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Development of the Dopaminergic Innervation in the Prefrontal Cortex of the Rat

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ABSTRACT

The pre- and postnatal development of the dopaminergic innervation in the prefrontal cortex (PFC) of the rat is described from embryonic day 14 through postnatal day 90. By embryonic day 15 the dopamine (DA)-containing fibers reach the anlage of the lateral neocortex; 2 days later the first fibers have reached the subplate of the future prefrontal cortex. The process of entering the cortical plate starts just before birth. Prenatally, some dopaminergic fibers can be observed in the marginal zone of both the lateral and the medial wall of the hemisphere. Within 48 hours after birth a large number of dopaminergic fibers can be observed in the marginal zone, i.e., the future layer I, in some subareas of the PFC. A transient appearance of DA-positive fibers is noticed in the late embryonic and early postnatal periods especially in the marginal zone and possibly in the superficial layers of the pregenual cingulate cortex. Changes in the morphology of DA fibers at P4 suggest that the actual DA innervation starts at this age. From postnatal day 6 the different subareas of the PFC can be recognized according to the characteristics of the topographical distribution of the dopaminergic fibers. Until postnatal day 60 the density of the dopaminergic fibers continues to increase. No difference in density and topography was observed between postnatal days 60 and 90.

Key words: cortical development, dopamine, mesocortical projection, prenatal development, postnatal development, transient DA positive fibers, growth cone

The prefrontal cortex (PFC) has a proposed role in higher associative functions, as can be deduced from its abundant interconnections with limbic structures (Van Eden, '85), and it has been implicated in a wide range of cognitive and emotional behaviors (Fuster, '80; De Bruin, '81; Kolb, '84). A characteristic feature of the PFC is that it receives projections from the mediodorsal nucleus of the thalamus (MDT) as well as a dense dopaminergic input originating in the ventral tegmental area (VTA) (Björklund et al., '78; Divac et al., '78; Beckstead, '79; Porrino and Goldman-Rakic, '82; Benjamin and Golden, '85). In the rat the PFC is virtually the only neocortical area which receives such a dense dopaminergic projection (Lindvall et al., '78; Björklund and Lindvall, '84).

Both dopaminergic and thalamocortical terminals are present early in the development of the PFC. As was recently shown in the rat, a considerable number of MDT fibers have already penetrated the future lower cortical layers and the cortical plate of the developing PFC by postnatal day 1 (Van Eden, '86). The first dopaminergic fibers appear to enter the future PFC on embryonic day 16 (E16), while at E21 a few presumed dopaminergic fibers are

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Abbreviations used: ACd, dorsal anterior cingular cortex; ACv, ventral anterior cingular cortex; AHC, anterior hippocampal continuation; AId, dorsal agranular insular cortex; AIv, ventral agranular insular cortex; CA, catecholamine; CP, cortical plate; CPu, upper cortical plate; DA, dopamine; E, embryonic day; 5-HT, serotonin; IG, indiseum griseum; IL, infralimbic cortex; IZ, intermediate zone; MDT, mediodorsal thalamic nucleus; MZ, marginal zone; NA, noradrenaline; PFC, prefrontal cortex; PL, prelimbic cortex; P, postnatal day; PrCm, medial precentral cortex; rs, rhinal sulcus; SP, subplate layer; STR, (developing) striatum; v, lateral ventricle; VTA, ventral tegmental area; VZ, ventricular zone; w, white matter.

seen in the marginal zone of medial and cingular cortex (Verney et al., '82). Through exogenous administration of α-methyl-noradrenaline, Schmidt et al. ('82) were able to demonstrate a remarkably well-established dopaminergic input to the frontal pole in newborn rats. Berger et al. ('85b) showed developmental differences in the postnatal dopaminergic innervation of the rat anterior cingulate cortex using antityrosine immunocytochemistry and catecholamine fluorescence histochemistry. There are indications that these early dopaminergic and thalamocortical fibers influence cortical development (Van Eden and Uylings, '86; Kalsbeek et al., '87a). Within each part of the PFC several subareas can be distinguished on the basis of cytoarchitecture from as early as postnatal day 6 (Van Eden and Uylings, '85a). A detailed study of adult rats showed clear differences in the pattern of dopaminergic fibers between the PFC subareas (Van Eden et al., '87). Little or no attention, however, has been paid to the development of the dopaminergic innervation in the various prefrontal subareas.

Difficulties previously encountered in processing immature brains (Loren et al., '76; Schmidt et al., '82) have now been overcome through the use of antibodies to glutaraldehyde-conjugated dopamine (Geffard et al., '84). The application of this antiserum has made it possible to combine an optimal fixation with the specific demonstration of endogenous dopamine (DA) without having to perform various pharmacological manipulations. The present study describes in detail the developmental changes of DA nerve fibers and terminals in the prefrontal cortical subareas and compares the pattern of the DA fibers with the cytoarchitecture in adjacent Nissl- and cresyl-violet-counterstained sections. This might give an indication of when and where DA exerts its possible trophic role (Schlumpf et al., '80a; Molliver, '82) on the development of the PFC.

MATERIALS AND METHODS

In this study 50 Wistar rats were analyzed for the postnatal developmental series; for the prenatal experiments several littermates from two different litters on each embryonic day were examined. All rats were bred at our own facility (lights on 10.00–22.00 for the prenatal experiments, 15.00–03.00 hours for the postnatal experiment). Food and water were delivered at libitum. The pregnant females were caged individually after insemination. The mothers were separated from the pups on postnatal day 21 (P21). The sexes were separated at P35. Males were housed in groups of four (maximum) and females in groups of six (maximum) to a cage.

Because of the rapid proliferation of the mesocortical DA projection, we used time-pregnant rats. The insemination was restricted to 2 hours, 0900–1100 (prenatal experiments) and 1300–1500 (postnatal experiments). For the prenatal series embryos and foetuses were examined on embryonic days E14–E21. For the postnatal series three to six animals of each of the following ages were examined: P0, P1, P2, P4, P6, P8, P10, P12, P20, P35, P60, and P90, P0 being the day of birth after a gestation of 21 days (so P0 = E22, E0 being the day of mating).

The pregnant rats and the postnatal pups were anesthetized with Nembutal (0.1 ml/100 g). Subsequently, the embryos and foetuses were removed one at a time and dissected from the amniotic membranes in a Ringer solution (± 35°C). Embryos of E14 and E15 were perfused through the transverse sinus with a glass microelectrode (with broken tip). At all other stages of development animals were perfused through the heart. Perfusion started with a rinse of Ringer (only in the prenatal experiment) or saline, followed by 5% glutaraldehyde in 0.05 M sodium-cacodylate + 1% $NA_2S_2O_5$, pH = 4–7.4. The fixed brains were removed from the skull and postfixed in the same fixative for another 1–3 hours. After 30-minute postfixation the postnatal brains were weighed. The prenatal brains were washed for several hours in Tris (0.05 M) buffer containing 1% $NA_2S_2O_5$ + 20% sucrose and embedded in gelatin. The gelatin was fixed for 6–7 hours in 2% glutaraldehyde in Tris (0.05 M)-buffered saline + 1% $NA_2S_2O_5$, after which the embedded brains were washed overnight in the aforementioned buffer containing sucrose. The following morning transversal and sagittal frozen sections, 40 μm thick, were cut on a sliding microtome. Series of Nissl-stained paraffin sections were made from immersion-fixed (Bouin's fixative) brains of littermates. The postnatal brains were cut on a vibroslice; coronal and sagittal sections, 50 μm or 100 μm thick, were processed for light microscopic immunocytochemistry and Nissl staining, respectively.

The preparation of the specific antibodies against dopamine has been described in detail elsewhere (Geffard et al., '84). The immunocytochemical procedure consisted of the following incubations: (1) dopamine antiserum diluted 1:2,000 in 0.05 M Tris buffer containing 1% $Na_2S_2O_5$ and 0.5% Triton X-100, pH 7.2, for 18 hours at 4°C; (2) rinse (rinses and subsequent incubations were in 0.9% NaCl-0.05 M Tris buffer, pH 7.6); (3) goat antirabbit 1:50; 60 minutes; (4) rinse; (5) peroxidase-antiperoxidase 1:1,000, 60 minutes; (6) rinse; (7) 0.5 mg/ml 3,3-diaminobenzidine (DAB) (Sigma) and 0.01% H_2O_2 (Merck) in Tris-saline, 10–20 minutes.

At a later stage of the project the buffer in the perfusion medium was changed to 0.05 M acetate, and DAB was dissolved in 0.05 M Tris-saline containing 6% $(NH_4)_2 \cdot SO_4 \cdot NiSO_4$. These modifications resulted in a considerable improvement in staining, especially in the visualization of the very fine DA fibers. Although only fibers that contain detectable levels of DA are observed, the present results, when compared with those of previous work employing DA-induced or TH immunocytochemistry (Schlumpf et al., '80b; Specht et al., '81; Verney et al., '82), give no reason to suppose that any significant amount of DA has been left unfixed.

In the present study the prefrontal cortex is defined as the cortical area receiving a strong projection from the mediodorsal nucleus of the thalamus. The nomenclature of the cytoarchitectonial PFC subareas used is that proposed by Krettek and Price ('77). The criteria according to which the different prefrontal subareas and layers are distinguished are described in Van Eden and Uylings ('85a). The prefrontal cortex in the rat consists of two spatially separated areas: namely, the medial and orbital prefrontal cortex. The medial prefrontal cortex is situated at the medial aspect of the hemisphere extending from the frontal pole to the level of the anterior commissure. The part rostral to the genu of the corpus callosum is referred to as the pregenual medial prefrontal cortex; the part extending from the genu caudally is the supragenual medial prefrontal cortex. Within the medial prefrontal cortex, three subareas are distinguished; the prelimbic (PL), the dorsal anterior cingulate (ACd), and the medial precentral area (PrCm). The orbital prefrontal cortex occupies the dorsal bank of the rostral part of the rhinal sulcus. Within this part of the prefrontal cortex two subareas are distinguished—the ven-

tral and dorsal agranular insular areas. A prominent feature of the PFC in several mammalian species is its dense dopaminergic innervation (Divac et al., '78). Although this DA innervation shows considerable regional differences, the DA fiber distribution coincides strikingly well with the cytoarchitectonically different prefrontal subareas (Van Eden et al., '87).

Drawings of representative sections stained for DA were made by camera lucida. Adjacent sections, either Nissl stained or counterstained according to the procedure described in Kalsbeek et al. ('87a), were used for identification of cortical layers.

RESULTS

Brain weight and body weight development

The postnatal development of the body weight and brain weight of the animals used in the present study is shown in Figure 1. Two phases can be distinguished in the postnatal development of the brain weight, viz., a period of rapid growth until P20, and a period of slower growth from P20 until P90, as reported by Van Eden and Uylings ('85b). The body weight increases steadily from birth until P90, the body weight of the male and female rats differing significantly at P35.

Development of prefrontal cortical cytoarchitecture from E15

Before describing the development of the DA fibers in the prefrontal cortical subareas, we shall indicate the early development of the cytoarchitecture. Regarding the description of the cortical development our terminology is largely derived from earlier studies of Crandall and Caviness ('84) and Van Eden ('86). The cytoarchitecture of the rat prefrontal cortex (PFC) develops from E15 as follows (Fig. 2): On E15–E18 a trilaminated pattern of the developing neocortex is present; a densely packed cellular cortical plate (CP) is bracketed between the marginal zone (MZ) above and the subplate (SP) below. An intermediate zone (IZ) and ventricular zone (VZ) are visible below the subplate. From E20 on the cortical plate greatly increases in thickness. At birth the cortical plate can be divided into two parts, viz., an upper zone of densely packed immature cells, the upper cortical plate (CPu), and a lower part in which after P2 two laminae can be recognized. These are the basal layers of the cortical plate and will be referred to as "developing layers V and VI. From P6 on the future layer IV can be recognized in the dorsolateral cortex, immediately below the cortical plate. This granular layer distinguishes the lateral neocortex from the agranular PFC. At this stage in the PFC the developing layer V can be distinguished from the upper cortical plate. From P10 the cortical plate has disappeared and the cortex matures to its adult pattern. For a detailed description of the postnatal maturation of the prefrontal cortical cytoarchitecture we refer to a previous report (Van Eden and Uylings, '85a).

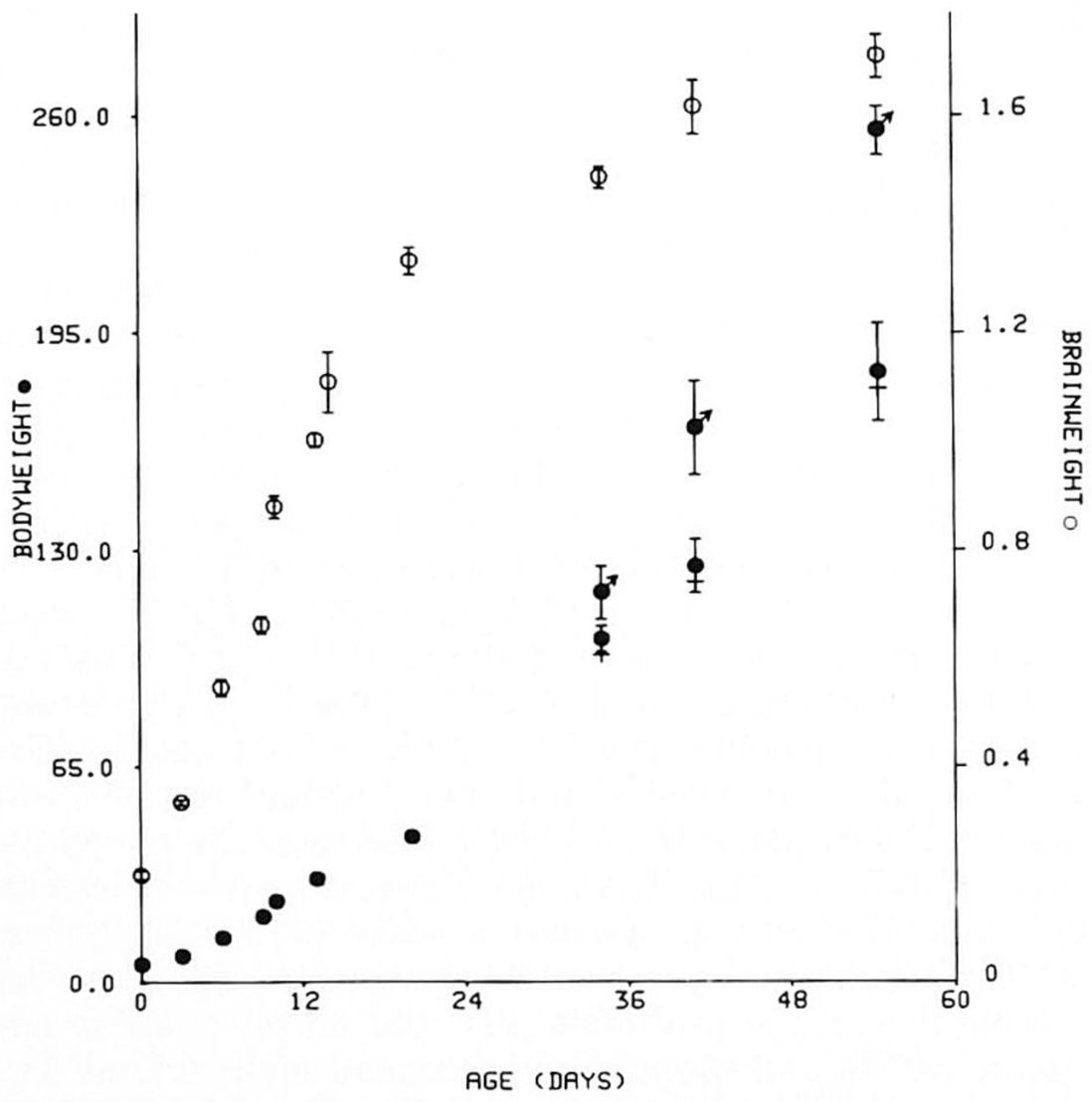


Fig. 1. Postnatal development of brain and body weight of the animals examined. ● Body weight, ○ Brain weight in grams (mean+S.E.M.). When not shown the S.E.M. is smaller than the the size of the dots.

Pre- and postnatal development of dopaminergic fibers in the prefrontal cortex

E14–E15. On E14 the DA fibers do not reach beyond the ventral ganglionic eminence. Before E15 no DA fibers are visible in the cortical anlage. On E15 the first DA-positive fibers pass through the developing striatum to cortical regions in the ventral part of the lateral wall of the frontal hemisphere. These fibers are situated in the intermediate zone beneath the subplate and cortical plate (Fig. 3).

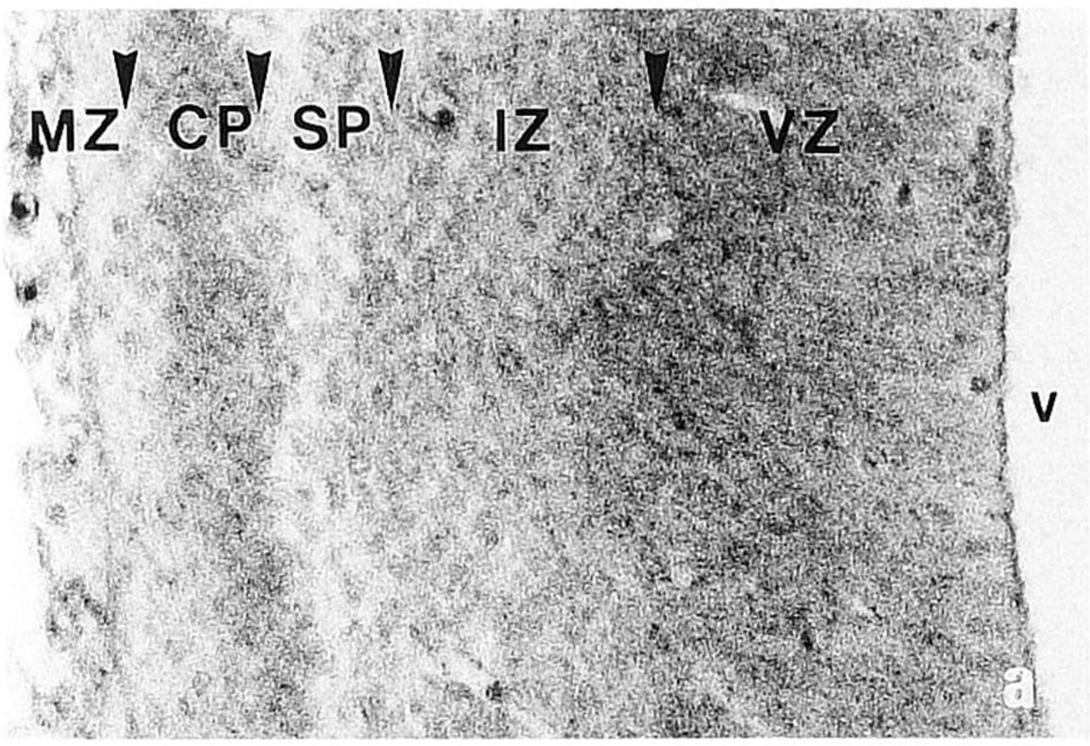
E16–E17. By E16 more fibers are passing through the developing striatum to the ventral part of the lateral wall. Most fibers are situated in the upper part of the intermediate zone and the subplate. From ventral to dorsal the fibers concentrate in the subplate. Some of the most rostral fibers cross the cortical plate to reach the marginal zone. At E17 the first DA fibers can be found in the subplate of the medial wall, coming from a fiber bundle situated ventromedially to the developing striatum (Fig. 4).

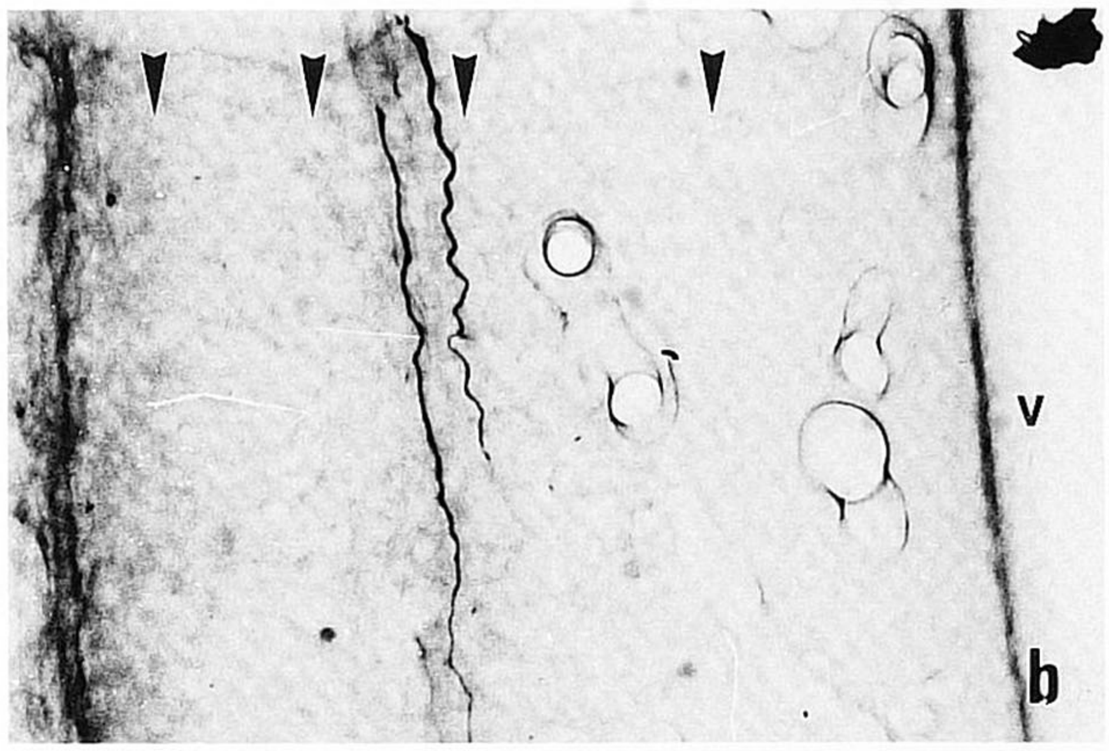
E18–E19. On E18 DA fibers in the lateral wall reach the dorsalmost part of the cortex via the subplate; in the medial wall they have advanced as far as the dorsal tip of the lateral ventricles, leaving only the shoulder region of the developing PFC free of DA positive fibers. At E19 the subplate in the medial and lateral wall is filled with DA-positive fibers. At this stage sparse DA fibers can be found throughout the greater part of the cortex, especially in somatosensory and occipital cortical areas both in the subplate and the marginal zone.

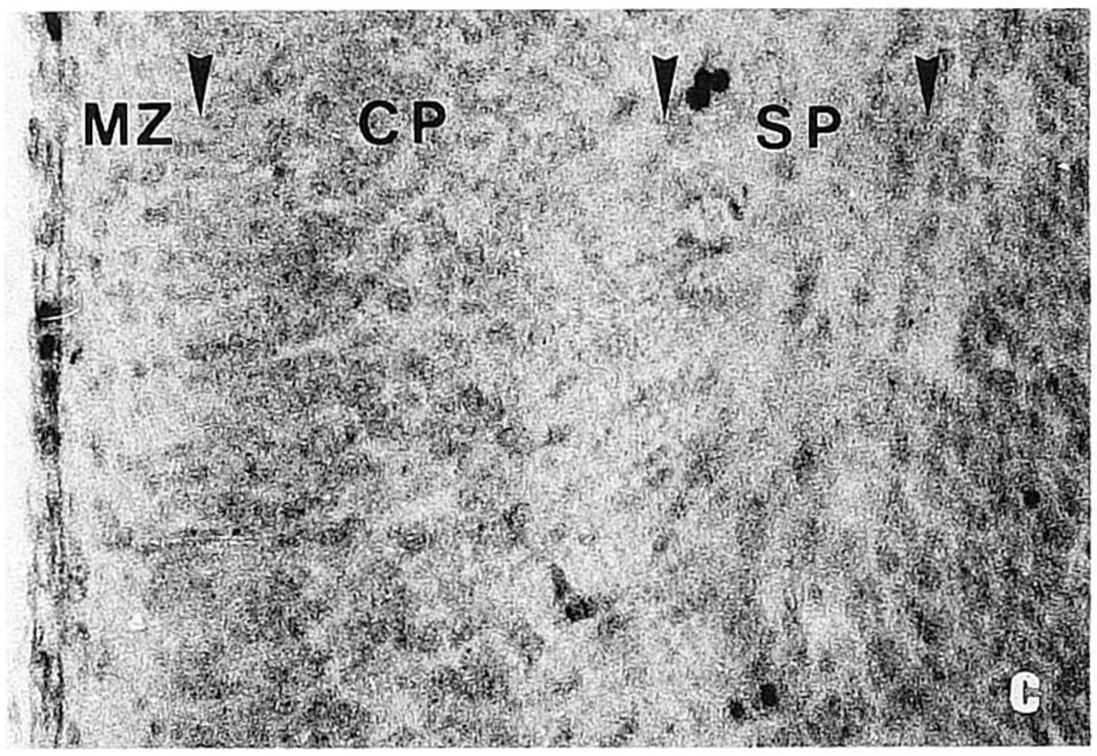
E20–E21. From E20 on the cortical plate increases in thickness. Most DA fibers are still found in the subplate, but their orientation is changing. In the medial wall fibers with a twisted form begin to appear, some of which can be seen running from the subplate to the cortical plate (Fig. 5). At this age DA fibers can be observed in the subplate and the marginal zone of the cortical area dorsal to the rhinal sulcus (Fig. 5).

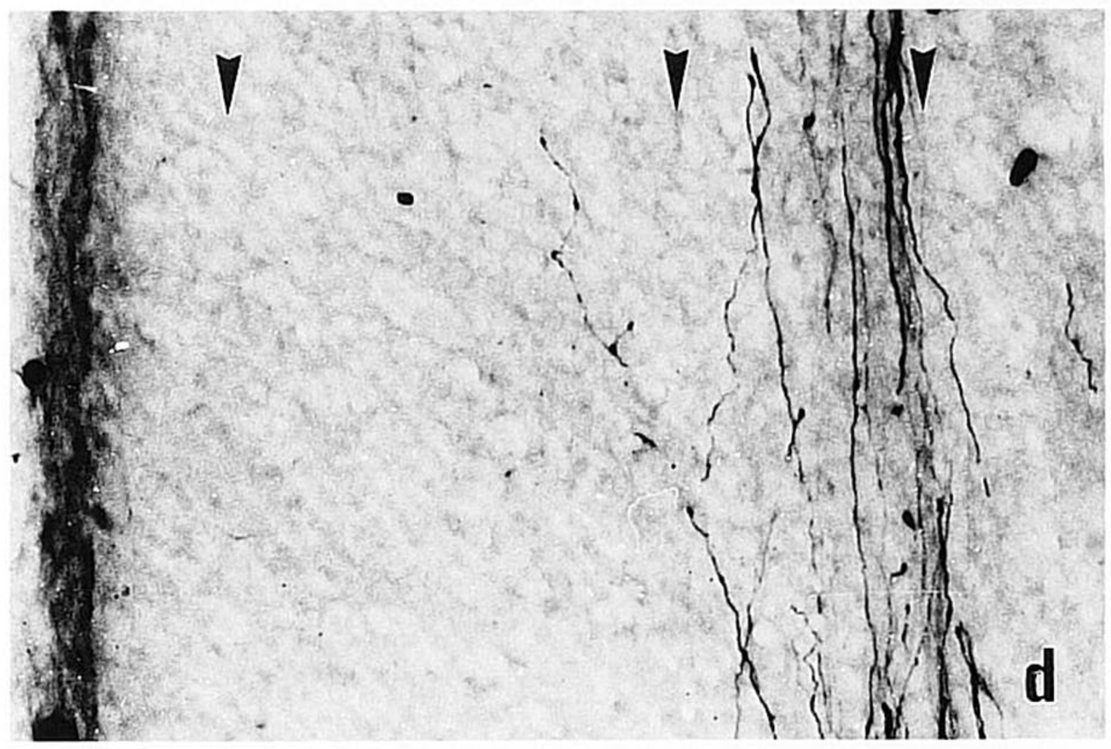
P0. In the medial and orbital cortex as well as in the supragenual part of the PFC, DA-positive fibers concentrate in the developing layer VI (Fig. 6). Most fibers in the future medial PFC are oriented parallel to the pial surface and are relatively thick and darkly stained, though thinner fibers can also be seen to enter the cortical plate. In the orbital cortex a great number of fibers with the typical twisted form are now appearing in the deeper layers.

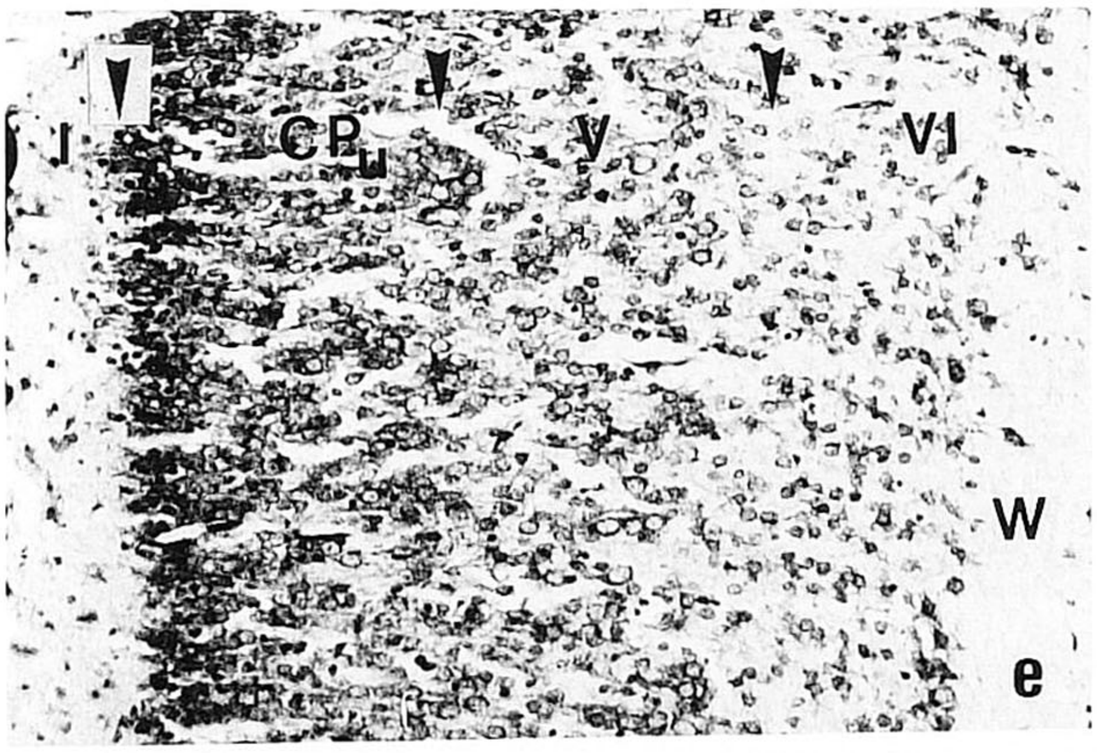
P1–P2. Twenty-four hours later the number of DA fibers has greatly increased, especially in the developing layer VI.

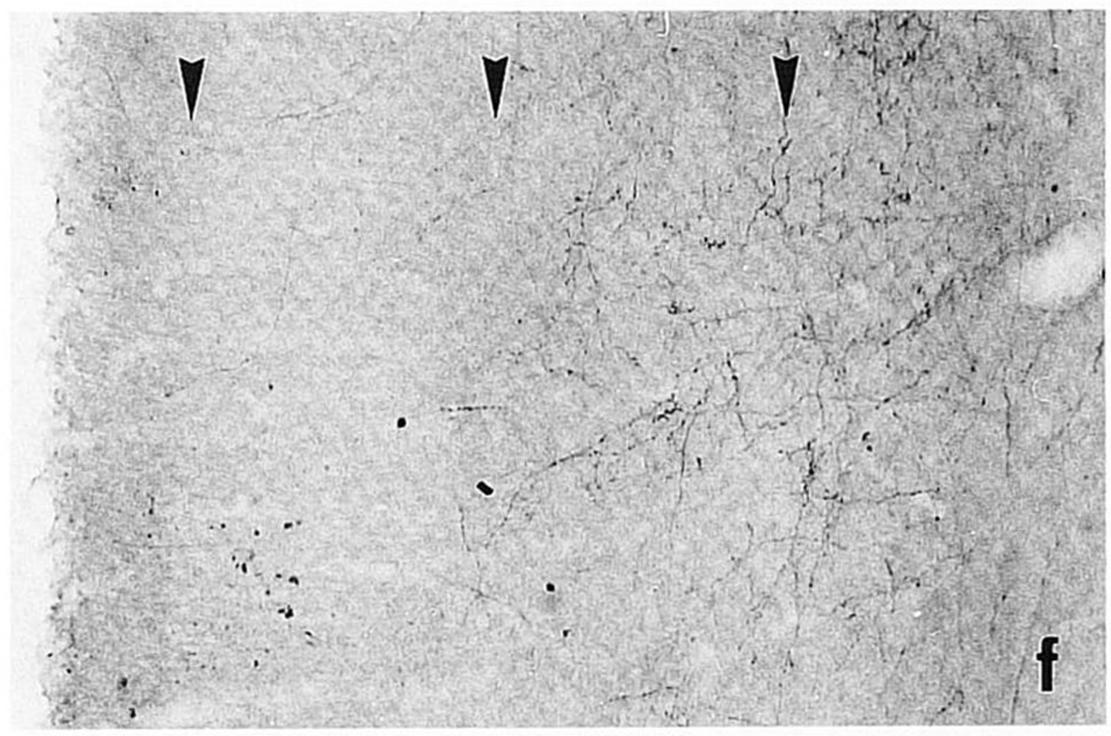


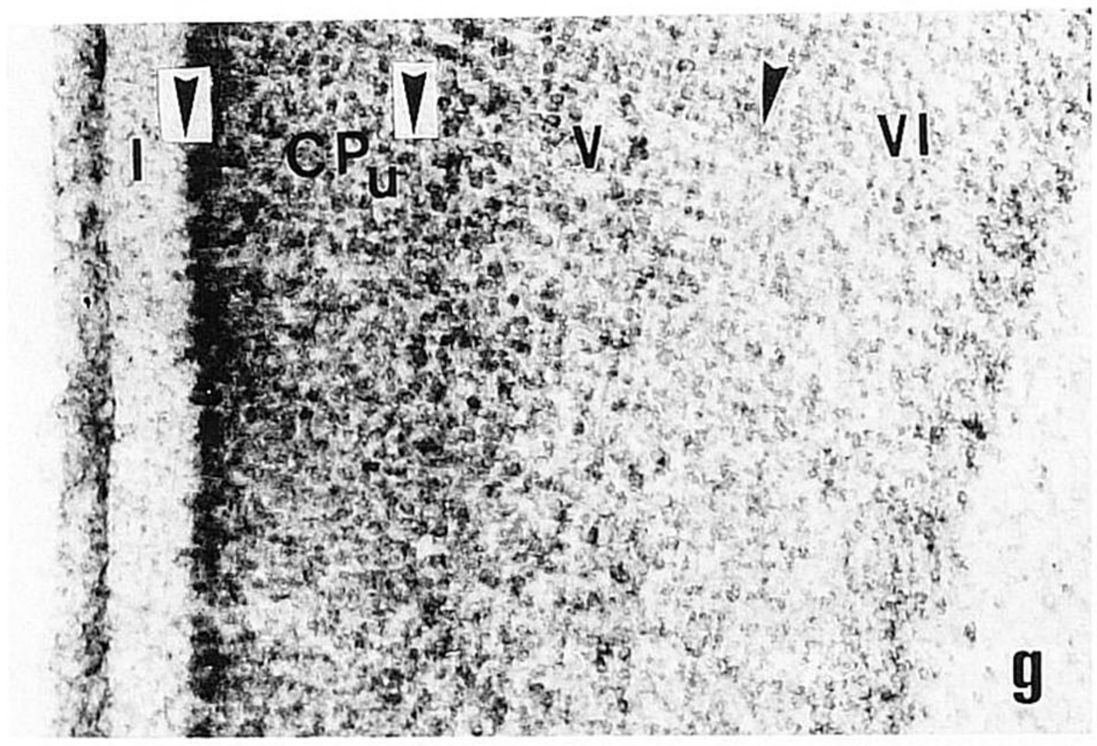












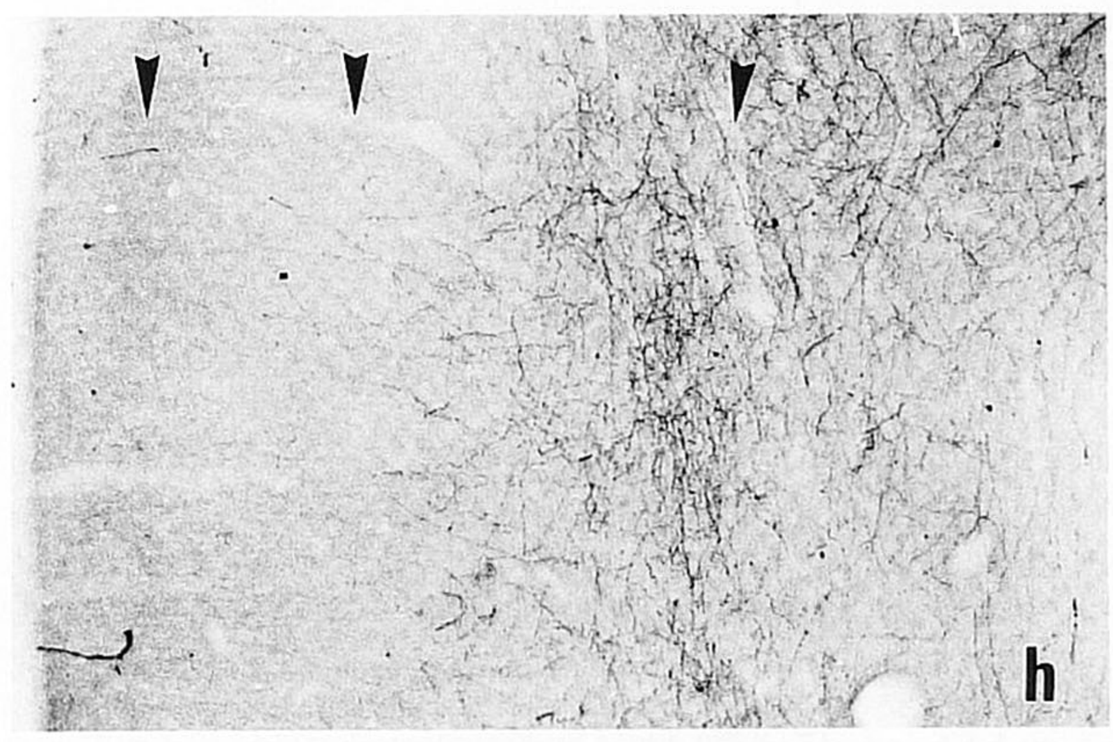
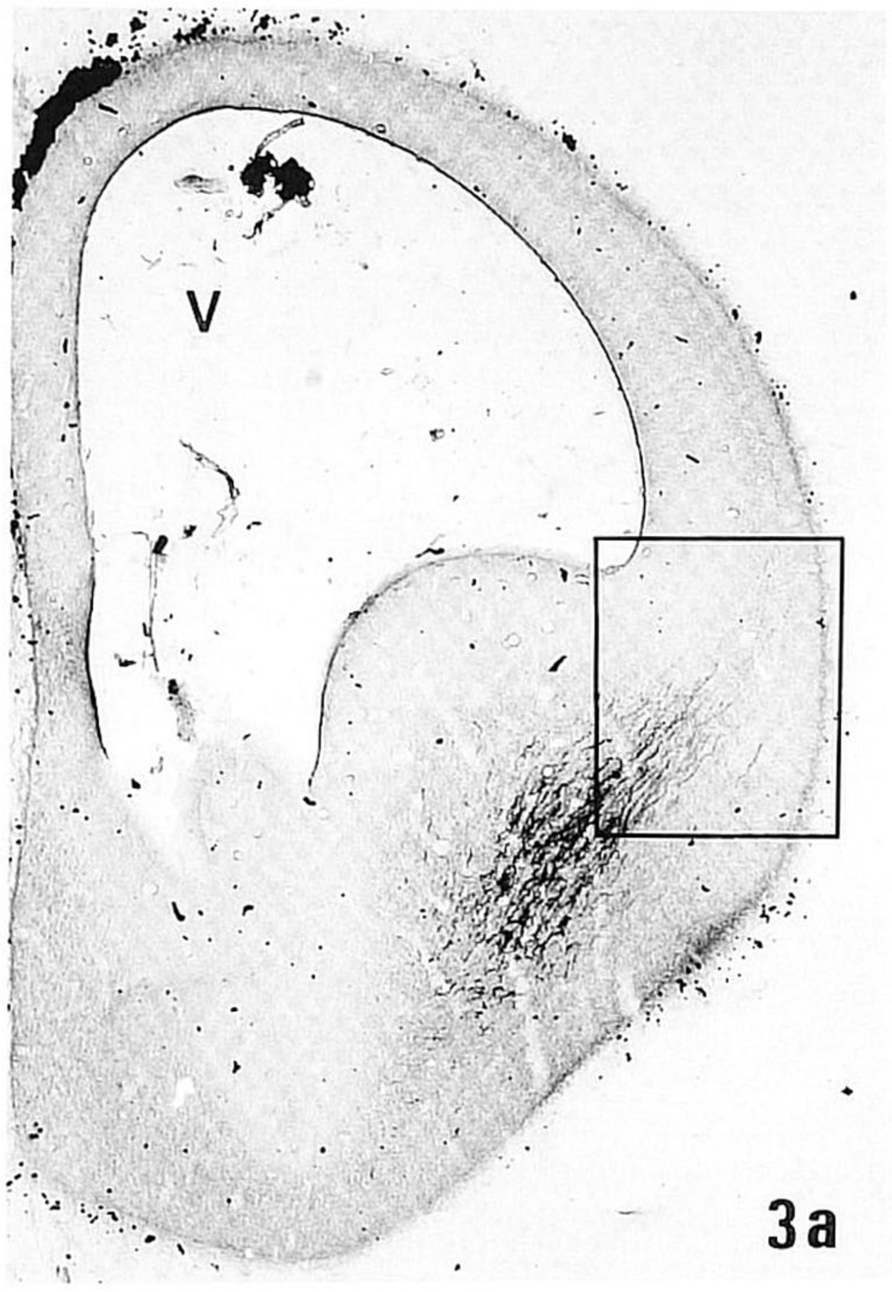
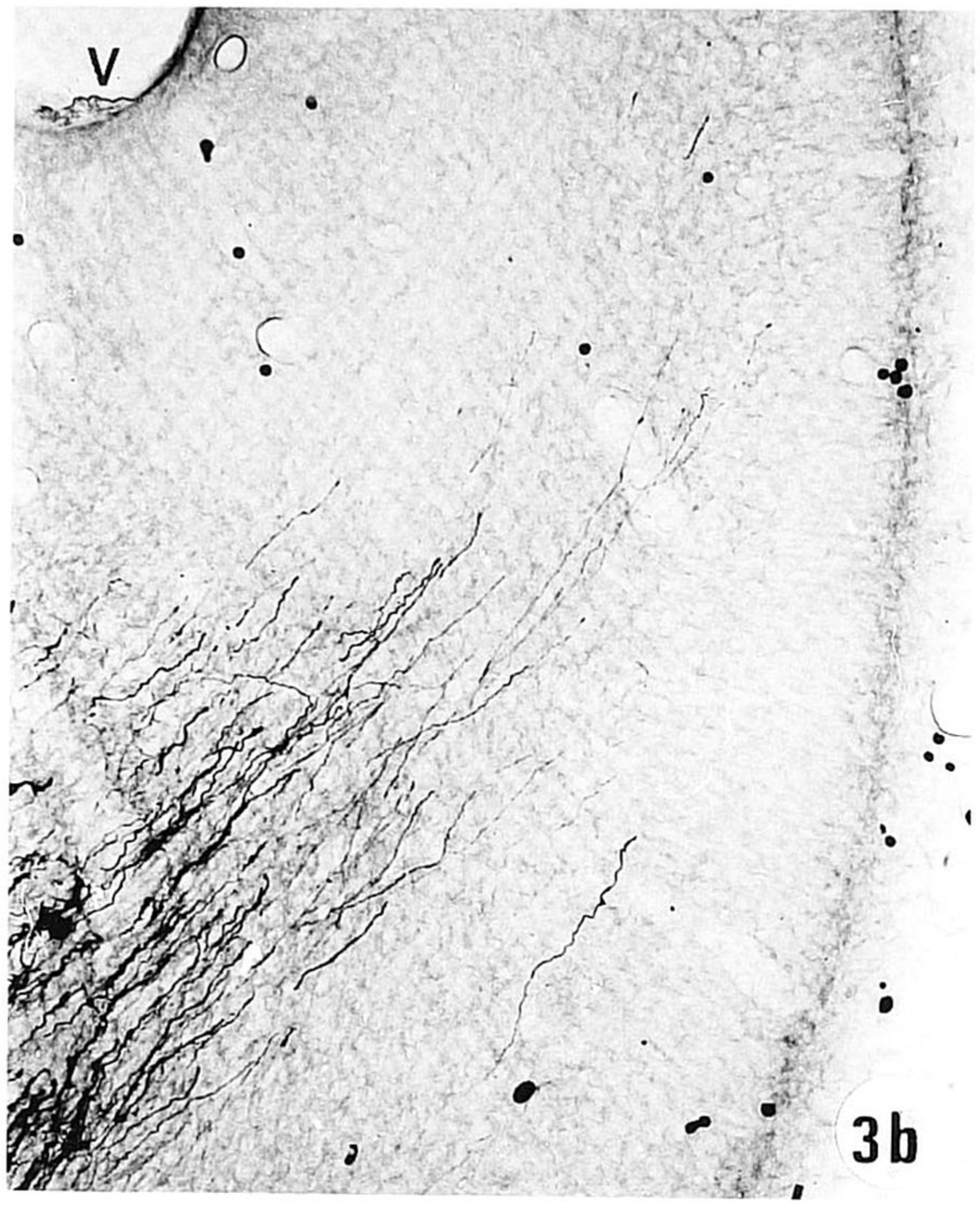
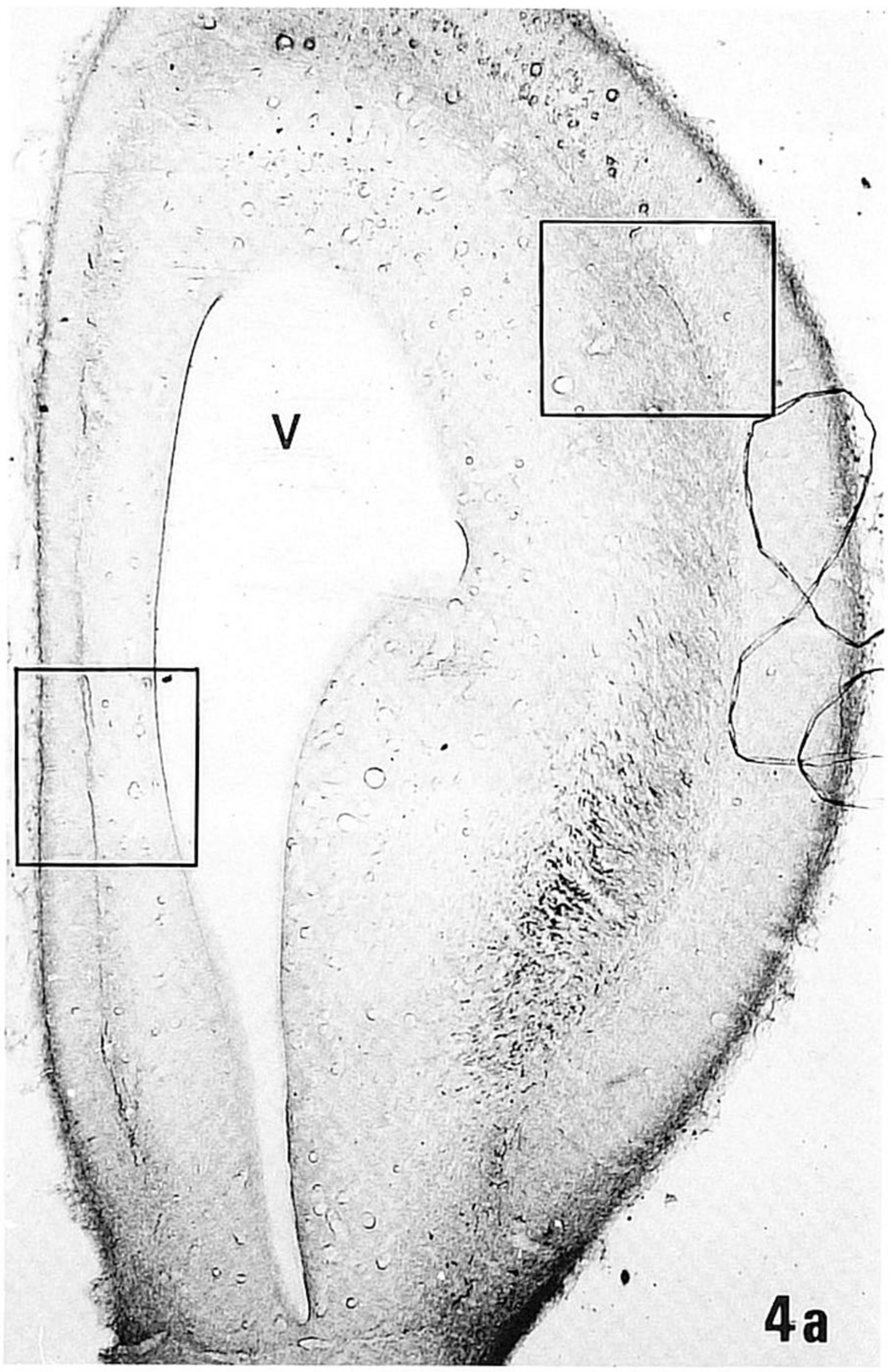
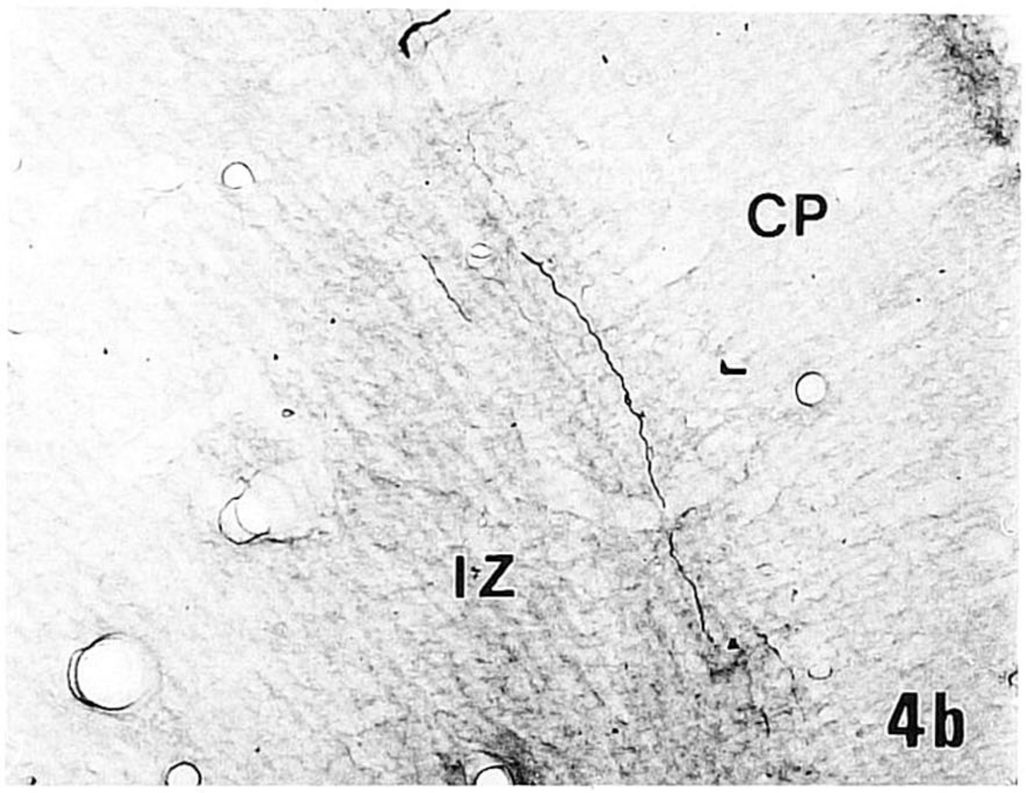


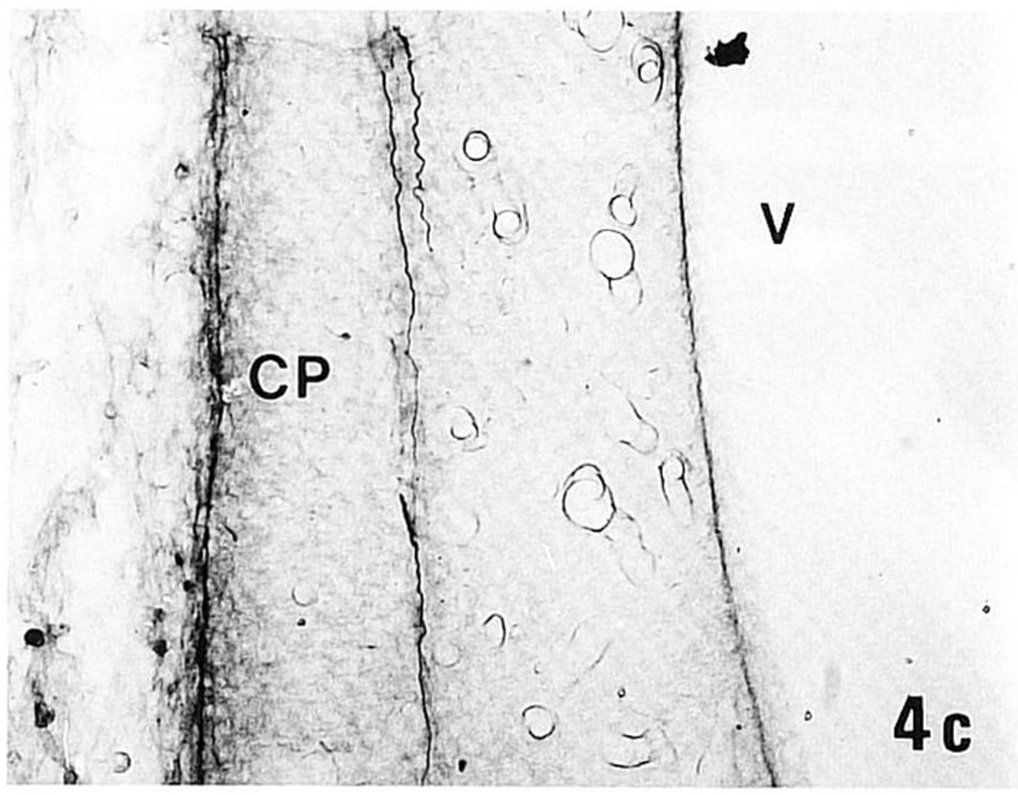
Fig. 2. Development of the medial prefrontal cortical cytoarchitecture (**a,c,e,g**) and the dopaminergic innervation (**b,d,f,h**). Cortical layers in b,d,f, and h are indicated in adjacent Nissl sections, a,c,e,g, respectively. a,b, E18 × 280; c,b, E20 × 200; e,f, P4; g,h, P10. ×100. Arrowheads indicate borderlines of the different cortical layers.



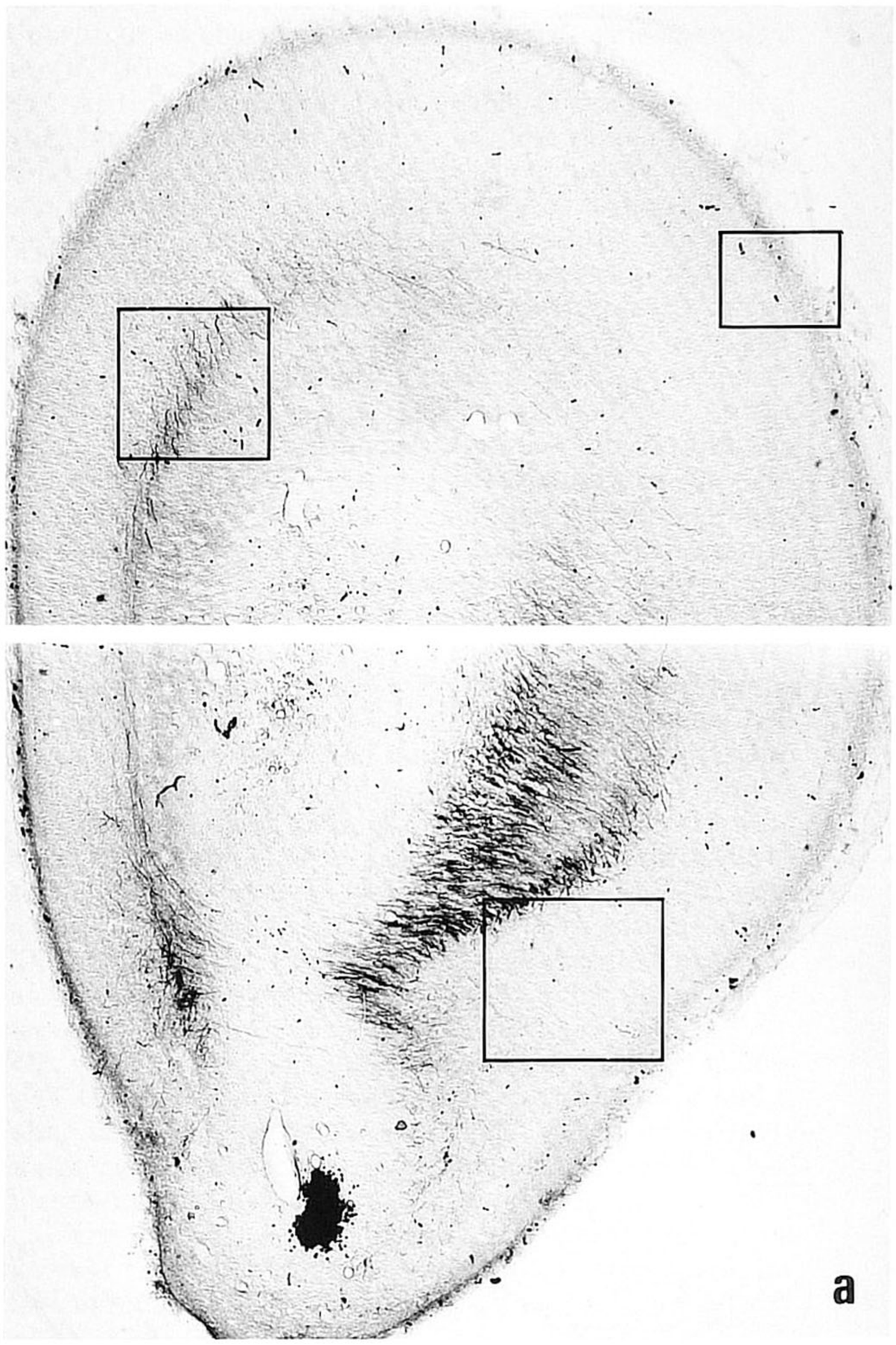


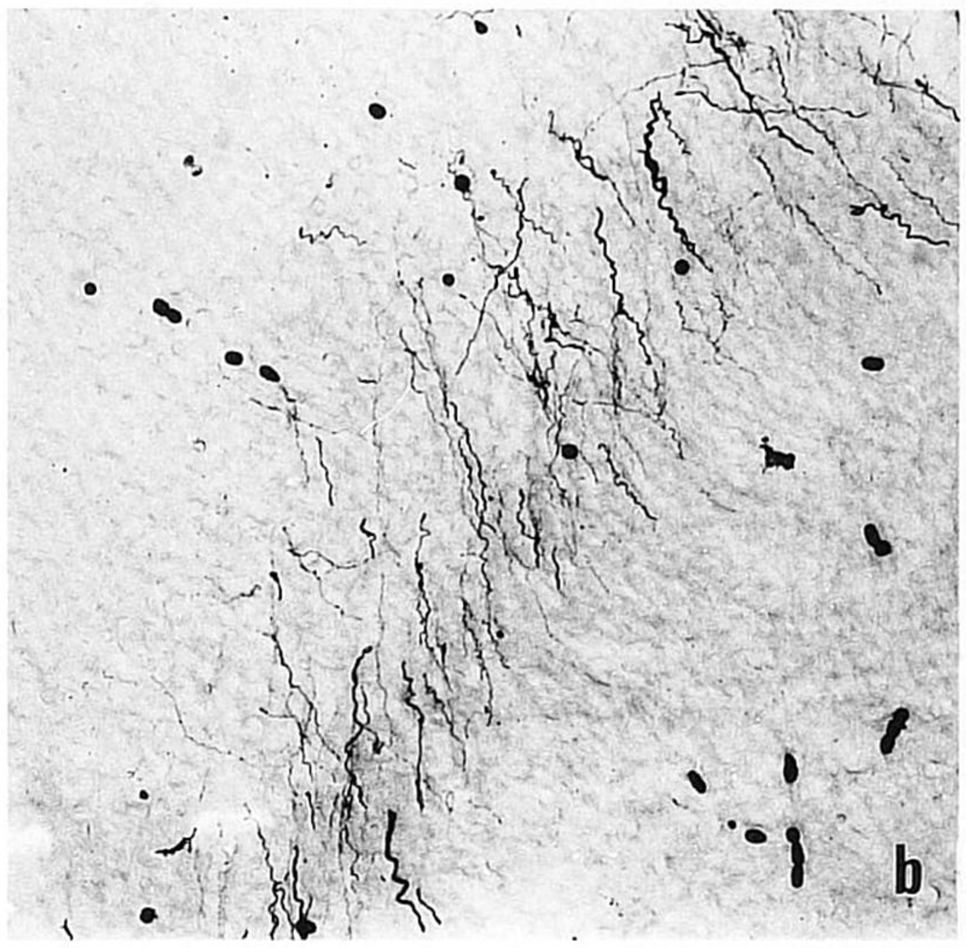


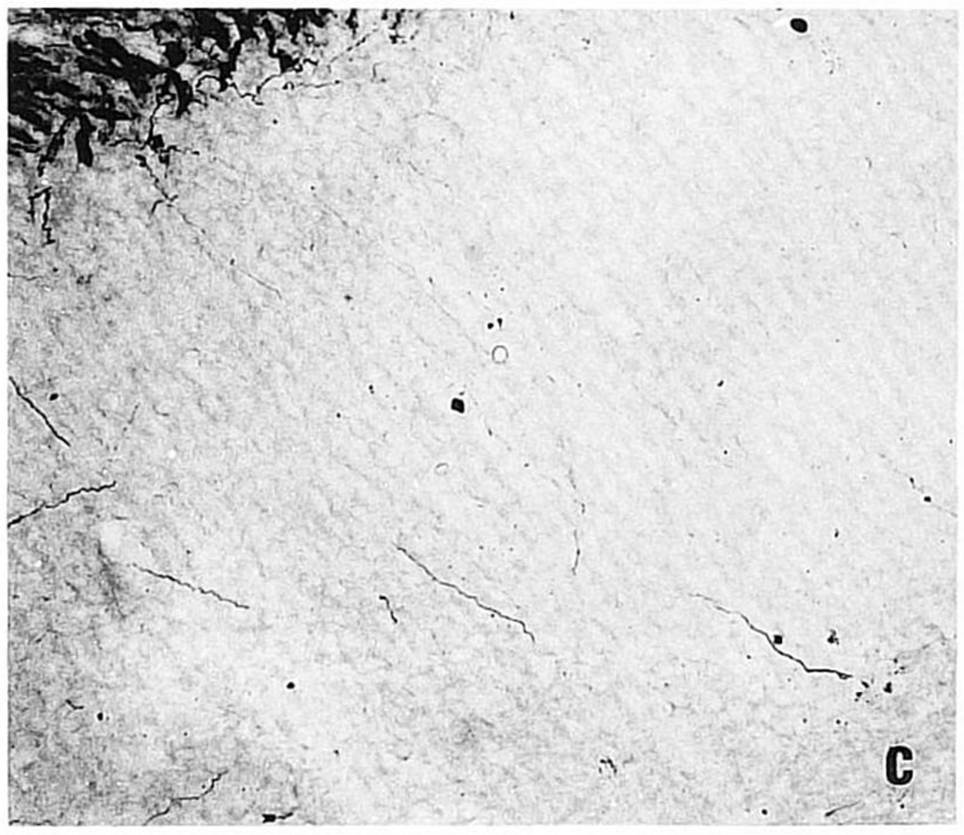




Figs. 3–5. Prenatal development of dopaminergic cortical innervation. Fig. 3. **a:** Embryonic day 16. × 25. **b:** DA-positive fibers passing through the devloping striatum reach the intermediate zone of the lateral cortex. ×160. Fig. 4. **a:** Embryonic day 18. ×40. **b,c:** Dopaminergic fibers in the subplate of the lateral (b) and medial (c) wall of the frontal cortex. ×120. Fig. 5. **a:** Embryonic day 20. ×40. **b,c:** DA-positive fibers invade the cortical plate in the medial (b) and orbital PFC (c). In some cases DA fibers can be seen in the marginal zone (d). ×180. Boxed areas in 3a, 4a, and 5a are represented in 3b, 4b and c, 5b, 5c, and 5d, respectively.







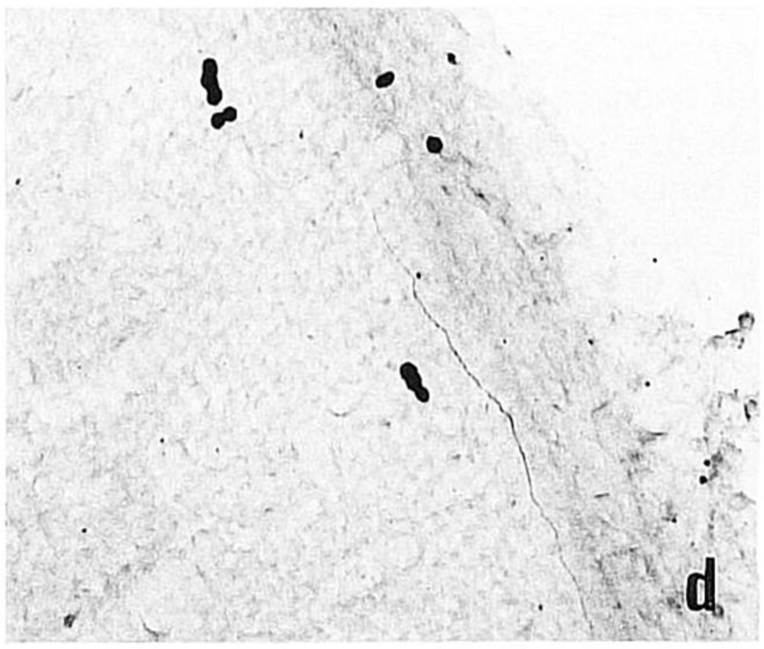


Figure 5

The orientation is no longer predominantly parallel to the pial surface. In the medial PFC many fibers are now also oriented in a transverse direction. In the future infralimbic area (IL) and the dorsal agranular insular area (AId) DA fibers can be observed in the marginal zone, i.e., the future layer I (Fig. 6).

P4. At this stage the deeper layers V and VI can be distinguished in Nissl-stained sections. Although most of the DA fibers are still concentrated in layer VI, layer V of the developing IL, PL, ACd, and AId also contains DA fibers. The future subareas IL and AId can be demarcated from neighbouring cortical areas because of the relative density of DA fibers in layer I. Some positive fibers can be observed in layer I of PL and ACd, running parallel to the pial surface. In the supragenual part there is an increasing density of immunoreactive fibers in the deeper layers, many of which have the typical twisted form (Fig. 7) observed at earlier stages in the pregenual medial (Fig. 5) and orbital

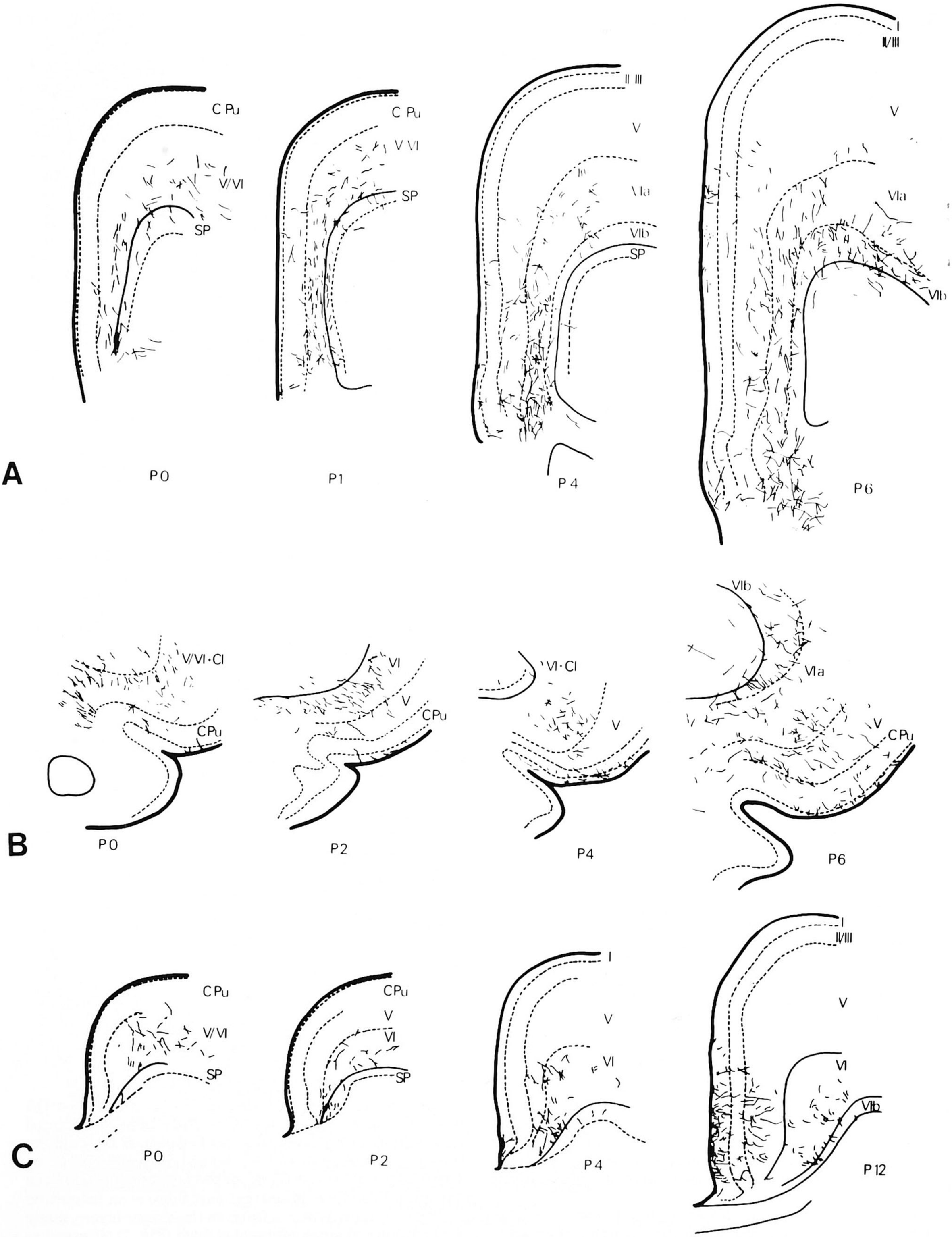


Fig. 6. Schematic presentation of postnatal development of dopaminergic cortical innervation. **A:** Medial PFC. **B:** Orbital PFC. **C:** Supragenual PFC.

cortex. On P4 the first DA fibers in layer I of the supragenual part can be observed, passing round the indiseum griseum (IG) (Figs. 6, 7).

P6. At this stage layer IV is visible in the dorsolateral cortex, immediately beneath the cortical plate. This enables a reliable cytoarchitectonic distinction to be made between the future PFC and the dorsolateral cortex. All PFC subareas that are discernible in Nissl staining can also be recognized now according to the characteristics of the DA fiber pattern (Fig. 8), although this pattern still differs greatly from the adult one. The infralimbic area is characterized by its seemingly adult pattern of DA fibers, with DA-positive fibers often reaching the pial surface. In the prelimbic area layer V is moderately innervated, while the upper cortical plate and layer I contain only a few DA-positive fibers. In the anterior cingulate area, however, layer I often contains DA-positive fibers that grow transversely from the deep dopaminergic field into the superficial layers where they branch. The density of DA fibers in the deeper cortical layers V and VI is equal in ACd and PL. In the medial precentral area (PrCm) no DA fibers can be observed in layer I. In the deeper cortical layers the DA-positive fibers are restricted mainly to layer VI, although in some cases DA fibers can be detected in layer V. In the orbital part the most distinct difference between the ventral (AIv) and dorsal (AId) insular area is again the DA fiber density in layer I—being pronounced in AId, with many ramifying fibers, but almost absent in AIv. DA fibers in the supragenual PFC are still concentrated predominantly in the lower cortical layers; only a few fibers can be observed in layer I.

P12. The most important changes are an increasing density of the DA fibers in the deeper cortical layers and a further development of the superficial cingular field. At this age most fibers in the supragenual ACd and ACv are still concentrated in layer I, but now layer III also contains DA fibers (Fig. 7). At this stage the general topological pattern of the dopaminergic innervation is almost similar to that found in the adult stage, although at a lower density level.

P20. At this age the DA fibers in layer I of the pregenual part of ACd have by and large disappeared, while the DAergic innervation of PL has reached the upper border of layer II and DA fibers in layer I are also visible. In the supragenual part of ACd the additional superficial innervation in layers II and III is now clearly visible, although the dense aggregations of DA fibers as observed in these layers in adulthood have not yet formed (Fig. 7).

P20–P60. In the supragenual part of the PFC the mature pattern of the DA innervation in the superficial cortical layers is established somewhere between P20 and P35. This dense innervation is predominantly concentrated in ACv and the ventral part of ACd. During this period the DA innervation of the superficial layers in the supragenual PFC develops into a mixture of two types of fibers: first, the relatively thick fibers that are present in other cortical areas, ascending through the anterior hippocampal continuation or coming from the lower cortical layers to reach layer I; second, a very thin type of DA fiber with short intervaricosity segments, forming dense aggregations in layers II and III (for detailed description see below). The density of the dopaminergic fibers in the other PFC subareas continues to increase until P60. This is most clearly observed in layers I–IV of PL and AId.

P60–P90. In this period no obvious changes in the density or the topography of the DA fibers can be observed.

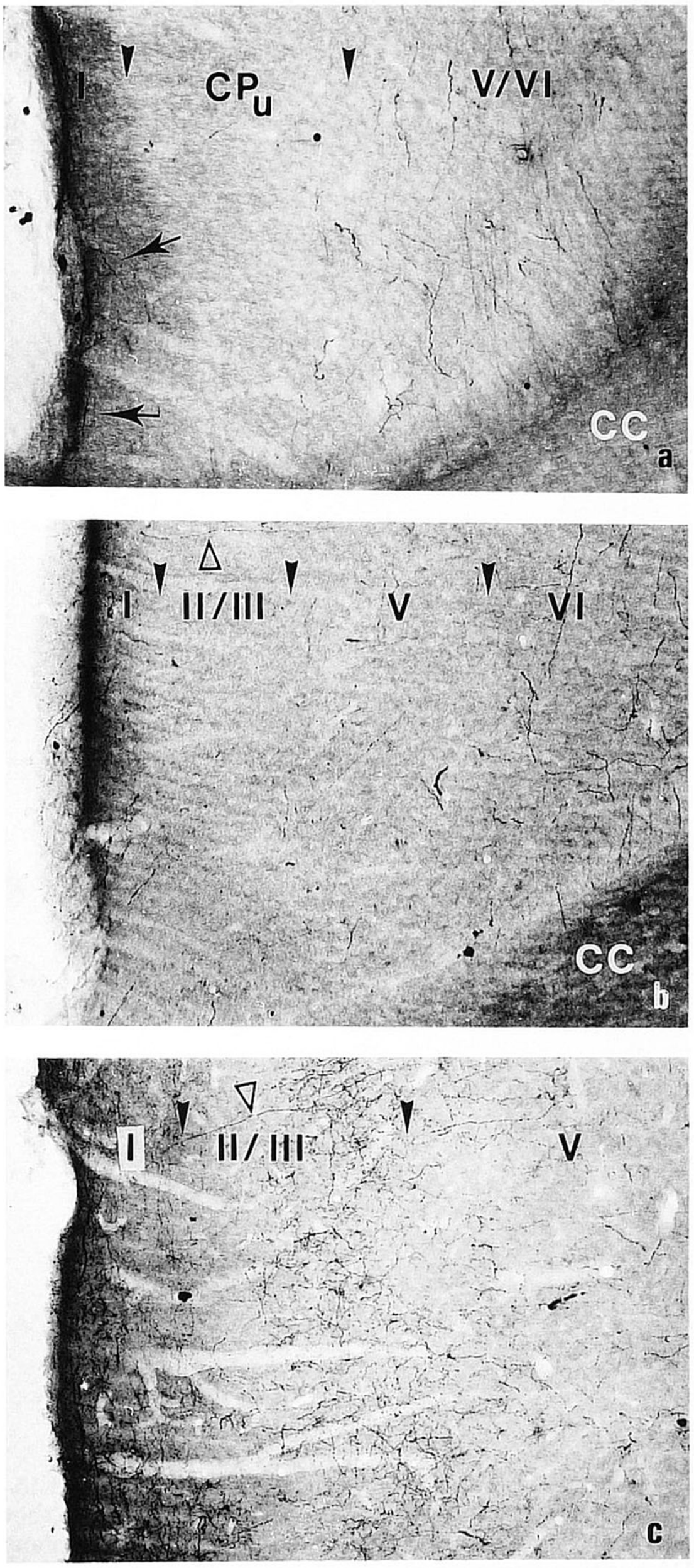
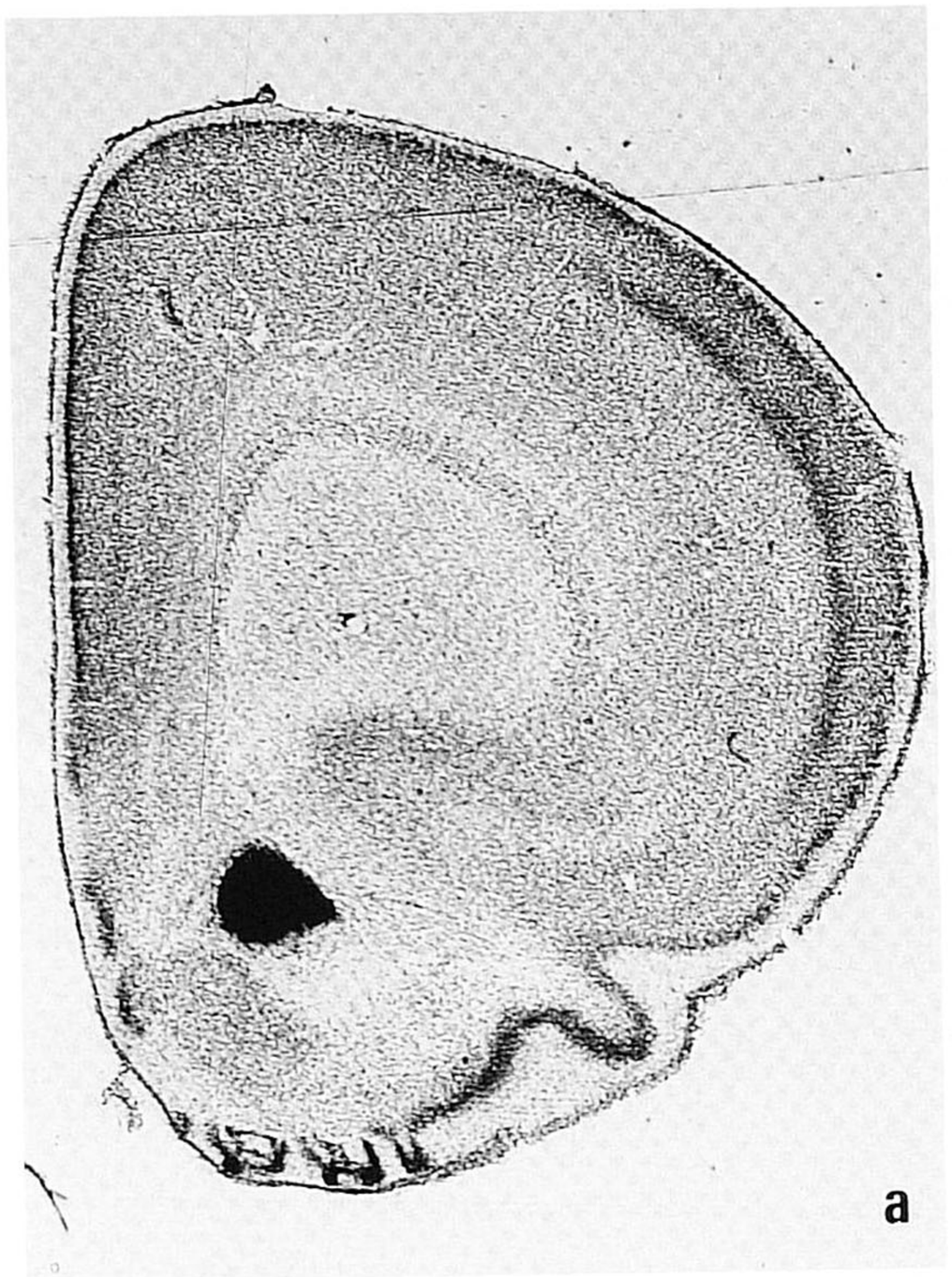
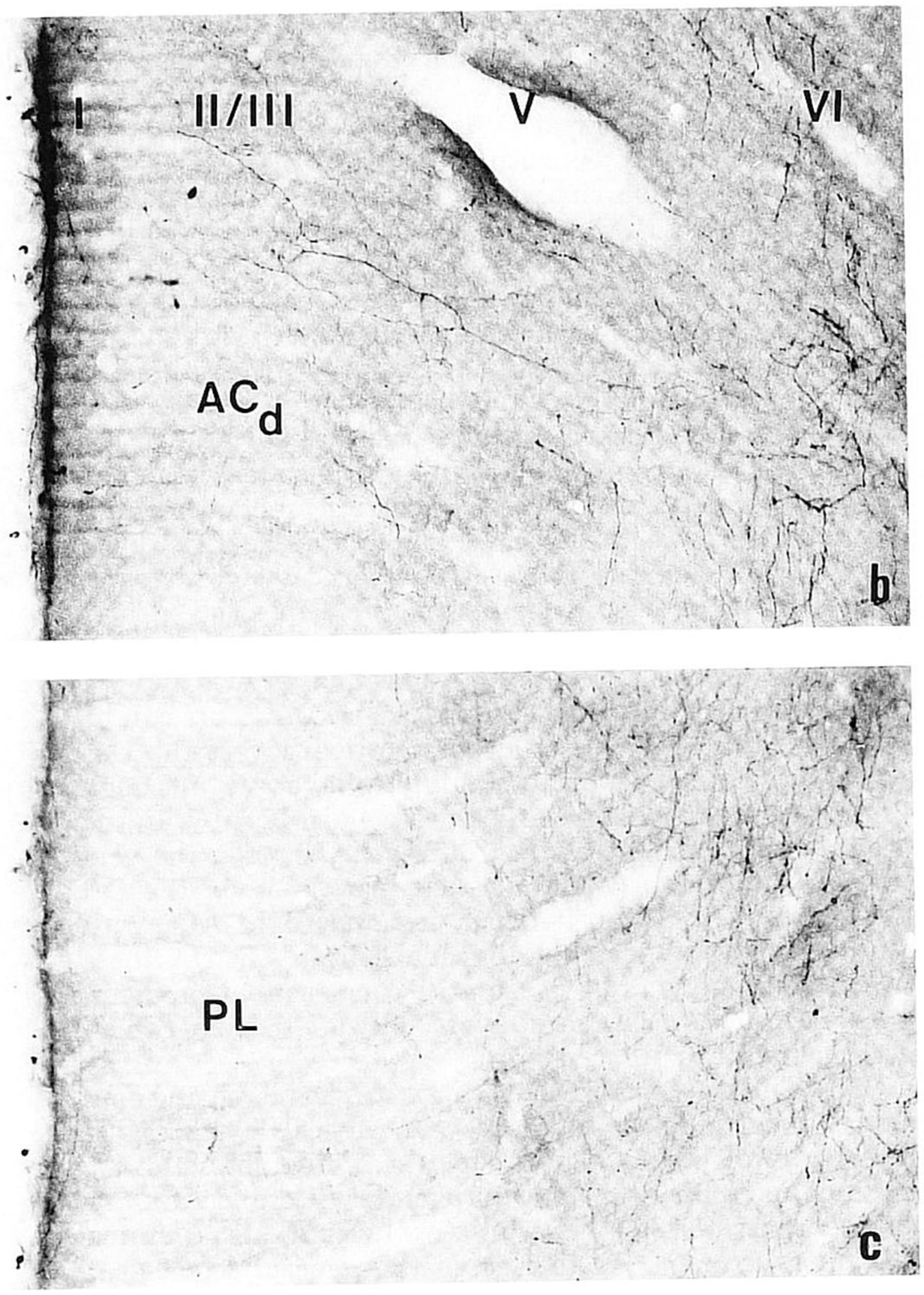


Fig. 7. Development of the superficial dopaminergic innervation in the supragenual PFC. **a:** P4, arrows point to dopaminergic fibers passing round the IG to reach layer I. ×170. **b:** P10, layer III only rarely contains a positive fiber. ×100. **c:** P20, a dense dopaminergic innervation is appearing in layers I and III. Open triangles (in a and b) point to fibers coming from the deeper layers to reach layer I. ×100. Arrowheads indicate borderlines of the different cortical layers.

Morphology of the DA fibers

The changes that occur in the shape and form of the dopaminergic fibers during the first period of their development are illustrated in Figure 9. In the first phase of the





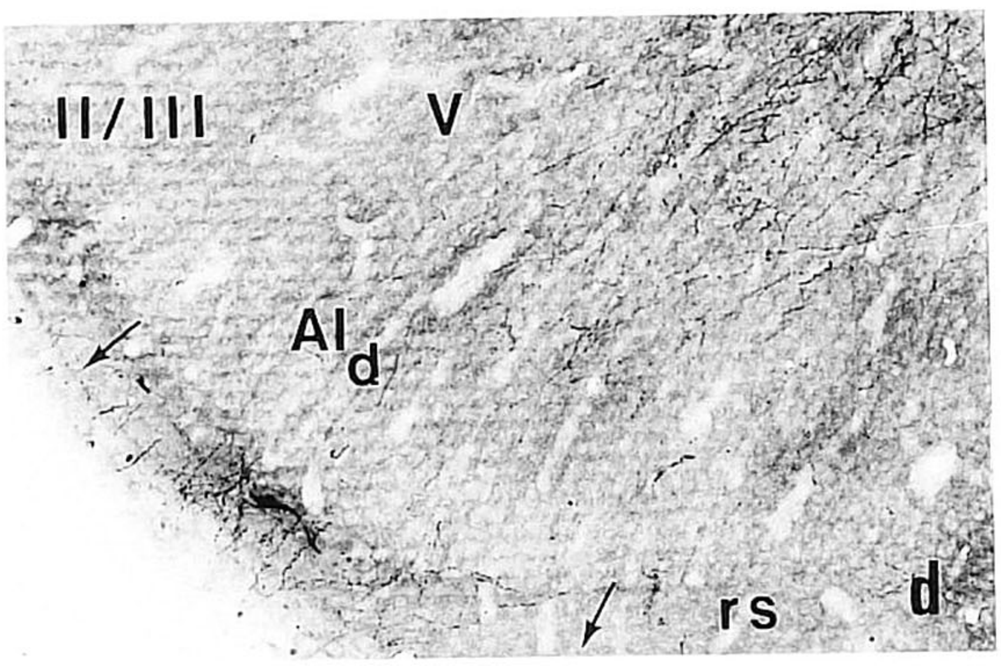


Fig. 8. Dopaminergic innervation pattern at P6. **a:** Layer IV is now clearly visible in the dorsolateral cortex. ×20. **b–d:** Show the characteristics of the dopaminergic innervation pattern as described in the text. Arrows in d indicate borderlines of AId. ×100.

development of the cortical dopaminergic innervation (E15–E20) the dopaminergic fibers are easily detected since they are relatively thick, straight, and darkly stained. In many DA-positive axons growth cones can be observed (Fig. 10). Just before DA fibers start to bifurcate and cross the cortical plate, many twisting fibers can be observed (E20–P2) (Fig. 5). Between P2 and P4 the morphology of the DA fibers drastically changes, with thick and darkly stained fibers being rarely observed after P4. Most DA-positive axons are now very thin and more difficult to detect, and irregularly spaced varicosities appear in most fibers. These morphological changes in the DA fibers suggest that from this point on the actual DA innervation begins. After P4 the shape of the DA-positive fibers does not change much; only the number of varicosities per fiber and the number of fibers increase strongly (Fig. 9). From P35 on the shape of the DA-positive axons has reached its adult morphology, i.e., thin fibers, with many varicosities. The morphological characteristics of the dopaminergic fibers in the superficial layers of the supragenual PFC, however, are quite different. The fine axons, sometimes hardly visible, have small, regularly spaced varicosities (Fig. 7). These fibers are concentrated in layer III, forming dense aggregations, but they can also be observed in layers I and II. In the upper part of layer I the thin fibers are intermingled with the aforementioned thicker DA fibers. The typical morphology of the adult DA fibers in layer III of the supragenual PFC develops between P20 and P35.

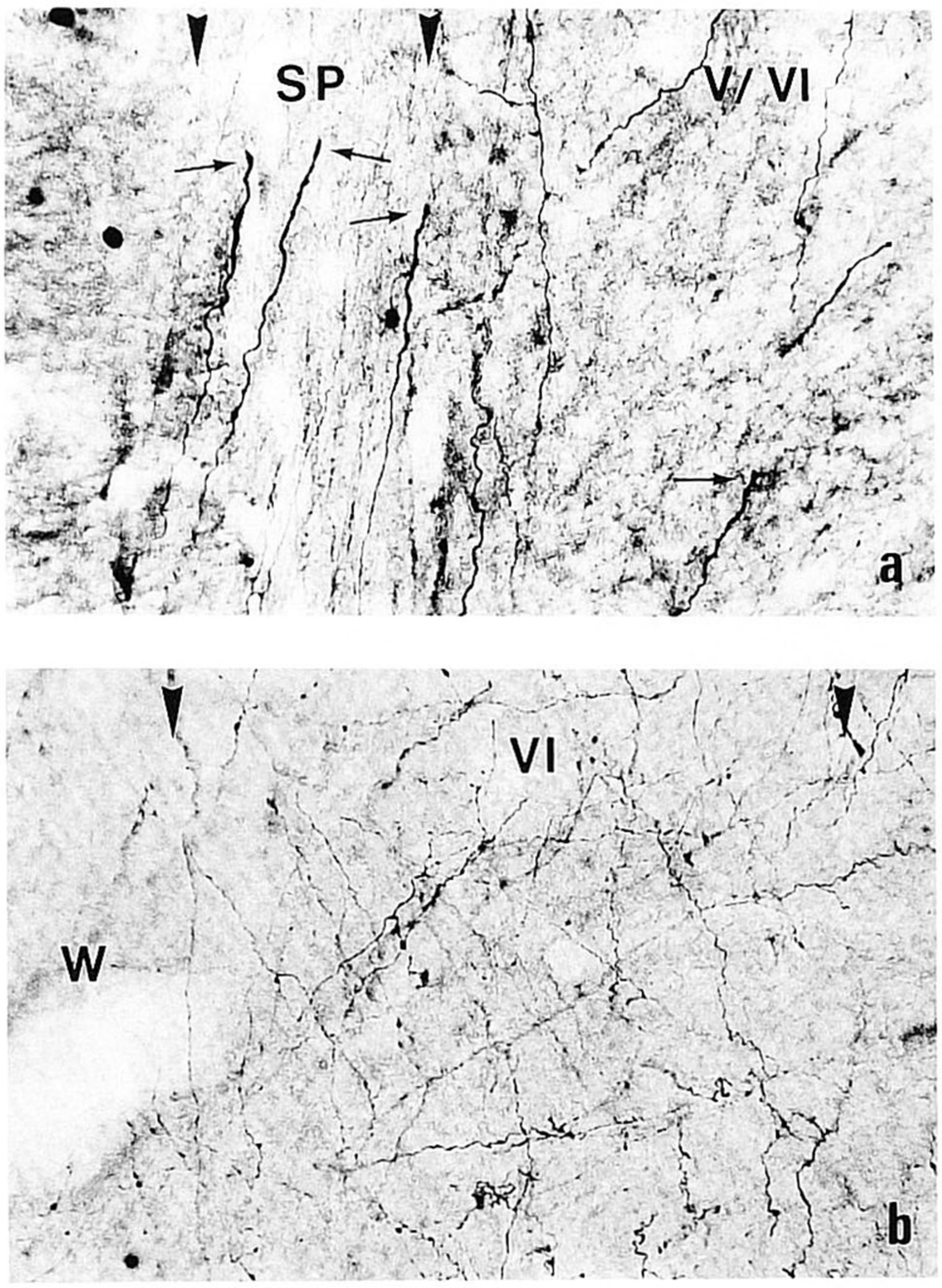


Fig. 9. Morphological changes in dopaminergic fibers during development. All photomicrographs were taken from the basal layers of PL. **a**, P0; **b**, P4; **c**, P12; **d**, P35; **e**, P90. Arrows in (a) point to growth cones. ×250. Arrowheads indicate borderlines of the different cortical layers.

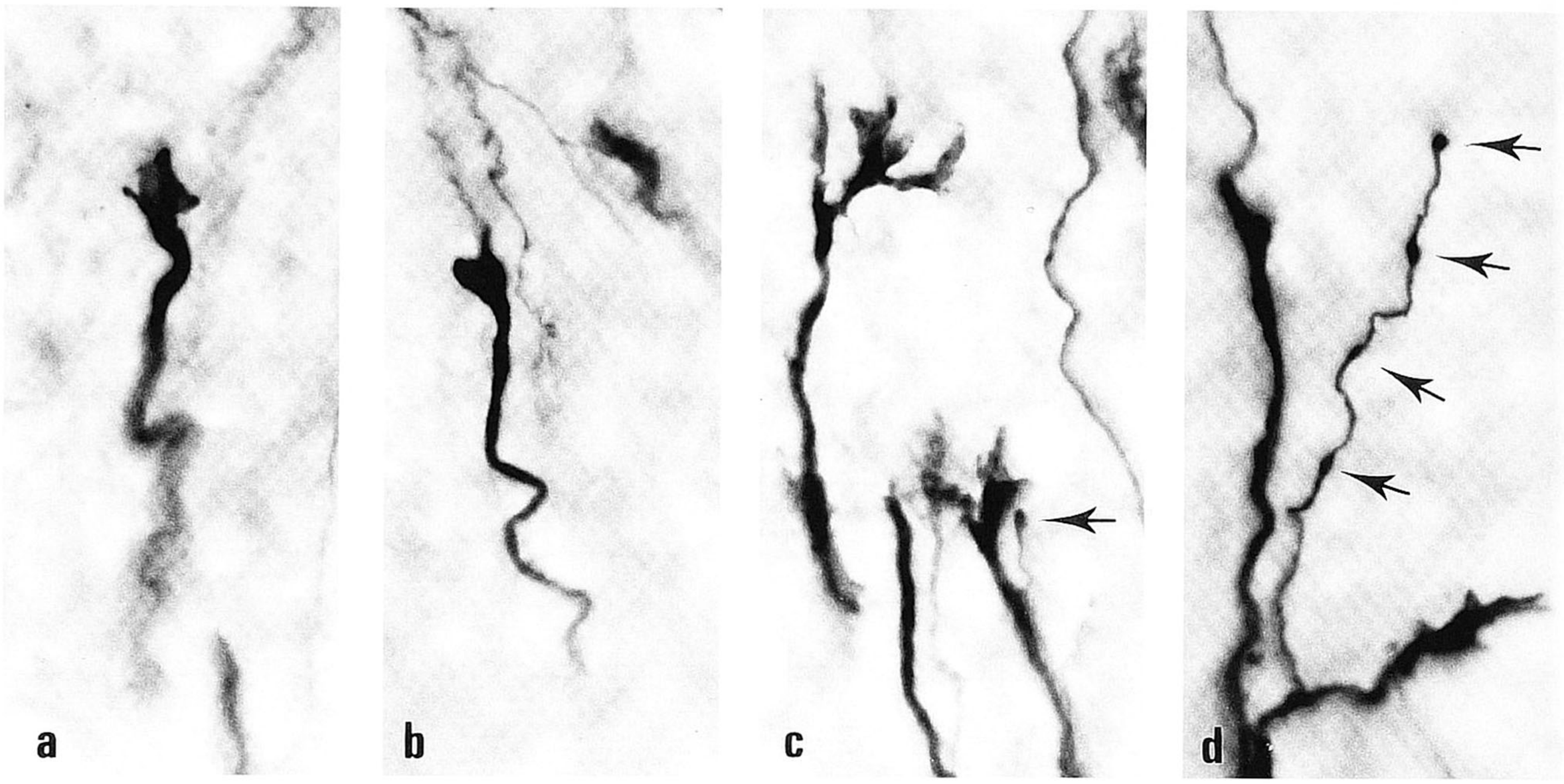
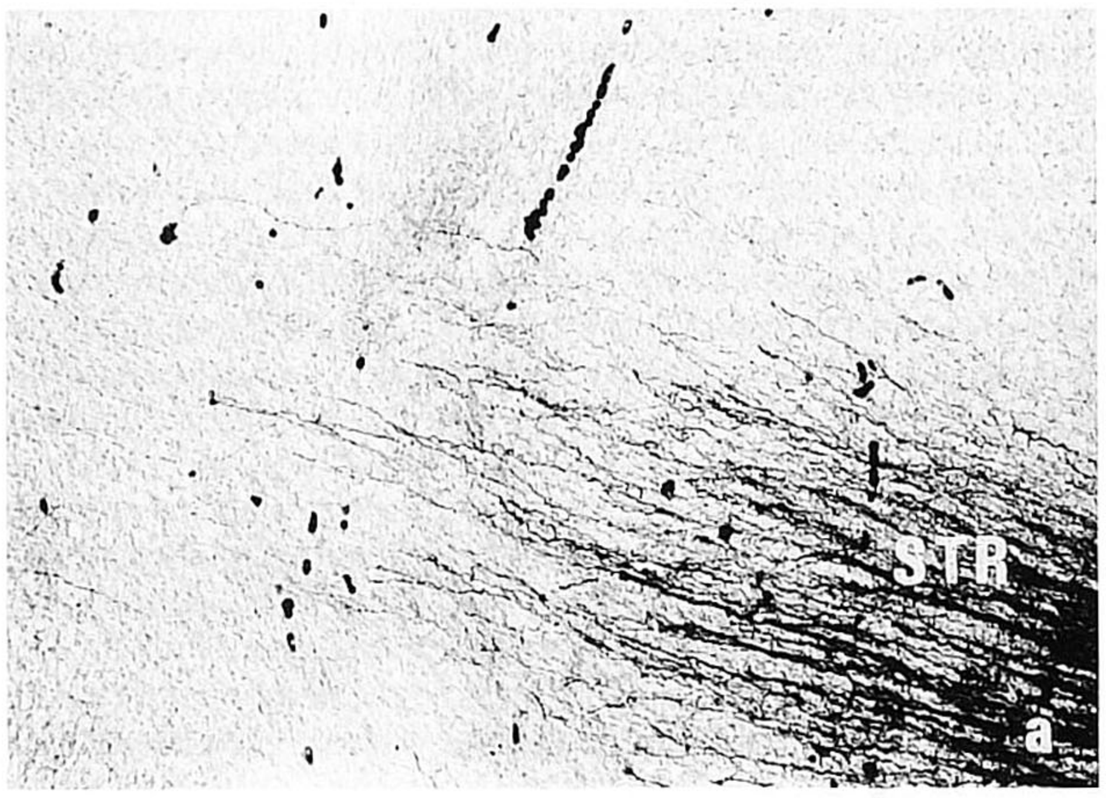


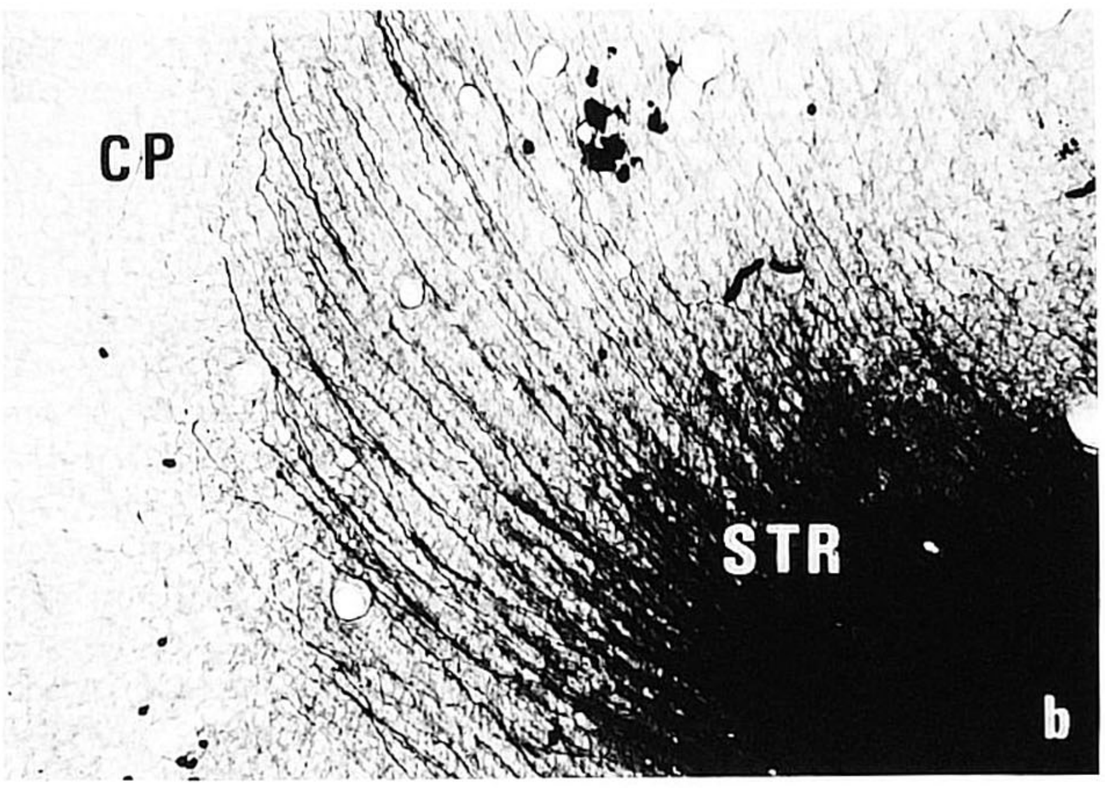
Fig. 10. In the growing axons DA is distributed all along the fiber, including the growth cone. **a**, E18; **b**, E18, **c**, E20, **d**, P0. Arrows point to varicosities. ×1000.

DISCUSSION

Development of cortical DA innervation

In the present study the distribution of DA immunoreactivity in the frontal cortex of pre- and postnatal rats was studied by using antibodies against DA. The specificity of the antibodies has been demonstrated previously by Geffard et al. ('84) and has been confirmed by the finding that preadsorption of the antiserum with DA coupled to Sepharose beads via a glutaraldehyde lysine bridge blocked all positive staining from the brain sections. On the other hand, serum absorbed with noradrenaline-covered beads revealed no differences in staining pattern as compared to the nonadsorbed antiserum (Buijs et al., '84). Only those fibers, however, that contain detectable levels of DA can be observed. It is therefore possible that future DA fibers are already present but are not observed because they contain no DA or undetectably low levels of DA. The results of the present study further extend the anatomical features of the development of the cortical DA innervation outlined in previous studies using immunocytochemistry of catecholamine-synthesizing enzymes (Schlumpf et al., '80b; Specht et al., '81; Verney et al., '82) or the selective uptake mechanisms of the DA system (Schmidt et al., '82) to visualize DA. These indirect methods, however, gave no definite answer to when DA itself is present. Our study shows some DA-positive fibers passing through the developing striatum to reach the anlage of the lateral cortex by E15. As the striatum develops the external capsule grows into a clear border, with a part of the DA fibers traversing through toward the frontal cortex (Fig. 11). For a detailed description of the pre- and postnatal development of the DA-positive fibers in the striatum we refer to the paper of Voorn et al. ('87). During development the dopaminergic fibers passing through the striatum concentrate more and more in the frontal cortex, as the direction of the fiber bundle shifts from rostrolateral to an almost entirely rostral direction. However, throughout development sparse DA fibers can be observed outside the PFC, as also described by Berger et al. ('85a) in young and adult rats. This rather widespread, although sparse, presence of DA fibers in the cortex might be an explanation for the recently demonstrated presence of D1 receptors throughout the forebrain in adulthood without a clear dopaminergic innervation (Boyson et al., '86; Martres et al., '85). As has already been mentioned in the present study, the first DA-positive fibers in the lateral cerebral wall could already be detected on E15, while other studies report E15–E16 (Schlumpf et al., '80b) or E16 (Verney et al., '82). In both studies E1 was designated as the day after the night of mating. Concerning the appearance of TH-like immunoreactive fibers in the medial wall, in the present study DA-positive fibers are also detectable one day earlier, E17 (in the present study) vs. E18 (Verney et al., '82). These findings indicate the DA is detected at an earlier stage than is one of its rate-limiting enzymes, TH. The same discrepancy is reported concerning the development of the noradrenergic cortical innervation. Schlumpf et al. ('80b), using fluorescence histochemistry, found the first positive fibers at E15–E16, whereas Verney et al. ('84), using dopamine-β-hydroxylase (the final enzyme in the synthesis pathway for noradrenaline) immunocytochemistry found the first NA fibers labeled on E17. The postnatal development of the dopaminergic innervation as described





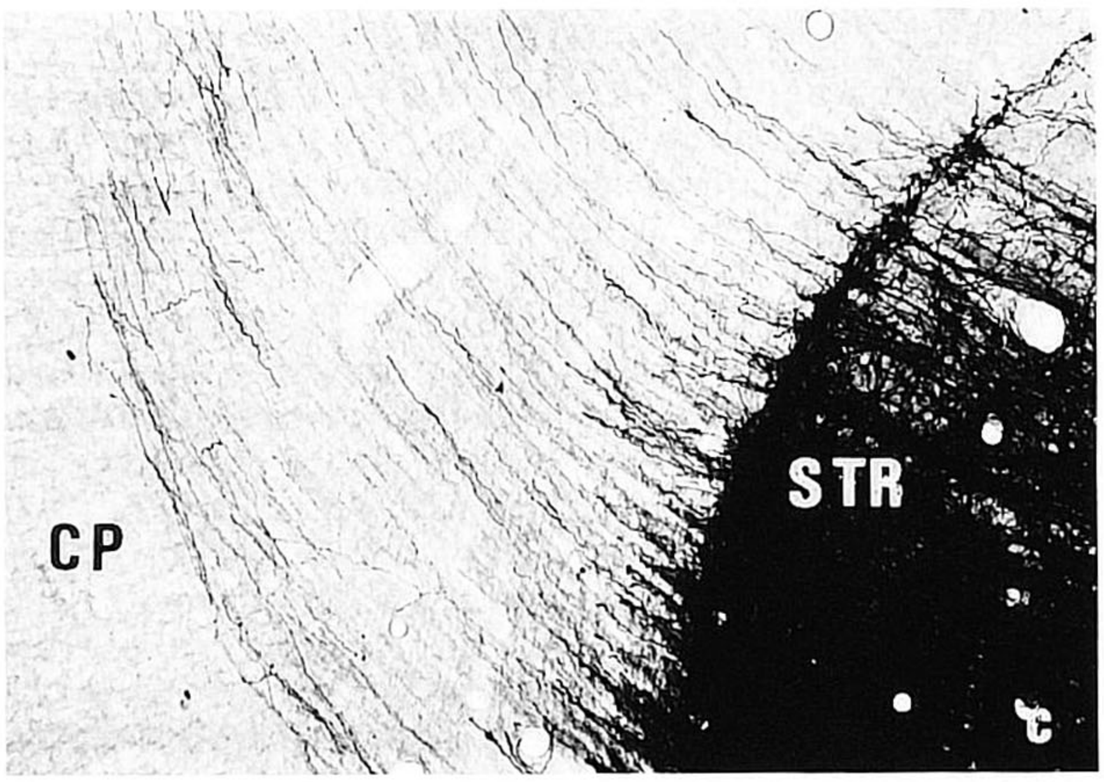


Fig. 11. DAergic fibers growing through the developing striatum. a, E16, b, E18, c, E20. ×100.

in the present study seems to be at variance with results of Schmidt et al. ('82), who described a density of the dopaminergic innervation in the lower cortical layers "well above that seen in the adult." They used, however, fluorescence histochemistry in combination with exogeneous administration of α-methylnoradrenaline, and thus showed fibers capable of DA uptake. This gives no indication of the endogenous DA level. As the maturation of the uptake mechanisms of monoamine terminals precedes and is independent of the mechanism for neurotransmitter synthesis (Coyle and Axelrod, '71; Coyle and Molliver, '77; Berger et al., '85), this results in a high dopamine uptake/endogenous dopamine ratio. It seems, therefore, that many future CA-containing fibers are already present in the frontal cortex, but these either do not yet contain the endogenous neurotransmitter or the major synthesizing enzymes, or they are present only in very low quantities. Because of the divergent methods employed and the different sensitivities of the immunohistochemical techniques, it is difficult to come to a definite conclusion. However, immunocytochemistry of CA-synthesizing enzymes (Molliver, '82) suggests a slower pattern of development of cortical innervation regarding the neurotransmitter of interest, whereas staining techniques using the selective uptake mechanism of the CA system (Schmidt et al., '82) suggest an earlier development.

In the present study DA-positive fibers could be detected before birth in the marginal zone (Fig. 5), which was also described by Verney et al. ('82) by using TH immunohistochemistry. However, the present study shows this location was transient and affected only the rostralmost fibers in the lateral and the medial cortical anlage of animals aged E17–E20. Not until after birth (P2–P4), is a permanent DA innervation of layer I established, and only in some subareas of the PFC. Before dopaminergic fibers start to cross the cortical plate around birth, many fibers can be observed with a typical twisted or helical shape in the subplate (Figs. 5,7). In the future medial PFC these typical fibers can be observed before birth (E20–E21), whereas in the orbital and supragenual part of the PFC these fibers are only observed after birth (P1–P2). The exact nature of this event is not known. It might reflect the interactive process of ascent into and through the cortical plate, and the onset of branching. It does not reflect the artifact of shrinkage, since in the same sections straight DA-positive fibers can be observed.

The regional differences in the distribution of dopaminergic fibers as observed in the adult rat (Van Eden et al., '87) develop in the first postnatal week. By the end of this week most of the adult characteristics of the topography of DA fibers can be recognized, despite the fact that the cortex has not yet developed fully and the cortical plate still contains cells of the developing layers II and III. The variation in the DA innervation of layer I and V makes possible a good distinction between the PFC subareas. The postnatal ontogenesis of the DA innervation in the anterior cingulate cortex has already been described in detail by Berger et al. ('85b). In the present study this developmental pattern is largely confirmed with respect to the appearance of DA fibers in the lower and superficial layers. The dopaminergic innervation of layer I in ACd, as was described by Berger et al. ('85b) in a young animal (P14), is somewhat surprising, since it is the reverse of the adult situation, in which the superficial layers I and II of the pregenual ACd contain only a few DA fibers (see also Van Eden et al., '87). In the present study, however, transverse DA-positive fibers in ACd could be observed to ascend through the cortical plate and branch in layer I only during the first week (Fig. 8), whereas later in development DA fibers were seen only rarely in this layer. This disappearance of DA fibers in layer I of the pregenual cingulate cortex could be an example of transient transmitter expression as also observed by Berger et al. ('85c) for TH in the medial and dorsolateral neocortex during postnatal development, or so-called trans-

mitter plasticity (Black et al., '84). Technical differences i.e., TH immunocytochemistry and catecholamine fluorescence histochemistry vs. DA immunocytochemistry or a somewhat different sensitivity of some noradrenergic fibers during development to the 6-OHDA treatment, might account for the difference in the postnatal period during which DA fibers can be observed in the superficial layers of the pregenual cingulate cortex as observed in this study and that of Berger et al. ('85b). In the second postnatal week an additional dopaminergic field develops in layer III of the supragenual PFC. The dopaminergic innervation, therefore, provides a clear distinction between the pre- and supragenual part of ACd from P12 on. In the superficial layers of PL and PrCm only a few DA fibers are present during early development. From P20 the density of DA fibers in the superficial layers of PL is clearly increasing, whereas in the superficial layers of PrCm only an occasional fiber is observed.

In the orbital cortex DA fibers have already reached the marginal zone a few hours after birth. But it is only after P6 that the number of DA-positive fibers is clearly on the increase. These frequently ramifying DA fibers are mainly restricted to layer I of the dorsal part of the agranular insular cortex.

The development of the cortical DA innervation as described in the present study differs strongly from that of the other monoamine systems. The developmental pattern of NA- or 5-HT-containing fibers consists of two tangential and parallel sheets of immunoreactive fibers above and below the cortical plate (Levitt and Moore, '79; Schlumpf et al., '80a,b; Specht et al., '81; Lidov and Molliver, '82; Verney et al., '84). There are also marked differences in the timetable of development (Crawford et al., '84; Verney et al., '84). The noradrenergic axons extend over the surface of the entire hemisphere at E20 and have reached adult densities by the end of the first postnatal week. Serotoninergic fibers, by contrast, do not cover all cortical areas until P3, and reach adult densities three weeks after birth (Lidov and Molliver, '82). The unique DA fiber pattern of the differing PFC subareas, as described in the foregoing section, also differs strongly from the fiber pattern of NA and 5-HT in these subareas. These last two systems are distributed over nearly the entire cortex and show quite a uniform distribution over the different PFC subareas (Lidov et al., '80; Steinbush, '81). Although NA shows a laminar distribution (Lindvall et al., '78; Lewis et al., '79) there are only minor regional differences in NA content (Palkovits et al., '79; Slopsema et al., '82).

Relationship between the development of the dopaminergic fiber pattern and the development of the prefrontal cortex

The DA-positive fibers seem to follow the prefrontal cortical development, as is indicated by the correlation between the cytoarchitectonial maturation of the PFC subareas and the dopaminergic fiber pattern. DA fibers invade only that part of the developing cortex that contains already-differentiated cells, while the upper cortical plate with undifferentiated, immature neurons remains free of DA fiber ingrowth (Fig. 2). The process of entering the cortical plate starts around birth as the future layer VI develops and lasts until about P12, when the cortical plate has disappeared, but before DA fibers have occupied all cortical layers. The same conclusion regarding the timetable of DA innervation and development of its target area was reached by Verney et al. ('87) with respect to the dopaminergic innervation of the septum and by Voorn et al. ('87) concerning the DA innervation of the striatum. It is difficult to correlate the developmental differences in the DA fiber pattern of the PFC subareas with earlier reports on functional and volumetric differences in the development of these subareas. These earlier observations indicated a later maturation of the orbital PFC as compared with the medial PFC, using purely volumetric development (Van Eden and Uylings, '85b). In addition, behavioral studies also suggest a later maturation of the orbital part (Nonneman and Corwin, '81). The cytoarchitectonic development, however, shows a somewhat different pattern; the cortical layers of the orbital cortex develop their adult characteristics at an earlier age as opposed to the medial PFC (Van Eden and Uylings, '85a). Although dopaminergic fibers start to invade the cortical plate somewhat earlier in the medial PFC, in later development there are no indications of differences in maturation of the orbital PFC compared with the medial PFC regarding the DA fiber pattern. The present study, coupled with biochemical data in rat (Loizou and Salt, '70; Keller et al., '73; Crawford et al., '84) and monkey (MacBrown and Goldman, '77; Goldman-Rakic and Brown, '82), point to a prolonged development of the dopaminergic innervation in the PFC. This might play a role in the relative late maturation and prolonged plasticity of the PFC in rats (Kolb, '84; Van Eden, '85) and monkeys (Goldman, '71), as opposed to other cortical areas. The dense and extensive innervation of both the prelimbic and dorsal agranular insular area is also striking. Both areas are innervated by the medial part of the thalamic mediodorsal nucleus, and they also have connections in common with several of the limbic structures. Therefore, DA might play an important role in the modulation of limboneocortical integration in these two areas.

Morphological changes in dopaminergic fibers during development

The beginning of the development of dopaminergic terminal field innervation is determined by the maturation of DA fibers to the adult form; the axons become much thinner and varicosities start to appear. The same morphological changes were observed by Fujimiya et al. ('86) in their study of the postnatal development of the serotoninergic innervation of mouse somatosensory cortex. In the 2 weeks that follow these changes in the rat, the number of varicosities per fiber is clearly increasing. Under the assumption that varicosities have synaptic contacts (Cooper et al., '82), these morphological changes may indicate that functional contact sites start to develop around birth. Although some studies indicate that monoaminergic terminal synapses are already present in newborn rats (Coyle and Molliver, '77) as well as functional monoaminergic postsynaptic receptors (Kellogg and Lundberg, '72), the maturation of the vesicular storage system of the catecholamines lags behind (Kirksey et al., '79). As dopaminergic receptors in the cortex are hard to detect with autoradiography even in adult animals (Altar et al., '85; Martres et al., '85; Dawson et al., '86), the precise moment at which dopaminergic receptors begin to appear in the cortex is still uncertain. Although dopaminergic receptors are present before birth in the striatum (Lanca et al., '86; Miller and Friedhoff, '86), recently Murrin et al. ('85) found no evidence of cortical DA receptors until P21. The morphological changes in the DA-positive fibers might suggest that specific neurotransmission sites

develop postnatally. In early prenatal development the dopaminergic axons in the subplate, however, might release their transmitter, which might then exert its action at a distance. As indicated by a study of Whitaker-Azmitia and Azmitia ('86), the interaction of neurotransmitters and their receptors may play an important role in the regulation of the direction and extent of axonal growth.

The present study shows DA is already present in the subplate of the developing PFC at E17 and that the unique pattern of dopaminergic innervation in the differing PFC subareas is established in early postnatal development along with the cytoarchitectonic development of the PFC. The reduction in cortical thickness of the PFC after neonatal lesioning of the DA mesocortical projection indicates a role of DA in the development of the PFC (Kalsbeek et al., '87a). Similarly, PFC-mediated behaviors may be disturbed after perinatal interference in the development of the DA mesocortical projection (Ahlenius et al., '77; Kalsbeek et al., '87b).

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