Neuropeptide Y (NPY) is a highly conserved, 36-amino acid neuropeptide, that was originally isolated from porcine brain (Tatemoto, 1982; Tatemoto et al., 1982). NPY is abundant in both the peripheral and central nervous system, and it is also present in the retina (Adrian et al., 1983; Bruun et al., 1984). NPY has multiple physiological actions, including potent effects on blood flow, memory retention, and food intake (Lundberg et al., 1996; Munglani et al., 1996). NPY acts both pre- and postsynaptically, and it is reported to inhibit cAMP and modulate both K⁺ and Ca²⁺ channels by means of multiple subtypes of G protein-coupled receptors (Balasubramaniam, 1997; Blomqvist and Herzog, 1997; Sun et al., 2001).

NPY is expressed in the retina of numerous vertebrate species (Bruun et al., 1984; Tornqvist and Ehinger, 1988;
Ferriero and Sagar, 1989; Straznicky and Hiscock, 1989; Hutslers et al., 1993; Hutslers and Chalupa, 1994). In mammals, NPY immunoreactivity has been reported in the mouse, rat, guinea pig, cat, monkey, and human retina (Bruun et al., 1984; Marshak et al., 1986; Tornqvist and Ehinger, 1988; Marshak, 1989; Ferriero and Sagar, 1989; Straznicky and Hiscock, 1989; Li and Lam, 1990; Hutslers et al., 1993; Hutslers and Chalupa, 1994, 1995; Jen et al., 1994; Jotwani et al., 1994; Sinclair and Nirenberg, 2001). In rat retina, NPY content is low to moderate compared with other brain regions (Ferriero and Sagar, 1989). In mouse retina, the NPY gene is expressed by cells in the inner nuclear layer (INL) and ganglion cell layer (GCL) (Ammar et al., 1998; Sinclair and Nirenberg, 2001). NPY immunoreactivity is localized predominantly to amacrine and displaced amacrine cells having a moderate to high cell density in most mammalian retinas. Immunoreactive amacrine and displaced amacrine cells are characterized by widely ramifying processes that are distributed to distinct strata of the inner plexiform layer (IPL). These processes form networks that cover the entire retinal surface. In cat and human retina, NPY immunoreactivity is also localized to ganglion cells (Straznicky and Hiscock, 1989; Hutslers et al., 1993; Hutslers and Chalupa, 1994).

A functional role for NPY in the neural retina is suggested by its potent inhibition of cAMP accumulation in whole rabbit retina (Bruun et al., 1984). Furthermore, endogenously applied NPY stimulates the release of glycerine, dopamine, and 5-hydroxytryptamine from the rabbit retina, and gamma-aminobutyric acid (GABA) and choline from the chicken retina (Bruun and Ehinger, 1993). Interestingly, NPY is released from the frog retina by high frequency light stimulation (Bruun et al., 1991).

Together, these findings indicate a role for NPY in visual processing in the inner retina. However, information on the retinal cell types containing NPY immunoreactivity and their synaptic connectivity is lacking. Therefore, the aim of the present study was to characterize the types of cells expressing NPY in the rat retina by examining their morphology, neurochemistry, and synaptic connectivity. Some of these observations have been reported in abstract form (D’Angelo and Brecha, 1999; Oh et al., 1999a).

MATERIALS AND METHODS

Animals

Adult Sprague-Dawley rats of either sex were used for these studies. They were housed and fed under normal conditions with a 12-hour light-dark cycle. The animals were treated according to the regulations of the Animal Research Committee of the University of California at Los Angeles, and the Catholic Ethics Committee of the Catholic University of Korea in conformity with all NIH guidelines.

Tissue preparation

Rats were deeply anesthetized with an intraperitoneal injection of 30–70 mg/kg pentobarbital and transcardially perfused with 50–100 ml of 0.1 M phosphate-buffered saline (PBS; pH 7.4) followed by 500 ml of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB; pH 7.4) or 2% PFA with 1.37% D, L-lysine and 0.214% NaI04 (PLP) in PB at room temperature. Each eye was removed, dissected, and the posterior eyecup containing the retina was immersed in 4% PFA or 2% PLP for 1–2 hours at room temperature. In some cases, the retina was removed from the eyecup. The eyecup or isolated retina was transferred to 25% sucrose in 0.1 M PB and stored overnight at 4°C. For Vibratome sections, the tissue was frozen in liquid nitrogen, thawed, and rinsed in 0.1 M PBS. The retinas were cut at 50 μm and stored in 0.1 M PB at 4°C.

For cryostat sections, the eyecup, which included the retina, was cut perpendicular to the vitreal surface at 12–16 μm. Sections were mounted onto gelatin-coated slides and stored at −20°C until staining. For sliding microtome sections, the isolated retina was cut parallel to the vitreal surface at 20–25 μm. Sections of the retina were stored in 0.1 M PB at 4°C until antibody staining.

Retrograde transport studies

Rats (n = 2) were deeply anesthetized with an intraperitoneal injection of 30–70 mg/kg pentobarbital. Bilateral stereotaxic injections of 1.79 mg/μl (total amount injected into each superior colliculus) of horseradish peroxidase (HRP) type VI (Sigma, St. Louis, MO) were made into the superior colliculus: two injections (2 × 2 μl) were made into the rostral superior colliculus and one (2 μl) was made into the caudal superior colliculus (Paxinos and Watson, 1986). Four days after the injections, the rats were deeply anesthetized and perfused with 4% PFA. Each eye was removed and dissected, and the posterior eyecup containing the retina was immersed in 4% PFA for 2 hours at room temperature. The isolated retinas were rinsed twice in PBS and three times in 0.05 M Tris-HCl buffer pH 7.4 (TB) for 5 minutes, incubated for 10 minutes in 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB) in TB, and subsequently incubated in DAB with a final concentration of 0.01% H2O2. The reaction was stopped after 1 to 2 minutes, depending on the amount of staining, with several washes of TB. The retinas were then processed for NPY immunoreactivity by using the indirect immunofluorescence procedure (see below).

To confirm the location of the HRP injections into the superior colliculus, brains were removed and immersion fixed in 0.1 M phosphate-buffered saline (16 M NPY) over-night at 4°C. For each eye, the isolated retina was cut perpendicular to the vitreal surface at 20–25 μm. Sections of the retina were stored in 0.1 M PB at 4°C until antibody staining.

Antibodies

An affinity purified rabbit polyclonal antibody directed against NPY (AB 8711) was produced by the Antibody/RIA Core of the CURE/Digestive Diseases Research Center and generously provided by H. Wong and Dr. J.H. Walsh of the University of California at Los Angeles. Specificity of immunostaining was evaluated by preadsorbing the antibody with 10−6 M NPY (Bachem, Torrance, CA) overnight at 4°C and by using it in the place of the primary antibody solution during the overnight incubation. Mouse monoclonal antibodies directed against GABA (Chemicon,
Temecula, CA), GAD_{65} (GAD6; Chemicon) and tyrosine hydroxylase (TH; Boehringer-Mannheim, Indianapolis, IN) were used to identify GABA- and TH-immunoreactive amacrine cells. A guinea pig polyclonal antibody to the vesicular acetylcholine transporter (VACHT) was used to establish the boundaries of strata 2 and 4 of the IPL, in relationship to the plexus of NPY-immunoreactive fibers. Vasoactive intestinal polypeptide (VIP; Casini and Brecha, 1992) and parvalbumin (PV; Sigma) antibodies were used to identify other amacrine cell populations.

**Immunohistochemistry**

Immunostaining was performed by using both the indirect fluorescence and the avidin-biotin-peroxidase complex (ABC) methods (Hsu et al., 1981; Casini and Brecha, 1991). Vibratome sections were rinsed in 0.1 M PB for 30 minutes and incubated for 1 hour at room temperature in 0.1 M PBS containing 10% normal goat serum (NGS) and 1% Triton X-100. Sections were subsequently incubated for 3 days at 4°C in rabbit polyclonal NPY antibody (1:1,000) in 0.1 M PB containing 3% NGS and 0.5% Triton X-100. After this incubation, the retinas were rinsed in several changes of 0.1 M PB for 1 hour, incubated for 24–36 hours in biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) in PBS solution at 4°C, rinsed in two changes of 0.1 M PBS and three changes of 0.05 M TB for 2 hours at room temperature, and incubated in 0.05% DAB in TB for 10 minutes. Hydrogen peroxide was added to the incubation medium to make a final concentration of 0.01% H2O2, and the sections were gently shaken during the reaction. After 10–20 minutes, as determined by the degree of staining, the reaction was stopped with several washes of 0.05 M TB followed by 0.01 M PBS. The retinas were attached to gelatin-coated slides with the GCL facing up, mounted in glycerol, and cover-slipped.

For indirect fluorescence immunostaining, cryostat sections were rinsed in 0.1 M PB for 30 minutes and incubated 12–16 hours in NPY (1,2,000) antibody with 0.5% Triton X-100 in 0.1 M PB at 4°C. Sections were rinsed for 30 minutes with 0.1 M PB and incubated in affinity purified goat anti-rabbit IgG fluorescein isothiocyanate (FITC; 1:100; Jackson Immuno Labs, West Grove, PA) for 1–2 hours at room temperature. Sections were washed for 30 minutes with 0.1 M PB, mounted onto gelatin-coated slides (if free floating), and air-dried. All sections were cover-slipped with 10% glycerol in 0.1 M PB containing 2% potassium iodide (KI).

For double-label studies, sections were incubated overnight in a mixture of the NPY (1,2,000) antibody and the GABA (1,2,0000), GAD_{65} (1:100) or TH (1:100) monoclonal antibody or the VACHT (1,500) guinea pig polyclonal antibody with 0.5% Triton X-100 in 0.1 M PB at 4°C. Sections were rinsed for 30 minutes with 0.1 M PB and incubated in affinity purified goat anti-rabbit IgG fluorescein isothiocyanate (FITC; 1:100; Jackson Immuno Labs) and goat anti-mouse or goat anti-guinea pig IgG tetramethylrhodamine B isothiocyanate (TRITC; 1:100; Jackson Immuno Labs) for 1–2 hours at room temperature. Sections were washed for 30 minutes with 0.1 M PB and cover-slipped with 10% glycerol in 0.1 M PB containing 2% KI. To determine that the secondary antibody did not cross-react with the inappropriate primary antibody, some sections were incubated in rabbit polyclonal primary antibody followed by anti-mouse secondary antibody, whereas other sections were incubated in mouse primary antibody followed by anti-rabbit secondary antibody. These sections did not show any immunostaining.

Immunostained retinas were evaluated by using a Zeiss Axiosplan 2 fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY). Immunostained was photographed by using either T-MAX 400 or Ektachrome 800/1600 color slide film. Photographic images were scanned with a SprintScan 35 Plus (Polaroid, Cambridge, MA) at 2700 DPI. Some sections were evaluated with a Zeiss 410 Laser Scanning Microscope with a krypton/argon laser. Confocal images were acquired with a Zeiss PlanApo 100× 1.3 NA objective at a magnification zoom of 4×. Usually 10–20 optical sections were taken with a z-axis of 1 μm. All images were scaled to final size, adjusted for contrast and brightness, labeled, and formatted by using Adobe Photoshop 5.5 (Adobe Systems, Mountain View, CA). Photographic images and confocal images were saved at 400 dpi, at their final magnification.

**Semi-quantitative analysis**

The analysis of the distribution of NPY-immunostained amacrine cells in the INL and GCL was conducted in two well-stained retinas. Immunolabeled cells were plotted by using camera lucida at low and medium magnifications. For the maps shown in Figure 7, fields of 200 × 200 μm² were sampled in 1-mm steps over the full extent of the retina. Nearest-neighbor analysis (Wassle and Riemann, 1978) was performed on the cells located approximately 2 mm from the optic disc in the temporal periphery. By using an image analyzer system (BMI-PLUS; Bumm Uni-verse Co., Ansan, Korea), soma size was measured in 50 NPY-immunoreactive cells in the INL and 50 immunoreactive cells in the GCL from both the central and peripheral retinal regions. Measurements were not corrected for the negligible shrinkage of the tissue from the mounting process.

**Electron microscopy**

The eyecups were fixed in a mixture of 4% PFA and 0.2% picric acid in 0.1 M PB for 30 minutes at room temperature. The retinas were then dissected, and small pieces were taken from the central region and fixed for an additional 2 hours at 4°C in the same fixative. After being washed in PB, the retinal pieces were transferred to 30% sucrose in 0.1 M PB at 4°C for 30 minutes. The sections were rinsed in 0.01 M PBS containing 3% NGS for 1 hour at room temperature. The sections were cut with a Vibratome at 50 μm, and sections were placed in 0.01 M PBS. After incubation in 0.01 M PBS containing 3% NGS for 1 hour at room temperature to block nonspecific binding sites, they were then incubated in the NPY antibody (1,1,000) for 16 hours at 4°C. The sections were washed in 0.01 M PBS for 45 minutes, incubated in biotin-labeled goat anti-rabbit IgG for 2 hours, and washed three times in 0.01 M PBS for 45 minutes. Sections were incubated in ABC solution for 1 hour, washed in TB, and then incubated in 0.05% DAB solution containing 0.01% H2O2. The reaction was monitored and ended by replacing the DAB and H2O2 solution with 0.05 M TB.

The stained sections were post-fixed in 1% glutaraldehyde in 0.1 M PB for 1 hour and, after being washed in 0.1 M PB containing 4.5% sucrose for 15 minutes, they were post-fixed in 1% OsO4 in 0.1 M PB for 2 hours, dehydrated
in a graded series of alcohol, and flat-embedded in Epon 812. After curing at 60°C for 3 days, well-stained areas were cut out and attached to an Epon support for further sectioning. Ultrathin sections (70–90 nm in thickness) were collected on one-hole grids coated with Formvar and evaluated by using a JEOL 1200EX electron microscope.

RESULTS

Localization of NPY immunoreactivity

NPY immunoreactivity was present in the inner retina in all retinal regions (Fig. 1A). NPY immunoreactivity was not observed in the outer nuclear layer, nerve fiber layer, or optic nerve head. Immunostaining was absent in sections incubated with the NPY antibody that was preadsorbed with the NPY peptide (Fig. 1B), indicating specificity of the antibody for NPY.

NPY immunoreactivity was localized to regularly distributed cell bodies located in the INL and GCL, and in processes that were mainly in strata 1 and 5 of the IPL (Figs. 1A, 2, 3, 8, 9). In most sections, processes were also in stratum 3 of the IPL (Fig. 3B), and in transverse sections, the plexus in this stratum was often discontinuous. NPY-immunostained processes were varicose and had an overlapping distribution. The location of NPY-immunoreactive fibers to strata 1, 3, and 5 of the IPL was confirmed by double-label studies in which antibodies to VACHT were used to define strata 2 and 4 of the IPL (data not shown; Koulen, 1997; Kim et al., 1998). NPY-immunoreactive fibers were not detected in strata 2 and 4. In some sections, rare varicose-immunoreactive fibers from cell bodies in the INL and from the IPL crossed the INL to enter the OPL. Sparsely occurring, varicose, immunoreactive fibers were observed in the OPL in all retinal regions.

NPY immunoreactivity was limited to the cytoplasm and processes, and it was absent from the prominent nucleus. Immunostaining was most intense in the region of the cell body where the primary processes emerged to enter the IPL and in primary processes located in the IPL. Secondary and other distal processes were more weakly immunoreactive. NPY-immunoreactive processes in the IPL and OPL were characterized by numerous varicosities (Fig. 2A,B). NPY-immunostained processes appeared to ramify over large distances in the IPL, and in strata 1 and 5, these processes formed overlapping plexuses in all retinal regions.

NPY-immunoreactive cells

Amacrine cells. In the INL, the most frequently observed immunostained cells were characterized by a small, round cell body located in the innermost row of the INL, adjacent to the IPL. These cells had multiple fine processes that were narrowly distributed in stratum 1 of the IPL and these processes appeared to be confined to this stratum (Figs. 1A, 3A).

An infrequently occurring NPY-immunoreactive cell with a small, slightly elongated cell body, located in the proximal INL was occasionally observed in transverse sections. This cell type was characterized by a single primary process that descended to and ramified narrowly in stratum 3 of the IPL (Fig. 3B). Immunostained processes in stratum 3 only appeared to originate from these cells. These cells were observed infrequently (less than 1 per 12 μm thick transverse retinal section). These cells could not be readily distinguished from other NPY-immunoreactive cell somata in the INL in whole-mount preparations (discussed below), and they were not further characterized in this study.

Fig. 1. Distribution of neuropeptide Y (NPY) immunoreactivity in a retinal section cut perpendicular to the vitreal surface. A: NPY immunostaining was localized to cell bodies in the proximal INL and varicose fibers distributed in strata 1, 3, and 5 of the IPL. B: NPY immunoreactivity was absent from a retinal section incubated with the NPY antibody that was preincubated with 10⁻⁶ M NPY. Cryostat sections. ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar = 30 μm in B (applies to A,B).
Fig. 2. Distribution of neuropeptide Y (NPY) immunoreactivity in the inner retina at different focal planes of a whole-mount retinal preparation. This field is from the central region of the retina near the optic nerve head. **A:** Focal plane through the proximal inner nuclear layer. Small, round cell bodies (arrows) showed NPY immunostaining. Small arrows point to varicose immunostained processes. **B:** Focal plane through the inner plexiform layer near the ganglion cell layer (GCL). Small processes (fine arrows) appear to emerge from immunostained cell bodies (open arrows) located in the GCL. These processes formed a complex network in stratum 5, illustrated here in B. **C:** Focal plane through the GCL. Several immunostained cell bodies (arrows) are present in this image. Scale bar = 50 μm in C (applies to A–C).
Displaced amacrine cells. In the GCL, NPY immunoreactivity was localized to small to medium cell bodies. Primary processes that entered the IPL and branched into secondary processes characterized these NPY-immunoreactive cells. These processes appeared to ramify extensively and diffusely in stratum 5 of the IPL (Fig. 3C). The possibility that the NPY-immunoreactive cells in the GCL were ganglion cells with small somata was investigated by retrograde labeling of ganglion cells with HRP and subsequently processing the retinas by immunohistochemistry with antibodies to NPY. Large bilateral injections of HRP were placed into the superficial layers of the superior colliculus, which receives projections from most, if not all, ganglion cells in the mouse retina (Hößbauer and Dräger, 1985).

We observed numerous ganglion cells retrogradely labeled with a prominent somal accumulation of HRP in all retinal regions after the injections of HRP into the superior colliculus. Retrogradely labeled ganglion cells (n = 500 from four retinas) did not contain NPY immunoreactivity (Fig. 4). These retrograde transport findings support the idea that the NPY-immunoreactive cells in the GCL are displaced amacrine cells.

NPY-immunoreactive cell populations

The size and distribution of NPY-immunoreactive amacrine and displaced amacrine cells were evaluated in whole-mount preparations. There were more NPY-immunoreactive amacrine cells in the INL than in the GCL. Overall, the highest density of immunoreactive cells in the INL and GCL was in central retinal regions near the optic nerve disc.

Soma size. Cell bodies of immunolabeled amacrine (n = 50) and displaced amacrine cells (n = 50) were measured in both the central and peripheral retina. Each cell body was drawn by camera lucida, and its diameter was measured. The mean somal diameter of NPY-immunoreactive amacrine cells was 8.8 ± 1.6 μm (SD) in the central retina and 9.6 ± 1.2 μm in the peripheral retina and that of NPY-immunoreactive displaced amacrine cells was 9.3 ± 2.2 μm in the central retina and 10.7 ± 0.9 μm in the peripheral retina (Fig. 5).

Cell density. In the INL, the maximum density of NPY-immunoreactive amacrine cells was 437.5 ± 32.1 cells/mm² (SD) in the retinal region temporal to the optic disc. Cell density decreased toward the peripheral retina, with densities of 119.4 ± 18.3 cells/mm² in the nasal and temporal periphery and 200 to 400 (234.6 ± 24.3 to 347.5 ± 34.7) cells/mm² elsewhere in the retina (Fig. 6, Top). In the GCL, the highest density of NPY-immunoreactive displaced amacrine cells was in the central and superotemporal regions of the retina. The highest density was 175.7 ± 20.4 cells/mm² in the central region around the optic disc and in the region superotemporal to the optic disc. The density decreased toward the periphery, with a minimum density of 25.3 ± 5.4 cells/mm² in the nasal and inferotemporal margins of the retina (Fig. 6, Bottom).

Cell distribution. Nearest-neighbor analysis was used to evaluate the distribution of NPY-immunoreactive cells in the INL and GCL (Wassle and Riemann, 1978) in a field of central retina that was 1 mm² (Fig. 7). The frequency histograms for the nearest-neighbor distances of these immunolabeled amacrine and displaced amacrine cells were better approximated by a Gaussian distribution, with similar mean distances and standard deviations (Fig. 7, lines without circles), than by the Poisson curve (lines with circles), which describes a random cell distri-

Fig. 3. Neuropeptide Y (NPY)-immunoreactive amacrine and displaced amacrine cells. A: Most NPY-immunoreactive amacrine cells in the INL were characterized by a small cell body (small arrow) in the proximal INL and processes that ramified in stratum 3 (arrowhead) of the IPL. B: A few NPY-immunoreactive amacrine cells in the INL were characterized by an ovoid cell body. These cells (arrow in B) were characterized by a single process that descended to and ramified in stratum 3 of the IPL (arrowhead). C: NPY-immunoreactive displaced amacrine cells characterized by a small cell body (small white arrow) and processes that ramified in stratum 5 of the IPL (arrowhead). INL, inner nuclear layer; IPL, inner plexiform layer. Scale bar = 25 μm in C (applies to A-C).
bution (Rayleigh distribution). These findings indicate that both NPY-immunoreactive amacrine and displaced amacrine cells have regular distributions across the retina.

A test of cell regularity, the mean of the nearest-neighbor distance divided by the standard deviation, was used to evaluate the regularity of NPY-immunoreactive amacrine and displaced amacrine cells. Higher values indicate a more regular spacing (Wassle and Riemann, 1978). The regularity values were 2.94 in the INL and 2.36 in the GCL, indicating the relatively regular spacing of NPY-immunoreactive cell bodies.

**Neurochemical characterization of NPY-immunoreactive amacrine cells**

The localization of other retinal transmitters to NPY-immunoreactive amacrine cells was evaluated by using well-characterized monoclonal antibodies to GABA, GAD<sub>67</sub>, and TH (Foster et al., 1985; Wässle and Chun, 1988; Nguyen-Legros et al., 1997). The GABA-immunostaining patterns were similar to earlier reports (Brecha, 1992; Nguyen-Legros et al., 1997), with numerous GABA-immunoreactive amacrine and displaced amacrine cells and a dense network of processes in the IPL. GABA immunoreactivity was observed in 97% of the NPY-immunoreactive cells (n = 250; 20 retinas) located in the INL and GCL (Fig. 8A,B). GAD<sub>67</sub> immunoreactivity was observed in all NPY-immunoreactive cell bodies (Fig. 8C,D).

Two types of TH-immunoreactive amacrine cells were observed in the INL, confirming earlier studies of the rat retina (Foster et al., 1985; Nguyen-Legros et al., 1997). One type (class 1) was characterized by prominent immunoreactivity, a large cell body and thick processes that ramified in stratum 1 of the IPL, whereas the second type (class 2) was characterized by weak immunoreactivity, a small cell body and processes that also ramified in the IPL, including stratum 3. Some class 2 TH-immunoreactive cells contained NPY immunoreactivity (Fig. 9). Twenty-two percent of the rarely occurring NPY-immunoreactive cells (n = 250; 20 retinas) that ramified in stratum 3 of the IPL contained TH immunoreactivity. NPY immunoreactivity was not colocalized with vasoactive intestinal polypeptide or parvalbumin, which are other neurochemical markers for amacrine cells in the rat retina (Terubayashi et al., 1983; Kondo et al., 1985; Sanna et al., 1990; Wässle et al., 1993).

**Synaptic connectivity of NPY-immunoreactive processes**

NPY immunoreactivity was identified as an electron-dense reaction product that was closely associated with...
the membranes of mitochondria, the nucleus, synaptic vesicles, and with amorphous matrices distributed in the cytoplasm. Pre- and postsynaptic contacts of NPY-immunoreactive processes were observed in the IPL (Table 1). Immunolabeled processes were identified as amacrine cell processes due to the presence of numerous vesicles and the lack of synaptic ribbons. Bipolar cell axonal terminals were identified by the presence of a ribbon.

A total of 233 synaptic specializations were identified in the IPL in these preparations; 54 were from other nonimmunostained amacrine cell processes and bipolar cell axon terminals onto NPY-immunostained processes, and 179 were from NPY-immunoreactive amacrine cell processes onto amacrine, ganglion, bipolar, and a few unidentified cell processes.

**Presynaptic labeling pattern.** NPY-immunoreactive processes (n = 175) formed conventional presynaptic specializations onto amacrine and ganglion cell processes, and rarely, onto bipolar cell terminals. These presynaptic contacts were observed in all regions of the IPL having NPY-immunoreactive processes. Approximately 50% (52%, n = 91) of the contacts by NPY-immunoreactive processes were onto nonimmunostained amacrine cell processes. In some cases, immunoreactive processes were presynaptic to a nonimmunostained amacrine cell process, which in turn formed a conventional synapse onto another nonimmunostained amacrine cell process (Fig. 10A). Approximately 40% (n = 71) of the presynaptic contacts of NPY-immunoreactive processes were onto ganglion cell dendrites. An example of a presynaptic contact onto a ganglion cell dendrite, identified by the presence of clear cytoplasmic matrix and microtubules, is given in Figure 10B. Infrequent synaptic contacts by immunostained processes onto bipolar cell terminals were also observed in the IPL (4%, n = 9). An example can be seen in Figure 10C of an NPY-immunoreactive process making a conventional synapse onto a bipolar axon terminal identified by a synaptic ribbon in its cytoplasm. There were a few (3.4%) NPY-immunoreactive processes that were presynaptic to small nonimmunostained processes. These were categorized as unidentified processes (not shown). In addition, there were a few (2%, n = 4) synaptic contacts between immunolabeled processes. Synaptic outputs from amacrine cells onto rod bipolar cell axons or axon terminals were not found.

**Postsynaptic labeling pattern.** The most common synaptic input (77.8%, n = 54) onto NPY-immunoreactive processes was from nonimmunostained amacrine cell processes. These synaptic inputs were observed in all regions of the IPL. An example of an immunostained process receiving presynaptic input from a nonimmunostained amacrine cell process in stratum 1 of the IPL is given in Figure 11A. Synaptic inputs from bipolar axon terminals onto NPY-immunoreactive amacrine cell processes were infrequent and composed approximately 22% (n = 12) of all synaptic inputs. In most cases, these synapses formed dyads, where one postsynaptic element was an NPY-immunoreactive process and the other was a nonimmunostained amacrine cell process or a ganglion cell dendrite. An example of a postsynaptic dyad of a bipolar cell terminal with an immunostained process and a ganglion cell dendrite in sublamina b of the IPL is given in Figure 11B. Rod bipolar cell axon terminals, identified by their size and location in stratum 5 of the IPL and the features of their postsynaptic dyads, were never observed to be presynaptic to NPY-immunoreactive processes.

In the OPL, there were rare varicose NPY-immunoreactive processes (Fig. 12). These processes appeared to originate from NPY-immunoreactive amacrine cells in the INL and processes in the IPL (data not shown). Neither presynaptic nor postsynaptic synaptic contacts to the immunolabeled processes were observed in these preparations.
DISCUSSION

NPY-immunoreactive neurons of the rat retina are amacrine cells with overlapping processes that ramify primarily in stratum 1 of the IPL, and displaced amacrine cells with overlapping processes in stratum 5 of the IPL. In addition, amacrine cells that only appear to ramify in stratum 3 were occasionally observed in transverse sections. The distribution of processes to the IPL was primarily determined in transverse sections. This determination is limited, because the cellular processes were often weakly immunolabeled. Furthermore, because these cells have wide-fields, some processes are likely to be out of the plane of section, and we cannot completely exclude the possibility that some processes ramify in other strata. Therefore, to fully ascertain the complete morphology of the NPY-immunoreactive cells, including the distribution of their processes to the IPL, cell labeling approaches like Golgi-staining, or intracellular dye labeling would be needed (Masland and Raviola, 2000). NPY-immunoreactive cells have a regular distribution in both the INL and GCL. These cells have a moderate overall cell density of approximately 257.4 ± 99.4 cells/mm² in the INL and...
85.7 ± 41.0 cells/mm² in the GCL. NPY-immunoreactive amacrine cells also contain GABA immunoreactivity, and they form most of their pre- and postsynaptic contacts with other nonimmunostained amacrine cells.

**NPY immunoreactivity in the mammalian retina**

The present findings are in general agreement with previous observations in other mammalian retinas (Bruun et al., 1984; Marshak et al., 1986; Tornqvist and Ehinger, 1988; Ferriero and Sagar, 1989; Li and Lam, 1990; Hutslers et al., 1993; Jen et al., 1994; Hutslers and Chalupa, 1994, 1995; Sinclair and Nirenberg, 2001), and they extend a prior study of the rat retina, which reported NPY-immunoreactive cells in the INL and GCL (Ferriero and Sagar, 1989), by providing detailed information about the size, density, distribution, neurochemical content, and synaptic connectivity of these cells. Our observations are also consistent with previous reports of NPY gene expression in cells in the INL and GCL of the mouse retina (Ammar et al., 1998; Sinclair and Nirenberg, 2001).

The predominant NPY-immunoreactive amacrine cell in the rat retina is characterized by a small, round cell body located in the inner row of cells in the INL and processes that are narrowly distributed in stratum 1 of the IPL. This cell type is remarkably similar in its morphology to NPY-immunoreactive amacrine cells described in the mouse (Sinclair and Nirenberg, 2001), guinea pig (Bruun et al., 1984), cat (Hutslers and Chalupa, 1994), and human (Tornqvist and Ehinger, 1988; Stratnick and Hiscock, 1989; Jotwani et al., 1994) retina. These cells are also similar to a recently described wide-field (WF1-1) cell characterized by a small cell body and very fine processes in stratum 1 of the rabbit retina (MacNeil et al., 1999). Additionally, NPY immunoreactive cell bodies in the GCL have been described in the mouse (Sinclair and Nirenberg, 2001), guinea pig (Bruun et al., 1984), cat (Hutslers and Chalupa, 1994), and human (Tornqvist and Ehinger, 1988; Stratnick and Hiscock, 1989; Jotwani et al., 1994) retina. In the mouse retina, these cells are reported to be displaced amacrine cells with processes that are distributed to stratum 5 of the IPL (Sinclair and Nirenberg, 2001). These NPY-immunoreactive cells are likely to correspond to the NPY-immunoreactive displaced amacrine cells in the rat retina.

However, there are also some differences in the NPY-immunoreactive pattern among different mammalian retinas. A counterpart to the rare NPY-immunoreactive amacrine cell we have observed in the rat retina, that appears to ramify in lamina 3, has not been reported in other mammalian retinas. There are, however, examples of amacrine cells that generally match the morphology of this cell type in the rabbit, cat, and primate retina (Cajal, 1893; Boycott and Dowling, 1969; Kolb and Nelson, 1981, Kolb et al., 1991; MacNeil et al., 1999). Another difference is that NPY immunoreactivity in certain species has been localized to ganglion cells and not displaced amacrine cells.
Fig. 10. A: A neuropeptide Y (NPY) immunoreactive process (star) with a conventional presynaptic contact (white arrow) onto a nonimmunostained amacrine cell process (A-) that is presynaptic (open arrow) to an unidentified cell process in stratum 1 of the inner plexiform layer (IPL). B: Ganglion cell dendrite (G), which is characterized by clear cytoplasm and microtubules is postsynaptic (arrow) to an immunostained process (star) in stratum 3 of the IPL. C: Immunostained process (star) synapsing (arrow) onto an axon terminal of a cone bipolar cell (CB) in the IPL. The arrowhead indicates a synaptic ribbon. Vertical ultrathin sections through the IPL. Scale bars = 0.5 μm.
In the cat retina, small gamma ganglion cells that contain NPY immunoreactivity have sparse dendritic processes that ramify in the ON sublamina of the IPL. These cells terminate in the C layers of the lateral geniculate nucleus and the superior colliculus (Hutsler and Chalupa, 1994). In human retina, NPY immunoreactivity is reported in large ganglion cells that are distributed in all retinal regions (Straznicky and Hiscock, 1989). These observations are in contrast to the findings of another study that reports the presence of NPY-immunoreactive amacrine and displaced amacrine cells in the developing human retina (Jotwani et al., 1994). In the rat retina, NPY immunoreactivity is reported in large ganglion cells that are distributed in all retinal regions (Strzynicky and Hiscock, 1989). These observations are in contrast to the findings of another study that reports the presence of NPY-immunoreactive amacrine and displaced amacrine cells in the developing human retina (Jotwani et al., 1994). In the rat retina, as in the mouse retina, NPY-immunoreactive cells appear to be exclusively displaced amacrine cells. Although unlikely, the possibility that the retrograde transport experiments did not label all ganglion cells and that a small number of the NPY-immunoreactive cells in the GCL of the rat retina may be ganglion cells cannot be definitely ruled out.

**NPY-immunoreactive amacrine and displaced amacrine cell populations**

In rat retina, NPY-immunoreactive amacrine and displaced amacrine cells form at least two cell populations based on their cell morphology and distribution. NPY-immunoreactive amacrine cells in the INL mainly ramified in stratum 1 of the IPL, and they had a moderate cell density that is less than the reported NPY-immunoreactive amacrine cell density in the mouse and cat retina (Hutsler and Chalupa, 1994; Sinclair and Nirenberg, 2001). NPY-immunoreactive amacrine cells in the INL of the rat have an average density of approximately 250 cells/mm². Compared with other amacrine cell populations in the rat retina, NPY-immunoreactive cells in the INL represent a moderately dense cell population. For example, the density of class 1 TH-immunoreactive cells is 20 to 30 cells/mm², and the density of the ChAT-immunoreactive cells in the INL is 530 to 590 cells/mm² in the rat retina (Versaux-Botteri et al., 1986; Voigt, 1986; Kim et al., 2000).

The NPY-immunoreactive amacrine cell population in rat, as in mouse, is distributed regularly across the retina; the regularity index in central retina of the rat is 2.94 in the INL, and in the mouse retina, it ranges from 2.6 to 3.0 in the INL (Sinclair and Nirenberg, 2001). In comparison, the NPY-immunoreactive amacrine cell population of the cat retina is less regular (Hutsler and Chalupa, 1994). Compared with other amacrine cell populations of the rat
retina, NPY-immunoreactive amacrine cells are quite regular. For example, both class 1 TH- and ChAT-immunoreactive amacrine cell populations are less regular than the NPY-immunoreactive amacrine cells (Versaux-Botteri et al., 1986; Voigt, 1986; Mitrofanis and Stone, 1988; Kim et al., 2000) in the rat retina.

There are a few NPY-immunoreactive amacrine cells that ramify in stratum 3 of the IPL, and these cells could not be readily distinguished from the more abundant NPY cells that ramify in stratum 1 of the IPL in whole-mount preparations. Therefore, we counted all NPY-immunoreactive cell bodies in the INL when evaluating the NPY cells in the INL. The regularity index of the NPY-immunoreactive amacrine cell population is comparable to the mouse NPY-immunoreactive amacrine cell population and higher than that of other amacrine cell populations. Therefore, we speculate that the infrequently occurring cells ramifying in stratum 3 have little influence on the NPY-immunoreactive amacrine cell regularity index, because their presence should have resulted in lowering the regularity index values.

NPY-immunoreactive displaced amacrine cells in rat ramified in stratum 5 of the IPL, and their cell density was less than the NPY-immunoreactive amacrine cell population with an average of approximately 90 cells/mm². Similarly, in the mouse retina the NPY-immunoreactive displaced amacrine cell population has a lower cell density than the NPY-immunoreactive amacrine cell population (Sinclair and Nirenberg, 2001). The displaced amacrine cell population is not randomly distributed and has a very similar regularity index in both rat and mouse. NPY-immunoreactive displaced amacrine cells regularity index was 2.36 in the rat retina and 2.35 in the mouse retina (Sinclair and Nirenberg, 2001). Compared with other displaced amacrine cell populations in the retina, NPY displaced amacrine cells are moderately dense and regularly distributed. For example, the ChAT-immunoreactive displaced amacrine cells are higher in density and are less regularly distributed than the NPY-immunoreactive displaced amacrine cells (Voigt, 1986; Mitrofanis and Stone, 1988; Kim et al., 2000).

**Neurochemical characterization of NPY-immunoreactive amacrine cells**

Nearly all NPY-immunoreactive amacrine cells contained GABA or the GABA synthesizing enzyme GAD₆₅, demonstrating that these cells form a subpopulation of the larger GABA-containing amacrine cell population. This observation is consistent with findings in the mouse retina; in the mouse retina, NPY-immunoreactive amacrine cells are likely to contain GABA, because they express the GABA transporter GAT-1 (Sinclair and Nirenberg, 2001). The colocalization of bioactive peptides, including vasoactive intestinal polypeptide and substance P, and of dopamine with GABA has been reported in wide-field amacrine cells in other mammalian retinas (Waßle and Chun, 1988; Vaney et al., 1989; Wulle and Wagner, 1990; Casini and Brecha, 1992; Young, 1994; Oh et al., 1999b). The present findings, together with previous observations, further support the idea that a general organizational feature of the mammalian retina is the coexpression of GABA with another neurotransmitter or neuromodulator in wide-field amacrine cells.

A small percentage of NPY-immunoreactive amacrine cells express TH immunoreactivity. These TH-immunoreactive cells are most similar to the previously described class 2 TH-immunoreactive amacrine cells in the rat retina (Nguyen-Legros et al., 1997), which are characterized by a small cell body and processes in stratum 3 of the IPL. The occurrence of NPY immunoreactivity in some of the class 2 TH-immunoreactive cells was unexpected and shows a neurochemical heterogeneity in TH-immunoreactive amacrine cells.
The colocalization of NPY and GABA suggests that these amacrine cells release both substances. Interestingly, in other systems, NPY and GABA may be released by different stimulus intensity or patterns, and they may act independently or in concert on their target sites (Hökfelt, 1991; Parker et al., 1998; Pu et al., 1999). Perhaps NPY and GABA are also released under different stimulus conditions by amacrine cells and act at different sites in the retina. For instance, GABA may have direct inhibitory effects on nearby postsynaptic sites, whereas NPY might diffuse in the extracellular space and influence neighboring neurons that are not directly postsynaptic to NPY-immunoreactive amacrine cells (D'Angelo and Brecha, 1999). NPY's action through a paracrine mode of action in the retina is consistent with other studies that report that the neuropeptides somatostatin and substance P may act in this manner, because there is a differential distribution of somatostatin- and tachykinin-containing processes and their respective receptors to different layers of the retina (Casini and Brecha, 1991; Rickman et al., 1996; Johnson et al., 1998). Therefore, the determination of the distribution of the different Y receptors in relationship to NPY-immunoreactive fibers would be of importance to better define NPY's physiological role in the retina and to determine whether NPY acts in a paracrine manner or in a direct, synaptic mode of action.

**Synaptic connectivity of NPY-immunoreactive amacrine cells**

The majority of NPY-immunoreactive processes form conventional presynaptic contacts onto nonimmunostained amacrine cell processes and ganglion cell dendrites in both the ON and OFF sublaminae of the IPL (Famiglietti et al., 1977). In addition, NPY-immunoreactive amacrine cell processes rarely form synaptic contacts onto bipolar cell axonal terminals. These findings indicate that NPY-immunoreactive cells influence both ON- and OFF-type ganglion cells, both indirectly by means of other amacrine cells and directly by means of synaptic contacts onto ganglion cells. The identity of the amacrine cells postsynaptic to NPY-immunoreactive processes is unknown, and more than one amacrine cell type is likely to receive synaptic input from NPY-immunoreactive processes, because NPY cells form synaptic connections with cell processes in different IPL strata. Determining the targets of these NPY-immunoreactive processes will be important for unraveling the microcircuits that are formed by these cells with other inner retinal cell types. To date, there are some clues as to the organization of these inner retinal pathways: an earlier pharmacologic study in the rabbit retina showed that endogenously applied NPY results in the release of dopamine, glycine, and serotonin from the retina (Bruun and Ehinger, 1993) indicating an action of NPY on several amacrine cell types, including the dopamine- and serotonin-containing wide-field amacrine cells (Brecha et al., 1984; Hokoç and Mariani, 1987; Mitrofanis et al., 1988; Dacey, 1990; Tauchi et al., 1990) and one or more of the glycine-containing narrow-field amacrine cell types (Pourcho and Goebel, 1985; Hendrickson et al., 1988; Wright et al., 1997; Menger et al., 1998). However, because of the time course and design of these pharmacologic studies in whole retina, questions remain regarding the direct action of NPY. Further experiments, thus, are needed to establish if these responses are occurring by means of polysynaptic pathways or by a paracrine action.

NPY-immunoreactive amacrine cell processes are primarily postsynaptic to nonimmunostained amacrine cell processes and some cone bipolar cell axonal terminals in all regions of the IPL. In addition, rod bipolar cell terminals that are identified by their cytologic characteristics in mammalian retinas (Famiglietti and Kolb, 1975; Kolb and Famiglietti, 1974; Kolb et al., 1979; McGuire et al., 1984, 1986) did not synapse onto NPY-immunoreactive processes. These findings indicate that NPY-immunoreactive amacrine cell processes specifically receive ON- and OFF-cone bipolar cell input; however, identification of the cone bipolar cell types that receive NPY input (Famiglietti and Kolb, 1975; Freed et al., 1987; Euler and Wässle, 1995; Hartveit, 1996) remains to be determined. These data agree with findings obtained for NPY in the monkey retina (Marshak et al., 1986). Overall, the pattern of synaptic connectivity indicates that the NPY-immunoreactive amacrine cells predominantly form both pre- and postsynaptic connections with other amacrine cells.

In conclusion, our findings show that NPY-immunoreactive cells are distributed in all retinal regions and they comprise at least two cell populations in the rat retina. One cell population is localized to the INL, and these cells mainly ramify in stratum 1 of the IPL. The second cell population is localized to the GCL, and these cells ramify in stratum 5 of the IPL. These cell populations have a moderate cell density and a regular distribution. These cells also contain GABA and the enzyme GAD$_{65}$, suggesting they are primarily inhibitory cells. Finally, NPY-immunoreactive processes form most of their synaptic contacts with other amacrine cells. Therefore, NPY-immunoreactive amacrine cells are components of inner retinal circuits involving other amacrine cells and are likely to contribute to the processing of visual information in the inner retina.

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**LITERATURE CITED**


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