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Neurotransmitters, receptors, and neuropeptides in the accessory optic system: An immunohistochemical survey in the pigeon (*Columba livia*)

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Abstract

Immunohistochemical techniques were used to survey the distribution of several conventional transmitters, receptors, and neuropeptides in the pigeon nucleus of the basal optic root (nBOR), a component of the accessory optic system. Amongst the conventional neurotransmitters/modulators, the most intense labeling of fibers/terminals within the nBOR was obtained with antisera directed against glutamic acid decarboxylase (GAD) and serotonin (5-HT). Moderately dense fiber plexuses were seen to label with antibodies directed against tyrosine hydroxylase (TH) and choline acetyltransferase (ChAT). GAD-like immunoreactivity (GAD-LI) was found in many small and medium-sized perikarya within the nBOR. Some of the medium-sized cells were occasionally positive for ChAT-LI. Cell body and dendritic staining was also commonly seen with the two tested antisera against receptors—anti-GABA-A receptor and anti-nicotinic acetylcholine receptor.

The antisera directed against various neuropeptides produced only fiber labeling within the nBOR. The densest fiber plexus staining was observed with antiserum against neuropeptide Y (NPY-LI), while intermediate fiber densities were seen for substance P (SP-LI) and cholecystokinin (CCK-LI). A few varicose fibers were labeled with antisera against neurotensin (NT), leucine-enkephalin (L-ENK), and the vasoactive intestinal polypeptide (VIP).

Unilateral enucleation produced an almost complete elimination of TH-LI in the contralateral nBOR. SP-LI and CCK-LI were also decreased after enucleation. No apparent changes were seen for all other substances.

These results indicate that a wide variety of chemically-specific systems arborize within the nBOR. Three of the immunohistochemically defined fiber systems (TH-LI, SP-LI, and CCK-LI fibers) were reduced after removal of the retina, which may indicate the presence of these substances in retinal ganglion cells. In contrast, the fibers exhibiting ChAT-LI, GAD-LI, 5-HT-LI, NPY-LI, NT-LI, L-ENK-LI, and VIP-LI appear to be of nonretinal origin. Two different populations of nBOR neurons exhibited GAD-LI and ChAT-LI. However, these two populations together constituted only about 20% of the nBOR neurons.

Keywords: Accessory optic system, Immunohistochemistry, Neuropeptides, Neurotransmitters, Visual system

Introduction

The vertebrate accessory optic system (AOS) consists of a specific population of retinal ganglion cells projecting to mesencephalic nuclei which, in turn, project to a variety of structures involved in visuomotor functions. The AOS has a significant role in the functional organization of optokinetic nystagmus (reviews in Fite, 1985; McKenna & Wallman, 1985; Simpson, 1984). The central nuclei of the AOS receive their afferents primarily from the contralateral retina. Other afferents, however, have been found to originate from diverse, nonretinal sources. Common sources of such connections among different vertebrate classes include visual areas of the telencephalon, specific pretectal nuclei, the ventral lateral geniculate nucleus, preoculomotor areas, and contralateral AOS structures (Brecha et al., 1980; Giolli et al., 1988; McKenna & Wallman, 1985; Miceli et al., 1987; Rio et al., 1983; Simpson, 1984).

Despite the increase in available data on the functional or-

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ganization of the AOS, there is little information available concerning the neurotransmitters and neuropeptides contained within its central nuclei or in its retinal cells of origin. A high proportion of cells in the mammalian AOS nuclei appear to contain glutamic acid decarboxylase (GAD), the synthesizing enzyme for the inhibitory neurotransmitter gamma aminobutyric acid (GABA). Furthermore, more than half of the neurons in those nuclei appear to be contacted by GABAergic terminals (Penny et al., 1984; Giolli et al., 1985*b*). Both the AOS and pretectal nuclei of the frog also contain substantial amounts of GAD (Yucel et al., 1988). Neurons immunoreactive for a GABA antibody were also reported within the AOS in a recent mapping study in the pigeon brain (Domenici et al., 1988). There appear to be only three reports on putative transmitters in the ganglion cells projecting to the AOS. The first indicates the occurrence of substance P-like immunoreactivity in such cells in the rabbit (Brecha et al., 1987). The second demonstrated that approximately 10% of the displaced retinal ganglion cells projecting upon the pigeon accessory optic nucleus exhibit tyrosine hydroxylase-like immunoreactivity (Britto et al., 1988). The third study reported the presence of a neurotensin-related peptide, LANT-6, in retinal ganglion cells of the turtle AOS (Eldred et al., 1988). Several recent studies have indicated the possible presence of other transmitter/peptide/receptor molecules in the AOS nuclei; e.g. an extensive AOS serotoninergic innervation was described in the chicken (Sako et al., 1986) and the lizard (Smeets & Steinbusch, 1988; Wolters et al., 1985), and binding sites for both neurotensin (Brauth et al., 1986) and opioids (Atweh & Kuhar, 1977; Atweh et al., 1978; Reiner et al., 1989) were shown to be present in AOS nuclei.

In the present study, we have used immunohistochemical techniques to survey the distribution of a wide variety of putative neurotransmitters and receptors in the pigeon basal optic root nuclear complex (nBOR), the central component of the avian AOS (Brecha & Karten, 1981). This nucleus provides a useful model for chemical anatomy studies, for a number of reasons. First, it receives retinal projections from a limited number (*ca.* 5000) of displaced ganglion cells (Fite et al., 1981). Second, the nBOR neuronal population can be readily segregated into several subtypes of neurons (Brecha et al., 1980). Third, the different nBOR neuronal types project to specific targets (Brecha et al., 1980; Brecha & Karten, 1981). Finally, both the connectivity and physiology of the avian nBOR are well-known (Britto et al., 1981; Morgan & Frost, 1981; reviewed in McKenna & Wallman, 1985).

The nBOR contains three major cytoarchitectonic components (Brecha et al., 1980). These subnuclei include: (1) nBORp, the spherical principal ventral division of the nBOR; (2) nBORd, a cellular cap covering most of the dorsal aspect of the nBORp; and (3) nBOR1, a laterally elongated portion lying over the optic tract. In order to demonstrate the boundaries of the nuclear complex in relation to the immunohistochemical staining, intraocular injections of a fluorescent tracer and cresyl violet staining were used. The effects of unilateral enucleation on the distribution of immunoreactive neural elements within the nBOR was also evaluated.

Materials and methods

Forty adult pigeons (*Columba livia*) were used in these experiments. Twelve birds received a single microinjection of colchicine to inhibit axonal transport and thereby enhance visualization of staining in cell bodies. These animals were anesthetized with ketamine (5 mg/100 g of body weight, i.m.) and xylazine (1 mg/100 g, i.m.) and placed in a stereotaxic apparatus device (Karten & Hodos, 1967). All incision and pressure points were infiltrated with lidocaine. The scalp was then cut at the midline and the skull opened. A solution of colchicine in buffered saline (1.0 μg/μl) was pressure-injected immediately dorsal to the nBOR in volumes ranging 0.3–0.5 μl. These pigeons were allowed to survive for 24 h after injections. In six other animals, rhodamine isothiocyanate (RITC) was injected into one eye in order to map the retinal projection to the nBOR (Thanos & Bonhoeffer, 1983; Thanos et al., 1987). In these cases, the birds were anesthetized with metoxyfluorane and 3–5 μl of a 5% RITC solution in phosphate buffer with 5% dimethyl sulfoxide were injected into the vitreous humor through a sterile, small gauge needle. Survival times for these animals ranged from 3–10 days. Four other pigeons were deeply anesthetized with ketamine and xylazine and subjected to unilateral enucleation. Lidocaine was injected intravitreally before the retina was removed. Sterile gelfoam was placed in the eye after completion of the procedure, and the birds were sacrificed after 7, 10, 14, and 28 days. Both the normal and the experimental pigeons were given an overdose of ketamine and xylazine and perfused through the left ventricle and aorta with 6% dextran in 0.1 M phosphate buffer solution (PB). This was generally followed by ice-cold 4% paraformaldehyde in PB. In a few instances, 1.0% paraformaldehyde or 1.0% glutaraldehyde were used instead. The brains were kept in the fixative for an additional 6–12 h, then transferred to a 30% sucrose solution in PB and maintained there for approximately 16 h to minimize damage resulting from freezing. The brains were frozen and cut at 30 μm on a sliding microtome in either the coronal or sagittal stereotaxic planes (Karten & Hodos, 1967). Two brains were cut in the horizontal plane.

Both the indirect fluorescence and the avidin–biotin (ABC) techniques were used in this study. In both procedures, we employed primary immunoglobulins directed against cholecystokinin (CCK), leucine-enkephalin (L-ENK), neurotensin (NT), serotonin (5-HT), vasoactive intestinal polypeptide (VIP), luteotropin-releasing hormone (LRH), somatostatin (SS), bombesin (BOM), beta-endorphin (END), vasopressin (VPR), and glutamate (GLUT), all from Immunonuclear Corporation (Stillwater, MN); choline acetyl transferase (ChAT, donated by M.Epstein; see Johnson & Epstein, 1986), glutamic acid decarboxylase (GAD, provided by W.Oertel; see Oertel et al., 1981), substance P (SP, Sera Lab, West Sussex , UK), tyrosine-hydroxylase (TH, Eugenetech, Allendale, NJ), glucagon (GLU, Chemicon Inc., El Segundo, CA), neuropeptide Y (NPY, Amersham, Buckinghamshire, UK), nicotinic acetylcholine receptors (nAChR, monoclonal antibodies mAb270 and mAb210, from J. Lindstrom; see Whiting & Lindstrom, 1986) and GABA-A receptors (From A. de Blas; see Vitorica et al., 1988). All antisera were diluted in 0.3% Triton-X in PB, and consistent results were usually obtained with dilutions ranging 1:1000–1:5000. Incubation times ranged from 16–48 h at 4°C. For the immunofluorescence experiments, sections were then incubated for 1 h at room temperature with an antiserum against the immunoglobulins of the animal in which the primary antibody was made and labeled with fluorescein isothiocyanate (FITC). In a few cases, in order to obtain double-labeling, we also used secondary antisera which were labeled with RITC. The secondary antisera were diluted 1:100 in 0.3% Triton X-100 in PB. Glyc-

erol-carbonate buffer was used as a mounting medium in this case. For ABC processing, a biotinylated secondary antiserum was used at a dilution of 1:200 in 0.3% Triton X-100 in PB. After 1 h of incubation at room temperature and washing in PB, the sections were placed in the avidin-peroxidase complex diluted 1:100 in 0.3% Triton X-100 in PB for 1 h at room temperature. Finally, the tissue was placed in a 0.05% diaminobenzidine solution in PB for 15 min and hydrogen peroxide was added (0.01% final concentration). After several washes in PB, the sections were mounted on gelatin-coated slides, dipped in 0.1% osmium tetroxide for 15–30 s, dehydrated, cleared, and a coverslip was applied (Permount, Fisher Sci., Fair Lawn, NJ).

Controls for immunohistochemistry included (1) the omission of the primary antibody incubation; (2) the preabsorption of the primary antibody with the appropriate antigen; and (3) the substitution of the primary antibody with normal serum from the same species. Under these conditions, staining was abolished for all antibodies used in this study. However, we will hereafter refer to immunostaining as neurotransmitter, receptor, or neuropeptide-like immunoreactivity (LI). In most cases, adjacent sections were stained with cresyl violet, in order to facilitate the identification of the nBOR boundaries. The relative densities of labeled perikarya could then be scaled according to the number of stained cell bodies in adjacent sections. The scoring system for the cell bodies was based on the percentage of labeled neurons compared to the total number of neurons seen using a Nissl stain. Low, moderate, or high numbers of immunohistochemically positive cells were ascribed to the following classes of percentages: less than 33%, between 33% and 66%, and more than 66%. In the case of fiber labeling, a far more subjective system had to be used. In this scaling system, we considered *high* the highest number of labeled fibers observed for any antiserum, and *low* the lowest number. Any intermediate labeling was scaled as *moderate*. These ratings proved to be sufficient in preliminary experiments. Figure 1 shows samples of low, moderate, and high density of fiber stain.

The immunofluorescent material was examined with a microscope equipped for epifluorescence and photographed with Kodak T-Max at ASA 400, 800, or 1600. Nikon filters B2 (excitation at 470–490 nm and barrier at 520 nm) and G (excitation at 546 nm and barrier at 590 nm) were used to visualize FITC and RITC, respectively. The sections processed with the ABC protocol were mainly observed and photographed using differential interference contrast optics.

Results

Many somata and fibers in the pigeon brain were clearly and specifically stained with each antibody used. No major differences were seen between sections processed for indirect fluorescence and those processed using the ABC technique. The latter method appeared, however, to increase the detectability of stained fibers. No apparent differences in the distribution of immunoreactive fibers and perikarya were observed between normal animals and those that were pretreated with colchicine or had been injected with rhodamine in the eye. However, the colchicine treatment clearly improved the staining of cell bodies over an approximately 2-mm diameter area around the injection site. Furthermore, despite an overall reduction of fiber labeling, this treatment produced a marked increase in both the number and intensity of GAD-LI terminals that were visualized. This effect was not observed for the other antisera used.

The intraocular rhodamine injections helped to clearly delineate the various components of the nBOR complex on the basis of its retinal input (Fig. 2). In many instances, observation of sections processed for immunofluorescence (FITC) and that also contained RITC-labeled retinal fibers was helpful for confirmation of whether or not labeled elements were actually located within the boundaries of the nBOR complex. This was especially true for the zones medial and dorsal to nBOR. These areas correspond mostly to the ventral tegmental area and lateral hypothalamic structures in the area just medial to the nBOR. Dorsally, the neural elements appear to correspond to

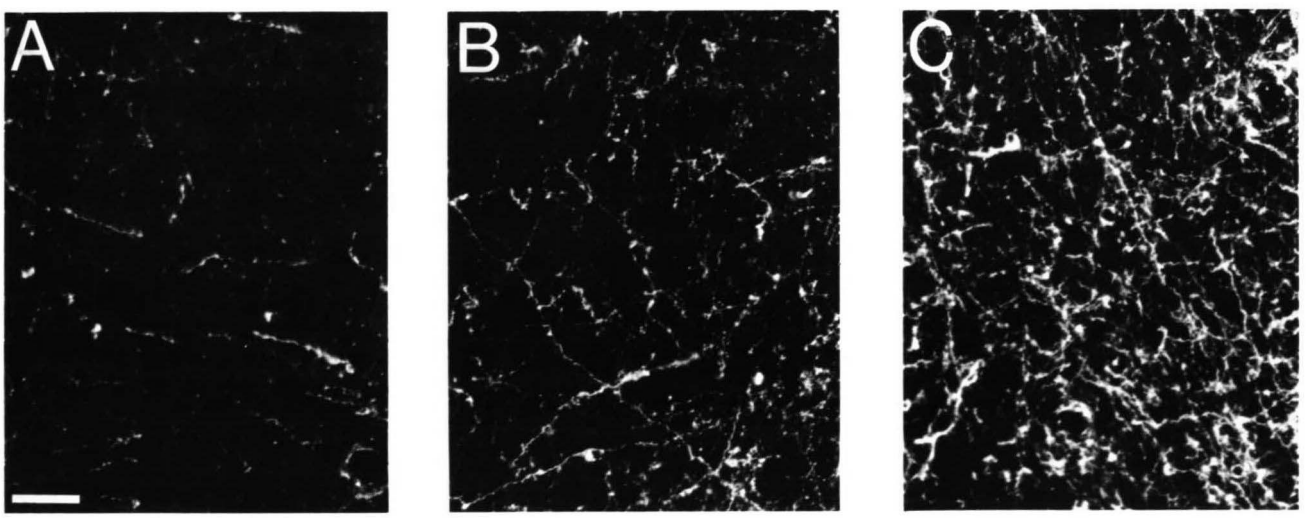

Fig. 1. Dark-field photomicrographs showing examples of *low* (A), *moderate* (B), and *high* (C) densities of immunostained fibers (ABC technique). These patterns were used as a reference for the classification of the fiber densities within the pigeon nBOR. All fibers in these photomicrographs were stained with an antiserum against TH, and were found in several regions of the mesencephalic tegmentum at the same level of the nBOR. Scale bar = 40 μm.

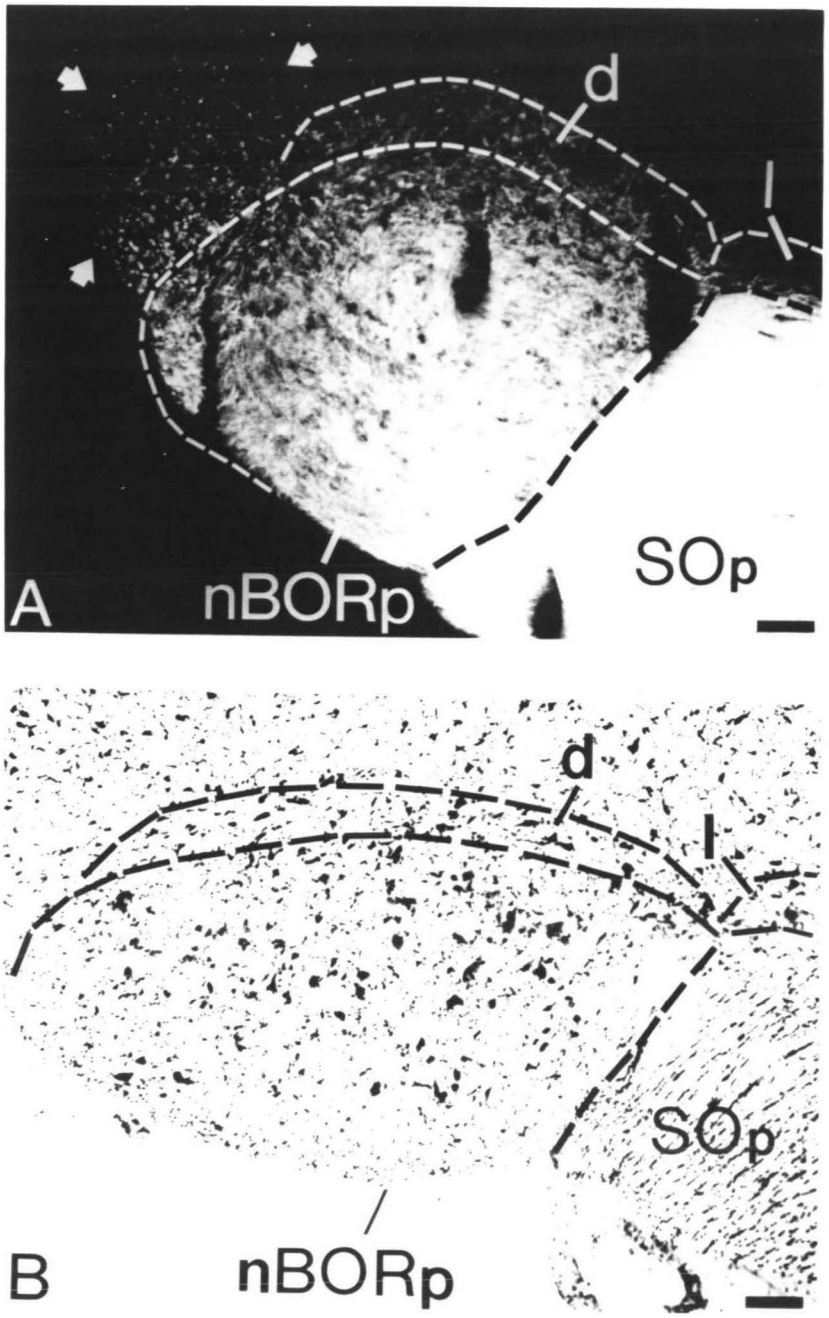

Fig. 2. A, a photomicrograph taken under rhodamine fluorescence illustrates the pattern of the RITC-labeled retinal fibers terminating within the nBOR (coronal section). Arrows indicate basal optic root axons entering the nBOR complex. B, photomicrograph of a Nissl-stained coronal section of the pigeon brain through the nBOR, at a level *ca.* 240 μm caudal to the one shown in A. SOp: stratum opticum of the tectum; p, d, and l indicate the principal, dorsal, and lateral subdivisions of the nBOR complex, respectively. Scale bar in A and B = 80 μm.

a ventro-rostral extension of the nucleus tegmenti pedunculopontinus, pars compacta, and to a ventral expansion of the nucleus mesencephalicus profundus (Karten & Hodos, 1967). Labeled elements within all these neighboring structures were not studied further.

Cresyl violet staining was also used to define the limits of the nBOR (Fig. 2) and permitted classification of labeled cells in relationship to the several types of neurons within that AOS nucleus (Brecha et al., 1980). Soma sizes within nBOR ranged from 6–26 μm in diameter along their largest axes. We classified as *small* those cell bodies ranging from 6 to 8 μm in diameter, *medium* the ones ranging from 9–15 μm in diameter, and *large* those between 16–26 μm in diameter. The shapes of those neurons varied from round to fusiform.

The nBOR appeared to be innervated by several of the chemically-specific systems examined. The distribution of the putative transmitters/modulators or receptors within the nBOR complex appeared rather homogeneous within its component

subnuclei along their rostro-caudal and medio-lateral extents. Thus, only one representative level through the nBOR is illustrated in Figs. 3 and 5 (and one photomicrograph in Figs. 4 and 6) for each antibody that produced labeling in that nucleus. In Table 1, the relative densities of immunoreactive processes or cell bodies are summarized for all of the antibodies used in this study.

Classical transmitters and receptors

GAD-LI and GABA-A-LI

The most intense immunohistochemical labeling within the nBOR complex was obtained with antibodies directed against GAD. Positive perikarya were distributed throughout nBOR. They were either medium- or small-sized, and both round and spindle-shaped. The large neurons in nBORp did not contain GAD, but their somata were always surrounded by punctate GAD-positive features, presumably terminals. These punctate features were found in high density, which made visualization of processes, and sometimes even cell bodies, rather difficult (Figs. 3A and 4A). The number of GAD-positive cells was classified as *low*, since they were estimated to represent approximately 12% of the total number of neurons in nBORp and 15–20% of those in nBORd or nBORl.

Labeling with the antibody against GABA-A receptors was mostly seen in the large perikarya within the nBORp and their large dendrites (Figs. 3B and 4B). Some medium-sized cells throughout the nBOR were also stained with this anti-

Table 1. *Relative densities for immunostained fibers/terminals and perikarya within the subnuclei of the nBOR complex*[a,b]

	nBORp		nBORd		nBORl	
Subnuclei	P	F/T	P	F/T	P	F/T
Classical transmitters and receptors						
GAD	+	+++	+	+++	+	+++
GABA-A	++	+	+	+	+	+
ChAT	+	++	−	++	−	++
nAChR	+	+	+	+	+	+
5-HT	−	+++	−	+++	−	+++
TH	−	++	−	++	−	++
Peptides						
NPY	−	+++	−	++	−	++
SP	−	++	−	++	−	++
CCK	−	+	−	++	−	++
VIP	−	+	−	+	−	+
NT	−	+	−	+	−	+
L-ENK	−	+	−	+	−	+

[a]The fibers/terminals density (1) was scaled compared to the density of NPY-labelled fibers, which was by far the most intense fiber labelling in the nBOR complex. The cell body density (2) was rated in relation to the total number of cresyl-violet stained cell bodies in each section.

[b](1,2) Relative fiber or cell densities: − absent; + low; ++ moderate; +++ high. P and F/T refer to perikarya and fibers/terminals, respectively.

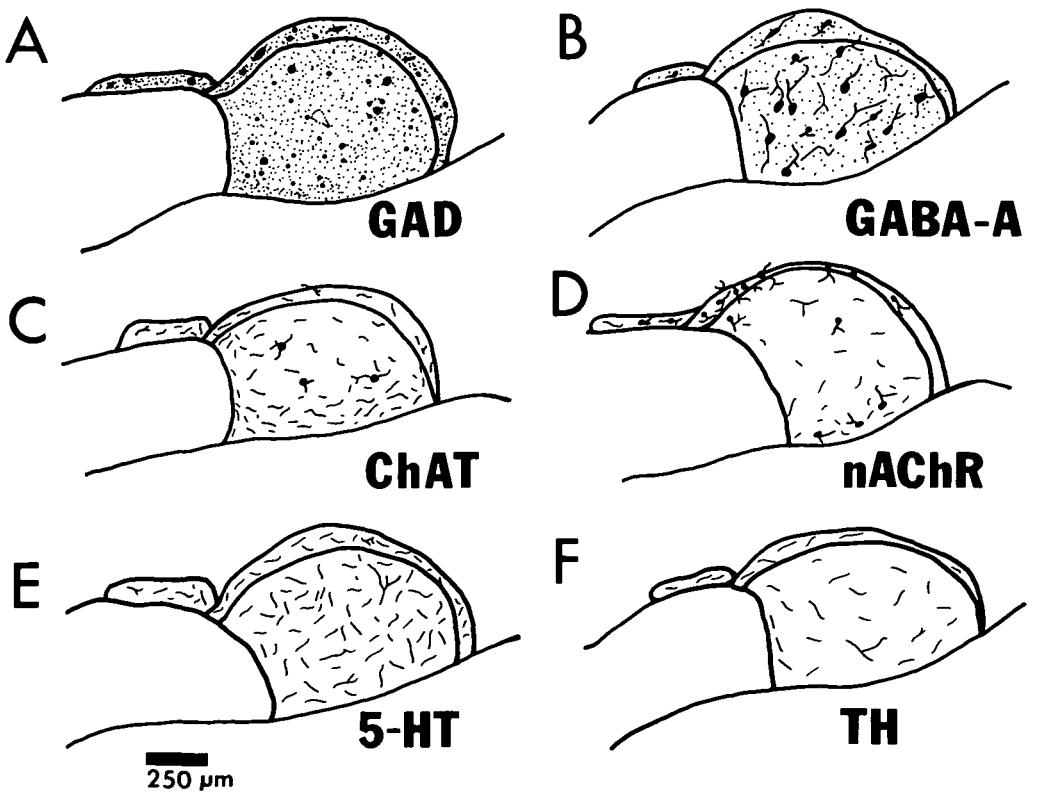

Fig. 3. A series of line drawings illustrating the distribution of cell bodies, fibers, and terminals which stained with antibodies against several classical transmitters and receptors within the nBOR. The representative coronal sections depicted in this figure correspond to intermediate nBOR levels (between the levels shown in Figs. 2A and 2B). See Fig. 2 for nBOR subdivisions.

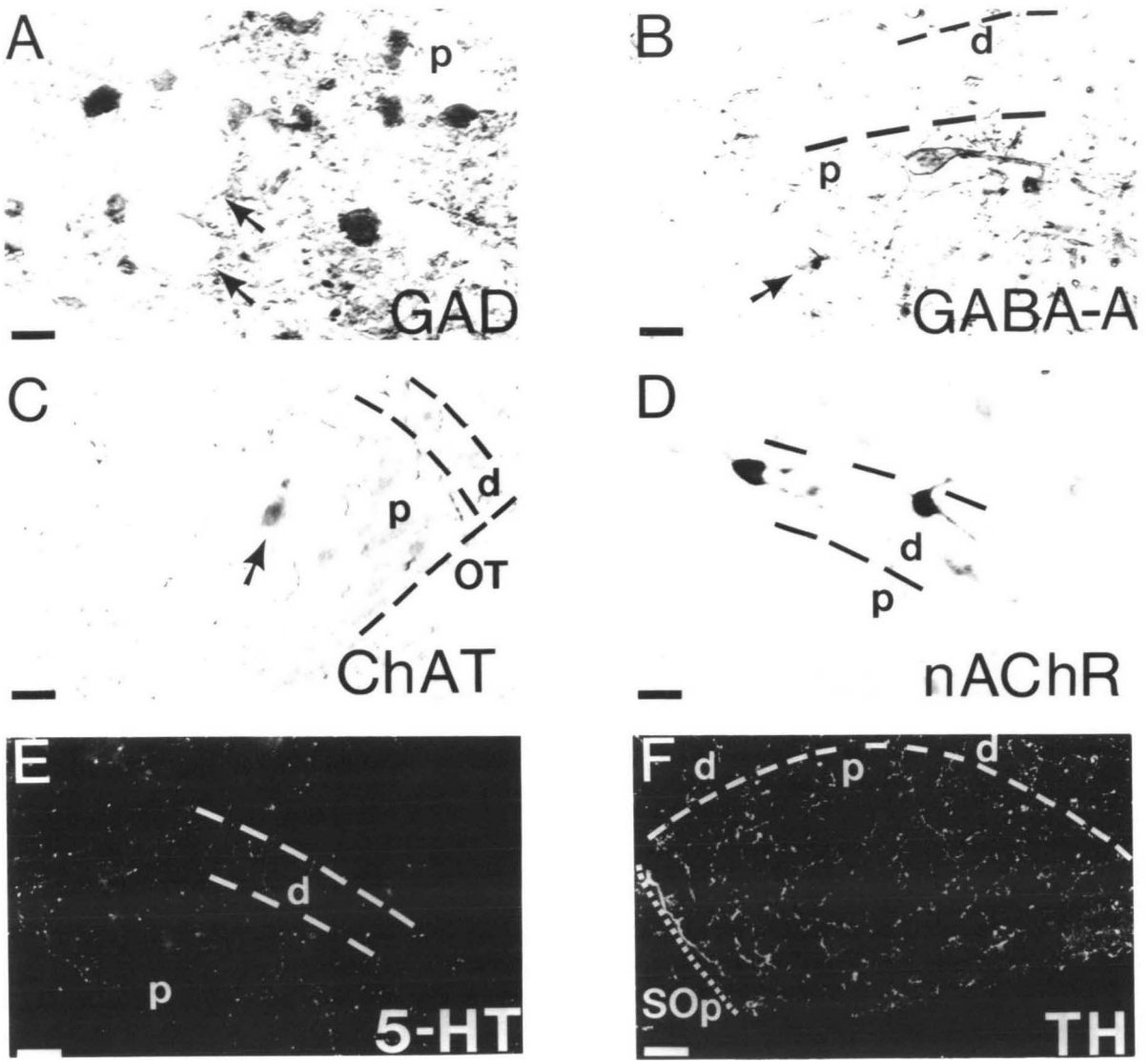

Fig. 4. Photomicrographs of immunoreactive nBOR perikarya, fibers, and terminals which were stained with antibodies against several classical transmitters and receptors (A–D and F, ABC technique; E, FITC technique). Part F shows a dark-field photomicrograph. All of these sections through the nBOR were cut in the coronal plane. Arrows in part A indicate large GAD-negative cells which are surrounded by GAD-LI puncta. In parts B and C, the arrows point to nBOR medium-sized neurons exhibiting GABA-A-LI and ChAT-LI, respectively. Dashed lines indicate the approximate boundaries of nBORp and nBORd, as determined by the pattern of RITC-labeled retinal fibers and Nissl staining of adjacent sections. OT: optic tract; SOp: stratum opticum of the tectum. Scale bar in A = 12 μm; B = 20 μm; C = 25 μm; D = 20 μm; E = 40 μm; and F = 100 μm.

body. The total number of labeled cells was estimated to represent at least 35% of all neurons which could be counted in the cresyl-violet-stained adjacent sections. Small cells were never seen to be labeled with antibodies against GABA-A receptors. The overall nBOR labeling found with this antibody was much less intense than with antibodies directed against GAD.

ChAT-LI and nAChR-LI

A moderately dense plexus of ChAT-LI varicose fibers was observed within the nBOR (Figs. 3C and 4C). The density of staining, however, was somewhat greater in the ventral and dorsolateral parts of the nBORp and in the nBORd than it was in other parts of the nBOR complex. Some of the medium-sized cells within the nBORp (fewer than 1%) were occasionally seen to stain with this antiserum. This stain was always faint, and consequently we were unable to decide whether they also contained GAD in doubly-labeled preparations. The pattern of ChAT-LI stain for fibers within the nBOR was consistent with the nAChR-LI labeling, since both staining patterns were observed in the same nBOR regions. Indeed, nAChR-LI was observed to occur mainly in small- and medium-sized neurons in nBORd and also in some of the medium cells in the ventral part of the nBORp (Figs. 3D and 4D). Both the cell bodies and proximal dendrites were seen to stain with the nAChR antibodies mAb270 and mAb210. No apparent axonal or terminal labeling was seen. The percentage of perikarya labeled with anti-nAChR was approximately 8%.

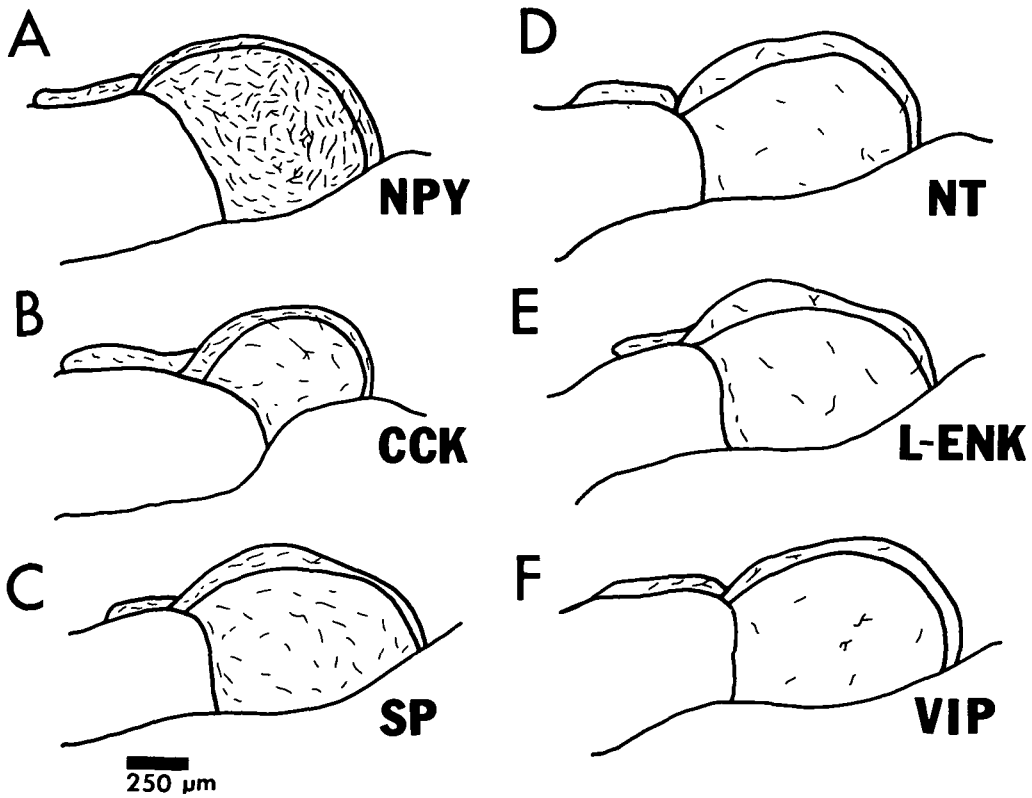

Fig. 5. A series of line drawings illustrating the distribution of neuropeptide-LI within the nBOR. The levels of these representative coronal sections correspond to those shown in Figs. 2 and 3, except for the drawing in part B. This section is rostral in relation to all others depicted in this figure and also in Figs. 2 and 3. See Fig. 2 for nBOR subdivisions.

Biogenic amines

Densely packed varicose fibers were observed to stain with the antiserum against 5-HT fibers in all subdivisions of the nBOR (Figs. 3E and 4E). Such fibers were found in notably lower density in the adjacent tegmentum. Putative catecholaminergic fibers labeled with the TH antiserum were also seen throughout the nBOR. The TH-LI fibers were found in moderate numbers in the three nBOR subnuclei (Figs. 3F and 4F).

Peptides

NPY-LI

A very dense plexus of NPY-LI varicose fibers was seen within the nBORp. The density of NPY-LI fibers in that subnucleus was taken as the reference for the *high* density in the scaling used in Table 1. The density of such fibers within the nBORd and nBORl was intermediate (Figs. 5A and 6A).

CCK-LI and SP-LI

Fibers exhibiting CCK-LI were found in moderate numbers within the nBOR (Figs. 5B and 6B). However, we observed a lower CCK-LI fiber density in nBORp when compared to nBORd and nBORl. A medium-dense SP-LI fiber plexus was also observed throughout the nBOR complex (Figs. 5C and 6C).

NT-LI, L-ENK-LI, and VIP-LI

Only a few varicose fibers were observed to contain NT-LI, L-ENK-LI, and VIP-LI. These fibers were distributed in approximately the same density in each subdivision of the nBOR (Figs. 5D–F and 6D–F).

Other peptides

No staining in nBOR was observed with antisera directed against GLU, LRH, SS, BOM, VPR, or END in either normal or colchicine-treated pigeons. All of these antisera produced, however, specific labeling in other brain structures.

Enucleation experiments

Examination of the brains of pigeons subjected to unilateral enucleation revealed a dramatic reduction in the number of TH-immunoreactive fibers throughout the contralateral nBOR. This effect was consistently seen following survival times of 1–4 weeks after enucleation. A few TH-positive axons were still present, however, at all survival times examined. The persisting TH-LI fibers were subjectively estimated to represent about 5% of the total TH-LI fibers. Two to 4 weeks after enucleation, reduced SP-LI and CCK-LI fiber staining was also observed within the nBOR (Fig. 7). The overall reduction in nBOR fiber labeling for these two neuropeptides represented about 70% of the total fiber labeling for SP-LI and CCK-LI. No evident alterations were observed for any of the other antibodies used in this study. Furthermore, the pattern of immunoreactivity in the region around the nBOR was not noticeably affected by enucleation in any case.

Discussion

The results of this study in the pigeon indicate that a wide variety of neurotransmitters, neuropeptides, and receptors are

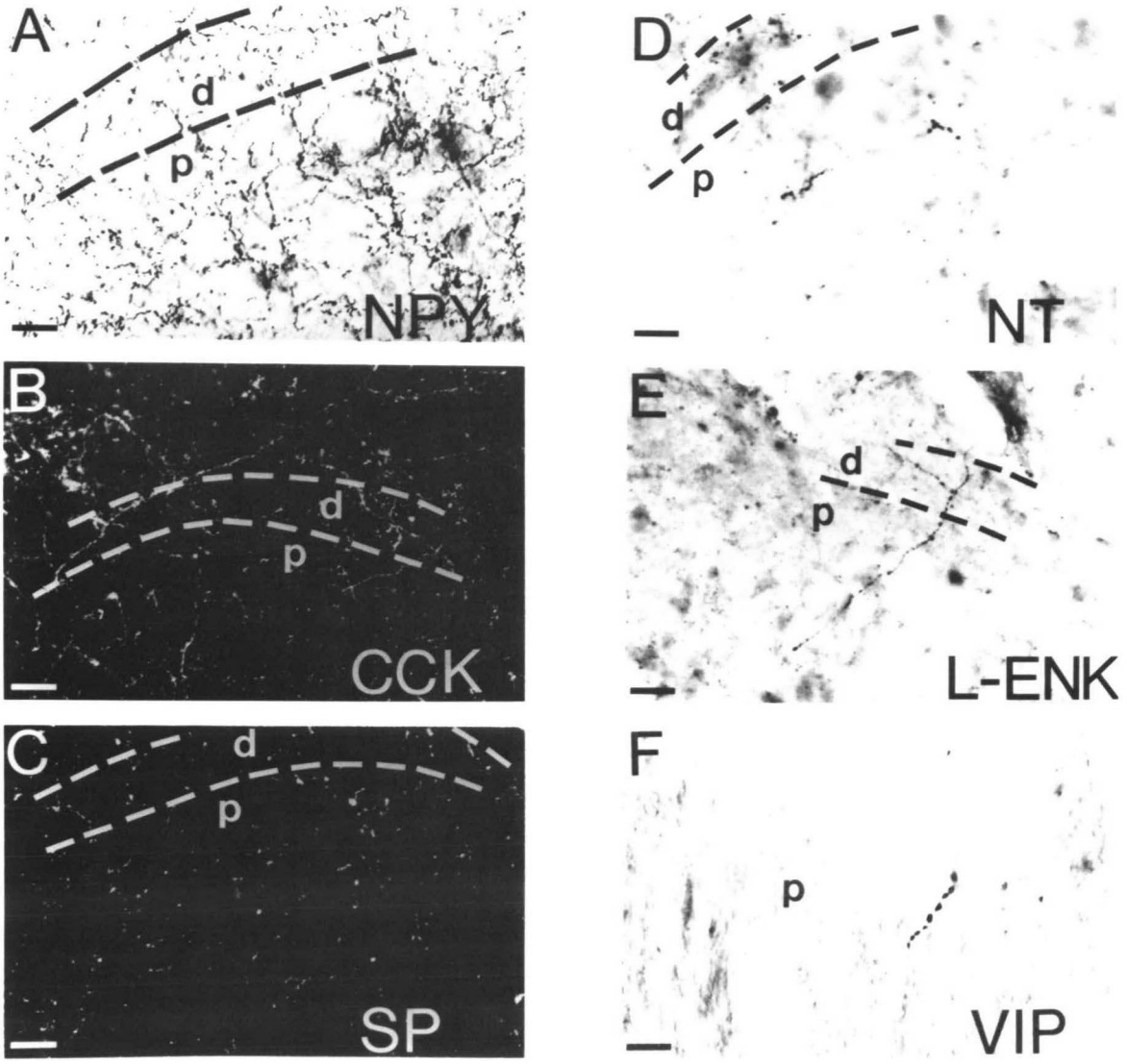

Fig. 6. Photomicrographs of fibers/terminals which were stained with antibodies against several neuropeptides within the nBOR (ABC technique). Photomicrographs depicted in parts A–E are taken from coronal sections, and that in part F is from a horizontal section through the nBOR. Parts B and C are dark-field photomicrographs. As in Fig. 4, the dashed lines indicate approximate boundaries of nBORp and nBORd. Scale bar in A–E = 40 μm; and F = 20 μm.

found within the nBOR. They are distributed in a characteristic pattern within its three cytoarchitectonic subdivisions. The majority of these substances were contained in fibers, presumably arising from cell groups extrinsic to nBOR. However, many somata throughout the nBOR complex were observed to be GAD-positive, and others were ChAT-positive. The majority of somata did not stain with any other of the antisera used in these experiments. This chemical diversity within a small nuclear complex in the visual system may reflect the heterogeneity of its afferent and efferent connections. Such multiplicity of neuroactive substances has already been described for the optic tectum (Karten et al., 1982; Karten & Kuljis, 1986), a very complex, large, and laminated structure.

The variety of the neurotransmitter-specific systems found within the nBOR contrasts with the apparent limited number of previously identified afferent sources of that nuclear complex. The retinal input is by far the dominant one, as demonstrated by the observation that early enucleation results in almost complete degeneration of the nBOR of birds (Karten, 1979; Peduzzi & Crossland, 1983; Rio, 1979) and the AOS nuclei of rats (Shen & Baisden, 1986). Other sources of afferents to nBOR include pretectal nuclei, the ventral lateral geniculate nucleus, visual areas of the telencephalon, preoculomotor nuclei, and contralateral AOS nuclei (Gamlin & Cohen, 1988; Giolli et al., 1988; McKenna & Wallman, 1985; Miceli et al., 1987; Rio et al., 1983; Simpson, 1984). The number of neuroactive substances found in the nBOR in the present study suggests that the inputs to that nucleus are chemically heterogeneous. This was further emphasized in our recent study demonstrating that a subpopulation of retinal displaced ganglion cells projecting to the pigeon nBOR were positive for TH. The TH-LI displaced ganglion cells represented only about 10% of the total number of the retinal neurons projecting to the nBOR, and also had a limited range of soma sizes. They were always medium-sized (14–20μm in the

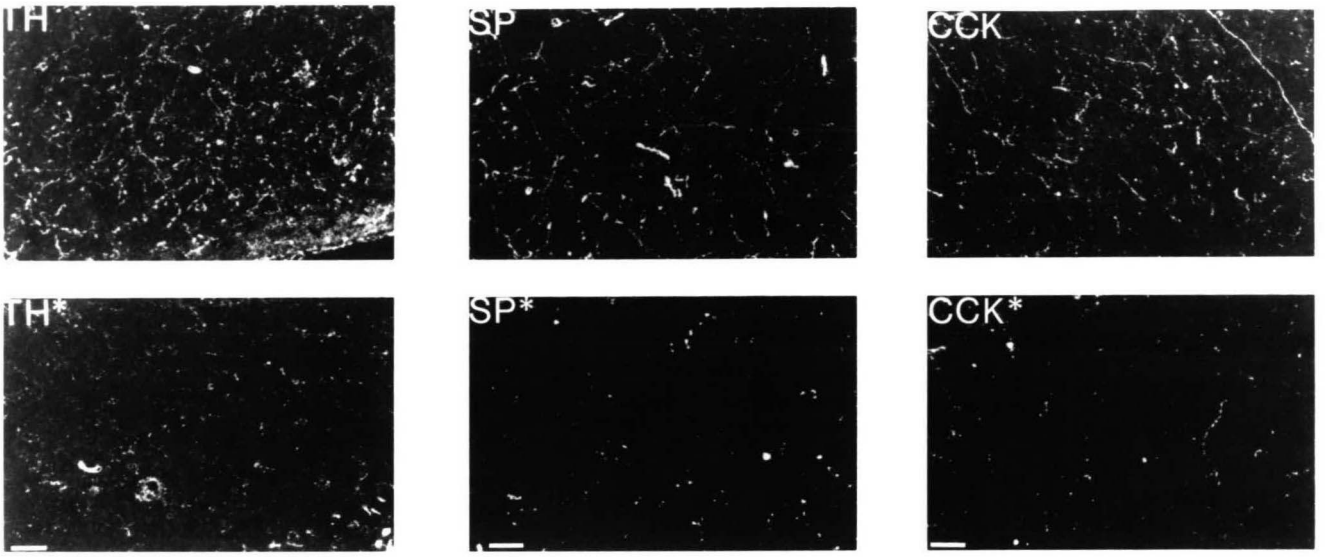

Fig. 7. Effects of unilateral enucleation upon the pattern of TH-LI, SP-LI, and CCK-LI fibers within the nBOR. Representative dark-field photomicrographs of TH-LI, SP-LI, and CCK-LI fibers (ABC technique) within the control (upper row) and deafferented (lower row, *) nBOR. All of these sections were cut in the coronal plane. There was a marked reduction of all three types of immunoreactive fibers in the nBOR after contralateral enucleation. No changes were noted for all other antibodies used in this study. Scale bar in TH-LI = 80 μm; and SP-LI, CCK-LI = 40 μm.

largest axis), whereas the small (10–13 μm) and large (21–28 μm) displaced ganglion cells were TH-negative. This chemical heterogeneity also may prove true for nonretinal nBOR afferent systems.

Classical transmitters and receptors

The most intense labeling within the nBOR complex was obtained with the antibodies against GAD, both in terms of presumed terminals and in the number of labeled cell bodies. A high density of both GAD-positive somata and GAD-recipient neurons in the AOS, and its allied pretectal nucleus of the optic tract, has previously been reported in rodents (Giolli et al., 1985*b*) and frogs (Yucel et al., 1988). Our results in the pigeon support the hypothesis of an important role for the inhibitory neurotransmitter GABA in the functional organization of the AOS and the pretectum. We also found large numbers of GAD-positive and GAD-recipient neurons in the mesencephalic lentiform nucleus, the avian equivalent of the nucleus of the optic tract (Azevedo et al., 1983; Brecha et al., 1980; Gottlieb & McKenna, 1986: McKenna & Wallman, 1985). Furthermore, at least the large nBOR neurons contain GABA-A receptors, although they are negative for GAD-LI. These large nBOR neurons are a demonstrated source of mossy fiber terminals in the vestibulo-cerebellum (Brecha et al., 1980). The GABA/benzodiazepine receptors, which represent a subtype of GABA-A receptors, have been recently described in the nBOR by means of binding experiments (Dietl et al., 1988*b*).

The labeling of nBOR with the anti-GABA-A receptor was much less impressive than that produced by anti-GAD. Several factors could account for these apparently conflicting results:

1. The GABA-A antibody is very sensitive to the methods of tissue fixation.
2. Both antibodies were made against mammalian antigens, and it is possible that the avian GAD is closer to its mammalian counterpart than is the GABA-A receptor.
3. The nBOR could also contain a different type of GABA receptor, other than GABA-A.
4. The GABA-A antibody could have recognized only a subset of the avian GABA-A receptors. Even under optimal circumstances, the GABA-A antibody only recognizes *ca.* 40% of GABA-A receptors in mammals (A. de Blas, personal communication).
5. The lack of correspondence between GAD and GABA-A densities within the nBOR may represent a situation in which there is a mismatch between neurotransmitter and receptor localizations. There are several reported mismatches for GABA systems in different brain structures (review in Herkenham, 1987). Further studies of receptor identity and characteristics are necessary to clarify this issue.

As in rodents, the size, shape, and the relatively high number of the GAD-positive neurons within the nBOR are all suggestive that some of them could represent projection neurons. For instance, the medium and the small spindle-shaped cells are known to project to the inferior olive (Brecha et al., 1980). The small round cells could well represent intrinsic circuit neurons (Rio, 1979). A combined retrograde transport-immunohistochemical study is needed to resolve this question.

The impact of GABA upon the AOS functional organization has been tested in preliminary physiological experiments, which suggested that GABA plays a role in the modulation of directional selectivity of nBOR neurons. The iontophoretic application of bicuculline, a GABA-A receptor blocker, resulted in changes of directional preferences in 14 out of 32 nBOR units tested so far. Yet at least part, if not most, of the properties of

nBOR neurons are established at the retinal level (Britto, 1983; Simpson, 1984). There is evidence, however, that both the pretectal nucleus of the optic tract and the visual cortex impose part of the directional selectivity of AOS neurons in the cat, rat, and pigeon (Grasse & Cynader, 1986, 1987; Hamassaki et al., 1988; Natal & Britto, 1987, 1988). These nonretinal AOS afferents could well exert their actions by modulating the nBOR intrinsic GABAergic system and/or by contributing GABAergic fibers themselves.

The ChAT-positive fibers and cells noted within nBOR in this study were not previously described and no information concerning their possible functional significance is presently available. Interestingly, on the basis of acetylcholinesterase and brain lesions, Shute and Lewis (1967) suggested that the medial terminal nucleus of the rat AOS could constitute the source of cholinesterase activity in the ipsilateral oculomotor nucleus. However, the medial terminal nucleus of the rat does not project directly to the oculomotor nucleus (Giolli et al., 1984, 1985*a*), and the decrease in the intensity of staining for cholinesterase in the oculomotor nucleus after ventral tegmentum lesions (Shute & Lewis, 1967) could be explained by the involvement of the oculomotor nerve in those lesions (see Brecha et al., 1980). In the pigeon, the nBOR projects bilaterally to the oculomotor complex, and at least those neurons projecting contralaterally appear to have approximately the same size as those reported to be ChAT-positive in this study. Based on the cell size, the nBOR ChAT-positive neurons could also project either to the cerebellum, pretectum, or contralateral nBOR (see Brecha et al., 1980). The nAChR staining indicates a role for nicotinic receptors in nBOR, similarly to what has been described for the rat medial terminal nucleus of the AOS (Swanson et al., 1987). Nicotine has recently been shown to increase the metabolic activity of rat visual neurons (London et al., 1988). It is important to note in this context that at least 40% of the retinal displaced ganglion cells projecting to the chick nBOR contain nAChRs (Keyser et al., 1988). This indicates that acetylcholine may constitute an important modulatory system for the AOS function, and that its actions may be mediated by nicotinic receptors. Further studies are needed to clarify such a role and also to characterize which nAChR subtypes (Lindstrom et al., 1987) are present in this system. However, the organization of the cholinergic system within the AOS may be even more complex, since muscarinic receptors were recently described in the avian nBOR (Dietl et al., 1988*a*).

The serotoninergic innervation of the nBOR was previously described in the chicken, and it was shown to develop mainly in the posthatching period (Sako et al., 1986). The presence of 5-HT terminals in the AOS nuclei was also reported in the lizard (Smeets & Steinbusch, 1988; Wolters et al., 1985). The source of this major input to nBOR is still unknown, and no data also exist on the type of receptors involved in this system. The 5-HT fibers within the nBOR may represent a gating control system similar to that described for the mammalian lateral geniculate nucleus (Mantyh & Kemp, 1983; Sherman & Koch, 1986).

In addition to the above-mentioned data on catecholaminergic displaced ganglion cells, the presence of catecholamines within the nBOR had previously been reported with both the Falck and Hillarp technique (Fuxe & Ljunggren, 1965) and immunohistochemistry (Kiss & Peczely, 1987). In contrast, in a study employing glyoxylic acid histofluorescence, the nBOR reportedly did not contain catecholamines (Bagnoli & Casini, 1985). Differences in sensitivity of the several techniques may account for these varying results. We recently demonstrated that the retina constitutes the main, if not the sole, source of TH-positive fibers in the pigeon nBOR (Britto et al., 1988). No information exists at present on the type of catecholamine involved in this projection. The available antibodies directed against other enzymes of the catecholaminergic biosynthetic pathway, such as the dopamine-B-hydroxylase and phenylethanolamine-N-methyl-transferase, have not yielded consistent results on avian brains (unpublished observations).

Peptides

Information concerning the occurrence of this class of putative neurotransmitters and/or modulators in the AOS is scant. Specific binding sites have already been shown in AOS structures for NT and opioids. A medium number of NT binding sites (Brauth et al., 1986) and several types of opiate receptors (Reiner et al., 1989) were recently shown in the pigeon nBOR. In the present study, the nBOR was shown to contain very few NT-LI and L-ENK-LI fibers, whereas the density of the corresponding receptors was low to medium (Brauth et al., 1986; Reiner et al., 1989). Such differences may represent mismatches in the distributions of these peptides and their receptors, similar to what has been described for the equivalent systems in the mammalian brain (reviewed by Herkenham, 1987). Mismatches between opiate receptors and endogenous opioid distributions have been also described in the pigeon brain (Reiner et al., 1989). A high density of potential presynaptic opiates receptors was previously demonstrated in the rat AOS nuclei (Atweh & Kuhar, 1977; Atweh et al., 1978). The levels of opiate receptors within the pigeon nBOR appear to be low to moderate (Reiner et al., 1989). The significance of this difference is uncertain. However, it is of interest that the opiate receptors in the primary sensory fibers of the rat brain appeared to show a rather specific localization to small caliber and unmyelinated fibers (Atweh et al., 1978), whereas the avian nBOR receives its retinal input mostly by way of medium-to-large myelinated fibers (Brecha & Karten, 1981). Further experiments are necessary to clarify the functional organization of the AOS opioid system. For example, it is of interest to study the occurrence of dynorphin peptides within the nBOR, since their highest affinity receptors (*kappa* type) are found in higher densities than the *mu* or *delta* opioid receptors in the pigeon nBOR (Reiner et al., 1989). It would be also important to evaluate the possibility that the opiate receptors in the pigeon nBOR could have a retinal source, similar to what was described for the rat AOS nuclei (Atweh et al., 1978). Presently, there is no information available on this issue or on the retinal ganglion cells which might be the source of the opiate receptors in the rat AOS.

Given the well-known co-occurrence of NT with catecholamines (e.g. Goedert, 1984), the possibility exists that some of the fibers which were stained for NT might also contain TH. Thus, NT-LI might prove to be of retinal origin as well. A neurotensin-related peptide (LANT-6) has been described in the turtle nBOR, and it originates from retinal ganglion cells (Eldred et al., 1988). However, we did not observe NT-labeled ganglion cells projecting to the pigeon nBOR in a recent study (Britto et al., 1988).

Substance P has been shown to occur in the retinal projection to the rabbit AOS (Brecha et al., 1987) and in the retinal projection to the tectal system in both anurans (Kuljis & Karten, 1983, 1986) and chickens (Ehrlich et al., 1987). The presence of SP-positive fibers has been reported in the AOS in a mapping study of the turtle brain, although the SP-positive fibers were restricted to a region that appears equivalent only to the avian nBORd (Reiner et al., 1984). In the present study, SP-positive fibers were found in all subdivisions of the nBOR complex. In a previous radioimmunoassay study, Reubi and Jessell (1978) have also reported the occurrence of SP within the pigeon nBOR.

Extensive NPY fiber staining was found in all subdivisions of the nBOR complex. The density of these NPY-positive fibers was very high and was matched only by the density of the 5-HT plexus. Thus, NPY appears to represent an important afferent system to the nBOR. No information is available concerning its possible source(s) or significance. This peptide is possibly present, however, in the projection of the ventral lateral geniculate nucleus to the lateral terminal nucleus of the AOS in the rat (Card & Moore, 1982). We were unable to detect NPY-positive cells in the pigeon ventral lateral geniculate nucleus in the present study. However, Karten and co-workers recently observed NPY-positive somata in a cell group interposed between the dorsal and ventral geniculate nuclei in the pigeon. These NPY-positive cells were shown to project to the optic tectum (preliminary observations). These neurons form the nucleus of the marginal optic tract and appear to represent the avian equivalent of the mammalian intergeniculate leaflet, which also contains many NPY-positive perikarya (Chronwall et al., 1985; De Quidt & Emson, 1986). We have not yet determined if the intergeniculate NPY neurons represent the source of NPY within the nBOR. It is worth mentioning that the ventral geniculate-lateral terminal nucleus projection in the rat brain was actually studied with an antiserum against APP, the avian pancreatic polypeptide, which was later shown to be absent from the brain and to cross-react with NPY (DiMaggio et al., 1985). In some recent mapping studies of the rodent brain, however, the AOS nuclei appeared almost devoid of NPY fibers, except for the medial terminal nucleus, where a few fibers appear to be present (Chronwall et al., 1985; De Quidt & Emson, 1986). The reason for such discrepancy is not clear at present.

Moderate numbers of CCK-positive fibers and a small number of VIP-positive fibers were also found in nBOR. No previous data on the presence of these peptides within the AOS of either birds or mammals have been reported. The possible sources of these peptides within the nBOR, as well as their presumptive roles, are also unknown. The presence of CCK-LI axons in the nBOR raises an interesting possibility in view of the reported co-occurrence of this peptide with SP in some central neurons and fiber systems (Reiner et al., 1985). Thus, both peptides could be contained in the same fibers within the nBOR as well. The approximately equivalent fiber densities for both antibodies observed in this study suggests that co-localization is also possible in the nBOR, but we were unable to confirm this hypothesis in our double-labeling experiments.

Enucleation experiments

Displaced ganglion cells (DGCs) constitute the sole source of retinal afferents to the nBOR (Fite et al., 1981; Karten, 1979; McKenna & Wallman, 1985). Although DGCs appear to be a singular homogeneous group of neurons, our recent studies indicate that DGCs may consist of two or more biochemically distinct subgroups. The results of the enucleation experiments confirmed that the retina is the main, although not necessarily the exclusive, source of TH-positive fibers within the pigeon nBOR. These fibers originate from a specific subpopulation of DGCs (Britto et al., 1988). The decrease in SP and CCK staining suggests that at least some of the fibers which stained with the antibodies against these peptides may also be of retinal origin. However, we have only occasionally observed presumptive SP- and CCK-positive displaced ganglion cells in the pigeon retina (unpublished observations). The time course of the modification in the SP and CCK immunostaining following enucleation is also compatible with trans-synaptic phenomena involving other nBOR afferents. Indeed, the nBOR contains afferents originating from other retinorecipient areas, such as the pretectal lentiform nucleus and the ventral lateral geniculate nucleus (Azevedo et al., 1983; McKenna & Wallman, 1985). During the course of the present study, however, we did not observe either SP- or CCK-immunoreactive neurons within these two retinorecipient nuclei in the pigeon brain. Thus, complex trans-synaptic interactions should be invoked in order to explain the present enucleation results. Further immunohistochemical-retrograde tracing studies are clearly needed to confirm the retinal origin of the SP- and CCK-positive fibers within the pigeon nBOR. In the rabbit, SP-positive retinal ganglion cells project to AOS nuclei (Brecha et al., 1987). This information, taken together with the present enucleation results, suggests that SP may be a peptide common to ganglion cells that project to the AOS in various vertebrates. The above-mentioned hypothesis of co-localization of SP and CCK is also supported by the present enucleation experiments, since fiber staining in the nBOR for both peptides showed approximately the same reduction after contralateral enucleation.

Conclusion

In this study, the nBOR was found to contain several neurotransmitter and neuropeptide systems. The functional significance of such a multiplicity of chemical systems is unclear at present. Three of these systems appear to originate, at least partially, from the retina (TH-LI, SP-LI, and CCK-LI fibers). Two chemically-specific neuronal populations, containing GAD or ChAT, were also characterized within the nBOR and which may represent projection neurons. However, the transmitter of the majority of nBOR neurons remains unknown. The sources of the other neurotransmitters and neuropeptides in nBOR also have to be determined. Since the AOS is involved in the control of oculomotor systems, it appears very promising to investigate not only the sources and targets of those neurotransmitters and neuropeptides within the nBOR, but also their impact on its functional organization.

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