

# Morphology and Dye-Coupling of Cells in the Pigeon Isthmo-Optic Nucleus

Wen-Chang Li Shu-Rong Wang

Laboratory for Visual Information Processing, Institute of Biophysics, Chinese Academy of Sciences, Beijing, P.R. China

## Key Words

Isthmo-optic nucleus · Ectopic cell region · Dye-coupling · Lucifer yellow · Neuronal morphology · Pigeon

## Abstract

Ground-feeding birds such as pigeons possess the most developed isthmo-optic nucleus in all classes of vertebrates. A previous study showed that this centrifugal or retinopetal nucleus modulates visual activity in tectal cells of pigeons; the present study aimed at revealing the morphology and possible dye-coupling of neurons in the isthmo-optic nucleus and in the ectopic cell region by intracellular injections of Lucifer yellow into neurons in slices. One hundred and twelve successfully labeled cells of the isthmo-optic nucleus were classified into bipolar (83%) and multipolar (17%) types, each of which was further divided into two subtypes, B and P and M and N, respectively. Neurons of B- and P-types are similar in that they have apical dendrites and axons usually arising from the opposite pole of piriform perikarya, but they differ in the length (20–120 vs. 10–20  $\mu\text{m}$ ) of their dendritic stems; M- and N-types possess polygonal perikarya giving rise to two to five primary dendrites either in the same orientation (M) or in a radiation fashion (N), and their axons originate from perikarya or occasionally from dendritic stems. Twelve single-injections resulted in the labeling of 26 cells, including 11 pairs and 1 quadruple labeling. About half of these are closely apposed 'twin-

cells'. Dye-coupling was found only between neighboring cells in the cell lamina. Thirteen cells in the ECR rostroventral to the ION were labeled and could be grouped into large or L- (46%) and small or S- (54%) types, mainly depending on the dendritic field size and the number of primary dendrites. No dye-coupling was observed between the presumptive ECR cells. The functional role of the ION and the significance of dye-coupling between neurons are discussed.

## Introduction

Almost all classes of vertebrates possess centrifugal pathways from the brain to the retina, the most prominent and extensively studied of which is the isthmo-optic nucleus (ION) in ground-feeding birds, including pigeons, chickens, and quails [Uchiyama, 1989; Feyerabend et al., 1994; Malz and Meyer, 1994; Miceli et al., 1993, 1997]. This nucleus is located medial to the caudal dorsomedial edge of the optic tectum. Several electrophysiological studies have shown that the visual field or the retina is mapped onto the nucleus in an orderly, topographical manner and that ION cells are sensitive to moving targets [Holden and Powell, 1972; Miles, 1972; Li et al., 1998]. Electrical stimulation of the isthmo-optic tract (IOT) enhances visual responses of retinal ganglion cells in decerebrate chicks [Miles, 1972], as confirmed by findings that stimulation of the centrifugal fibers enhances ganglion cell responses to sinusoidal gratings over a wide

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Shu-Rong Wang  
Laboratory for Visual Information Processing, Institute of Biophysics  
Chinese Academy of Sciences, 15 Datun Road  
Beijing 100101 (P.R. China)  
Tel. +86-10-64889858, E-Mail [wangsr@mmi.cnc.ac.cn](mailto:wangsr@mmi.cnc.ac.cn)

range of spatial frequencies in quail [Uchiyama and Barlow, 1994]. On the other hand, reversible cooling of the ION in pigeons causes a decrease in visual responsiveness of retinal ganglion cells without changing their receptive field properties [Pearlman and Hughes, 1976]. Recently, we have shown that blocking ION by lidocaine significantly reduces visual responses of tectal cells whose receptive fields (RFs) are located within or overlap the receptive field of the ION cell where lidocaine is injected [Li et al., 1998].

On the basis of electrophysiological and neuroanatomical findings, some hypotheses concerning the functional significance of centrifugal pathways have been proposed [Uchiyama, 1989; Holden, 1990; Woodson et al., 1995; Clarke et al., 1996]. Within the feedback loop, tectal neurons projecting to ION are located in layer 9 and at the border between layers 9 and 10 of the tectum. They do not have ascending dendrites extending into the superficial layers but do have descending dendrites branching in the deep layers, with axons bifurcating horizontally [Uchiyama and Watanabe, 1985; Uchiyama et al., 1996]. However, this description is in disagreement with that by other authors [Woodson et al., 1991; Miceli et al., 1997]. According to Miceli and colleagues, the tectal-ION neurons possess radially oriented apical dendrites. The number of tectal-ION cells approximates that of ION cells, suggesting that a 1:1 tectal-isthmus connection is established within the nucleus [Woodson et al., 1991; Uchiyama et al., 1996]. Centrifugal fibers terminate in convergent and divergent modes on amacrine cells and displaced ganglion cells in the retina [Maturana and Frenk, 1965]. It was recently shown that the ION issues mainly centrifugal fibers of the convergent type, whereas the ECR around the ION gives rise only to retinopetal fibers of the divergent type [Woodson et al., 1995].

Until now, however, only a few studies have focussed on a morphological description of centrifugal neurons, and these have been based on Golgi material [Cowan, 1970; Crossland, 1979; Gunturkun, 1987]. With Golgi preparations, it is very difficult to see clearly the branching patterns and appendages of neuronal processes [Crossland, 1979]. Also, we wondered whether there exist dye-coupling junctions in the attachment plaques between ION cells [Angaut and Reperant, 1978] and in the dense dendritic arborization network of the opposite lamina neurons [Gunturkun, 1987]. Therefore, we used Lucifer yellow as an intracellular marker to study the morphology and dye-coupling of ION cells and of presumptive ECR cells in vitro preparations, because this fluorescent dye is an excellent tool for staining cellular morphology and revealing dye-coupling between neurons [Stewart, 1978; Peinado et al., 1993; Onn and Grace, 1994; Hatton and Yang, 1996].

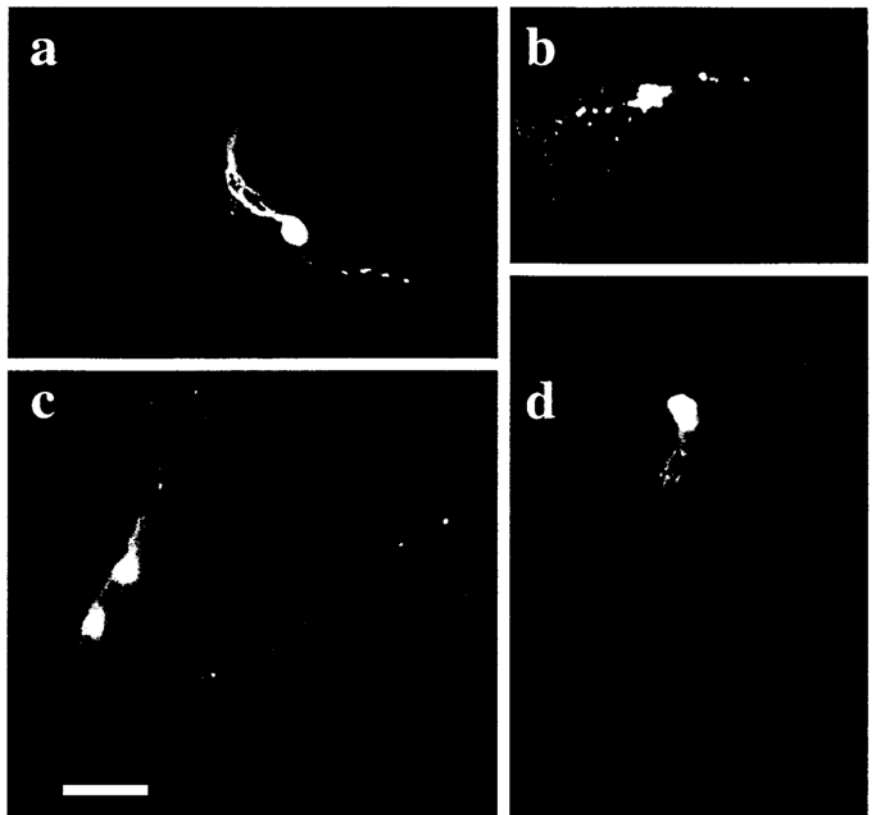
## Materials and Methods

Twenty-two homing pigeons (*Columba livia*) weighing 250–350 g and representing both sexes were used in this study. The original research reported here was performed following the Policy on the Use of Animals in Neuroscience Research approved by the Society for Neuroscience in 1995. The animal was anesthetized with ketamine hydrochloride (30 mg/100 g b.w.) and then decapitated. The brain was immediately removed from the skull and washed in ice-cold Krebs-Ringer solution consisting of (in mM) NaCl, 124; KCl, 5; CaCl<sub>2</sub>, 2; MgSO<sub>4</sub>, 2; KH<sub>2</sub>PO<sub>4</sub>, 1.25; NaHCO<sub>3</sub>, 26; glucose, 10 [Hardy et al., 1987]. The midbrain containing the ION was blocked and cut midsagittally into two halves. Each half-block was glued onto the stage of Vibroslice (Campden Instruments 752M). Slices including the nucleus were sectioned sagittally at 300  $\mu$ m in thickness, then transferred from a storage container into the recording chamber (BSC-HT, Medical System Corp.) and perfused with Krebs-Ringer solution bubbled with a mixture of 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The slices were incubated for 40–60 min and maintained at 34 °C.

For intracellular staining, a micropipette filled with an aqueous solution of Lucifer yellow (5%, dilithium salt, Sigma) was used. The electrode tip was broken by touching it against the edge of a glass slide with a micromanipulator under a microscope to about 0.5–1  $\mu$ m. The micropipette was advanced into the ION or into the neighboring ECR in slices under visual control. Isthmo-optic neurons were impaled by applying brief positive current pulses (4 nA, 0.3 s in duration). Intracellular impalement was signalled by a sudden d.c. drop of 20–65 mV. The dye was injected by passing negative current of 2–4 nA through the electrode for 2–10 min. In cases for study of dye-coupling between ION cells, only one injection of Lucifer yellow was made in each slice; otherwise, two to three injections were made in sites about 300  $\mu$ m apart. After 0.5–2 h survival, the slices were removed from the recording chamber and fixed in 4% paraformaldehyde overnight in a refrigerator. The slices were rinsed with physiological saline and then placed in 100% dimethylsulfoxide (DMSO) for 20 min [Onn et al., 1993]. The DMSO-mounted slices containing Lucifer yellow-marked isthmo-optic cells were covered and observed or photographed with a fluorescence microscope. In some experiments, the fluorescence image of the ION and ECR cells was acquired and saved by a computer using Neuro-Lucida software to aid in their morphological reconstruction.

## Results

A total of 112 ION cells and 13 ECR cells were successfully labeled with Lucifer yellow and their morphologies examined (fig. 1). According to the shape of perikarya, dendritic arborizations, and origin of axons, the ION cells could be divided into two main types: bipolar and multipolar. Bipolar cells have an apical dendrite and axon and could be further divided into subtypes B and P. The B type cells (40/112 = 36%) possess piriform perikarya and longer apical dendrites (20–120  $\mu$ m). Each apical dendrite forms a simple branching pattern in a slender column (fig. 2B<sub>1-2</sub>). Axons arise from the opposite pole or side of the perikarya and take tortuous routes along the perimeter of the nucleus

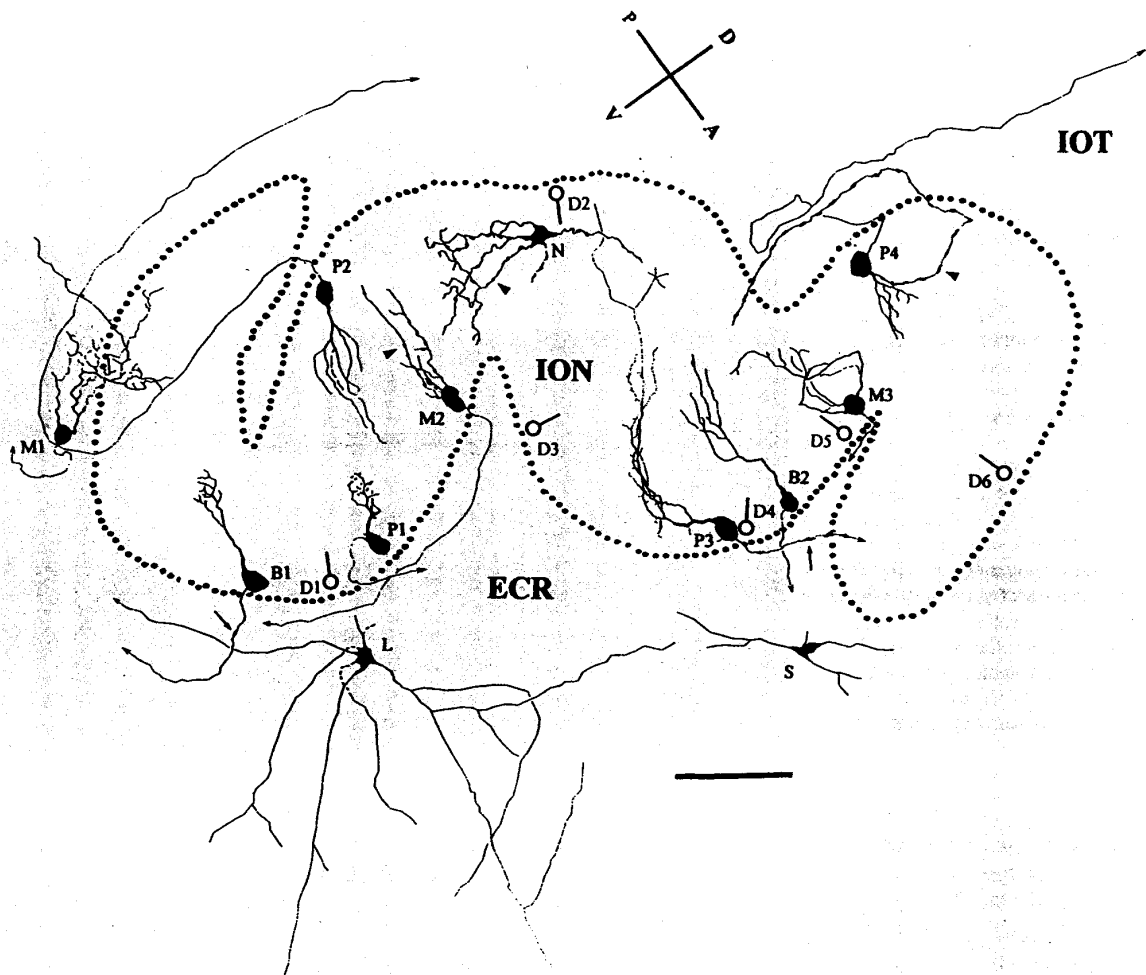


**Fig. 1.** Photomicrographs showing morphological features of some ION neurons labeled by Lucifer yellow. Single-labeled cells in **a** and **b** are drawn in figures 2P<sub>3</sub> and N, respectively; multilabeled cells in **c** and **d** are drawn in figures 3D<sub>4</sub> and D<sub>2</sub>, respectively. Scale bar: 75  $\mu$ m in **a**; 60  $\mu$ m in **b, c, d**.

to participate in the isthmo-optic tract (IOT). The perikarya and dendrites of type B cells are smooth, but some axons bear varicosities. The P type bipolar cells (53/112 = 47%) also have piriform perikarya but shorter apical dendrites (10–20  $\mu$ m) (fig. 2P<sub>1–4</sub>). An apical dendrite of a type P cell may ramify into fine branches bearing some spines (P<sub>1</sub>) or into several longer branches without further branching (P<sub>2</sub>). The neuron shown in figures 1a and 2P<sub>3</sub> possesses a thin and long dendritic field which covers almost the whole anterior-posterior extent of the nucleus. Its axon, with varicosities, originates from the basal pole of the piriform perikaryon and extends rostradorsally to participate in the IOT. It is worth mentioning that some P type cells issue their axons from apical dendrites close to the perikarya, and some of their axons may form pin-like loops on the way to the IOT. Figure 2P<sub>4</sub> shows an interesting cell giving rise to an axon-like process with varicosities. This process goes back to the interior of the nucleus, whereas the axon originates from the opposite pole of the perikaryon, makes a loop in the depression of the nucleus contour, and then goes to the IOT. Multipolar cells were also divided into two main types: M type (15 cells, 13%) and N type (4 cells, 4%). Neurons of type M

cells have round or elongated perikarya (fig. 2M<sub>1–3</sub>), from which two to four primary dendrites originate and go in the same direction (M<sub>2</sub>), or in divergent and convergent modes (M<sub>3</sub>). These dendrites could extend up to 200  $\mu$ m and sometimes bore varicosities. The cell shown in figure 2M<sub>1</sub> has its cell body located outside the nucleus, with its dendritic tree spreading into the nucleus. Its axon issues from the opposite pole of the perikaryon and then goes likely to the IOT. From its cellular morphology and axonal trajectory, it appears to be a displaced ION cell instead of an ECR element. Neurons of the N type usually have polygonal perikarya radiating three to five primary dendrites, some of which are appended with varicosities (fig. 1b, 2N). However, no discernible axons could be stained in this type of neuron. Their perikarya are located in the neuropilar zone of the nucleus, with a lower membrane potential of about –20 mV.

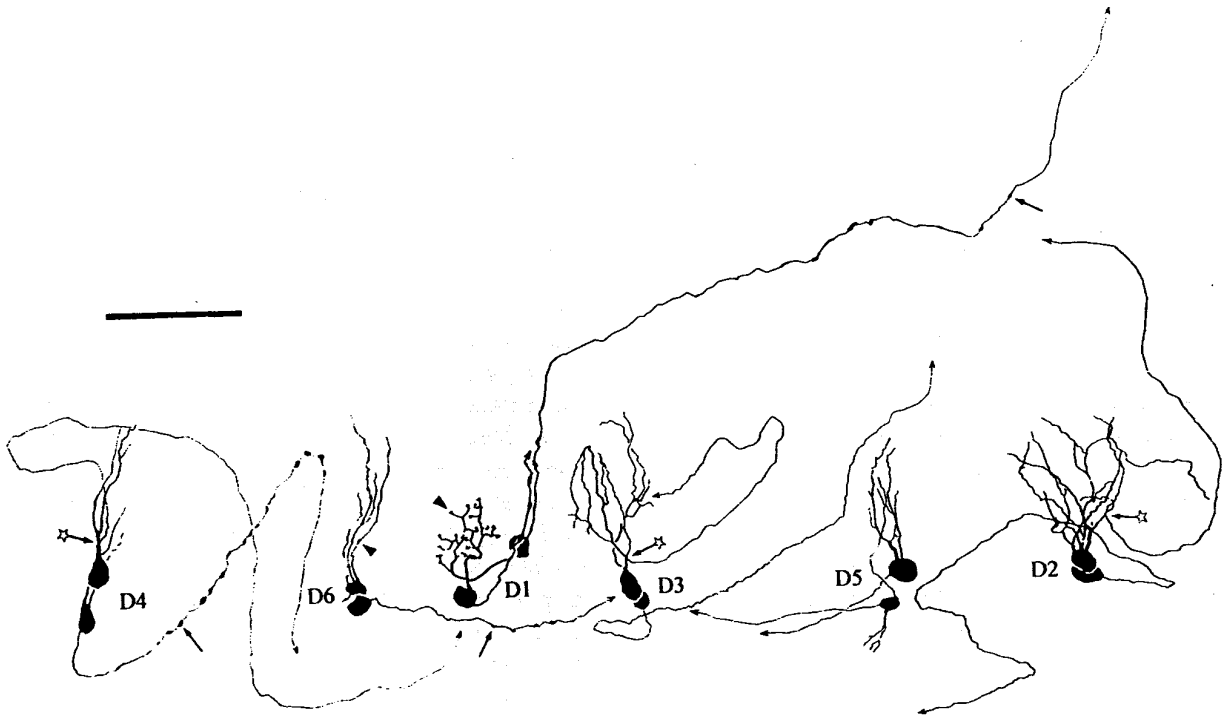
Thirteen cells were labeled in the ECR rostroventral to the ION, and they are quite different morphologically from the ION cells. On the basis of the size of perikarya and dendritic fields, these cells can be classified as large, or L-type, and small, or S-type. Morphologically speaking, S-type neurons (7/13 = 54%) are somewhat similar to ION neurons of



**Fig. 2.** Computer-aided morphological drawings of some labeled neurons in the isthmo-optic nucleus (ION), outlined with dotted lines, and in the ectopic cell region (ECR). ION neurons B<sub>1,2</sub> and P<sub>1-4</sub> represent two subtypes of bipolar cells; M<sub>1-3</sub> and N represent two subtypes of multipolar cells. Photomicrographs of cells P<sub>3</sub> and N are shown in figures 1a and b, respectively. ECR neuron L represents large cells; neuron S is an example of small cells. Their axons (arrows) converge to form the isthmo-optic tract (IOT). Short arrows point to varicosities on axons; arrowheads indicate varicosities on dendrites. 'Rackets' signify the locations of some multilabeled cells and their dendritic orientations. These cells are drawn in figure 3, with corresponding letters and numbers. A, D, P, and V indicate anterior, dorsal, posterior, and ventral, respectively. Scale bar: 100  $\mu$ m.

the N type in that they have polygonal perikarya radiating three to five primary dendrites that are short (25–180  $\mu$ m) and give off few branches, and they have no discernible axons (fig. 2S). Cells of the L-type (6/13 = 46%) also have polygonal perikarya that radiate three to six primary dendrites that can extend up to 350  $\mu$ m, branching 1–3 times; dendrites

oriented towards to the ION, however, are much shorter (30–100  $\mu$ m) than those oriented in other directions (fig. 2L). Axon-like processes could be traced in some cells. The axons of L cells tend to embrace the nucleus border and run together with isthmo-optic fibers coming out of the nucleus (cells B<sub>1</sub> and M<sub>2</sub>). In addition to morphological differences



**Fig. 3.** Computer-aided morphological drawings of some multilabeled ION neurons. Their locations and dendritic orientations are indicated by 'rackets' in figure 2, with corresponding letters and numbers. Cells  $D_{2,3,6}$  are twins, and  $D_{1,4,5}$  are doubles. Curved arrows represent axon trajectories. Short arrows on axons and arrowheads on dendrites point to varicosities; stars point to the origin of axons from dendrites. Photomicrographs of cells  $D_4$  and  $D_2$  are shown in figures 1c and d, respectively. Scale bar: 100  $\mu\text{m}$ .

between ION cells and presumptive ECR cells in their dendritic arborization patterns, ECR cells also differ in lacking varicosities and appendages on the perikarya and processes.

Furthermore, no dye-coupling was found between presumptive ECR cells in 13 single-injections, each of which was made in one slice. This is not the case with ION cells: twelve of 43 (28%) single-injections resulted in the labeling of 26 cells. Each of 11 injections labeled two cells, and one injection labeled four cells (fig. 3). Closely apposed twin-cells were labeled in 50% of the cases (fig. 3 $D_{2,3,6}$ ). The  $D_2$  twin-cells (also see fig. 1d) give rise to several dendrites and issue an axon from the perikaryon, which runs tortuously along the caudal side, and another axon from a secondary dendrite, which extends along the rostral side of the nucleus. The  $D_3$  cells possess willow-like dendritic branches, one of which appears to be an axon recurrent to dendrites; another axon arises from the opposite pole, makes a hairpin loop

near the perikaryon, then runs along the rostral side of ION. In  $D_6$  cells, the dendrites bearing varicosities form a thin field; an axon runs dorsally to the IOT, and an apparently truncated axon could be visualized originating from the second perikaryon. Two twin-cells were found to possess smooth dendrites and morphologically different axons, varicose and smooth; these axons took divergent then convergent routes to the IOT. Another group of multilabeled cells can be termed double-cells; their perikarya are 5–50  $\mu\text{m}$  apart (fig. 3 $D_{1,4,5}$ ). The dendrites of all of these cells are smooth, with the exception of those of  $D_1$  double-cells whose dendritic branches bear some spines. The two axons of most double cells are similar morphologically and in their trajectories; an exception is the  $D_4$  double-cells (also see fig. 1c), where a smooth axon arises from the apical dendrite of one cell while a varicose axon arises from the perikaryon of the other. These axons have quite different trajectories to

the IOT. Some multilabeled cells revealed no axons; the reasons are unknown, although the possibility of incomplete filling can be not excluded. It appears that all these multilabeled cells are located in the cell lamina, and the dendrites of a pair of cells usually have similar orientation. Dye-coupling between cells in the opposite cell lamina was never observed in this study.

## Discussion

The present study shows the morphology of isthmo-optic neurons in more detail than reported in previous studies [Cowan, 1970; Crossland, 1979; Gunturkun, 1987; Miceli et al., 1993, 1997]. Our fluorescent labeling indicates the existence of two main types of ION cells: bipolar and multipolar, each of which could be further divided into two subtypes. There also exist two types of cells in the ECR: large or L-type and small or S-type. Golgi material shows only a single type of ION cells, characterized by round to oval perikarya and thick dendrites that may give off claw-like branches [Cowan, 1970; Gunturkun, 1987]. The appearance of these cells is similar to that of the principal neurons but different from that of the second type of neurons in the ION of chicks [Crossland, 1979]. Golgi-impregnated cells look like some ION neurons as shown in figures 2B<sub>1</sub>, B<sub>2</sub> and M<sub>1</sub>, but their dendritic trees and distribution of spines are much less detailed than those in our fluorescent materials. Axons and their trajectories can be traced for much longer distance (1300  $\mu\text{m}$ ) in Lucifer yellow stained preparations than in Golgi materials (75  $\mu\text{m}$ ). Based on general description, our N type neurons seem similar to the second type of neuron found in the ION of chicks [Crossland, 1979]. They are located within the neuropilar zone and do not have discernible axons, suggesting that they may be interneurons, based on their similarity to GABA-immunoreactive interneurons, which represent less than 2% of the total population of ION cells [Miceli et al., 1995]. The discrepancies of results based on dye-labeled cells and those based on Golgi-impregnated cells could be explained by the finding that Golgi impregnation is not suited for showing a dense network of dendritic branches and appendages such as the dendritic arborization patterns of ION cells [Crossland, 1979]; also, Golgi-stained cells have thicker processes and more numerous spines than those stained with intracellular horseradish peroxidase [Wang et al., 1983].

The present study also provides convincing evidence that dye-coupling connections exist in about 30% of the ION cells. The possibility that dye-coupling between ION cells is formed by an electrode bridge may be excluded, at least in

most cases, for two reasons: firstly, perikarya of dye-coupled cells are up to 50  $\mu\text{m}$  distant from each other; secondly, dye-coupled cells are usually not requisitely arranged in the electrode track direction. It appears that dye-coupling is not an artifact, and it has been shown in various regions of the nervous system [Stewart, 1978; Grace and Bunney, 1983; Wang and Matsumoto, 1990; Peinado et al., 1993; Onn and Grace, 1994; Wu and Wang, 1995]. It has been suggested to reflect electrotonic connections between cells [Peinado et al., 1993]. This electrical communication may be involved in modulating burst firing and in synchronizing transmitter release [Grace and Bunney, 1983]. Within the ION, dye-coupling frequently occurs between two closely apposed cells, probably 'twin-cells' with large attachment plaques between them [Angaut and Reperant, 1978], suggesting that these cells may form somatosomatic electrical synapses. Other dye-coupled neurons may form dendrodendritic gap junctions [Peinado et al., 1993], as their dendrites are usually intermingled in a column-like region, whereas their axons do not contact each other. The axons of dye-coupled cells run in the same direction, or they first diverge then converge to participate in the IOT. In some cases, two axons originating from a dye-couple are quite different morphologically, one being smooth and the other bearing varicosities. This morphological difference is reminiscent of two branches arising from a single axon of pretectal neurons. However, the functional significance of this morphological difference is unknown [Ito et al., 1997]. Although Golgi materials [Gunturkun, 1987] and our own horseradish peroxidase tracings (unpublished observations) have shown that dendritic branches of cells in the opposite lamina of the nucleus intermingle to form networks in the central region, it is surprising that no dye-coupling is found between the opposite neurons. This unexpected finding may imply that transmission between the opposite neurons, if any, is probably different from that among neurons in the same layer. In some cases, 'twin-cells' have axons running separately along the opposite sides of the nucleus, opposing the notion that ION cells in the opposite layers exert dichotomous excitatory and inhibitory actions on retinal cells [Holden and Powell, 1972]. In fact, several electrophysiological studies [Miles, 1972; Pearlman and Hughes, 1976; Uchiyama and Barlow, 1994; Li et al., 1998] also do not support this hypothesis.

Most ECR neurons lie within an area medial, ventral, rostral and caudal to the ION, and they are quite different morphologically from ION cells in their multipolar appearance [Hayes and Webster, 1981; O'Leary and Cowan, 1982; Wolf-Oberhollenzner, 1987]. The sole reliable criterion for identifying ECR neurons is retrograde labeling of these cells

by tracers applied in the eye or in the IOT. However, this tracing method can not determine whether dye-coupling exists between ECR neurons. Though the present study does not show the existence of dye-coupling between neurons in the ectopic region, it also did not confirm which cells labeled outside the ION are really ECR cells. Considering the heterogeneity of ECR cells [Miceli et al., 1993, 1997], we believe that at least some of the cells labeled outside ION are ECR cells, based on their location and morphology. Neurons of type S appear to be similar to those labeled by retrograde transport of Fast blue in pigeons [Wolf-Oberholzer, 1987] or by wheat germ agglutinin-horseradish peroxidase in chicks [O'Leary and Cowan, 1982], but without discernible axons. On the other hand, type L neurons in the present study also appear to be ectopic neurons, due to their multipolar perikarya and the fact that their presumptive axons run the same direction as ION axons: for example, axons of B<sub>1</sub> and M<sub>2</sub> neurons (fig. 2). O'Leary and Cowan [1982] postulated that ectopic neurons are displaced ION cells based on several considerations. The present study not only shows morphological differences between ION and ECR neurons; it also shows the existence of dye-coupling connections in the ION and not in ectopic region.

The ION in birds is the most extensively studied retinal-tectal-ION-retinal feedback loop in all classes of vertebrates, although its functional significance is not yet clearly

known. Several hypotheses have postulated that this nucleus may be involved in visual attention, search, and discrimination [Uchiyama, 1989; Holden, 1990; Clarke et al., 1996]. In recent years, accumulating evidence supports the hypothesis that this centrifugal system may act as a 'searchlight' in ground-feeding birds, highlighting or alerting them to threatening targets in the upper visual field [Uchiyama, 1989; Holden, 1990]. In fact, this system has a heterotopic organization: i.e., the ION receives its major input from the dorsal retina via the tectum and projects primarily to the ventral retina [Holden, 1990; Woodson et al., 1995; Clarke et al., 1996]. The two parts of the retina might be connected by intrinsic interneurons [Woodson et al., 1991] or by the propriorectal neurons which project long axons from the ventral retina to the dorsal retina. Therefore, local enhancement of the ventral retina and long-range inhibition in the dorsal retina would result in the switching of visual attention switching [Clarke et al., 1996]. Our recent electrophysiological findings [Li et al., 1998] lend strong support to this notion.

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