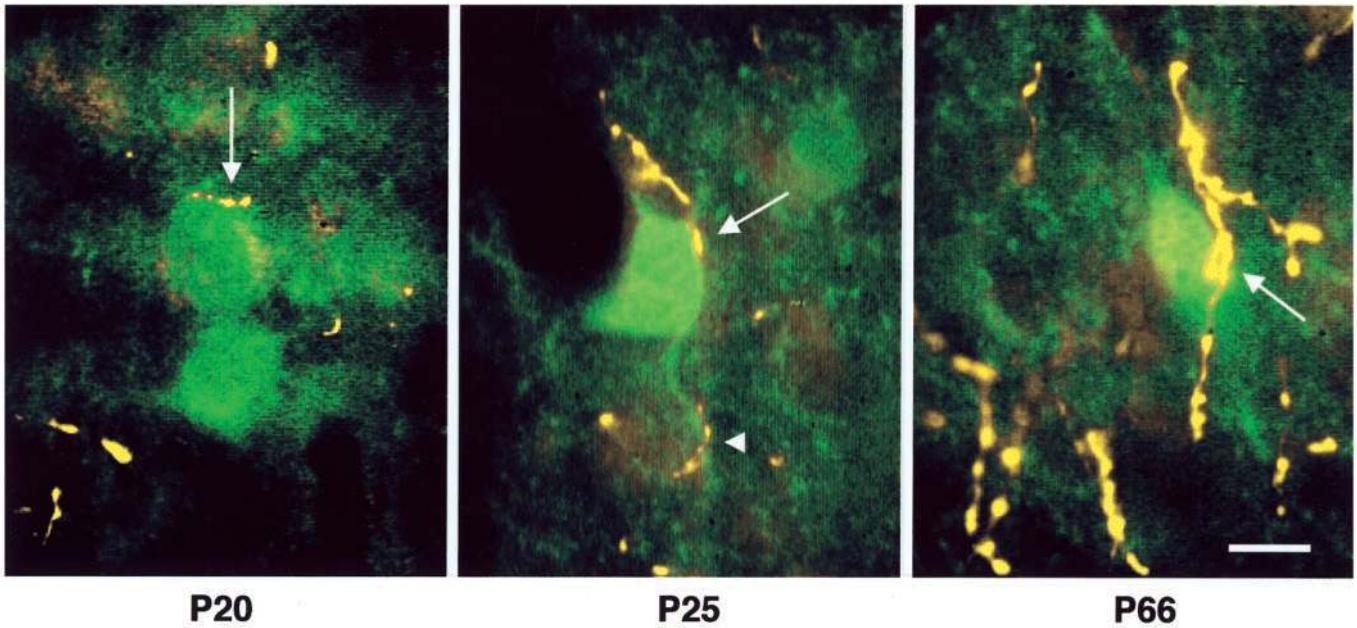
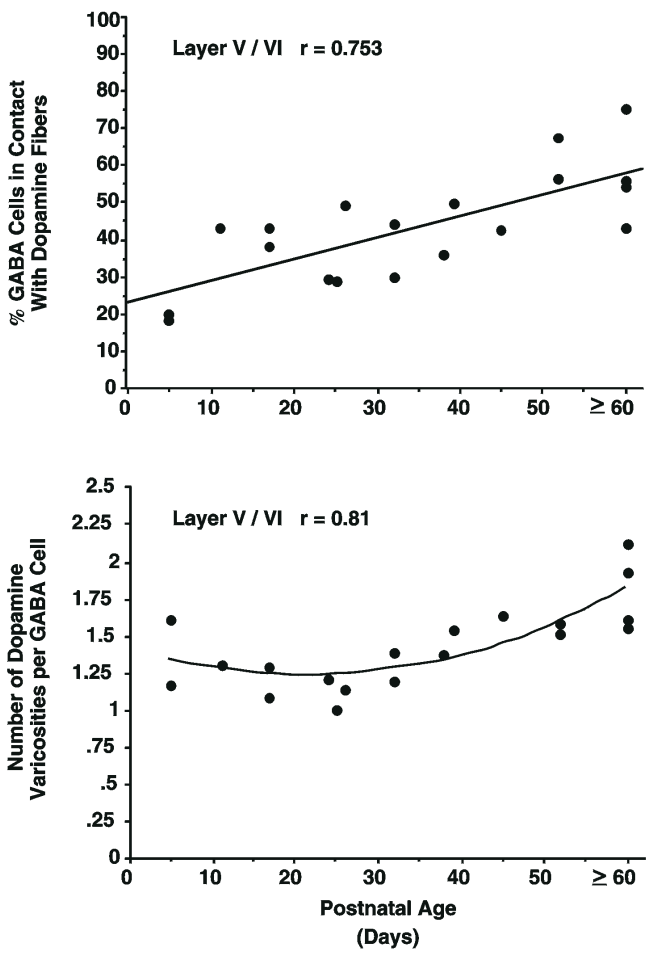


Figure 4. A set of digital confocal photomicrographs of a double-immunofluorescence localization of dopamine-IR fibers (yellow) and GABA-IR (green) somata in layer VI of the rat mPFCx at three different postnatal ages (P20, P25 and P66). By P20, dopamine-IR varicosities are already forming contacts with GABAergic cell bodies (arrows). Within a few days of weaning (P25), there are still no obvious changes in the extent of interaction, although some varicosities may be forming contacts with a dendritic shaft (arrowhead). By P66, however, there are several varicosities in apposition with the GABA-containing cell body. Magnification = ×1238.



during the postnatal period, particularly since DA receptors are localized on nonpyramidal cell bodies in the rat mPFCx (Vincent *et al.*, 1993; Vincent and Benes, 1995). Moreover, both agonists and antagonists of DA can alter the postsynaptic potentials recorded in GABAergic interneurons in the pyriform (Gellman and Aghajanian, 1993) and frontal (Zhou and Hablitz, 1999) cortices. Organically synthesized agonists of the D_2 receptor (i.e. RU24926 and LY171555) have been found to inhibit the release of [^{3}H]GABA (Tam and Roth, 1990; Retaux *et al.*, 1991a,b) and dopamine itself can influence the firing of GABAergic neurons (Penit-Soria *et al.*, 1987).

The Influence of Serotonin on Dopamine Fiber Ingrowth

Recent evidence from studies of the anterior cingulate region of post-mortem brain has suggested that the interaction of dopamine fibers with intrinsic cortical neurons may be abnormal, as the distribution of TH-IR varicosities appears to be shifted from pyramidal to nonpyramidal neurons in layer II of schizophrenics (Benes *et al.*, 1997a,b). Although many different neurotrophic mechanisms could potentially contribute to the induction of such a change, a facilitatory effect associated with the serotonin system presents an intriguing possibility because these latter fibers have been found to promote the ingrowth of afferents

Figure 5. A set of bivariate plots showing the interaction of dopamine-IF varicosities with GABA-IF somata in the rat mPFCx at different postnatal ages. Upper panel: the percentage of GABA-IF cell bodies having an apposed dopamine-IF varicosity. Lower panel: the number of dopamine-IF varicosities per GABA-IF cell body. Between P5 and P60, there is a progressive increase in the percentage of GABA-IF cell bodies in apposition with dopamine-IF varicositeis ($r = 0.75$, $P = 0.0005$ using a first-order polynomial equation). During this same period, there is, on average, only one dopamine-IF varicosity in apposition with any given GABA-IF neuron; however, after weaning (P24), the number of varicosities per cell shows a curvilinear rise toward adult levels ($r = 0.81$, $P = 0.0005$ using a second-order polynomial equation). Repoduced with permission (Benes *et al.*, 1996).

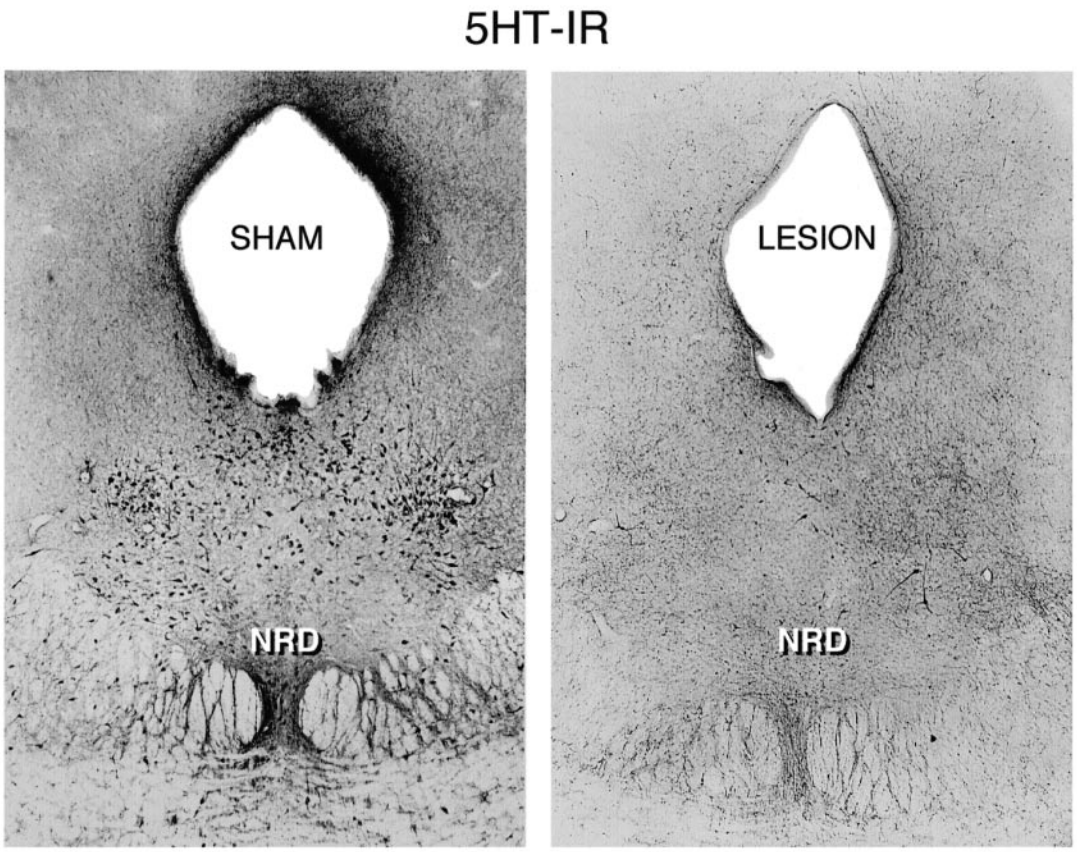


Figure 6. Brightfield photomicrographs of 5-HT-IR neuronal cell bodies in the NRD in ventral portions of the PAG of sham- (left) and 5,7-DHT-lesioned (right) rats.

originating in the thalamus during cortical development (D'Amato *et al.*, 1987). This possibility seems particularly intriguing because a significant number of cortical neurons probably receive a convergence of these two monoaminergic systems (see above). To assess the nature of this relationship, a series of experiments in which the 5-HT projections from the nucleus raphe dorsalis (NRD) were lesioned during the neonatal period using the selective toxin, 5,7-dihydroxytryptamine (5,7-DHT) were recently undertaken (Taylor *et al.*, 1998). As shown in Figure 6 (left), the sham-lesioned rats in which only vehicle was injected (n = 4) showed a dense distribution of 5-HT-IR neuronal cell bodies in ventral portions of the peri-aqueductal gray (PAG) at the level of the decussation of the superior cerebellar peduncle. The NRD is particularly prominent at this brainstem level and lesioned rats show a marked reduction in the number of immunoreactive cells (Figure 1, right). At more rostral levels of the midbrain, both sham and lesioned rats show abundant TH-IR fibers in the substantia nigra (SN) and ventral tegmental area (VTA). It seems unlikely that 5,7-DHT adversely affects dopamine neurons, since the rats have been treated with nomifensine, which blocks its uptake into these latter cells. It is hypothetically possible that this treatment could potentially have had an adverse effect on other neuronal populations, particularly those that receive an abundant serotoninergic innervation; however, there was no obvious indication of this. Rather than being decreased, TH-IR fibers appeared to be increased in dopaminergic nuclei of 5,7-DHT-lesioned rats, suggesting that the NRD may exert a suppressive effect on the projection neurons of the SN and VTA. In the mPFCx of sham-lesioned rats, 5-HT-IR varicose fibers are distributed throughout the cortical mantle (Figure 7, left), while the 5,7-DHT-lesioned rats show almost a complete absence of stained fibers (Figure 7, right). When TH-IR is visualized in this region, a rich plexus of varicose fibers is seen throughout the cortical mantle in both the sham- and 5,7-DHT-lesioned groups (Figure 8, left and right, respectively), particularly in layers V and VI, where DA fibers are typically most abundant. As with the SN and VTA, visual inspection suggests that the lesioned rats may have a higher density of TH-IR fibers in layer V and possibly also layer VI. Consistent with this impression, a computer-assisted microscopic analysis (Figure 9) has revealed that the density of TH-IR fibers in layer II showed no difference in the sham- versus 5,7-DHT-lesioned groups, while a twofold increase on neuronal cell bodies (t = 5.35; P = 0.0007) and in the neuropil (t = 4.08; P = 0.0035) was observed in layer V (Figure 5); a significant increase has also been observed in the neuropil of layer VI (t = 2.63; P = 0.03). When the distribution of TH-IR fibers is compared for different neuronal subtypes in layer V, the density is increased by 100% on neuron somata >100 μm^2 in size (P = 0.002) and 60% on those <100 μm^2 in size (P = 0.02) in the mPFCx of the lesioned group (not shown). There were no obvious differences in the size of varicosities, suggesting that an increase in the content of TH per varicosity cannot explain the current findings. In a controlled series of experiments in the frog neuromuscular junction, the size of axon terminals was found to increase in proportion to the number of synaptic vesicles and their content of neurotransmitter-synthesizing enzyme (Benes and Barrnett, 1978).

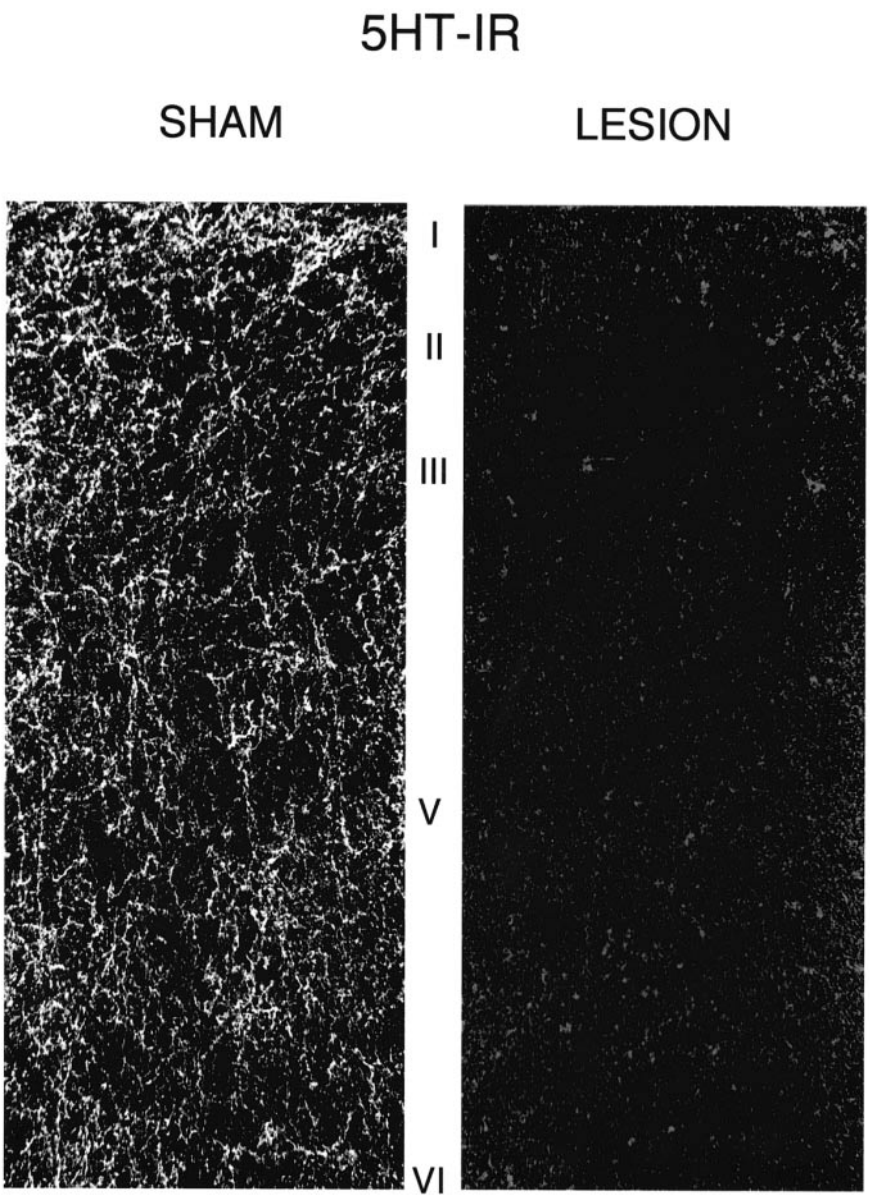


Figure 7. Darkfield photomicrographs of 5-HT-IR fibers in the mPFCx of a sham- (left) and 5,7-DHT-lesioned rats (right). There are abundant fibers present in the specimen from the sham-lesioned rat, while there is a paucity of such fibers in the 5,7-DHT-lesioned animals. Reproduced with permission (Taylor *et al.*, 1998).

Consistent with the above findings, DA fibers are known to interact extensively with dendritic processes throughout the neuropil (Seguela *et al.*, 1988; Goldman-Rakic *et al.*, 1989; Verney *et al.*, 1990; Smiley and Goldman-Rakic, 1993), although the somata of both pyramidal and nonpyramidal neurons probably also serve as non-random targets for these fibers in the rat mPFCx (Verney *et al.*, 1990; Huntley *et al.*, 1992; Benes *et al.*, 1993; Vincent *et al.*, 1993, 1995a; Taylor and Benes, 1996; Davidoff and Benes, 1998; Davidoff *et al.*, 2000). In the primate PFCx, TH-IR varicosities have not been found on neuron somata (Krimer *et al.*, 1997); however, in the PFCx of the human brain, TH-IR varicosities are present on the somata of both pyramidal and nonpyramidal neurons (Todtenkopf and Benes, 1998). As noted above, it appears that the association of dopamine fibers with neuronal cell bodies may vary in degree from one species to another. Based on rodent studies, serotoninergic fibers appear to have a distribution that is similar to that of TH-IR fibers, although the latter also include some noradrenergic elements, particularly in the superficial layers where DA fibers are quite sparse. Serotonergic fibers probably interact with both projection cells and interneurons (Sheldon and Aghajanian, 1991; Morilak *et al.*, 1993; Smiley and Goldman-Rakic, 1996; Taylor and Benes, 1996).

It is noteworthy that thermal ablation of the VTA in neonatal rats has been associated with a 30% decrease of basal dendritic branches of pyramidal neurons (Kalsbeek *et al.*, 1989b). This latter treatment resulted in a depletion not only of dopamine levels, but also those of serotonin (Kalsbeek *et al.*, 1989a). It seems likely that an interruption of fibers originating in the raphe nuclei and traveling *en passage* through the midbrain could have contributed to this change in the cortical serotoninergic projections. If so, it is uncertain as to whether one or both

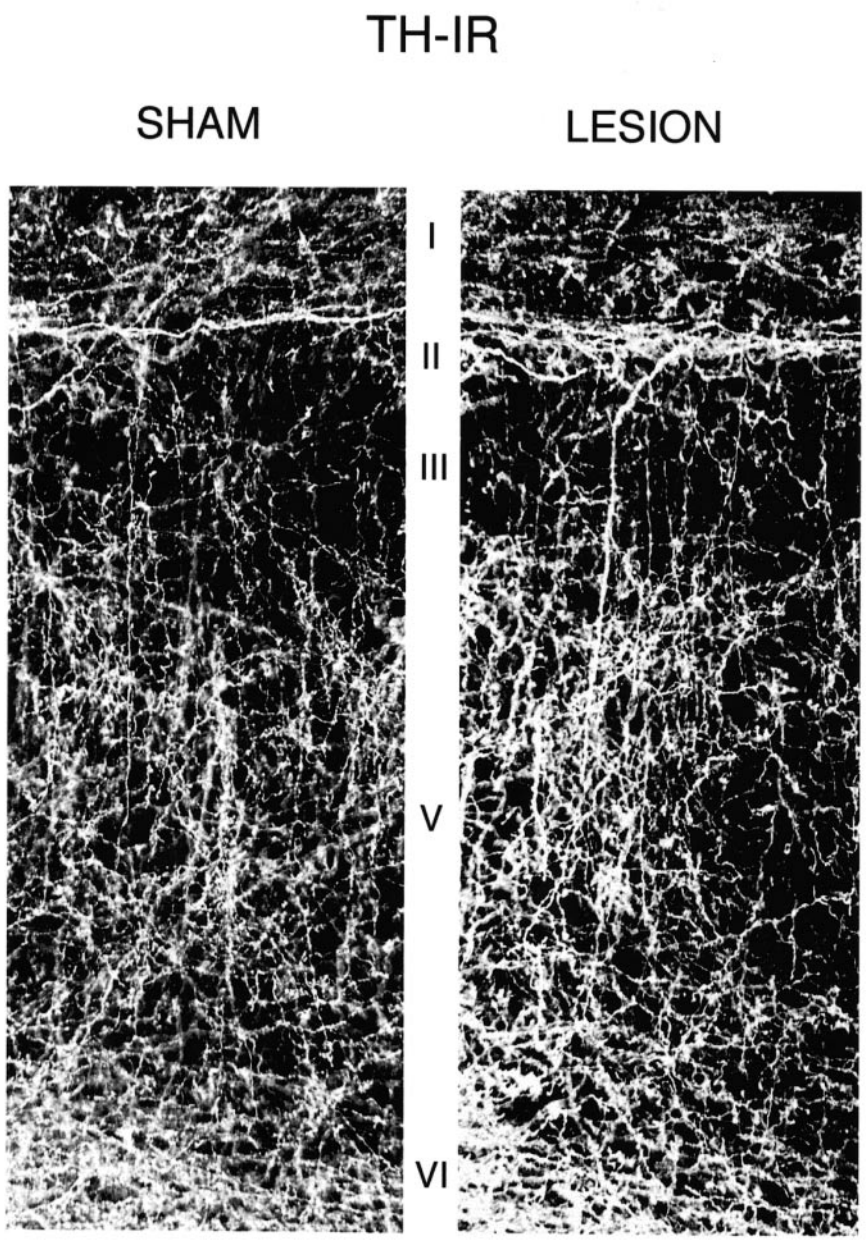


Figure 8. Darkfield photomicrographs of TH-IR varicose fibers in the mPFCx of sham- (left) and 5,7-DHT-lesioned (right) rats. There are abundant fibers present in the cortical mantle with both treatments; however, the mPFCx of a 5,7-DHT-lesioned rat appears to have a higher density of TH-IR fibers in layer V and possibly also layer VI. Reproduced with permission (Taylor *et al.*, 1998).

monoaminergic systems contributed to the observed decrease in pyramidal cell dendrites. It is important to emphasize that in the studies described herein, nomifensine was co-administered with 5,7-DHT to prevent the uptake of this latter toxin into midbrain dopamine cells. This pharmacologic strategy has made it possible to lesion the serotoninergic projections to the mPFCx, whilst preserving the dopaminergic ones originating in the VTA.

Overall, the present findings are not consistent with the idea that 5-HT may act trophically to facilitate the ingrowth of DA fibers during the late post-weanling and early adult periods. Rather, it seems more likely that the opposite is the case, i.e. the 5-HT system seems to be exerting an inhibitory trophic effect on the normal postnatal ingrowth of TH-IR fibers. One interpretation of the findings described above is that the 5-HT and DA systems may be competing with one another for functional territory on the surface of intrinsic cortical neurons within the rat mPFCx. An interaction of this type would tend to produce a reciprocal relationship between the two systems. An alternative possibility, however, is that the 5-HT and DA systems mainly influence one another at the level of their respective brainstem nuclei. Accordingly, lesioning of the NRD may result in a stimulation or release of dopaminergic neurons within the VTA to sprout the distal portion of their fiber projections in various termination sites, such as the mPFCx. Consistent with this idea, an increase of TH-IR staining was observed in the SN, VTA and PAG of 5,7-DHT-lesioned rats. Physiological studies have yielded contradictory results regarding the manner in which the DA and 5-HT systems may be influencing one another. On the one hand,

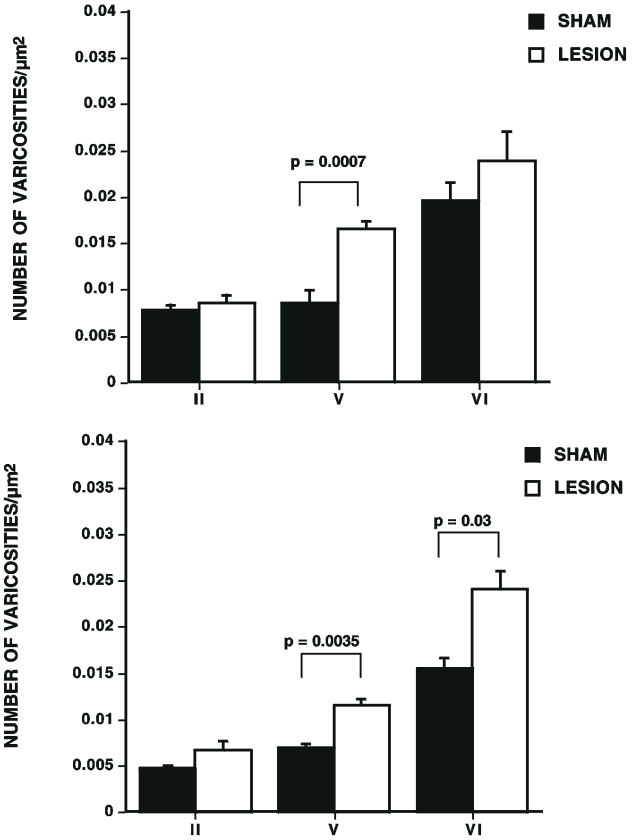


Figure 9. A set of bar graphs showing the density of TH-IR varicosites in apposition with neuronal cell bodies (upper panel) and in the neuropil (lower panel) of sham and 5,7-DHT lesioned rats. The data are expressed as the average number of varicosities per $\mu m^2 \pm$ SEM in the sham versus lesioned groups. Although there are no differences in layer II, both layers V and VI show a marked increase in the density of TH-IR varicosities, particularly in neuropil. Reproduced with permission (Taylor *et al.*, 1998).

some believe that 5-HT can increase the release of DA in the nucleus accumbens (Van Bockstaele *et al.*, 1994; Broderick and Phelix, 1997), corpus striatum (Gudelsky and Nash, 1996; West and Galloway, 1996; Broderick and Phelix, 1997) and prefrontal cortex (Gudelsky and Nash, 1996; Iyer and Bradberry, 1996). Contrariwise, some studies suggest that 5-HT may actually decrease the release of DA, since exposure to selective 5-HT receptor antagonists has been associated with an increase of extracellular DA concentrations (Pehek, 1996; Howell *et al.*, 1997). The latter pattern is consistent with the idea that there may be a competitive interactive between these two monoaminergic systems. This idea is a particularly appealing one because the VTA receives a direct input from serotoninergic fibers (Van Bockstaele *et al.*, 1994).

Conclusions

The studies described above provide evidence in support of the idea that the dopamine and serotonin systems show a significant degree of convergence and plasticity in the rat mPFCx, and the degree to which this occurs is probably similar for both pyramidal cells and GABAergic interneurons. Particularly noteworthy is the fact that the dopamine system may be capable of considerable plasticity, at least until the start of the early adult period. If the dopamine system in the human brain exhibits similar characteristics, the maturation of the limbic cortex during adolescence and early adulthood may potentially provide 'a window of opportunity' for the induction of abnormal interactions of the monoaminergic systems with one another and with their intrinsic cortical targets. Indeed, some experimental evidence suggests that exposure to adrenal steroids during the postnatal period can result in an increase of dopamine-IR varicosities on interneurons in the mPFCx of rats also exposed to these hormones prenatally (Benes, 1997). Based on the studies discussed above, an important question to ask is whether pre- and/or postnatal stress might also result in an altered distribution of serotoninergic projections to the rat mPFCx [for a review see Stanford (Stanford, 1993)], one that is reciprocal in nature to that observed for the dopamine system. Since a combination of pre- and postnatal stress is believed to play a central role in the pathophysiology of some neuropsychiatric disorders (Benes, 1997; Walker and DiFiorio, 1997), it is plausible that changes in the way these two monoaminergic systems interact with one another might ultimately influence the activity of the individual cortical neurons upon which they both converge. Future studies will be directed at identifying further what effect dopaminergic fibers in the mPFCx might have on convergent serotoninergic inputs and their shared target neurons, and how this interaction may be influenced by psychotropic drugs.

Notes

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Address correspondence to: Francine M. Benes, MD, PhD, McLean Hospital, 115 Mill Street, Belmont, MA 02478, USA. Email: benesf@mclean.harvard.edu.

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