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The development of commissural connections of somatic motor-sensory areas of neocortex in the North American opossum

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Summary. The North American opossum does not have a corpus callosum; neocortical commissural axons are contained entirely within the anterior commissure. We have used axonal transport techniques to study the origin and distribution of commissural axons from somatic motor-sensory cortex in developing and adult opossums. Neocortical axons grow into the anterior commissure by postnatal day (PND) 12, the contralateral external capsule by approximately PND 19, the area deep to the contralateral homotypic cortex by approximately PND 26 and the cortex proper by approximately PND 35. Commissural neurons were first demonstrated at about PND 26, when they form a fairly continuous band in the cortical subplate (presumptive layers V–VI). By at least PND 37, commissural neurons are also present in layers II and III, where they form a continuous band, and in layer IV, where they are sparse. In older pouch young and adult opossums the bands of commissural neurons, especially in layers V–VI, are interrupted, and commissural neurons are rare in layer IV. In general, commissural axons in both pouch-young and adult opossums innervate areas containing commissural neurons as well as layer I.

In the acallosal opossum as well as in the callosal rat, the development of commissural connections from somatic motor-sensory cortex is characterized by pauses during the growth of axons into the opposite cortex, by a general inside-out-gradient, and by a transition from continuous bands to patchy, radial columns of commissural neurons and axons. This suggests that similar mechanisms govern the formation of commissural connections in the two species.

Key words: Development – Motor-sensory cortex – Commissural connections – Opossum

Introduction

The organization and development of interneocortical connections have received considerable attention in recent years. In the rat, for instance, axons from the somatosensory cortex have been shown to cross in the corpus callosum and to reach the contralateral homotypic cortex where they distribute in radial columns (Jacobson and Trojanowski 1974; Wise and Jones 1976, 1978; Akers and Killackey 1978; Ivy et al. 1979). Neurons giving rise to commissural axons are found in layers II–VI, but they are not uniformly distributed through all layers and are virtually absent in the barrel field of layer IV (Wise and Jones 1976; Akers and Killackey 1978). Callosal axons in the rat have reached the opposite side at birth, but do not grow into the cortex until about postnatal day 5 (Wise and Jones 1976; Killackey and Akers 1979). From birth to postnatal day 4, neurons giving rise to callosal axons are located only in the cortical subplate (presumptive layers V–VI) and, later on, in layers developed from the cortical plate, where they are found in clusters separated by gaps (Wise and Jones 1976; Ivy et al. 1979; Ivy and Killackey 1981). In both the rat (O'Leary et al. 1981; Ivy and Killackey 1982) and cat (Innocenti 1981),some of the neurons projecting through the corpus callosum in the neonate eventually lose or retract their callosal axon.

The North American opossum, like other marsupials, does not have a corpus callosum; interneocortical connections are contained entirely within the anterior commissure (Loo 1931; Ebner and Myers 1967; Martin 1967; Granger and Glendenning 1983). At birth (12 days after conception; McCrady 1938; Hartman 1952), the opossum's neocortex is very immature and an anterior commissure is not present. The entire development of interneocortical connections occurs postnatally. We have used axonal transport techniques to study the development of such connections in the opossum, specifically those from motor-sensory areas of neocortex. We sought to determine how the development of those connections in the acallosal opossum compares with that described for the callosal rat.

Materials and methods

Pouch-young opossums (*Didelphis virginiana*) were obtained from females captured in the wild or bred in captivity. The Snout-Rump length (S-R length) of each animal was measured by stretching it as much as possible on a ruler. Pouch-young from our colony were of known age, but their S-R length was measured to establish a growth curve. Our growth curve was comparable to that published

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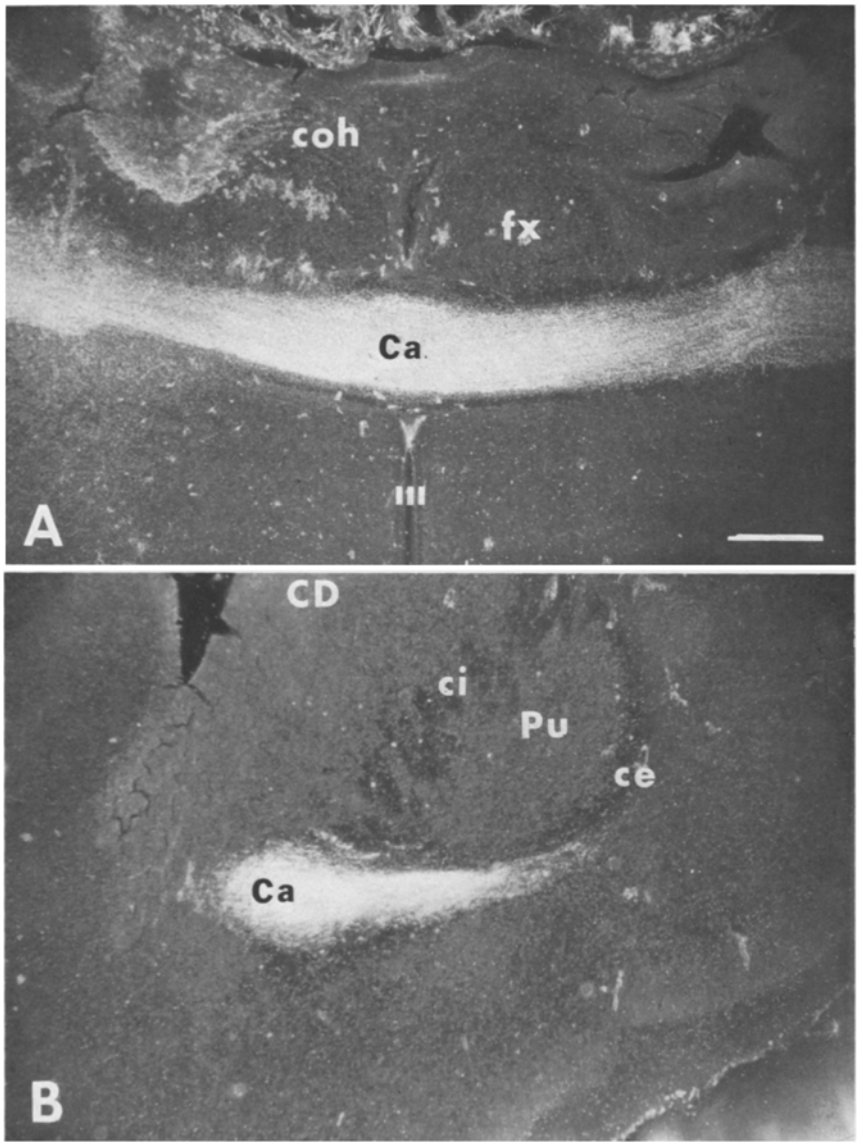

Fig. 1 A, B. Darkfield photomicrographs showing, in **A**, labeled axons crossing the midline in the anterior commissure (*Ca*) and, in **B**, entering the contralateral external capsule (*ce*) at a level rostral to the commissure. The micrographs were taken from an opossum subjected to injections of WGA-HRP at approximately PND 19 (41 mm S-R length). *CD* caudate nucleus; *ci* internal capsule; *coh* hippocampal commissure; *fx* fornix; *Pu* putamen; *III* third ventricle. The bar in **A** equals 220 μm

by Cutts et al. (1978) and both were used to estimate the age of animals obtained in the wild. Commissural connections of somatic motor-sensory cortex were studied in 51 pouch young from postnatal day (PND) 10 (28 mm S-R length) to estimated PND 87 (190 mm S-R length), about the time of weaning, with the following distribution: 7 animals from PND 10 to 20 (28 to 42 mm S-R length), 9 animals from PND 21 to 30 (43 to 56 mm S-R length), 13 animals from PND 31 to 40 (57 to 70 mm S-R length), 8 animals from PND 41 to 50 (72 to 85 mm S-R length) 7 animals from PND 51 to 60 (86 to 99 mm S-R length) and 7 animals from PND 61 to 87 (100 to 190 mm S-R length). Due to the variation in the size of littermates, which increases as the animals grow older, the size of littermates was averaged to estimate their age.

Wheat-germ agglutinin conjugated to horseradish peroxidase (WGA-HRP, Sigma type VI) was employed as a tracer of axonal connections. Pups were removed from the pouch and anesthetized with Metofane (2,2-Dichloro-1,1-difluoro ethyl ether) inhalation. Multiple injections of 10% WGA-HRP were made into the presumptive somatic motor-sensory cortex as identified in the adult opossum by Lende (1963a, b). The injections were made by pressure through a micropipette pulled to a tip diameter of 20–40 μm and attached to a 1 μl Hamilton syringe.

Following surgery the animals were kept in an incubator for 20–28 h after which they were anesthetized for sacrifice. Buffered saline at room temperature was injected intracardially followed by a cold solution of 1% paraformaldehyde-1.25% glutaraldehyde-1% sucrose in phosphate buffer. The brain was removed and kept in the cold fixative for 4–6 h before being transferred to a 20% sucrose-phosphate buffer solution.

The brains were sectioned transversely or sagitally at 40 μm on a freezing microtome and all sections were reacted for HRP using tetramethyl benzidine (TMB, Sigma) according to the technique of Mesulam (1978). In most cases the sections were mounted onto subbed slides prior to processing because sections from immature brains are very friable and difficult to mount subsequent to processing. This procedure was as sensitive as that applied to free-floating sections when the incubation times were doubled. The processed

Fig. 2. Montage of darkfield photomicrographs showing retrograde and orthograde labeling in the presumptive somatic motor-sensory cortex of an opossum subjected to injections of WGA-HRP in the contralateral cortex at approximately PND 26 (50 mm S-R length). Labeled neurons are seen in the cortical subplate (*CSP*), just beneath the cortical plate (*CP*). Labeled axons are seen deep to the cortical subplate. The *arrows* in the upper left indicate the dorsal (*D*) and lateral (*L*) directions. Note the ventrolateral to dorsomedial gradient in the density of labeling. The *curved arrow* points to the rhinal fissure. *"WM"* presumptive white matter. The bar equals 200 μm

tissue was counterstained with Neutral Red and examined with a Leitz microscope using both light and darkfield condensers.

Five adult opossums were subjected to cortical injections of 2% WGA-HRP. They were anesthetized with sodium pentobarbital (40 mg/kg) and stabilized in a stereotaxic frame. Multipe injections were made into all areas of motor-sensory neocortex (Lende 1963a, b) with a glass pipette attached to a 1 or 10 μl Hamilton syringe. Three days later the animals were anesthetized, perfused and sacrificed as were the pouch-young. Their brains were sectioned and processed free-floating. The delineation of cortical laminae in the adult opossum was after Gray (1924) and Walsh and Ebner (1970). Nissl preparations of brains from pouch-young and adult opossums were available for reference.

Results

In Nissl preparations evidence for an anterior commissure can be found by postnatal day (PND) 4 (22 mm S-R length). Although large cortical injections were made as early as PND 10 they did not label the anterior commissure. At PND 12 (31 mm S-R length), comparable injections labeled axons which could be traced across the midline, but not into the contralateral external capsule. When injections were made at approximately PND 19 (41 mm S-R length)

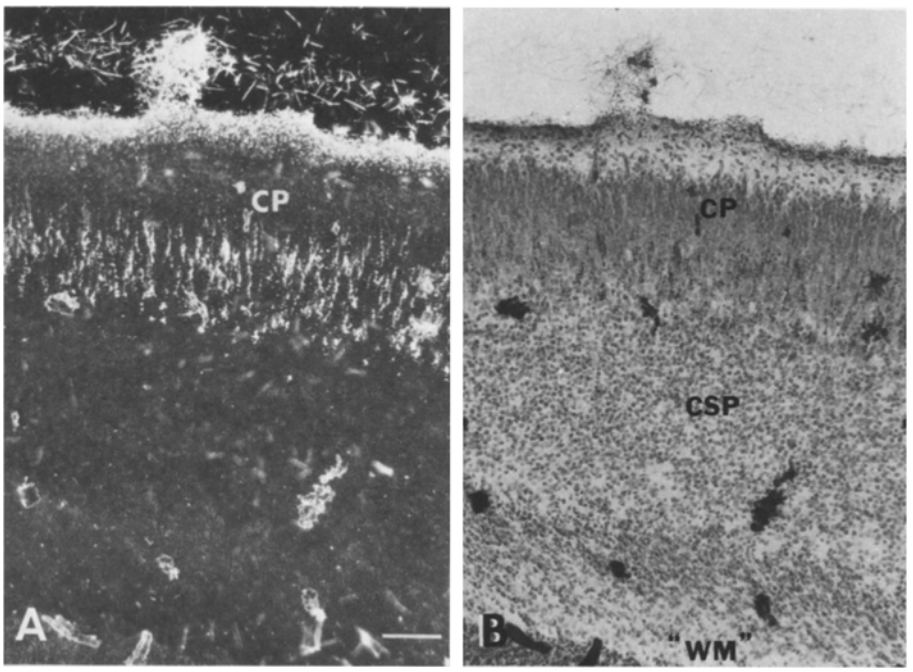

Fig. 3A, B. Darkfield (**A**) and lightfield (**B**) photomicrographs showing labeled commissural neurons in the cortical subplate (*CSP*) of a 50 mm (S-R length) littermate of the one used for Fig. 2. *CP* cortical plate, *"WM"* presumptive white matter. The bar in **A** equals 80 μm

labeled axons extended across the anterior commissure (Fig. 1A) and into the ventral part of the contralateral external capsule (Fig. 1B). It was not until about PND 26 (50 mm S-R length), however, that labeled axons were found deep to the homotypic cortex on the contralateral side.

Commissural neurons were first labeled at approximately PND 26 (50 mm S-R length). In cross sections of the presumptive somatic motor-sensory cortex, labeled neurons (Figs. 2, 3) formed a band just deep to the cortical plate (the cortical subplate of Kostovic and Molliver 1974). The neurons labeled in the deeper portion of the band were less densely packed and smaller than those in the outer portion. Processes of labeled neurons often extended between unlabeled neurons of the cortical plate. Our observations on the pattern of commissural labeling in gradually older pouch-young led us to conclude, as did Ivy and Killackey (1981) for the rat, that the outer portion of the band becomes the outer part of layer V and the inner portion becomes deep layer V and outer layer VI.

Nissl preparations revealed that neocortical maturation followed a general ventrolateral to dorsomedial gradient. This gradient was reflected in the pattern of commissural labeling. At about PND 26, the band of labeling was thickest and most dense ventrolaterally (Fig. 2), and in that area an occasional neuron was labeled in deep parts of the cortical plate.

By approximately PND 35 (64 mm S-R length) commissural neurons could be labeled in large numbers in the cortical subplate where they formed a wide bilaminar band (Fig. 4). The outer lamina (presumptive outer part of layer V) was thick and densely packed with labeled neurons whereas the inner one (presumptive deep layer V and outer layer VI) was thinner and less densely packed. Columns of labeled neurons bridged the two laminae. Pyramidal-shaped neurons were labeled along the interface between the cortical subplate and the cortical plate proper. These neurons were smaller than those belonging unequivocally to the outer subplate (presumptive outer layer V) and they probably belonged to layers IV and/or III. Such labeling was most obvious ventrolaterally. It was sometimes difficult to distinguish orthograde from retrograde labeling (see Discussion) but, when distinct, labeled axons were seen in areas which contained labeled neurons and in layer I.

In a slightly older animal (estimated PND 37, 66 mm S-R length) injected with a lesser amount of the marker, neuronal labeling was not obscured by axonal labeling (see Discussion). The two laminae described above were present (Fig. 5) but a third one could also be distinguished just deep to layer II (open arrow in Fig. 5A), and on that basis was judged to be layer III. It was separated from the cortical subplate (presumptive layers V and VI) by an area relatively free of labeling which we presume to be layer IV. Layers III and IV could not be distinguished histologically from one another. Gaps devoid of labeling were present in the deepest lamina (presumptive inner layer V and outer layer VI). The three laminae were occasionally bridged by radial columns of labeled neurons and axons.

By about PND 42 (77 mm S-R length) all cortical layers could be distinguished easily. Labeled neurons were numerous and formed a dense, continuous band in layer III and, to a lesser extent, in layer II (Figs. 6, 7). In contrast, labeled neurons were less numerous in layers V and VI, particularly layer V, and gaps devoid of labeling were present. Labeled neurons were generally sparse, sometimes absent, over large areas of layer IV, but in some regions they formed dense columns which bridged patches of labeling in supra- and infragranular layers (arrows in Figs. 6, 7).

The pattern of labeling in older pouch young and adult (Fig. 8) opossums was essentially the same as that described for the 77 mm pouch-young and conforms to that reported by Foster et al. (1981) and Granger and Glendenning (1983). Labeled axons were found in regions containing labeled neurons as well as within layer I. In the adult opossum, however, both neuronal and axonal labeling appeared less dense than in the pouch young.

Individual variation in the pattern and density of labeling was observed in pouch young from the same or different

Fig. 4. Montage of darkfield photomicrographs showing retrograde and orthograde labeling in the somatic motor-sensory cortex of an opossum subjected to injections of WGA-HRP in the contralateral cortex at approximately PND 35 (64 mm S-R length). Labeled commissural neurons form a bilaminar band beneath the cortical plate (*CP*). The outer lamina of the band (*ol*) is presumed to differentiate into outer layer V and the inner lamina (*il*) into deep layer V and outer layer VI. The *arrow* points to the rhinal fissure. "*WM*" presumptive white matter. The bar equals 200 μm

litter(s). Although littermates vary in size, especially as they grow older, it is worth noting that larger littermates did not necessarily display a more mature pattern of labeling than smaller ones. In addition to genuine individual variation, differences in the pattern and density of labeling could be due to the variable amounts of WGA-HRP injected as well as to technical variations. The latter are discussed below. In spite of the individual variations, the sequence of development seemed obvious.

Discussion

Technical considerations

Use of WGA-HRP together with TMB processing allowed us to label both afferent and efferent connections in the same experiment. In some cases, however, orthograde labeling was poor even though retrograde labeling was good. The converse was never observed. The presence of good orthograde labeling seemed to be related to the amount of WGA-HRP injected. However, in cases with massive injections, orthograde labeling was so dense that it obscured the pattern of retrograde labeling.

Effective inclusion of the somatic motor-sensory cortex in the injection was assessed by examining the thalamic labeling except in the youngest animals, in which thalamic labeling was not present. The injections were considered sufficient only when the ventrobasal complex was entirely labeled (retrogradely and orthogradely). Small injections produced labeling restricted to the contralateral homotypic cortex, but the labeling pattern was better evidenced when the injections covered all of the motorsensory cortex. Spread of the marker to cortical areas outside the somatic motorsensory cortex or to the basal ganglia did not produce a pattern of labeling in the contralateral neocortex different from that obtained when there was no such spread. Occasionally the tracer was inadvertently injected into the lateral

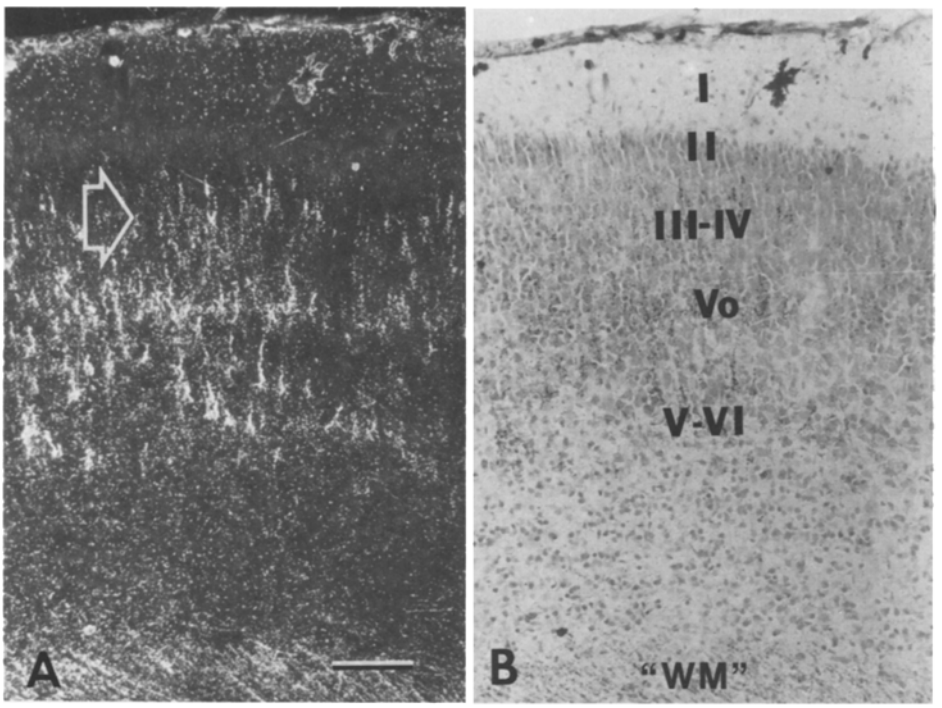

Fig. 5A, B. Darkfield (**A**) and lightfield (**B**) photomicrographs showing retrograde and orthograde labeling in the somatic motor-sensory cortex of an opossum subjected to injections of WGA-HRP in the contralateral cortex at approximately PND 37 (66 mm S-R length). Labeled commissural neurons are seen in presumptive layers *V* and *VI* of the cortical subplate *Vo* in **B**: presumptive outer layer *V* and in presumptive layers *III–IV* (*arrow*) of the cortical plate. "*WM*" presumptive white matter. The bar in **A** equals 100 μm

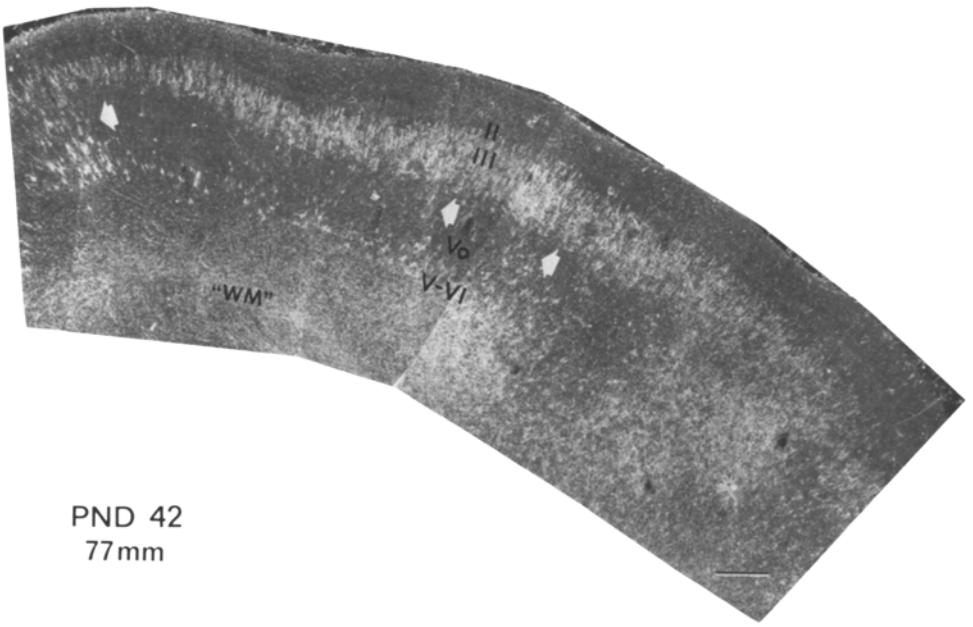

Fig. 6. Montage of darkfield photomicrographs showing retrograde and orthograde labeling in the somatic motor-sensory cortex of an opossum subjected to injections of WGA-HRP in the contralateral cortex at approximately PND 42 (77 mm S-R length). Labeled commissural neurons are seen mostly in layers *II–III* and *V–VI*, but also in layer *IV* where in some regions they form bridges (*arrows*) between supra- and infragranular layers. "*WM*" white matter. The bar equals 200 μm

ventricle, resulting in spread through the ventricular system. When artifactual, background labeling obscured the true pattern of commissural labeling, the cases were rejected. Nevertheless, moderate amounts of ventricular contamination did not produce artifactual labeling.

The development of commissural connections

In the pouch-young opossum, commissural axons enter the anterior commissure by at least PND 12, but they are not found deep to the contralateral homotypic cortex until about PND 26 or in the cortex proper until approximately PND 35. The development of commissural connections occurs more rapidly in the rat (Ivy and Killackey 1981), but in both the opossum and rat, it is characterized by pauses. Possibly, commissural axons wait for their targets to reach the appropriate stage of maturation before growing into them.

Commissural neurons can be retrogradely labeled about one week before their axons penetrate the cortex proper. The first commissural neurons labeled were located deep to the cortical plate, in an area presumed to differentiate into layers V and VI (Rice and Van der Loos 1977). Judging by their relative maturity, such neurons were probably not migrating to the cortical plate. Migration seems to have largely terminated at that stage of development.

Although it is difficult to define exactly when neurons of the cortical plate are first labeled, they are labeled later

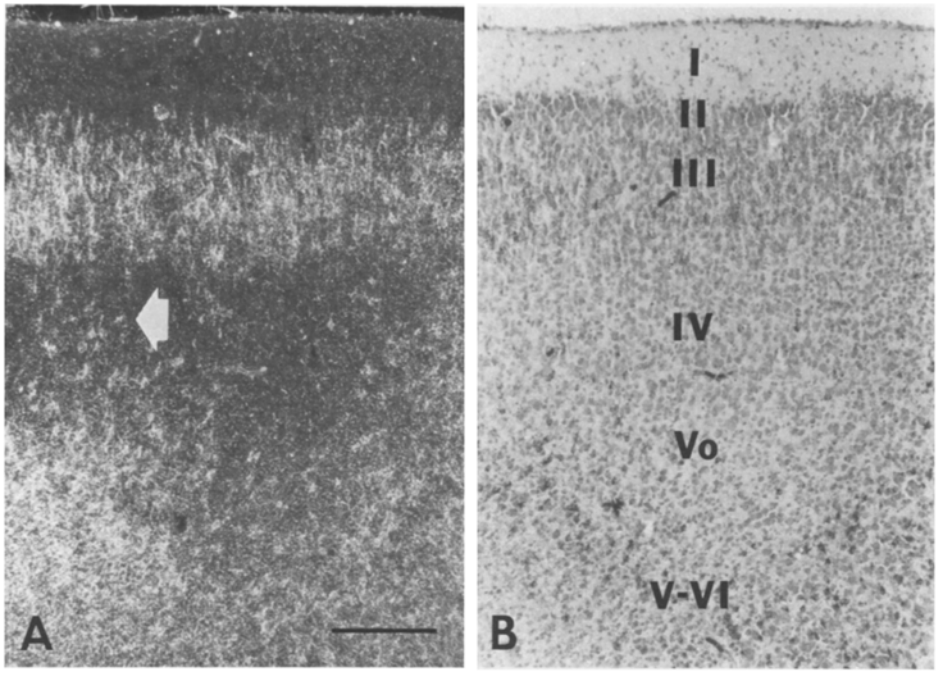

Fig. 7A, B. Higher power darkfield (**A**) and lightfield (**B**) photomicrographs of a field from Fig. 6. The bar in **A** equals 200 μm

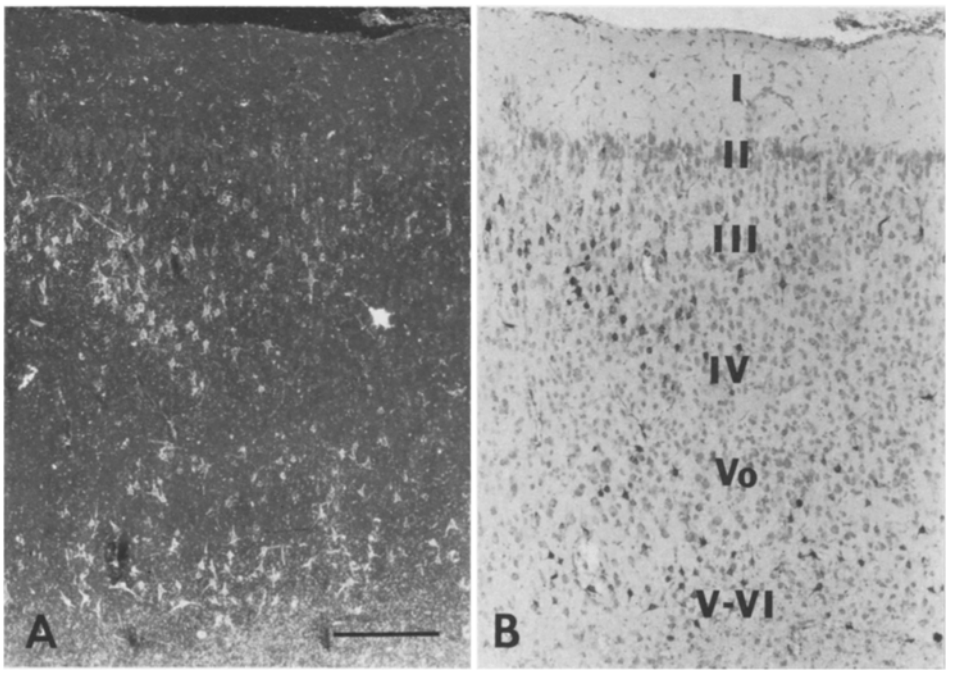

Fig. 8A, B. Darkfield (**A**) and lightfield (**B**) photomicrographs showing the retrograde and, to a lesser extent, orthograde labeling in the somatic motor-sensory cortex of an adult opossum subjected to injections of WGA-HRP in the contralateral cortex. The bar in **A** equals 200 μm

than those in the subplate. The development of commissural connections in the opossum thus follows a general inside-out-gradient, as reported for the rat (Ivy and Killackey 1981). It is generally accepted that the migration and maturation of cortical neurons follows an inside-out gradient (Angevine and Sidman 1961; Berry and Rogers 1965), so it is not surprising that a similar gradient exists in the formation of commissural connections.

Initially, numerous neurons give rise to commissural projections, resulting in a continuous, dense band of retrograde labeling. As development proceeds, the density of labeled neurons decreases and gaps interrupt the continuous band. The decrease in labeling density of deeper layers is already apparent when superficial layers are just beginning to be labeled. The transition from a continuous to a patchy pattern of labeling has been described for commissural projections of the somatosensory cortex of the rat (Ivy and Killackey 1981) and cat (Innocenti and Caminiti 1980) as well as the visual cortex of the rat (Olavarria et al. 1983) and cat (Innocenti et al. 1977). The apparent decrease in the number of labeled commissural neurons could be due to cell death and/or the loss of commissural collaterals from neurons which do not die but retain axonal projections to other targets. The latter possibility has been shown to

occur in the developing rat (O'Leary et al. 1981; Ivy and Killackey 1982) and cat (Innocenti 1981). It is of interest that removal of an eye in the neonatal rodent results in the maintenance of the continuous pattern of commissural labeling in the visual cortex (Rhoades and Dellacroce 1980; Rothblatt and Hayes 1982; Olavarria et al. 1983), suggesting that it is possible to induce retention of transient connections and that plasticity exists in the development of commissural connections.

Interneocortical connections in the rat and the opossum take different routes. Nevertheless, their development follows the same general pattern, suggesting that similar mechanisms govern the formation of commissural projections in both species. In the opossum, however, the development of interneocortical connections occurs postnatally and over a relatively long period of time. It would appear, therefore, that the opossum would make a good model for studies of commissural plasticity.

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