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POSTNATAL CHANGES IN THE DISTRIBUTION AND MORPHOLOGY OF RAT SUBSTANTIA NIGRA DOPAMINERGIC NEURONS

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Abstract—Significant changes in the neurophysiology and neuropharmacology of nigral dopaminergic neurons take place in the first postnatal month. In order to correlate these changes with the postnatal development of dopaminergic neuron morphology and substantia nigra cytoarchitecture, brains from Sprague–Dawley rat pups of age postnatal days 1, 7, 14, 21 and 28 and adult rats were sectioned and processed for tyrosine hydroxylase immunocytochemistry.

At postnatal day 1, pars compacta and pars reticulata were not clearly delineated; tyrosine hydroxylase positive neurons and a dense plexus of fibers were scattered throughout the substantia nigra. By day 7 the density of tyrosine hydroxylase positive neurons decreased markedly in ventral substantia nigra, and a dopaminergic pars compacta and a non-dopaminergic pars reticulata could be more clearly distinguished. By day 14 the substantia nigra appeared essentially as it does in the adult. Cell counts during development revealed that the number of tyrosine hydroxylase positive neurons/section in both pars compacta and pars reticulata decreased significantly from postnatal day 1 to postnatal day 14, while those in pars lateralis did not change.

Tyrosine hydroxylase-positive somatic size increased modestly but significantly from postnatal day 1 to day 14 as did the diameter of the proximal and distal dendrites. However, even at day 1, the morphology of tyrosine hydroxylase positive neurons appeared essentially the same as in adults. Dendritic arborizations were well developed. The dendrites were non-varicose and modestly branched, with some of the longer ventrally directed dendrites passing through pars reticulata into the crus cerebri.

The most significant postnatal changes in the rat substantia nigra take place in its cytoarchitectural organization, whereas only modest changes occur in the morphology of individual dopaminergic neurons. The distribution of dopaminergic neurons first resembled that in adults by around 14 days of age, the same time at which these neurons begin to display physiological and pharmacological responses similar to those seen in adults.

Dopaminergic neurons are among the earliest in the brain to differentiate, express their transmitter phenotype and send axons to innervate their target regions.[15,21,22,33] Combined thymidine pulse labeling and immunocytochemical studies indicate that dopaminergic nigrostriatal neurons are the earliest neurons born in substantia nigra, between embryonic day 12 and 13 in the rat.[25] Many of these neurons exhibit tyrosine hydroxylase immunoreactivity while still migrating to their final locations within the midbrain.[20]

Recent studies of the *in vivo* postnatal development of the electrophysiological properties of dopaminergic neurons in the rat substantia nigra have revealed significant differences in various electrophysiological parameters including spontaneous firing rate and pattern, conduction velocity and response to stimulation of neostriatum compared to those obtained in adult rats.[18,28–30] During the course of our original nigral neonatal recordings, particularly during the first postnatal week, it was noticed that antidromically identified nigrostriatal dopaminergic neurons were encountered over a dorsoventral extent that appeared to be much less restricted than is the case in adult animals in which the vast majority of nigral dopaminergic neurons are located in a relatively narrow band in the pars compacta. Based on Pontamine Sky Blue marking of the recording sites and Neutral Red counterstaining, many of these cells were located within what would be considered pars reticulata in adult animals, yet tyrosine hydroxylase immunostaining revealed a dense plexus of tyrosine hydroxylase-immunoreactive (TH^+) neurons and dendrites throughout substantia nigra.[28] This suggested that the substantia nigra and in particular, nigrostriatal neurons, may exhibit a cytoarchitectonic organization in the early postnatal period that differs from that seen in the adult.

Furthermore, the period around postnatal day 14 (P14) seems to represent an important milestone for the shift from immature electrophysiological properties of nigrostriatal neurons to those more closely

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Abbreviations: P, postnatal day; TH^+, tyrosine hydroxylase immunoreactive; PBS, phosphate-buffered saline.

resembling the physiological properties seen in adults.[28,29] This time-period also represents a critical juncture for the development of the response of dopaminergic neurons to amphetamine; during the first postnatal week a significant proportion of electrophysiologically identified nigrostriatal dopaminergic neurons respond to systemic administration of amphetamine with paradoxical increase in spontaneous activity,[31] rather than the decrease that is seen in adult animals.[1] By the end of the second week, however, the response of nigrostriatal neurons to amphetamine is essentially the same as in adult animals.

For these reasons, it was of interest to examine the postnatal development of the substantia nigra, particularly the pars compacta, in an attempt to relate the observed postnatal changes in neurophysiology and neuropharmacology with morphological development. Portions of these data have been previously reported in abstract form.[30]

EXPERIMENTAL PROCEDURES

Subjects

Subjects consisted of 15 Sprague–Dawley rat pups of both sexes derived from pregnant dams bred at The Institute of Animal Behavior at Rutgers University. Pregnant females were checked daily for the presence of new litters, and the day of birth was considered to be P1. For purposes of comparison with nigrostriatal dopaminergic neurons from adult rats, additional data were collected from three male Sprague–Dawley rats, over 75 days of age (body weight > 240 g).

Tyrosine hydroxylase immunocytochemistry

Rat pups on P1, P7, P14, P21, P28 and adult rats were deeply anesthetized with urethane and perfused intracardially with a saline rinse followed by 4% paraformaldehyde and 0.2% glutaraldehyde in 0.15 M sodium phosphate buffer, pH 7.4. Brains were postfixed for 24–72 h and 50-μm frozen sections were collected. Sections were rinsed repeatedly in 0.15 M sodium phosphate buffered saline (PBS), incubated in 0.1 M glycine for 5 min, rinsed in PBS followed by 0.15% H_2O_2, and incubated overnight in PBS containing 0.3% Triton X-100 and 3.3% normal goat serum. Sections were incubated for 24–48 h in a well-characterized commercially available antibody to tyrosine hydroxylase (Eugene Tech., 1:3000 dilution) containing 2% bovine serum albumin and 10% fetal calf serum and processed for immunocytochemical visualization of tyrosine hydroxylase by the avidin-biotinylated peroxidase method (Vector ABC kit) using 3, 3′-diaminobenzidine as a chromogen. Sections were mounted, dehydrated in an ascending series of ethanol, cleared, and cover slipped. The specificity of the immunoreactivity was checked in several ways including omitting the primary antibody or normal serum from the protocol. Omission of the primary antibody eliminated all immunostaining.

Measurements

Each age group was comprised of three rat pups from at least two litters. All measurements were taken from five consecutive 50-μm coronal sections through the rostral portion of the substantia nigra surrounding the medial terminal nucleus of the accessory optic tract in order to control for regional differences in cytoarchitecture.[6] Somatic and dendritic sizes were measured on a Nikon Optiphot microscope using a 60× oil immersion objective with the aid of a Quantex QX-7 image analysis system. Somatic sizes were measured only from neurons in which the nucleus was visible. Measurements of somatic size consisted of the length of the longest axis and the length of the perpendicular to that axis so that neuron sizes could be compared directly with measurements taken from electrophysiologically identified intracellularly labeled nigrostriatal dopaminergic neurons in adults from a previous study.[26] Proximal dendritic diameter measurements were made at 25 μm from the center of the soma, and distal dendritic diameter measurements were made from randomly selected dendrites within pars reticulata at least 400 μm from the soma. Because of the limits of resolution of the light microscope, all measurements were rounded to the nearest 0.25 μm before statistical analyses. No corrections were made for shrinkage.

To ascertain postnatal changes in their distribution, TH^+ neurons were parsed into three regions: pars compacta, pars reticulata and pars lateralis, and the numbers of TH^+ neurons per area per section were counted. Measurements and cell counts were analysed with a one-way analysis of variance, and individual groups were compared to their appropriate adult control group with Schéffé's F-test with the alpha level set to 0.1.[13]

RESULTS

Tyrosine hydroxylase-positive neuron distribution

At P1, it was difficult to delineate clearly a dopamine neuron-rich pars compacta from a dopamine neuron-poor pars reticulata, and TH^+ neurons and dendrites were found scattered throughout the ventral tegmentum as shown in Fig. 1. By P7 the density of TH^+ neurons appeared to increase dorsally and decrease ventrally, and the pars compacta could be more easily distinguished from the pars reticulata. By P14 a dense, cellular pars compacta could be easily distinguished from the TH^+ cell-poor pars reticulata, and the organization of the substantia nigra did not appear markedly different from that in older neonates or adults.

In order to quantify these cytoarchitectural changes, the number of TH^+ neurons in a representative region of substantia nigra were counted. For three animals of each age listed, five consecutive 50-μm coronal sections centered around the medial terminal nucleus of the accessory optic tract were examined, and each TH^+ neuron was parsed into pars compacta, pars reticulata or pars lateralis. There was an overall age-dependent decrease in the number of TH^+ neurons in pars compacta ($F = 5.10$, d.f. = 5,89, $P < 0.01$), pars reticulata ($F = 24.41$, d.f. = 5,89, $P < 0.01$) and in the overall number of TH^+ neurons in substantia nigra ($F = 15.01$, d.f. = 5,89, $P < 0.01$), but not in pars lateralis. Most of this variance was accounted for by the large decrease in the number of TH^+ neurons in pars reticulata from P1 to P7. These data are shown in Fig. 2.

When all TH^+ neurons located in pars reticulata, pars compacta or pars lateralis were counted in the five sections from each brain and divided by the total number of TH^+ neurons from the five sections of that brain, it became apparent that the relative proportion

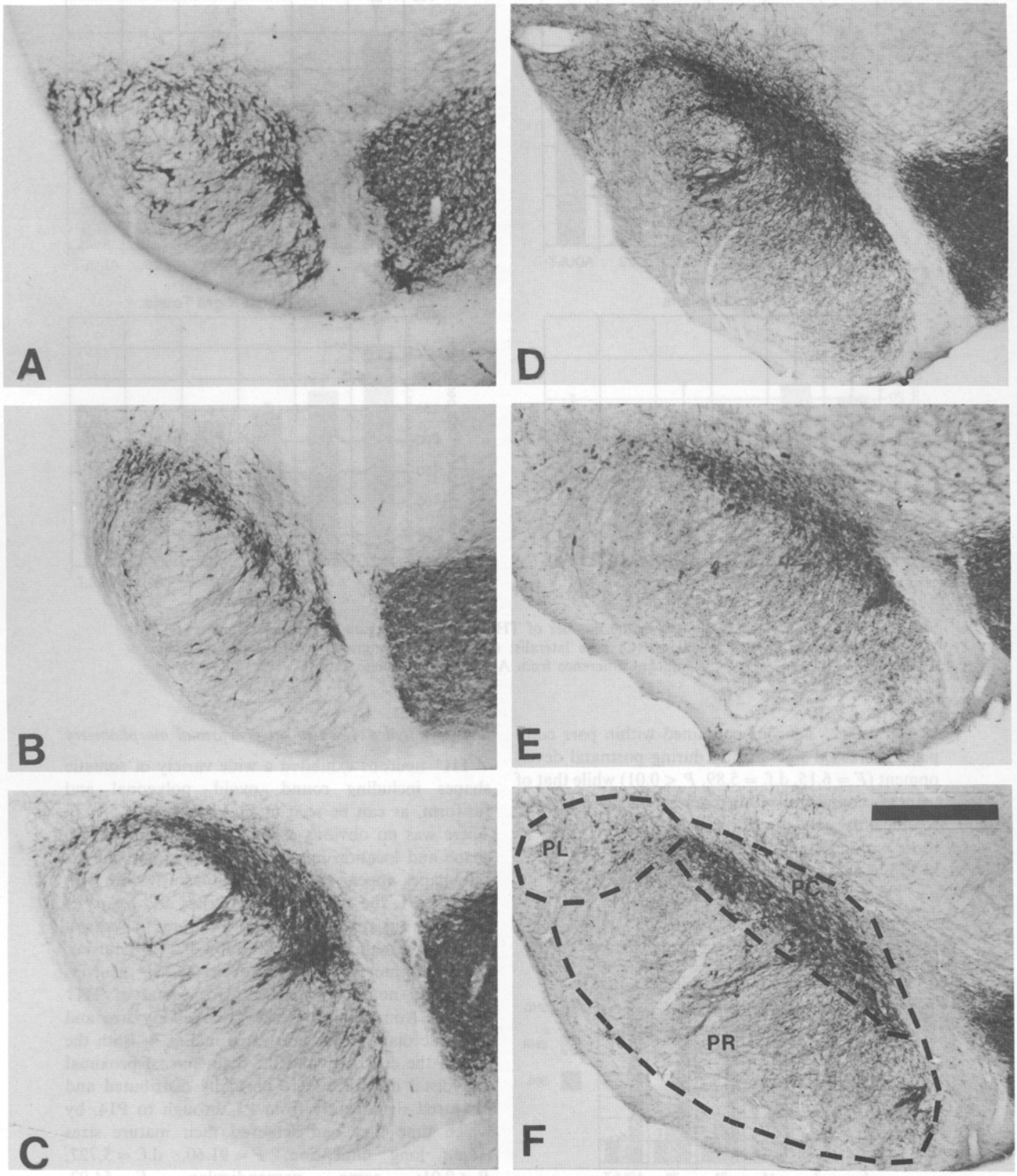

Fig. 1. Representative 50-μm coronal sections through substantia nigra at the level of the medial terminal nucleus of the accessory optic tract processed for tyrosine hydroxylase immunocytochemistry. The substantia nigra is parsed into pars compacta (PC), pars reticulata (PR) and pars lateralis (PL) by the dashed lines in F. These parcellations were used for the analysis of cell distribution reported in Figs 2 and 3. (A) P1; (B) P7; (C) P14; (D) P21; (E) P28; (F) adult. Scale bar = 500 μm.

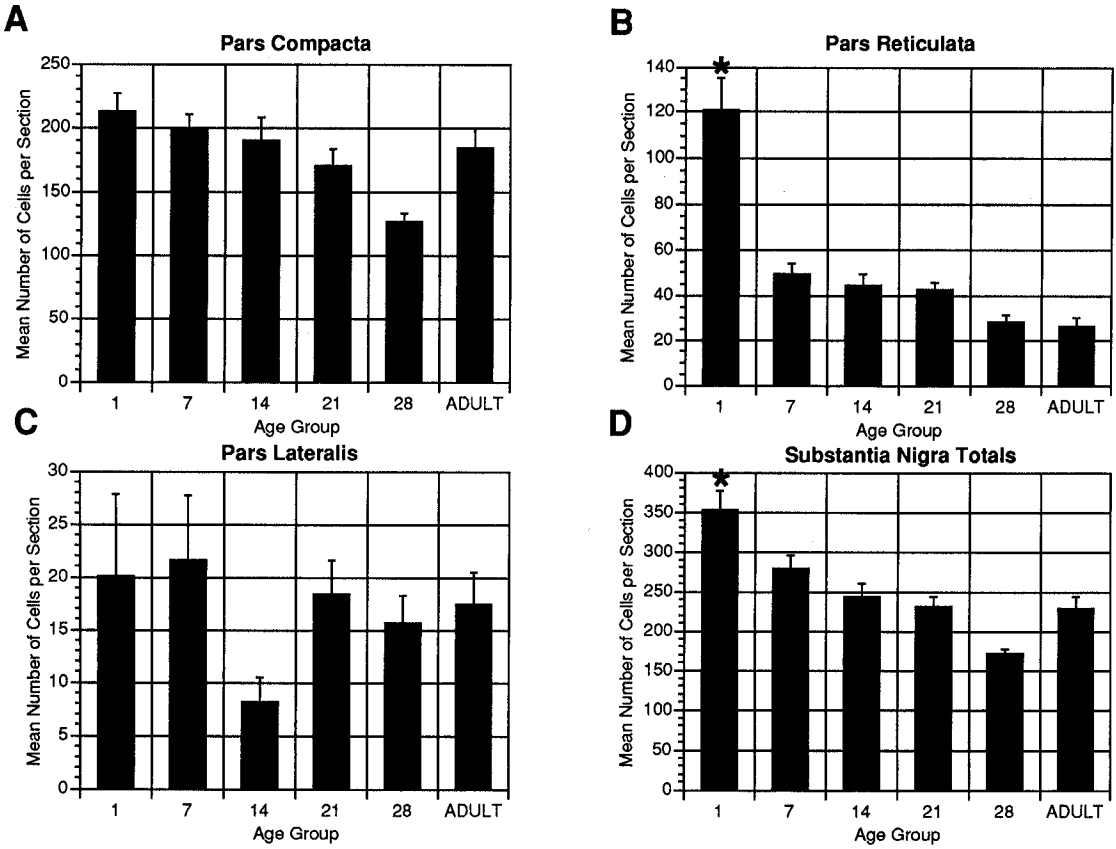

Fig. 2. (A) Postnatal changes in the number of TH$^+$ neurons by region in substantia nigra. (A) Pars compacta; (B) pars reticulata; (C) pars lateralis; (D) substantia nigra overall. Asterisk indicates a significant difference from Adult group (Schéffé F-test).

of nigral TH$^+$ neurons contained within pars compacta increased significantly during postnatal development ($F = 6.15$, d.f. = 5,89, $P < 0.01$) while that of neurons contained within pars reticulata decreased significantly ($F = 13.71$, $df = 5,89$, $P < 0.01$) and those within pars lateralis did not change, as shown in Fig. 3.

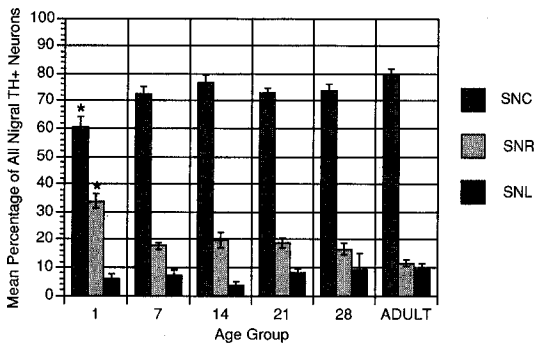

Fig. 3. Postnatal changes in the relative distribution of TH$^+$ neurons in substantia nigra. Proportions were calculated by counting the number of TH$^+$ neurons within pars compacta, pars reticulata or pars lateralis within each brain and dividing by the total number of substantia nigra TH$^+$ neurons counted in that brain.

Tyrosine hydroxylase-positive neuronal morphometry

TH$^+$ neurons exhibited a wide variety of somatic shapes including round, ovoid, polygonal and fusiform, as can be seen in Figs 4A, B and 5A, B. There was no obvious association between somatic shape and location in substantia nigra, nor did the cell shapes appear to change significantly with age. Even at P1, the cell bodies, dendrites and axons of TH$^+$ neurons appeared relatively mature. In contrast to the cytoarchitectural distribution of TH$^+$ neurons, the overall morphology of most nigral TH$^+$ neurons at P1 did not differ markedly from that of TH$^+$ neurons from adults, except in cell body size and dendritic diameter, as illustrated in Fig. 4. Both the size of the cell body and the diameters of proximal and distal dendrites were normally distributed and increased significantly from P1 through to P14, by which time they had achieved their mature sizes (soma long dimension, $F = 91.60$, d.f. = 5,727, $P < 0.01$; soma perpendicular, $F = 54.02$, d.f. = 5,727, $P < 0.01$; proximal dendrites, $F = 17.71$, d.f. = 5,727, $P < 0.01$; distal dendrites $F = 27.60$, d.f. = 5,1396, $P < 0.01$).

Even at P1, the dendritic arborization was well developed, and the dendrites appeared mature in most respects. Most of the TH$^+$ dendrites in neonates

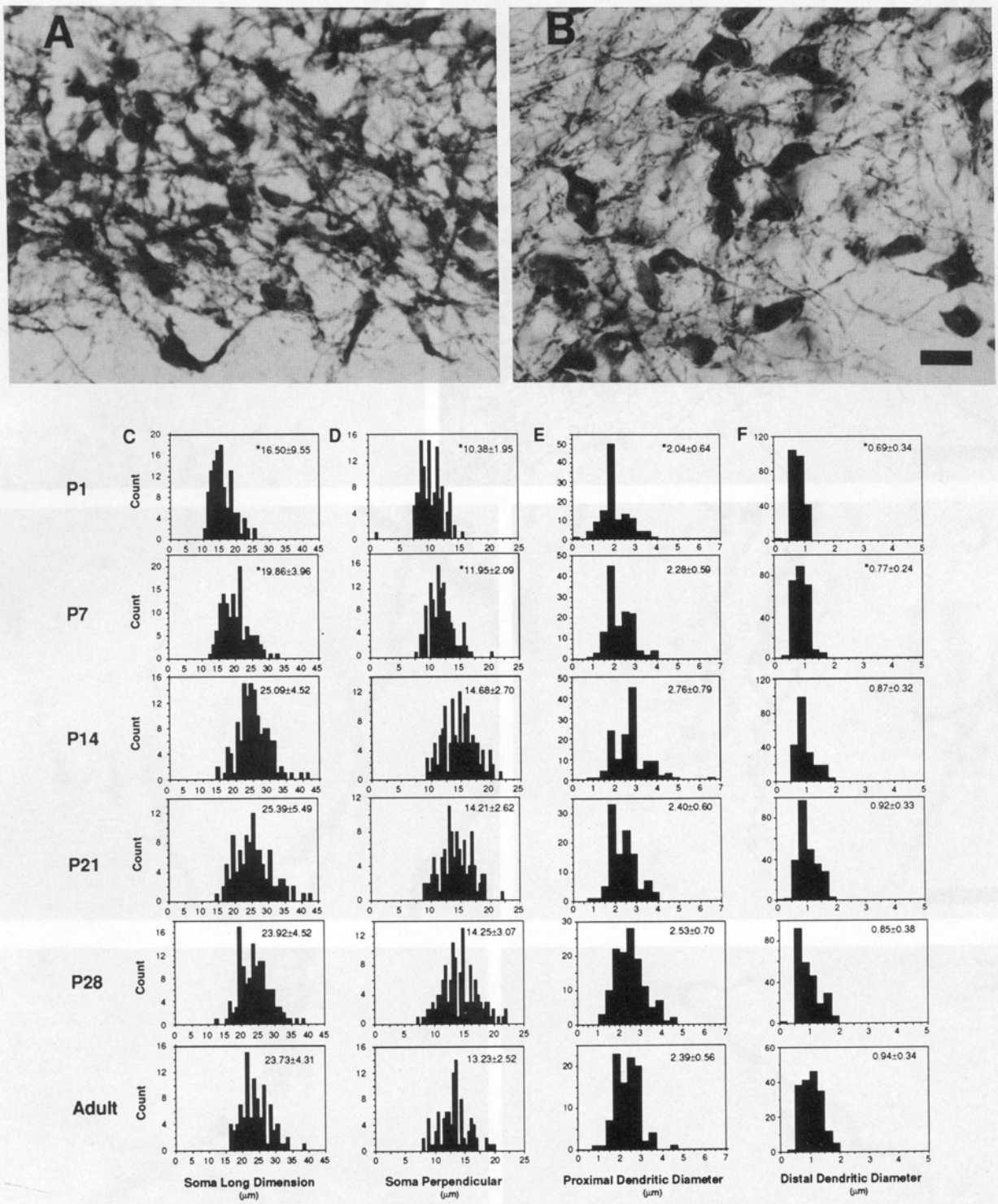

Fig. 4. Postnatal changes in somatic size and proximal and distal dendritic diameter of substantia nigra TH^+ neurons. (A, B). TH^+ neurons from similar regions of substantia nigra in a P1 rat (A) and an adult rat (B). Scale bar = 25 μm. (C–F) Postnatal changes in somatic and dendritic measurements of TH^+ substantia nigra neurons. Numbers at the top right of each histogram represent mean $\pm$ standard deviation. Asterisk indicates a significant difference from corresponding Adult group (Schéffé F-test).

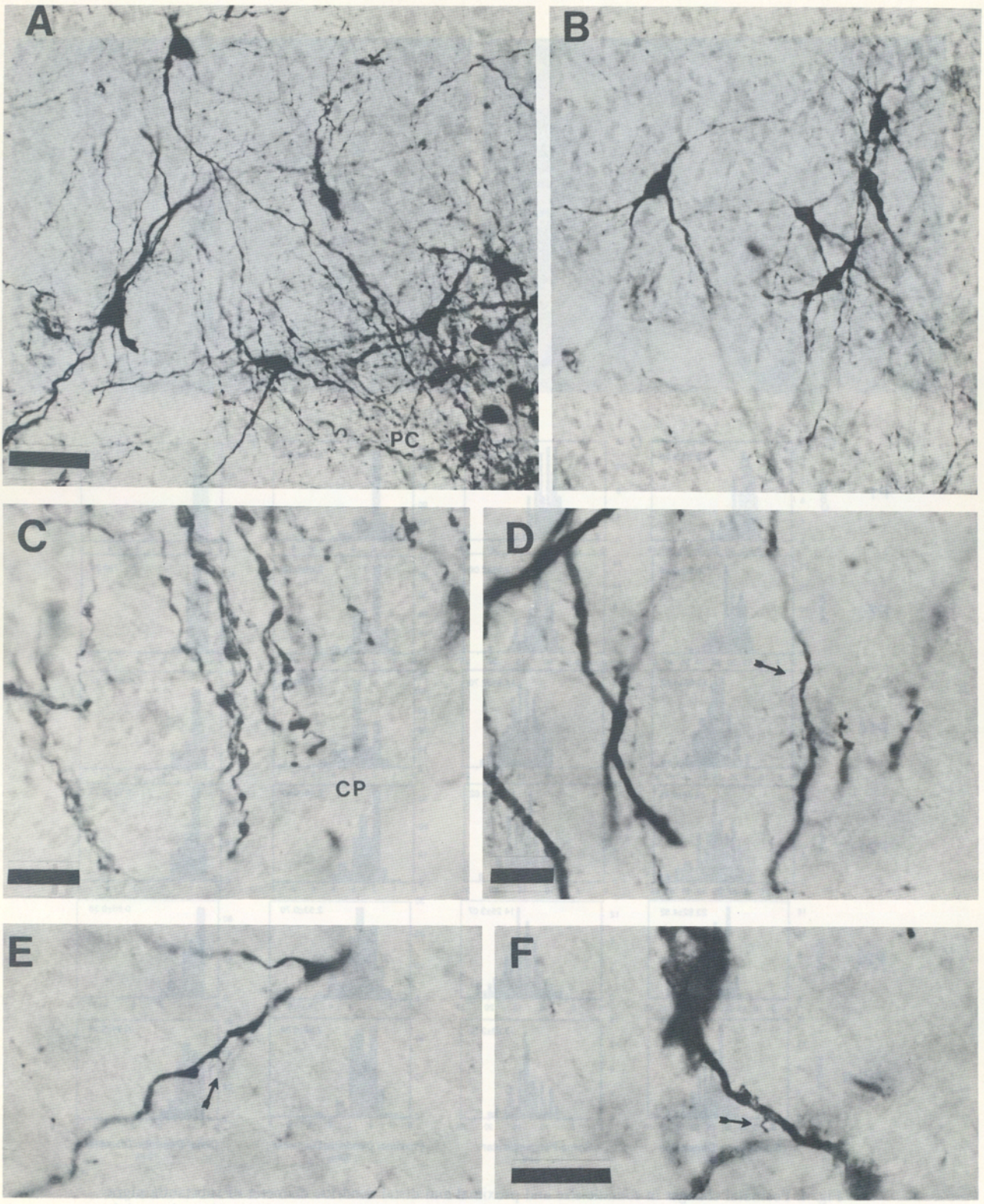

Fig. 5. Somatic and dendritic morphology of TH^+ neurons. (A, B) TH^+ neurons just dorsal and lateral (for the sake of clarity) to pars compacta (PC) from two different PD1 rats exhibit a variety of somatic shapes including fusiform, ovoid and polygonal. Note the long, well-developed non-varicose dendrites and mature branching patterns. The fine, beaded processes are axons. (C) Distal TH^+ dendrites in ventral pars reticulata are grouped into small fascicles and terminate in or near the cerebral peduncle (CP) in a P1 rat. (D–F) TH^+ dendrites from three different P1 rats exhibit sparse filiform processes (arrow in D) and spine-like appendages (arrows in E and F). Scale bar = 50 μm (A, applies also to B); 10 μm (C, D, F); Scale bar in D applies also to E.

were non-varicose, and showed branching patterns that were not markedly different from those in adults, as illustrated in Fig. 5A, B. At P1, as in adults, many nigral TH+ neurons possess one or two extremely long ventrally directed dendrites that are several hundreds of microns in length, some of which extend throughout the pars reticulata to reach the crus cerebri, as shown in Fig. 5C.

As in adults, the dendrites of neonatal dopaminergic neurons were sparsely invested with long filiform processes and dendritic appendages, but lacked true dendritic spines.[8,11,26] Examples of these are shown in Fig. 5D–F. Although a quantitative analysis of these dendritic specializations was not performed, they appeared to be more common in neurons from P1 animals than in older neonates or adults. A similar developmental trend was reported in a Golgi study of kitten substantia nigra,[16] but feline substantia neurons appear to be more heavily invested with these dendritic appendages than those in rat.

The dendrites of dopaminergic neurons have long been known to band together in fascicles that extend ventrally in the pars reticulata of the adult rat,[3] and may be important loci for dendrodendritic neurochemical and electrotonic interactions.[7,9] Such fascicles exist throughout the postnatal developmental period, exhibiting little apparent change in structure at the level of the light microscope, although the frequency of the fascicles appears to decrease in parallel with that of the density of TH^+ neurons and dendrites during development (c.f. Fig. 1). The majority of the dendrites within the fascicles in neonates, like most TH^+ dendrites in the adult substantia nigra, were non-varicose.

DISCUSSION

Postnatal changes in distribution of tyrosine hydroxylase-positive neurons

Although it has sometimes been claimed that the cytoarchitectural organization of the rat substantia nigra matures quite early in development,[20,22,23] it is clear that the substantia nigra undergoes substantial postnatal development in the rat. At the day of birth, TH^+ neurons are distributed throughout a large expanse of the ventral tegmentum; segregation of TH^+ neurons into a clearly defined cell-dense pars compacta and a cell-sparse pars reticulata does not occur until seven to 14 days after birth. This is largely attributable to a significant decrease in the number of TH^+ neurons located within the pars reticulata. Thus the formation of the substantia nigra into a dopamine cell-rich pars compacta and a dopamine cell-poor pars reticulata is a relatively late event in ontogenetic terms, and most likely does not result from a differential prenatal migration of cells as has been previously suggested.[12]

A number of dopaminergic neurons can normally be found within substantia nigra pars reticulata in adult animals, particularly within the caudal regions of the nucleus,[6] and some of these neurons have been shown to project to the neostriatum.[2] In the present study, both the absolute numbers of TH^+ neurons as well as the proportional distribution within pars reticulata is far greater during the first postnatal week, even when measured from the rostral portion of the substantia nigra surrounding the medial terminal nucleus of the accessory optic tract. At P1, fully one-third of all the TH^+ neurons in substantia nigra are found within pars reticulata, a proportion that shrinks to just over 10% in the adult. Thus, it would appear that the reason that such an unusually large number of putative dopaminergic neurons, all antidromically activated from neostriatum at latencies consistent with those of dopaminergic neurons, was recorded within pars reticulata of neonatal rats in an earlier study[28] was because there are more of these neurons present in neonates than in adults.

Whether these neurons die off during postnatal development or simply continue to migrate into the pars compacta cannot be determined from the present results. However, a reduction in the B_{max} for tegmental spiperone binding[14] as well as for $GABA_B$ receptors[5] from neonates to adults may be evidence for the former hypothesis, and a recent study revealed a large number of unidentified degenerating neurons in rat substantia nigra pars compacta during the first two postnatal weeks.[10] Furthermore, D_2 mRNA levels, which increase from birth, fall markedly in substantia nigra after P14 in contrast to their continued rise in neostriatum at least until P28[24,37] which also argues for a developmentally dependent death of midbrain dopaminergic neurons. Finally, since in our study the number of TH^+ neurons per section decreased significantly in pars compacta as well as within pars reticula, but did not change in pars lateralis during development, some type of developmental artifact, for instance, an overall decrease in the packing density of neurons as they grow larger does not seem likely as the cause of the reduction in cell counts, and cell death of the TH^+ nigrostriatal neurons during early postnatal development remains a likely possibility.

Postnatal changes in morphology of tyrosine hydroxylase-positive neurons

The basic cytological appearance of nigral dopaminergic neurons in rat does not appear to change appreciably after birth, as reported in an earlier study[20] although there are modest, but statistically significant increases in the sizes of TH^+ cell bodies and proximal and distal dendrites during postnatal development, as has been previously reported for the feline substantia nigra.[16,17] By P14, the mean soma size ($25.1 \pm 4.5 \times 14.7 \pm 2.7\ \mu m$) did not differ from the adults in this study ($23.7 \pm 4.3 \times 13.2 \pm 2.5\ \mu m$) or from measurements obtained from electrophysiologically identified rat nigrostriatal dopaminergic neurons intracellularly labeled with horseradish peroxidase in a previous study ($24.8 \pm$

$1.7 \times 12.7 \pm 3.4\ \mu m$).[26] The postnatal development of the feline substantia nigra is apparently slightly different from the rat since in the early postnatal feline nigral neurons possess markedly varicose dendrites in Golgi and intracellularly labeled material, as well as a large number of long, filiform spine-like appendages, and continue to mature morphologically up through the eighth postnatal week.[17,34] However, in rat, even by P1, the dendritic arborization of these neurons is well developed, and the dendrites do not appear markedly different from TH^+ dendrites from adult animals or those of electrophysiologically identified adult nigrostriatal neurons intracellularly labeled with horseradish peroxidase or Lucifer yellow.[8,26] Most dopaminergic dendrites from early neonates, like those from adults, are non-varicose,[11,26] and can extend for several hundred micrometers throughout the ventral tegmentum. These findings are consistent with previous electrophysiological studies which showed that even by P1, rat substantia nigra dopaminergic neurons exhibit spontaneous activity, as well as neostriatal evoked antidromic and orthodromic responses similar to those seen in adults.[28,29]

The apparent cytological maturity of nigral dopaminergic neurons during the early postnatal period is in contrast to what is seen in the rat neostriatum during this time. Neostriatal medium spiny neurons intracellularly labeled with biocytin exhibit a marked morphological and electrophysiological immaturity at birth. The normally spiny dendrites are markedly varicose and almost completely aspiny during the first 10–12 postnatal days.[27,32] Their electrophysiological properties are also markedly different from those of adults until after late in the fourth postnatal week, by which time they have achieved the morphology of adult medium spiny neurons. Thus, both from a morphological and electrophysiological perspective, the midbrain component of the nigrostriatal system appears to mature significantly in advance of the neostriatal component.

Correlation between morphology and physiology

The time-course of the postnatal maturation of substantia nigra cytoarchitecture closely parallels that of the development of the neurophysiological and neuropharmacological properties of nigrostriatal neurons. In particular, the development of the typical inhibitory responses of nigral dopaminergic neurons to systemically administered amphetamine does not appear until after P14, by which time nigral TH^+ neurons have achieved their adult distribution. Prior to this, many dopaminergic neurons exhibit an anomalous excitatory response to amphetamine[31] that may have relevance to the mechanism of action of stimulants in children with attention deficit disorder with hyperactivity. Although the mechanism(s) underlying the paradoxical response to amphetamine in early neonates is unclear, an absence of autoreceptors, or decreased autoreceptor function have been ruled out since these neurons exhibit equivalent inhibitory responses to apomorphine at all ages.[31]

It seems more likely that the anomalous response is a function of the immature state of the nigrostriatal/striatonigral system as a whole. One possible mechanism involves increased dendritic dopamine release in pars reticulata. Dopamine has been shown to suppress the inhibitory effects of endogenous and exogenous GABA stimulation on pars reticulata GABAergic output neurons.[35,36] The overabundance of dopaminergic neurons early in the postnatal period could alter basal ganglia output both tonically and under the influence of amphetamine by reducing striatal and/or pallidal GABAergic inhibition of pars reticulata projection neurons. Another possibility is that there is a perinatal reorganization of striato- and/or pallidonigral afferents[4] concomitant with the loss of the extraneous dopaminergic neurons such that descending inhibitory effects following amphetamine administration[19] become more potent on the remaining neurons.

CONCLUSION

Some of the postnatal changes in the neuropharmacological properties of midbrain dopaminergic neurons may be related to the distribution and density of the neurons, and /or their afferents, rather than solely to properties of individual neurons. Since the normal postnatal developmental sequence of the substantia nigra involves a reduction in the number of dopaminergic neurons, particularly within pars reticulata, these data suggest the possibility that attention deficit disorder with hyperactivity and/or the paradoxical effects of amphetamine in children with this disorder could be related to an anomalous distribution and/or number of nigral dopaminergic neurons.

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