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Organization of pioneer retinal axons within the optic tract of the rhesus monkey

(retinal axon ingrowth/pathfinding/decussation pattern/prenatal development)

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ABSTRACT **Retinal ganglion cell axons must make a decision at the embryonic optic chiasm to grow into the appropriate optic tract. To gain insight into the cues that play a role in sorting out the crossed from the uncrossed optic axons, we investigated the sequence of their initial ingrowth in rhesus monkey embryos. Two carbocyanine dyes, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate and 4-(4-dihexadecylaminostyryl)-*N*-methylpyridinium iodide, were placed, respectively, into the left and right retinas to identify the course of uncrossed and crossed retinal axons through the optic chiasm and tract. Our results show that at embryonic day 36 the most advanced retinal projections are uncrossed. At this age the leading crossed axons are just reaching the chiasmatic midline, whereas the uncrossed fibers have already entered the optic tract. This indicates that the pathfinding of these pioneer uncrossed fibers does not require the presence of retinal axons from the opposite eye. At subsequent stages of development (embryonic days 40 and 42) there is a clear partial segregation of the uncrossed and crossed retinal axons within the optic tract: the uncrossed-component course is in the deeper portion of the optic tract, whereas the crossed component lies in a more superficial region. Thus, the spatial organization of retinal axons within the primordial optic tract reflects the sequential addition of the uncrossed and crossed retinal fibers. The orderly and sequential ingrowth of these pioneer retinal axons indicates that specific chiasmatic cues are expressed early in development and that such pioneer fibers may serve as guides for the later-arriving retinal fibers.**

In primates the chiasmatic routing of retinofugal axons is highly stereotyped, so that ganglion cells located in the temporal retina send uncrossed axons, whereas all ganglion cells in the nasal retina send crossed axons (1). Retrograde tracing experiments have revealed that this nasotemporal decussation pattern is remarkably precise in fetal rhesus monkeys (2). Thus, about 100 days before birth, at embryonic day 69 (E69), very few retinal axons were found to project to the inappropriate hemisphere. Clearly, the retinal location of ganglion cells can provide an important clue to the chiasmatic choice of axons.

However, at earlier stages of development retinal ganglion cells which form the initial contingent of the crossed and uncrossed fibers in the optic tract are largely intermingled within the embryonic primate retina (3). Such an intermingling has also been reported in the mouse (4, 5) and could constitute a general characteristic of the early projection pattern of mammalian retinal ganglion cells. Thus, if the chiasmatic decision of retinal axons is critically dependent upon the location of their parent ganglion cell in the developing retina, it might be expected (given the initial intermixing of the ipsilaterally and contralaterally projecting ganglion cells) that the first ingrowth of retinal fibers into the tract would be largely random in organization. On the other hand, if the early retinal fibers are organized in an orderly fashion within the optic tract, this would argue that retinal position is not a critical factor in the chiasmatic choice of the first contingent of retinal fibers. As yet, however, nothing is known about the organization of uncrossed and crossed retinal axons that form the primordial optic tract.

In primates, retinal ganglion cells are generated over an extended period of time (6) which offers an opportunity of examining the chiasmatic choice of the very first retinal axons forming the optic tract. In the present study, we have used carbocyanine dyes to examine the organization of the initial contingent of retinal axons that form the optic tract of the rhesus monkey. A major advantage of such dyes is their passive diffusion along axonal membranes in fixed specimens (3). Consequently, they can be used to study very early projection patterns in fixed tissue obtained from embryos too fragile to withstand injections of conventional anatomical tracers. In particular, we sought to compare the temporal and spatial properties of crossed and uncrossed axons in order to relate the ingrowth pattern of such pioneer fibers to the mechanisms that have been postulated to underlie the formation of retinal decussations.

MATERIALS AND METHODS

Four fetal rhesus monkeys were employed in the present study at E30, E36, E40, and E42 (gestational period, 165 ± 2 days). Timed-pregnant monkeys were obtained from the California Primate Research Center. Gestational ages were confirmed before surgery in pregnant monkeys anesthetized with ketamine hydrochloride (10 mg/kg). An estimation of the crown–rump length of each embryo was performed by ultrasonographic measurements (Table 1). Subsequently, under halothane and nitrous oxide anesthesia, the embryo was removed by caesarean section. A detailed description of the surgical procedures has been reported elsewhere (7). After the amniotic membrane and the visceral yolk were taken away, embryos were placed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and stored at 4°C for several days. Cornea and lens were then discarded and crystals of two lipophilic carbocyanine dyes, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (diI) and 4-(4-dihexadecylaminostyryl)-*N*-methylpyridinium iodide (diA), were implanted in the left and right retinas, respectively. For the youngest embryo (E30), crystals of 3,3′-dioctadecyloxacarbocyanine perchlorate (diO) were used instead of diA. Heads were stored in fixative in the dark for 5–11 weeks at 37°C. The storage time varied with the size of the specimen (Table 1). Subsequently, the whole head or only the brain was embedded in 4% agar, depending on age. Horizontal sections

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Abbreviations: diI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; diA, 4-(4-dihexadecylaminostyryl)-*N*-methylpyridinium iodide; E*n*, embryonic day *n*.

Table 1. Ages of the fetuses, their crown–rump length, and the storage time with dyes

Age	Length, mm	Storage time, weeks
E30	9	5
E36	15	5
E40	20	6
E42	22	8

were cut at a thickness of 100–200 μm with a Vibratome through the whole head of the E30 and E36 embryos to examine labeled fibers in the optic stalk and in the chiasm. For the older specimens (E40 and E42), a two-step procedure was performed to section the brain. Horizontal sections were first taken from the ventral aspect of the brain at the level of the optic chiasm. The block was then removed, trimmed, and rotated so that the optic tracts could be cut coronally. Sections were collected in phosphate buffer and mounted in the same medium onto gelatinized slides for observation. They were examined with an epifluorescence microscope (Nikon) equipped with two sets of filters. A rhodamine filter set was used to view dil-labeled fibers and a fluorescein filter set to observe diA-labeled axons. The distribution of labeled retinal fibers was recorded with Ektachrome 800 or 1600 film.

RESULTS

To characterize the sequence of uncrossed and crossed retinal axon ingrowth during early development, we examined the organization of retinal projections within the optic chiasm and tract from E30 to E42. In the rhesus monkey the production of the first ganglion cells occurs at about E27 (6). Retinal axons enter the optic stalk—the precursor of the optic nerve—shortly after the first ganglion cells are born (8). At E30 we failed to observe any labeled axons in the optic stalk even though the dil injection covered the entire retina. Since the lateral diffusion of the dye spread to the base of the optic stalk, this could have masked the very first retinal fibers within the stalk. By E36, the trajectories of individual axons within the optic stalk could be traced to the level of the future optic chiasm. By this age, the optic stalk was clearly obvious, with its lumen in continuity with the third ventricle (data not shown). Fig. 1*A* shows that at a position where the optic stalk joins the diencephalic wall, the labeled retinal axons were highly dispersed. This particular section illustrates the distribution of optic fibers within the ventral part of the optic stalk where most of the retinal growth cones are located at early stages (9). Examples of labeled fibers ending with a growth cone are shown in Fig. 1*B*. The trajectory of the labeled axons, located in the medial aspect of the optic stalk, was deflected at the entry point of the diencephalic wall (Fig. 1 *A* and *B*). These pioneer retinal fibers were seen to make a turn laterally and caudally to reach the pial margin of the brain. This reorientation occurred 150–430 μm from the midline. More caudally, the labeled retinofugal fibers formed a fasciculated bundle lying near the pial margin of the diencephalic wall (Fig. 1*A*). These tightly bundled axons formed the initial contingent of the ipsilateral optic tract. This uncrossed component of the retinal projections continued up to 600 μm within the optic tract (data not shown). Importantly, this uncrossed pathway was formed before any crossed fibers had reached the optic tract. Fig. 1*B* shows that labeled retinal axons, at varying distances from the ipsilateral optic tract, occasionally made a turn back in the rostromedial direction to reach the vicinity of the midline. A few labeled fibers could be followed ventrally in the vicinity of the future chiasm, ending in growth cones just across the midline (Fig. 1 *C* and *D*). These leading fibers represent the pioneer crossed retinal axons. The crossed axons grew in close apposition to the marginal zone, near the pial surface of the diencephalon (Fig. 1*D*). This suggests that at E36 the first retinal fibers to cross the future chiasm grow into an extracellular space which includes radial glial endfeet (10). Within the vicinity of the future chiasm, clusters of labeled cells were observed in the vicinity of the labeled axons (Fig. 1*C*). These labeled profiles could represent transcellularly labeled neurons or radial glial cells.

By E40 and E42 numerous retinal axons had crossed the midline. These crossed axons lay within the rostrolateral portion of the optic tract, near the pial surface of the brain (Fig. 2*A*). In contrast, the uncrossed component of the retinal projection was located mainly in the caudomedial region of the optic tract, corresponding to its deeper portion. Fig. 2*A* clearly indicates that the crossed retinal axons were partially segregated from the uncrossed retinal fibers along the deep–superficial axis of the optic tract. To determine whether this partial segregation of retinal axons observed at the entry point of the optic tract was maintained along the entire tract, the spatial distributions of uncrossed and crossed axons were analyzed in coronal sections. In this orientation the total extent of labeled retinal axons could be observed in single sections (Fig. 1 *E* and *F*). The trajectory of crossed retinal fibers was mainly confined within the lateral portion of the optic tract, up to their distal end (Fig. 1*E*). In contrast, the uncrossed component lay in its whole extent in the innermost part of the optic tract (Fig. 1*F*). A few crossed and uncrossed retinal axons appeared in close apposition within the marginal zone (Fig. 1 *E* and *F*). Occasionally, labeled fibers were seen to make a sharp turn medially, toward the ventricular zone (Fig. 1*F*). The composite drawing shown in Fig. 2*B* indicates that the most numerous uncrossed retinal fibers tend to avoid the superficial portion of the optic tract where the crossed component is located. The overlap zone, defined as the region of the optic tract where uncrossed and crossed retinal axons are intermixed, is restricted to its intermediate portion and to the marginal zone (Fig. 2*B*). Thus, these results demonstrate that the initial contingent of crossed and uncrossed retinal fibers remain largely segregated from their entry point to their distal end, within the developing optic tract.

DISCUSSION

In this study we have described the organization of the initial contingent of retinal fibers which navigate through the optic chiasm to form the primordial optic tract of the embryonic rhesus monkey. The ingrowth pattern of these pioneer retinal fibers strikes us as remarkably specific in two major respects. First, the uncrossed fibers enter the optic tract well in advance of the crossed fibers, an observation which has been previously reported in the embryonic mouse (3). Indeed, some 6 days after crossed axons have reached the chiasmatic midline, there are still only a few such fibers in the tract compared with the substantial number of uncrossed axons. Second, when the crossed axons begin to enter the tract they are largely segregated from the uncrossed contingent of fibers. This provides evidence that crossed and uncrossed retinal fibers are spatially segregated within the primordial optic tract. Thus, the crossed and uncrossed retinal projections, which initially form the optic tract of the primate, are characterized by distinct temporal and spatial ingrowth patterns.

This organization of the initial contingent of optic fibers in the rhesus monkey embryo has several developmental implications. First, it implies some type of recognition among fibers which form the initial crossed as well as the uncrossed components of the optic tract. The orderly distribution of both contingents as they first grow into the optic tract is

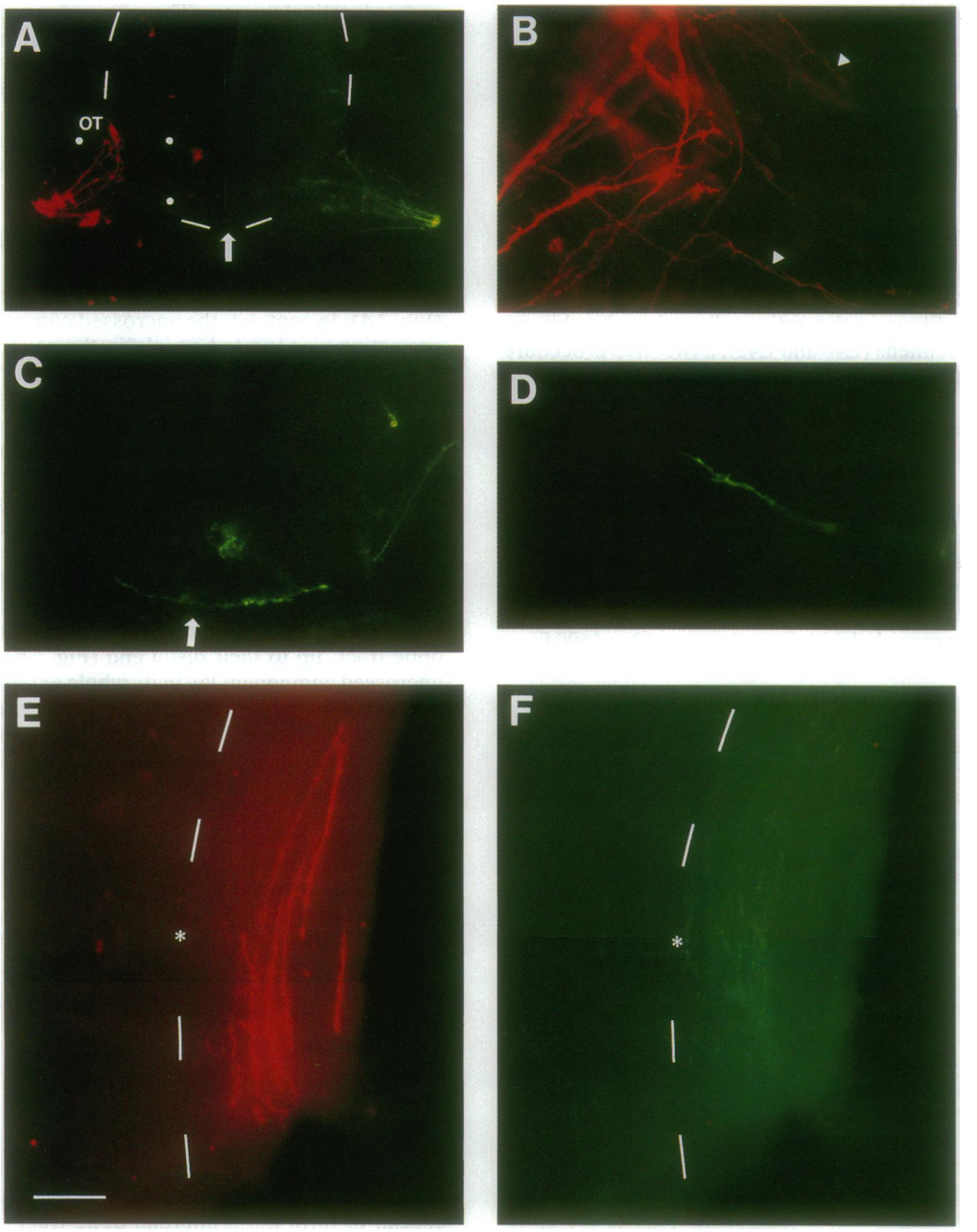

FIG. 1. Distribution of uncrossed and crossed retinal axons in two E36 and E42 embryos after labeling of the entire left and right retinas with diI (red fluorescence) and diA (green fluorescence), respectively. In each photomicrograph from A–D anterior is located at the bottom. (*A*) Horizontal view of the ventral part of the optic stalks at their junction point with the diencephalon, showing the trajectory of labeled retinal axons as they first reach the brain (by E36). The left side of the photomicrograph illustrates diI-labeled retinal axons viewed with the rhodamine filter, whereas on the right are diA-labeled fibers from the opposite eye observed at the same focus with the fluorescein filter. Uncrossed labeled retinal axons can be seen entering the optic tract (OT) at that stage. Arrow points to the midline, and the dashed line indicates the lateral and ventral borders of the diencephalon. (*B*) Higher magnification of the region delineated in *A* by white points. The trajectory of diI-labeled retinal axons (some of them tipped with growth cones) is illustrated at the site where putative crossed retinal axons (arrowheads) are turning back toward the diencephalic midline. (*C*) Horizontal section through the ventral part of the presumptive optic chiasm, showing the most advanced labeled crossed axons. The chiasmatic midline is delineated by the arrow. (*D*) Higher magnification of the diA-labeled axon illustrated in *C*, tipped with a growth cone. (*E*) Coronal section viewed with the rhodamine filter through the rostral portion of the right optic tract in an E42 embryo. The diI-labeled crossed axons come from the lower right, a region of the optic tract located 200 μm above the chiasm, and extend dorsally (upper right). Asterisk shows diverging retinal axons labeled with diA, indicating that there is some bleed-through of the green emission when viewed with the rhodamine filter. (*F*) Same coronal section as in *E*, observed at the same focus with the fluorescein filter, showing diA-labeled uncrossed axons. As in *E*, the diverging diA-labeled axons are shown with an asterisk. In *E* and *F* the dashed line indicates the inner border of the optic tract. [Bar (in *E*) = 250 μm (*A*), 50 μm (*B*), 100 μm (*C*), 25 μm (*D*), or 100 μm (*E* and *F*).]

unlikely to be due to random events. More likely, crossed and uncrossed fibers are able to recognize other members from their contingents as they navigate through the optic chiasm. Second, the ganglion cells from which these initial fibers are derived have been shown to be regionally intermingled within the embryonic retina (5). Consequently, at this early stage of development, it seems unlikely that retinal position can provide the relevant cues for the navigational strategies employed by these pioneer axons. Third, the temporal and spatial segregation of crossed and uncrossed fibers within the optic tract suggests that the ingrowth of axons into the tract is not dependent upon binocular interactions among ipsilat-

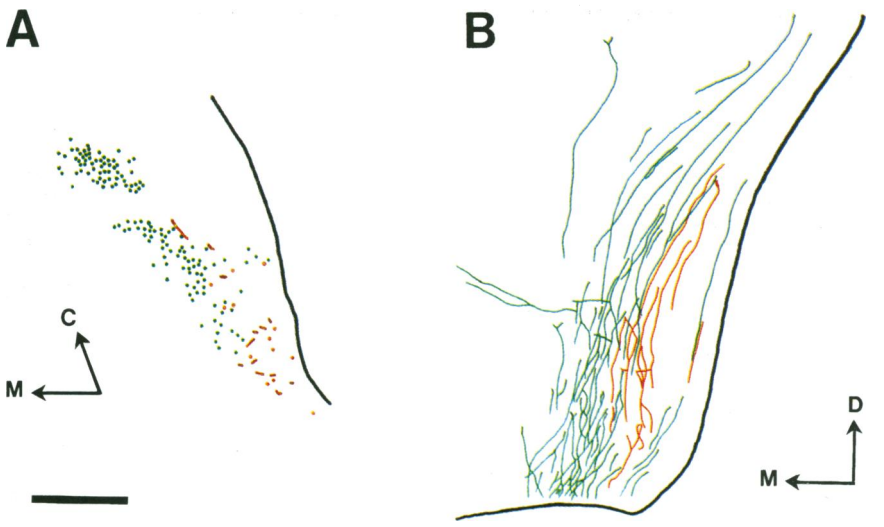

FIG. 2. Spatial organization of crossed and uncrossed retinal axons within the optic tract of an E42 embryo. In each drawing the black line indicates the pial surface of the diencephalon. (*A*) Perspective view of a horizontal section through the most ventral part of the right optic tract. Note the different spatial distribution of crossed (red dots and short lines) and uncrossed (green dots) retinal fibers. C, caudal; M, medial. (*B*) Composite drawing from serial coronal sections through different levels of the right optic tract shown in *A*. Micrographs were made of the serial sections and sketches of the labeled fibers were subsequently drawn. Landmarks, such as the outline of the diencephalic wall and the third ventricle, were reported from camera lucida drawing of each section onto the corresponding sketch. The sketches were then superimposed according to these landmarks to reconstruct a composite drawing of the labeling zone. The trajectory of the labeled retinal axons is shown from the ventral aspect—corresponding to the horizontal drawing in *A*—to their distal end. Note that crossed retinal axons (red lines) remain partially segregated from the uncrossed retinal fibers (green lines). D, dorsal; M, medial. [Bar = 100 μm (*A*) or 200 μm (*B*).]

erally projecting fibers from one eye with those projecting contralaterally from the other eye. Such an interaction between the fibers stemming from the two eyes has been inferred from studies in the mouse which demonstrated that monocular enucleation, performed before the retinal axons arrive at the chiasm, altered the normal development of the uncrossed pathway (11, 12). Recently, however, Sretavan and Reichardt (13) have questioned this notion on the basis of time-lapse video analysis of the navigational patterns of crossed and uncrossed fibers in an *in vitro* embryonic mouse preparation.

It is commonly assumed that within the vicinity of the chiasm, specific signals are expressed which guide the decussation process. Our results suggest that such signals must be present very early in development, when or shortly before the initial contingent of optic axons reach the future chiasmatic region. In particular, the ingrowth of the first uncrossed optic axons into the optic tract suggests some sort of repulsive mechanism. A recent *in vitro* study has in fact demonstrated the presence of a repulsive signal during a brief time window, specifically recognized by uncrossed retinal axons of rat embryos (14). The temporal separation of the uncrossed and crossed fiber ingrowth into the optic tract could signify the delayed expression of an "attraction" signal, inducing the growth cones of retinal fibers to subsequently cross the midline. If this line of reasoning is correct, it raises the problem of the cues employed by the much larger contingent of later-arriving optic axons. Given the fact that the putative chiasmatic cues have been shown to be transiently expressed during development (14, 15), it has to be assumed that the chiasmatic choice of the later component of the retinal projections is governed mainly by the retinal location of the parent ganglion cells. A plausible explanation is that the retinal cue provides a molecular identity to each subset of retinal axons (uncrossed and crossed), so that this underlies a specific recognition among the early and later components of the retinal fibers. Thus, the later-arriving fibers may follow cues provided by the initial crossed and uncrossed contingents described in the present study. If this were the case, the early contingents of fibers would serve the role of pioneer axons, as has been demonstrated in invertebrate (16–18) and mammalian (19) developmental studies. This notion is attractive for two reasons. It provides commonality with guidance mechanisms which have been demonstrated in other biological systems. Furthermore, the merits of this idea are testable. Indeed, it has been shown in the peripheral nervous system of invertebrates that when the differentiation of the pioneer neurons is perturbated, the sensory axons fail to reach the central nervous system (20). Because of the pronounced central-to-peripheral gradient in the generation of retinal ganglion cells (6), it should be possible to delete the initial contingent of optic axons in the primate so as to determine to what degree this perturbs the chiasmatic choice of the axons of later-born retinal ganglion cells situated in the nasal and temporal hemiretinas.

It must be emphasized that our observations apply only to the initial contingent of retinal axons, those that navigate through the chiasm between E36 and E42. At later ages, the crossed and uncrossed fibers are intermingled within most of the tract (unpublished observations), which could reflect binocular interactions among later-generated fibers. Such intermingling also characterizes the organization of the adult monkey optic tract (21, 22), although a limited pure crossed component is situated within the deepest region of the tract. In the monkey (22, 23), as previously shown in other mammalian species (24, 25), a chronotopic order has been suggested to characterize the adult organization of the optic tract, so that the axons stemming from the first-generated retinal ganglion cells course in the deepest region of the tract, whereas the later generated fibers lie closer to the pial surface of the brain. Interestingly, we have found that the uncrossed fibers, which first innervate the tract, lie in the deepest region, with the later-arriving crossed axons located superficially. Thus, the ingrowth pattern of the pioneer fibers described in the present study appears to follow the chronotopic regional distribution that has been inferred from studies of the adult monkey. The organization of the adult optic tract, in which the deepest region contains only crossed fibers, would seem to argue that the uncrossed contingent of pioneer optic fibers is eliminated at later stages of development. Such

an elimination of pioneering pathfinding fibers takes place during the formation of specific projections in nonmammalian nervous systems (20, 26).

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