Postnatal Development of the Retinal Projection to the Nucleus of the Optic Tract and Accessory Optic Nuclei in the Hooded Rat*

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Summary. Retinal projections to the nucleus of the optic tract (NOT) and accessory optic nuclei (AON) were studied in the postnatal hooded rat after monocular injection of cholera toxin B subunit (CTB) into the vitreous chamber of the eye. At all postnatal ages, retinal axons were labeled sensitively; they revealed dense projections to the contralateral, and sparse but distinct projections to the ipsilateral, NOT and AON. The CTB labeling enabled the first delineation of the complete morphology of developing retinal axons in the ipsilateral NOT and AON. From postnatal day (P) 1 to P3, axons with complex growth cones were seen, and unbranched collaterals with simple growth cones increased and extended gradually. At P6, complex growth cones disappeared while branched collaterals with simple growth cones as well as small-sized varicosities increased. By P12 (two days before eye-opening) the adult-like pattern of terminal arbors appeared. The branched collaterals with tiny, small-sized varicosities present probably represented developing synaptic boutons. At P16 (after eye opening), the pattern of terminal arbors was well developed, almost to the same extent as in the adult. By contrast, a broadly distributed, transient retinal projection around NOT and AON was gradually eliminated; it started to disappear during the first few postnatal days, and was fully retracted by the time of eye-opening time to a pattern normal for the adult.

For many years, studies have been carried out on various aspects of the retinal projection to the nucleus of the optic tract (NOT) and accessory optic nuclei (AON) in mammals. The NOT is located

within the brachium of the superior colliculus (SC), beginning anterior to the SC and extending along its lateral side for some distance (SCALIA, 1972; SCALIA and ARANGO, 1979). The AON consists of three pairs of terminal accessory optic nuclei designated as the dorsal, lateral, and medial terminal nuclei (DTN, LTN, MTN). The MTN is further divisible into ventral and dorsal sectors (HAYHOW et al., 1960; GIOLLI et al., 1984).

As for the functions of these nuclei, it has been repeatedly reported that the responses of neurons of the NOT and DTN, MTN and LTN show a marked similarity in directional responsiveness. Cells in the NOT and DTN are related to horizontal optokinetic nystagmus (OKN), and those in the MTN and LTN are related to vertical OKN (SIMPSON, 1984; SIMPSON et al., 1988). Through their connections with the brainstem, it appears that both the NOT and AON are involved in the stabilization of the drift of image position on the retina (SEFTON and DREHER, 1995). Thus, the predominance of GABAergic interconnections between some of the accessory optic nuclei (GIOLLI et al., 1992; VAN DER WANT et al., 1992) may contribute to the fine-tuning of responses by reducing activity orthogonal to the plane of retinal slip, as well as that in the same plane but in the opposite direction (SEFTON and DREHER, 1995). The NOT-DTN complex has been demonstrated to be an essential element in the generation of slow eye movements during OKN and after-nystagmus in the rat (CAZIN et al., 1980a, b). Both anatomical and electrophysiological studies have shown that the NOT and AON are strongly

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interconnected (BLANKS et al., 1982; GIOLLI et al., 1984, 1985a, b; NATAL and BRITTO, 1987; GRASSE and CYNADER, 1991; SCHMIDT et al., 1995, 1998).

Although there have been no studies reporting on the postnatal development of the retinal projection to the NOT in the rat, retinal projections to the MTN were first identified at embryonic day (E) 17 following intra-ocular injections of horseradish peroxidase. BUNT et al. (1983) determined that the optic axons reach the DTN and LTN by E18. They also were the first to show that a retinofugal projection to the pretectal complex is present at E18, and that separate fields of axon terminals in pretectal constituents are first distinguishable at E20 (BUNT et al., 1983).

The experiments reported here were performed to study the processes involved in the postnatal development of characteristic sets of retinal ganglion cell connections in the rat. We labeled retinal axons with the cholera toxin B subunit (CTB) at different postnatal ages. The morphologies, trajectories, and branching patterns of the axons were studied at the level of the NOT and AON. The NOT and AON were selected for analysis because these nuclei are related to the OKN, and they have been relatively less studied in terms of their postnatal development.

MATERIALS AND METHODS

Animals

The material for this study was drawn from 15 hooded (Long Evans) rat pups taken at times ranging from a few hours after birth to adulthood (Table 1). The first 24-hour period after birth was designated as postnatal day (P) 0, and the following days were numbered in sequence. The surgery was performed under aseptic conditions on all animals. The care and handling of the animals followed the Principles of Laboratory Animal Care, as put forth by NIH, with the experimental protocols approved by the Niigata University Animal Care Committee.

Axonal labeling methods

Animals were anesthetized by hypothermia (ages P0–P5) or with sodium pentobarbital (ages P6–adult). In animals in which the eyes were not yet open (P0–P11), the eyelid was swabbed with alcohol, and an incision was made along the future opening. The tip of a microsyringe was inserted into the vitreous chamber, and a volume of 0.5% CTB (List Biological Laboratories) solution (ranging from 0.3–1.0 μ l) was injected very slowly into the eye. The needle was left in place for 1 min after the injection, removed, and the eyelid

of the animal (P0–P11) was then closed. After recovery from anesthesia, the animal was returned to its mother. After a 24-hour period of survival, the animal was reanesthetized with an overdose of sodium pentobarbital (80 mg/kg) and transcardially perfused with saline, followed by 100–200 ml of 3.7% paraformaldehyde in 0.1 M phosphate buffer (PB) at pH 7.4, for 20–30 min. The brains were then removed and postfixed in the same paraformaldehyde solution for 1 h at room temperature. Brains were cryoprotected in 20% sucrose with 0.1 M PB overnight at 4°C. Frozen sections were cut at 30–35 μ m with a freezing microtome using coronal sectioning, and these were collected in 0.1 M PB in four or six similar series of free-floating sections.

Immunohistochemistry

For the CTB reaction, sections were washed 3 times in 0.1 M PB, and then incubated overnight at room temperature on a shaker in goat anti-CTB (List Biological Laboratories) solution diluted 1:8000 in phosphate-buffered saline (PBS) containing 2% Triton X-100, 0.3% carrageenan, and 3% normal donkey serum. After three washes in PBS, the sections were incubated for 2 h in biotinylated donkey anti-goat IgG (Jackson Immuno Research) 1:200 in the same solution as above, then again washed in PBS three times. Sections were placed into a solution of ABC (avidin-biotin-peroxidase complex, Vector Laboratories) for 45 min diluted from 1:100 in PBS, then washed once

Table 1. Summary of tracer injected (CTB), postnatal age, day of the injections and amount of tracer injected

Age	Postnatal day of tracer injection	Amount of tracer (μ l)	Number of animals
P1	0*	0.3**	2
P2	1	0.3	2
P3	2	0.5	2
P6	5	0.5	2
P12	11	0.5	2
P16	15	0.8	2
P21	20	0.8	1
P28	27	0.8	1
Adult		1.0	1

*The first 24 hour period after birth was designated as postnatal day 0, and the following days were numbered in sequence.

**CTB was injected into the right eye.

in PBS, twice in Tris buffer (50 mM, pH 7.4), and immersed for 5 min in 0.02% DAB solution (3,3'-diaminobenzidine tetrahydrochloride) in Tris buffer. Sections were then placed into 0.02% DAB with 0.06% hydrogen peroxide (H_2O_2) solution for 2-5 min to generate the reaction product. After the reaction was complete, sections were washed three times in Tris buffer, and immediately mounted in Tris buffer on gelatine-coated glass slides, air-dried overnight, dehydrated in a series of alcohols, cleared in xylene, and coverslipped.

Selected sections were documented photographically, using TMAX 100 black and white film.

RESULTS

Following the injection of CTB into the vitreous chamber of the right eye of the rats (from P0 to adult), the CTB-immunopositive axonal terminals were observed to be filled completely with reaction product. The staining revealed heavy anterograde axonal transport to all retinal targets such as the lateral geniculate nucleus, SC, pretectal nuclei, and AON (Fig. 1), and offered us the opportunity of studying the postnatal developmental morphology of retinal axons in the hooded rat. The present anatomical findings in rats after P16 were in general accord with descriptions of the adult hamster (LING et al., 1998). The reaction product of the CTB was seen in axons and terminals throughout the optic system, and slight differences in the quality of staining between the various terminal regions were observed.

In the NOT and AON receiving input from the contralateral retina, the density of retinofugal fibers was very high, thus making it hard to follow single fibers for long distances (Fig. 2A). By contrast, in less densely innervated ipsilateral retinal targets, the morphology of axon arbors and their terminal specializations was visible in striking detail (Fig. 2B). Therefore, in order to determine the morphological characteristics of the retino-NOT and retino-AON projections in the developing rat, the ipsilateral NOT and AON projections were assessed. In addition, there were retinal projections to a number of other regions not usually associated with the visual system by way of the NOT and AON during the first postnatal days, though these gradually retracted to form their normal adult pattern. These transient retinal projections were observed mainly on the contralateral side.

Fig. 1. Photomicrographs showing the density and distribution of labeled axons and terminals seen on coronal sections through the brainstem at three different levels (rostral-caudally, **A**, **B**, and **C**; *L* indicates left side, *R* right side). In this case at P2, a rat had received an injection of CTB into the right eye. *LGB* lateral geniculate body, *LP* lateralis posterior thalamic nucleus, *LTN* lateral terminal nucleus, *MGB* medial geniculate body, *MTN* medial terminal nucleus, *NOT* nucleus of optic tract, *OPT* olivary pretectal nucleus, *PED* pedunculus cerebri, *SC* superior colliculus. Bar = 1 mm

Fig. 2. Retinal projections to the contralateral (A) and ipsilateral (B) MTN of P2. The contralateral labeling is so dense that the morphology of axon arborization and their terminals cannot be described in detail. On the other hand, the ipsilateral labeling is rather light; thus axon arborization and their terminals are seen in detail. Note that the contralateral accessory optic tract (*arrows*) is densely labeled. Bar = 50 μ m

Developmental morphological characteristics of retinal axons in the NOT and AON

From P1 onwards, labeled retinal axons were observed to be widely distributed on both sides, covering the NOT and AON, though retinal axons in the NOT and AON still existed in a developmental status. During the procession of development, the morphological characteristics of retinal axonal growth cones, collaterals, varicosities, and terminal arbors differed at each ontogenetic stage in the NOT and AON (Table 2).

At P1, 2 and 3 stages, the retinal projections entered the NOT via the brachium of the SC, whereas the AON was supplied via the accessory optic tract. Most axons had a single main trunk crossing the full extent of the nucleus. Axons terminating in a complex growth cone having thin veil-like extensions were also found in the NOT and AON (Fig. 3B), as well as on the pathway between the DTN and LTN, the LTN and MTN, and even from the ventral MTN to dorsal MTN. Some axons had already begun to give off collaterals to the body of the nucleus. Some unbranched collaterals in the NOT and AON displayed simple growth cones with lanceolate endings (Fig. 3A). At this same stage, some branched collaterals with small varicosities emerged from the parental axon trunk. The number and length of collaterals increased and extended progressively from P1 to P3.

By P6, there were no more complex growth cones such as those seen at P1, 2, 3 at the tips of elongating retinal axonal trunks. The number of collaterals terminating in simple growth cones increased. Unbranched collaterals and branched collaterals from

the same parent axons were present simultaneously, with the branched type being predominant. The number of varicosities on the collaterals and arbors increased, though they were generally small. Occasionally simple terminal arbors were noted. At the end of the first postnatal week, the morphological characteristics of retinal axons in both the NOT and AON were developed nearly to the same extent (Fig. 4).

By P12 (two days before eye opening), collaterals were still present with the same range of lengths, terminal specializations, and branching characteristics as were seen on P6. At this age, simple growth cones and unbranched collaterals disappeared and the number of collaterals from the same parent axon decreased from that seen at P6. Collaterals were for the most part branched and derived from different parental axons. Most collaterals gave rise to simple terminal arbors, and the number of varicosities exceeded what was seen at P6. Furthermore, some

Table 2. Morphological characteristics of retino-NOT and retino-AON projections in the developmental rat (ipsilateral)

	P1, 2, 3	P6	P12	P16
Growth cones	++	+	—	—
Collaterals	+	+++	++	+
Varicosities	+	++	+++	++++
Terminal arbors	—	+	++	+++

Fig. 3. Photomicrographs showing developmental morphological characteristics of the ipsilateral retinal projection to the NOT and AON. **A.** MTN of P1 indicating two simple growth cones with short terminal expansions (*arrowheads*). These growth cones are shown at higher magnification in the **inset** above. Bar = 20 μm (main figure), 10 μm (inset). **B.** LTN of P3 indicating a complex growth cone with long terminal expansions as well as filopodial extensions (*arrowhead*). This growth cone is shown at higher magnification in the **inset** at right. Bar = 20 μm (main figure), 10 μm (inset)

Fig. 4. Photomicrographs showing the ipsilateral retinal projection to MTN (**A**) and LTN (**B**) at P6. **A.** Moderate numbers of varicosities (*arrowheads*) are observed on axonal branches. **B.** *Arrow* indicates a branching point of an axonal collateral. Bar = 20 μm (**A**), 10 μm (**B**)

Fig. 5. Photomicrographs showing the ipsilateral retinal projection to the MTN (**A**) and NOT (**B**) at P12. **A** and **B** show a nearly adult pattern of terminal arbors: branched collaterals with different sizes of varicosities (*arrowheads* in **A** and **B**), and glomerulus-like boutons (*arrow* in **B**). This glomerulus-like structure is shown at higher magnification in the *inset* above. Bar = 20 μ m (main figure), 10 μ m (inset)

axonal terminal arbors were denser and more elaborate in the pattern and complexity of their branching, and contained both small and very small varicosities (Figs. 5A, B). Glomerular-like boutons that constituted the terminal arbor and probably represented developing synaptic boutons, were common (Fig. 5B). The pattern of retinal axons in the NOT and AON that was observed at P12 was similar to that of the adult.

By P16 (after eye opening), the collaterals resembled those seen at P12. Long axons in the NOT and AON, however, were difficult to follow in the same section, and short and multiply-bent collaterals with complex terminal arbors were notably increased in number. The adult pattern of terminal arbors was well developed by this age (not illustrated). By P21, the density of retinal terminals with CTB reaction product was rather high, and the morphological characteristics of the terminal arbors were difficult to discriminate from each other in the ipsilateral NOT and AON.

Transient retinal projection to regions around NOT and AON

The CTB method not only revealed heavy anterograde axonal transport to all retinal targets, but also demonstrated that the labeled axons are distributed broadly around those retinal targets at early post-

natal stages, and that this aberrant projection gradually retracts to the normal adult pattern. This kind of transient retinal projection phenomenon has also been seen in results from labeled contralateral NOT and AON areas of the postnatal developmental rat.

At the P1, P2, and P3 stages, labeled retinal axons were distributed in NOT and AON. In addition, they extended well beyond their "correct" terminal zone (Fig. 6A). Extensive retinofugal axons were seen around the contralateral NOT and AON. These retinal axons from the NOT, LTN, and MTN traveled into the deep layers of the SC, the caudolateral part of the posterior thalamus, and the ventral tegmental area, respectively. These extensively projecting axons gradually disappeared or retracted into the NOT and AON at P6 (Fig. 6B). After the time of eye opening, the transient axons were eliminated, and had retracted to the normal adult pattern (Fig. 6C).

DISCUSSION

The present results utilizing a sensitive method to reveal the presence of CTB gave a clearer picture of the extent of the postnatal retinal projections than was possible using the degeneration techniques previously employed. CTB labeling delineated the complete morphologies of developing retinal axons in the ipsilateral NOT and AON, offering hitherto unknown

views of postnatal developing retinal axons from the rat.

The present anatomical findings in the NOT and AON of the hooded rat are in broad accord with the description of the mammalian accessory optic system given by HAYHOW and co-workers (HAYHOW et al., 1960), and are partly in agreement with the description of the rodent pretectum by SCALIA (1972) and SCALIA and ARANGO (1979). In this respect, the observations confirmed the results of the previous degeneration studies. Furthermore, this study showed that the retinal axons reach the NOT bilaterally from P1, and then gradually developing the adult pattern. At these postnatal times, the density of the ipsilateral retinal projection from birth was always much lighter than the contralateral optic projection. This lighter ipsilateral retinal projection to the NOT was not detected previously in the prenatal rat (BUNT et al., 1983) or in the adult rat (SCALIA, 1972).

Labeling techniques

Various methods have been attempted in order to facilitate the visualization of the terminations of optic nerve fibers in mammals, such as degeneration after unilateral eye enucleation (HAYHOW et al., 1960; LATIES and SPRAGUE, 1966; KOSTOVIC, 1971), selective silver impregnation for degenerating axons (SCALIA, 1972), autoradiographic and silver impregnation techniques (SCALIA and ARANGO, 1979), autoradiography (WREE and ZILLES, 1983), horseradish peroxidase (BUNT et al., 1983; TERUBAYASHI and FUJISAWA, 1984), and wheat germ agglutinin-horseradish peroxidase conjugate (FROST, 1984); all have been used for tracing or detecting degenerating retinal axons and their terminals. Some of these methods provide good sensitivity but result in a granular reaction product, at times accompanied by a relatively high amount of background staining. With such techniques, it is especially difficult to distinguish axon terminals from fibers of passage, particularly in regions that receive only minor projections (AN-

Fig. 6. The elimination of transient collaterals. The extensive retinofugal axons around the contralateral MTN extend well beyond their correct terminal zone at P1 (**A**, arrows); these extensive axons gradually disappeared or retracted to the MTN at P6 (**B**, arrow). After eye opening, the transient axons were eliminated, and these retracted to the normal adult pattern (**C**). Bar = 200 μ m

GELUCCI et al., 1996), although some of these techniques well define terminal fields and growth cones on retinal axons (BUNT et al., 1983; SCALIA and MATSUMOTO, 1985).

With particular regard to the mammalian retinofugal pathway, CTB proved to be one of the best tracers suited for labeling the retinal projections as a whole, and for the detailed analysis of small fiber contingents such as the accessory optic system and retino-pretectal circuit (MIKKELSEN, 1992; ANGELUCCI et al., 1996; NAKAGAWA et al., 1998). After injections in the eye, CTB yields complete and highly detailed anterograde axonal labeling which stains axons and terminals in great detail, so that single axons can be followed over long distances and their arborization reconstructed in their entirety.

Recently, CTB has been used frequently to demonstrate the morphologies of retinal arborization in the midbrains of adult animals such as the rat (MIKKELSEN, 1992), hamster (JOHNSON et al., 1988; LING et al., 1997a, b, 1998), mouse (MIURA et al., 1997), ferret (ANGELUCCI et al., 1996), and monkey (NAKAGAWA et al., 1998). In studies of the retinofugal pathway, it is apparent that the CTB method has more advantages than do horseradish peroxidase or autoradiographic methods. Axons and terminals stained with CTB in this study have revealed a high level of anterograde labeling, so that the developmental morphological characteristics of retinal axons in the NOT and AON of the rat can be assessed in greater detail.

Axonal growth states

Previous reconstruction studies have suggested that developing retinal axons pass through at least three successive stages: elongation, collateralization, and arborization (LANGDON and FROST, 1991). They first elongate to reach their most distal targets in the midbrain, then they emit unbranched or poorly branched collaterals to multiple targets, and finally they elaborate terminal arborization in their definitive targets and eliminate their other collaterals. These stages may provide a framework for investigating the physiological events and cellular and molecular mechanisms that orchestrate the maturation of individual axons (BHIDE and FROST, 1991, 1999; LANGDON and FROST, 1991). Because it is sometimes difficult to distinguish the last two successive stages from each other, JHAVERI et al (1991) proposed that the collateralization phase is actually an early stage of axonal arborization.

Retinal axons entered the contralateral NOT and bilateral AON before birth (BUNT et al., 1983). According to the present observations, developing

retinal axons in NOT and AON of the hooded rat do follow the three successive stages of elongation, collateralization, and arborization. Postnatal development of the optic fibers appears to occur in a regular and progressive fashion. There is a gradual increase in the pattern of fiber growth throughout the postnatal period through to adulthood.

The timing of attainment of the adult pattern and visual experience

Birth does not appear to be a critical time in the development of the optic projection in the rat (BUNT et al., 1983). The distribution patterns of crossed and uncrossed retinal projections are established prior to the time of eye opening in the Syrian hamster, as has been determined through autoradiographic and anterograde degeneration techniques (FROST et al., 1979). As in the case of the hamster, the present study showed that CTB is anterogradely transported to all retinal targets before the time of eye-opening in the rat. Furthermore, a near-adult pattern of terminal arborization was formed in the NOT and AON by P12. A retinocollicular projection study performed in the rat showed that a mature pattern of this projection is present before eye-opening (YHIP and KIRBY, 1991). An electrophysiological study in rats has indicated that the first light-evoked responses arise around P12-P13 in both the retina and SC. The maturation of visual receptive field properties in the SC suggests that, although some aspects of sensory properties are modified during development, a high level of sophistication is already present in young animals even before the time of eyelid opening (FORTIN et al., 1999). In the SC, visual experience does not seem to be important for the maturation of visual receptive field properties. In our study on the retinal projection to NOT and AON, although a near-adult pattern of retinal axons is formed before eye-opening, the adult pattern of retinal axons subsequently developed further. The number of varicosities and terminal arborization observed at P16 was clearly greater than at P12, suggesting that visual experience is still an important factor for the development of the retinal projection to the NOT and AON. It might also be possible that the formation of OKN in the rat takes a longer time to develop after eye-opening, although it is still unclear as to which day OKN first occurs.

Elimination of transient collaterals

During development, a neural pathway may distribute broadly and then retract to its normal adult

pattern (LAND and LUND, 1979). The formation and elimination of transient connections is a widespread phenomenon in the normal development of the central nervous system (INNOCENTI, 1981). This phenomenon can be perceived clearly from our study on postnatal retinal projections. The retinal projection was more extensive around the contralateral NOT and AON at the P1, 2, and 3 stages than in the normal adult, and gradually retracted to its normal adult pattern. In addition, the present study showed that transient retinofugal axons project to a number of other regions not usually associated with the visual system — for example, the caudolateral part of the posterior thalamus and ventral tegmental area. This transient projectional phenomenon has been observed on the principal thalamic somatosensory (ventrobasal) nucleus in juvenile hamsters (FROST, 1984; LANGDON and FROST, 1991; BHIDE and FROST, 1999). Some of these connections disappear during development, while others persist into adulthood, albeit having a diminished density and a relatively reduced volume. Some retinofugal axons traverse their definitive targets in the visual system and temporarily penetrate structures associated with other sensory systems.

Neuronal death seems to be the major mechanism contributing to the elimination of most transient retinal projections (JEFFERY, 1984). In the pigmented rat, during the first few days following birth, cell death has been shown to occur in the ganglion cell layer of the retina (JEFFERY, 1984). As indicated in the present study, transient collaterals of retinal axons are gradually eliminated during the first few postnatal days. Thus, this activity appears to follow the process of retinal ganglion cell death (CRESPO et al., 1985; O'LEARY et al., 1987). The death of retinal ganglion cells during development thus seems to serve two purposes. It provides for the quantitative matching of the ganglion cell population to the needs of its central projection fields, and at the same time it serves to selectively eliminate those cells whose axons project to inappropriate targets or to inappropriate regions within the correct target fields (O'LEARY et al., 1987). On the other hand, the disappearance of each of these transient axonal populations apparently occurs simultaneously with the onset of the elaboration of terminal arbors (INNOCENTI, 1981; FROST, 1984). This phenomenon can be seen in the NOT and AON by P12. By this time, near-adult terminal arbors have formed in the NOT and AON. At the same time, some axons without significantly elaborating terminal arbors apparently have already disappeared. After eye-opening and at P16, the number of complex terminal arbors increases to a level

higher than before, and the majority of the retinofugal fibers make topographically appropriate terminations within the NOT and AON.

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