

## CHAPTER 2

# The Mosaic of Amacrine Cells in the Mammalian Retina

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## CONTENTS

1. Introduction . . . . .	50
2. Cholinergic Amacrine Cells . . . . .	50
2.1. Rabbit Retina: <i>Ca</i> and <i>Cb</i> Amacrines . . . . .	51
2.2. Cat Retina: Irregular Distribution . . . . .	54
2.3. A Third Type of Cholinergic Amacrine? . . . . .	56
2.4. Coexistence of Acetylcholine and GABA . . . . .	57
3. Serotonin-Accumulating Amacrine Cells . . . . .	59
3.1. Rabbit Retina: S1 and S2 Amacrines . . . . .	60
3.2. Displaced and Misplaced Amacrine Cells . . . . .	62
3.3. Cat Retina: Two or Three Types? . . . . .	65
3.4. Dendritic Overlap and Spatial Regularity . . . . .	68
4. Glycinergic Amacrine Cells . . . . .	68
4.1. Localization of Accumulated and Endogenous Glycine . . . . .	69
4.2. AII Amacrines in Cat and Rabbit . . . . .	70
4.3. A New Type of Glycinergic Amacrine . . . . .	72
5. GABAergic Amacrine Cells . . . . .	74
5.1. Morphological and Neurochemical Diversity . . . . .	74
5.2. Localization of GABAergic Markers . . . . .	74
6. Peptide-Immunoreactive Amacrine Cells . . . . .	76
6.1. Substance P and VIP . . . . .	76
6.2. Somatostatin . . . . .	77
7. Catecholaminergic Amacrine Cells . . . . .	79
7.1. Rabbit Retina: CA1, CA2 and CA3 Amacrines . . . . .	79
7.2. Cat Retina: One Type, Several Morphologies . . . . .	81
7.3. Monkey Retina: CA1 and CA2 Amacrines . . . . .	82
7.4. NADPH-Diaphorase Cells: ND1 and ND2 Amacrines . . . . .	82
7.5. Other Low-Density Amacrine Cells . . . . .	83
8. The Mosaic of Amacrine Cells . . . . .	85
8.1. How Many Types of Amacrines? . . . . .	85
8.2. Modularity of the Amacrine Mosaic . . . . .	86
9. Direction Selectivity: An Hypothesis . . . . .	90
9.1. Cholinergic Fascicles Follow DS Dendrites . . . . .	90

9.2. Cotransmission Model of Direction Selectivity . . . . .	92
Acknowledgements . . . . .	94
References . . . . .	94

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## 1. INTRODUCTION

The concept of neuronal type has been a unifying theme in retinal research: each cell type is defined by the regular association of particular morphological, synaptic, neurochemical and physiological properties, which may change systematically across the retina as a function of neuronal density (reviewed by Sterling, 1983). A local region of the retina will contain all neuronal types in characteristic proportions, thus forming a functional "module". The complexity of the module may approach that of a central ganglion in the insect nervous system, and some of the techniques that have proved powerful for analyzing "identified neurons" in invertebrates are applicable to the retina. In particular, intracellular dye injection of microscopically identified retinal neurons has enabled whole population and single cell studies to be combined in the same tissue (Vaney, 1984; Tauchi and Masland, 1984).

The interneurons of the inner retina, which Cajal (1893) called amacrine cells, are noted for their morphological and neurochemical diversity (reviewed by Massey and Redburn, 1987). Consequently progress in identifying different types of retinal amacrine cells has been more rapid than for the bipolar cells or retinal ganglion cells. Although only a minority of the estimated 40 to 50 amacrine types in mammalian retina have been characterized with respect to their dendritic morphology, neurotransmitter content and topographic distribution, the colour-by-colour tiling of the amacrine mosaic is sufficiently advanced that recurring patterns of amacrine organization are becoming evident (Masland, 1988). This is particularly so in rabbit and cat retinae and, throughout this review, the organization of each amacrine system is illustrated with reference to these two species. Emphasis is

placed on the diverse spatial strategies of different amacrine types and their possible implications for complex visual processing.

## 2. CHOLINERGIC AMACRINE CELLS

Although the cholinergic amacrines are as well documented as any retinal amacrine type, new findings continue to surprise us and challenge our assumptions about amacrine function, some ten years after the acetylcholine-synthesizing cells were first identified in chicken (Baughman and Bader, 1977) and rabbit retinae (Masland and Mills, 1979). Five characteristics in particular stand out as novel and unexpected. First, the cholinergic neurons exist as two mirror-symmetric populations of amacrine cells, one in the inner nuclear layer and the other displaced to the ganglion cell layer (Hayden *et al.*, 1980; Vaney *et al.*, 1981). Second, the dendritic field overlap of their distinctive "starburst" dendritic trees is an order of magnitude greater than that of integrating retinal neurons (Vaney, 1984; Tauchi and Masland, 1984; Famiglietti, 1985). Third, despite this coverage, the cholinergic dendrites do not blanket the retina but show a striking fasciculated topology (Tauchi and Masland, 1985). Fourth, the inputs of these amacrines are spatially segregated, with synapses on ganglion cell dendrites confined to the varicose distal zone (Famiglietti, 1983b). Fifth, the cholinergic cells appear to contain, synthesize and accumulate GABA, suggesting that they are both excitatory and inhibitory in function (Vaney and Young, 1988a; Kosaka *et al.*, 1988; Brecha *et al.*, 1988).

Although these special characteristics of the cholinergic amacrines were first described in

rabbit retina, they have been mostly confirmed in a wide variety of mammals, as typified by Voigt's (1986) study on rat retina. Thus, these interneurons may be a ubiquitous component of the retinal circuitry, performing complex functions that are conserved from species to species. It is not implied, however, that the cholinergic cells are atypical amacrices: their apparent complexity has been recognized only through intensive study, such as accorded few other amacrine types. Furthermore, it is becoming apparent that there are significant species differences in the organization of this amacrine system, which may prove useful in characterizing cholinergic function in the retina. For example, only the displaced population of cholinergic amacrices is well developed in rhesus monkey retina, the inner nuclear population appearing vestigial (Mariani and Hersh, 1988). The substantial literature on the physiology and pharmacology of cholinergic mechanisms in the retina has been reviewed by Neal (1983) and Hutchins (1987).

## 2.1. Rabbit Retina: *Ca* and *Cb* Amacrices

Acetylcholine is now firmly established as a neurotransmitter in rabbit retina (Masland and Tauchi, 1986). Two distinct populations of amacrine cells have been shown to accumulate [<sup>3</sup>H]choline, synthesize acetylcholine, and release the transmitter in response to flashed illumination (Masland and Mills, 1979; Masland *et al.*, 1984a,b). The cell bodies of the type *a* cholinergic (*Ca*) amacrices are conventionally located at the inner margin of the inner nuclear layer (the amacrine sublayer) and their dendrites stratify in sublamina *a* of the inner plexiform layer; the cell bodies of the type *b* cholinergic (*Cb*) amacrices are displaced to the ganglion cell layer and their dendrites stratify in sublamina *b*. The mirror-symmetry of these two matching populations, first described from the autoradiographic localization of [<sup>3</sup>H]choline uptake (Masland and Mills, 1979), is readily apparent from the distribution of choline acetyltransferase immunoreactivity (ChAT-IR) in transverse retinal sections

(Brandon, 1987a; Famiglietti and Tumosa, 1987; Millar and Morgan, 1987) (Fig. 1). The *Ca* and *Cb* amacrices transiently release acetylcholine at light Off and light On respectively (Masland *et al.*, 1984b; Massey and Redburn, 1985), thus conforming to the functional stratification described for Off- and On-centre ganglion cells (Nelson *et al.*, 1978; Peichl and Wässle, 1981; Bloomfield and Miller, 1986).

The cell mosaic of *Ca* and *Cb* amacrices was first mapped in wholemounts stained with classic neurofibrillar methods, which selectively label three amacrine populations in peripheral rabbit retina, namely the presumptive cholinergic amacrices and a distinct type of long-range amacrine (Vaney *et al.*, 1981, 1988) (Fig. 35). Subsequently, Masland *et al.* (1984a) showed that acetylcholine-synthesizing amacrices throughout rabbit retina could be brightly labelled with the fluorescent nuclear stain, diamidino-phenylindole (DAPI). However, two other types of conventionally-placed amacrices also accumulated DAPI (Vaney, 1984; Tauchi and Masland, 1984) and, thus, only the *Cb* amacrices could be mapped in isolation. More recently, both populations of ChAT-IR amacrices have been stained in rabbit retina, their cell mosaic mapped (Brandon, 1987b), and their density distributions plotted from central to peripheral retina (Famiglietti and Tumosa, 1987). Thus, the *Ca* and *Cb* amacrices in rabbit retina can be selectively labelled by either neurofibrillar staining, DAPI fluorescence or ChAT immunohistochemistry, as confirmed by double-label experiments showing DAPI staining of both the ChAT-IR amacrices (Brecha *et al.*, 1988) and those neurofilament-IR amacrices with the type C morphology (D.I. Vaney and H.M. Young, unpublished).

The density of *Ca* amacrices increases sixfold from the superior periphery to the visual streak, reaching a peak density of 750 to 950 cells/mm<sup>2</sup> in adult retinae (Vaney, 1984; Masland *et al.*, 1984a; Famiglietti and Tumosa, 1987). Neurofibrillar staining indicated that the *Ca* and *Cb* amacrices were present in about equal numbers in peripheral retina, but that the gradient of cell density was slightly greater for the *Cb* amacrices (Vaney *et al.*, 1981). Counts of ChAT-IR cells confirmed and extended this finding, showing that the *Cb* cells



FIG. 1. Cholinergic amacrine cells in a transverse section of rabbit retina, as labelled by choline acetyltransferase immunohistochemistry. The cholinergic amacrices in the inner nuclear layer (INL) stratify in sublamina *a* of the inner plexiform layer (IPL), while the displaced cholinergic amacrices in the ganglion cell layer (GCL) stratify in sublamina *b*.

Scale bar, 25  $\mu\text{m}$ . (From Brandon, 1987a.)

are 10 to 15% more common than *Ca* cells within 2 mm of the peak visual streak (Famiglietti and Tumosa, 1987). There are some 150,000 *Cb* amacrices in total, at a mean density of 330 cells/ $\text{mm}^2$  in a retina of 450  $\text{mm}^2$  area (Hughes, 1985; Masland and Tauchi, 1986). In DAPI-stained retina, at a mid-peripheral density of 350 *Cb* cells/ $\text{mm}^2$ , there are 11,000 cells/ $\text{mm}^2$  at the inner margin of the inner nuclear layer (D.I. Vaney, unpublished). It is calculated therefore that the *Cb* amacrices account for 3.0% of the 4,900,000 presumptive amacrine cells in rabbit retina, a proportion somewhat higher than our previous estimate. The *Ca* amacrices are distributed more regularly than the *Cb* amacrices, with a small proportion of the displaced cells occurring as closely-spaced pairs (Vaney *et al.*, 1981).

The DAPI-labelled amacrine cells were visualized microscopically in isolated retinal preparations and then injected with Lucifer yellow under direct visual control (Vaney, 1984; Tauchi and Masland, 1984). These definitive experiments established that the cholinergic amacrices have a distinctive unistratified morphology corresponding, as suggested by Famiglietti (1983a), to the "starburst" amacrine cells

impregnated by Golgi methods in rabbit retinal wholemounts. Mirror-symmetric amacrices with this dendritic morphology were first described by Perry and Walker (1980) in Golgi-stained rat retina. Four Lucifer-filled *Cb* amacrices from central rabbit retina are shown in Fig. 2. Each cell usually produces three to five primary dendrites that branch regularly and repeatedly, forming a stratified arborization with as many as 80 terminal dendrites. The dendritic field diameter of *Cb* amacrices increases almost fourfold from the peak visual streak to the superior periphery (180–640  $\mu\text{m}$ , Vaney, 1984; 230–830  $\mu\text{m}$ , Tauchi and Masland, 1984; 150–600  $\mu\text{m}$ , Famiglietti, 1985); at each retinal eccentricity, the *Ca* amacrices are about 13% wider than the overlying *Cb* amacrices.

The dendritic field overlap of the *Cb* amacrices ranges from 25 on the peak visual streak to about 70 in the far periphery; the overlap of the wider *Ca* amacrices may be correspondingly greater, reaching values of 90 or more (Vaney, 1984). Tauchi and Masland (1984) have calculated that two to four metres of *Cb* dendrites are packed into each square millimetre of retina, the highest values being located 1 mm inferior of the peak visual

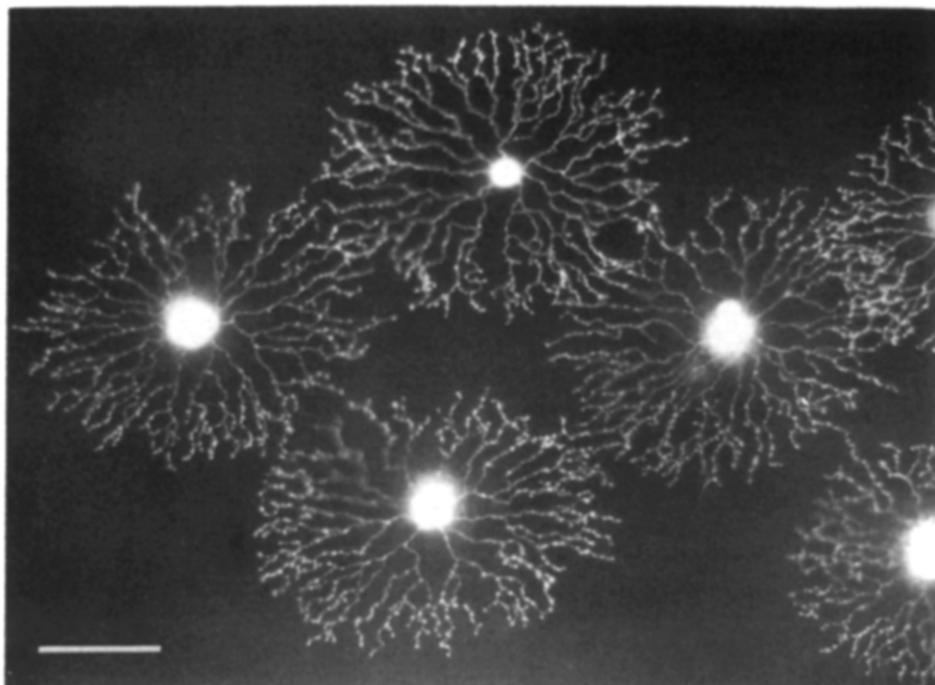


FIG. 2. Displaced cholinergic (*Cb*) cells in central rabbit retina injected with the fluorescent dye, Lucifer yellow. The cholinergic amacrices have a radially-symmetric "starburst" morphology, with their output synapses to ganglion cells confined to the varicose distal zone. There are about 250 *Cb* amacrices in the micrograph field, with each point in central retina being overlapped by the dendritic trees of about 30 *Cb* amacrices. Scale bar, 100  $\mu\text{m}$ . (D. I. Vaney, unpublished.)

streak. The cholinergic dendrites do not blanket the retina as originally proposed, but show a fasciculated topology with cords of dendrites surrounding lacunae of 10 to 50  $\mu\text{m}$  diameter. This has been demonstrated with either Lucifer injection of overlapping *Cb* amacrices (Tauchi and Masland, 1985), neurofibrillar staining (Wieniawa-Narkiewicz, 1983; Vaney *et al.*, 1988) or ChAT immunohistochemistry (Brandon, 1987b; Famiglietti and Tumosa, 1987): the latter method shows that the lacunae in the two cholinergic strata generally correspond in size and position.

Rabbit cholinergic amacrices receive sparse bipolar and amacrine input over the whole dendritic tree, but their synaptic output to retinal ganglion cells is clustered within the varicose annular zone covering the distal third of their dendrites (Famiglietti, 1983b; Brandon, 1987b). In addition, it has been reported that cholinergic-cholinergic synapses are common in rabbit and other retinae (Millar and Morgan, 1987; Millar

*et al.*, 1987; Mariani and Hersh, 1988), but it is not clear whether they account for all of the amacrine input to the cholinergic dendrites. The question of whether the cholinergic amacrices also synapse onto non-cholinergic amacrices is addressed elsewhere (Famiglietti, 1989; Millar *et al.*, 1989).

Although it is generally supposed that there is an invariant relationship between cell body position and stratification level for rabbit cholinergic amacrices, Lucifer injection of hundreds of DAPI-labelled cells revealed an occasional displaced amacrine that branched inappropriately in the *Ca* stratum (D.I. Vaney, unpublished). This suggests that cell body position does not determine stratification level *per se*, but that cells destined to branch in stratum *Cb* are segregated accordingly; a *Ca* amacrine misplaced to the ganglion cell layer might then branch in a stratum different from its neighbours. Although negligible in rabbit retina, such discordant stratification is common in goldfish retina

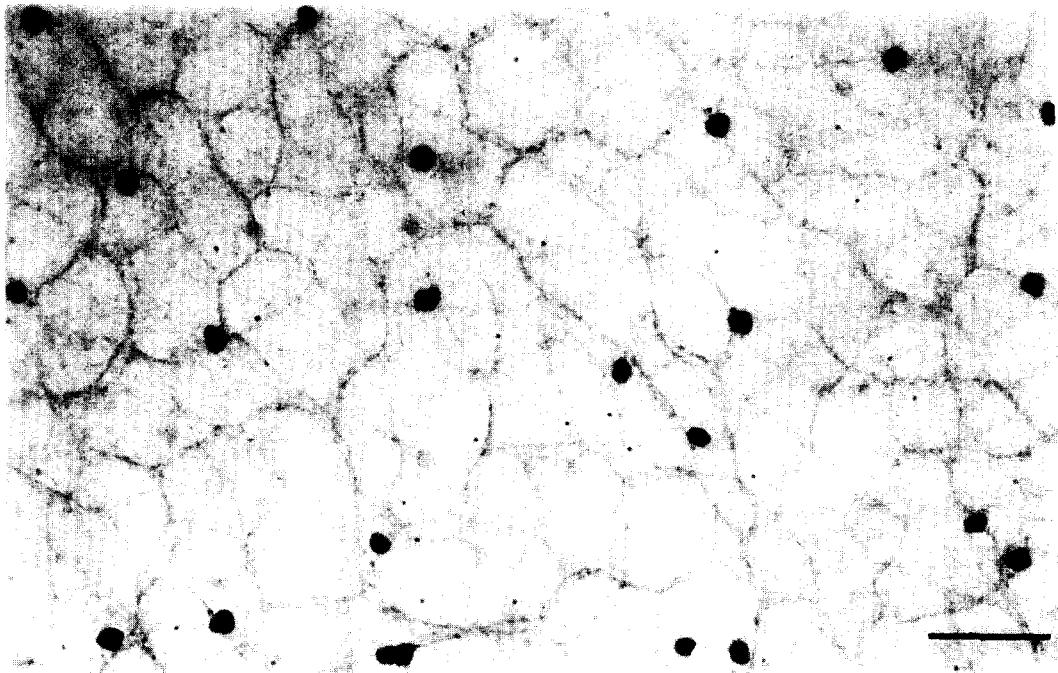


FIG. 3. Cholinergic amacrine cells in a wholemount of peripheral cat retina, as labelled by choline acetyltransferase immunohistochemistry. The cholinergic plexus has a striking fasciculated topology, with cords of dendrites surrounding lacunae of 15 to 50  $\mu\text{m}$  diameter. Scale bar, 50  $\mu\text{m}$ . (From Vanney *et al.*, 1989a.)

(Tumosa *et al.*, 1984) and may underly the irregular distribution of cholinergic cells in cat retina.

## 2.2. Cat Retina: Irregular Distribution

ChAT immunohistochemistry labels two distinct populations of amacrine cells in cat retina, one conventionally located in the inner nuclear layer and the other displaced to the ganglion cell layer (Schmidt *et al.*, 1985, 1987; Pourcho and Osman, 1986a; Vanney *et al.*, 1989a) (Fig. 3). The ChAT-IR strata are located around depths 20% and 49% of the inner plexiform layer (Pourcho and Osman, 1986b), compared with depths 23% and 70% for rabbit retina (Brandon, 1987a; Famiglietti and Tumosa, 1987). Two retinal wholemounts sampled on a millimetre grid contained 126,000 and 137,000 ChAT-IR cells, with a maximum central density of 2,000 and 2,600 cells/ $\text{mm}^2$  respectively, and a minimum density of 100 cells/ $\text{mm}^2$  in far-superior retina

(D. I. Vanney and G. E. Whitington, unpublished; cf. Schmidt *et al.*, 1987). The region of maximum density overlays the area where ganglion cells are stacked two deep, with displaced cholinergic amacrinies being located in both levels of the ganglion cell layer (Vanney *et al.*, 1989b). The 20-fold density range of cholinergic amacrinies in cat retina is much greater than the 6-fold range in rabbit retina, or the 3- to 4-fold range in rat retina (Voigt, 1986; Mitrofanis *et al.*, 1988b). Mapped distributions of ChAT-IR cells in cat retina show a pronounced streak topography, with the 500 cells/ $\text{mm}^2$  isodensity line extending 14 mm horizontally, but less than 5 mm vertically (Fig. 40). In addition, the 300 contour bulges towards superior retina, sometimes pulling the 400 contour with it (D. I. Vanney and G. E. Whitington, unpublished; cf. Schmidt *et al.*, 1987). The contours above 600 cells/ $\text{mm}^2$  are kite-shaped, becoming round within 0.5 mm of the peak area centralis (Fig. 4).

In cat retina, there are many more ChAT-IR cells in the ganglion cell layer than in the inner

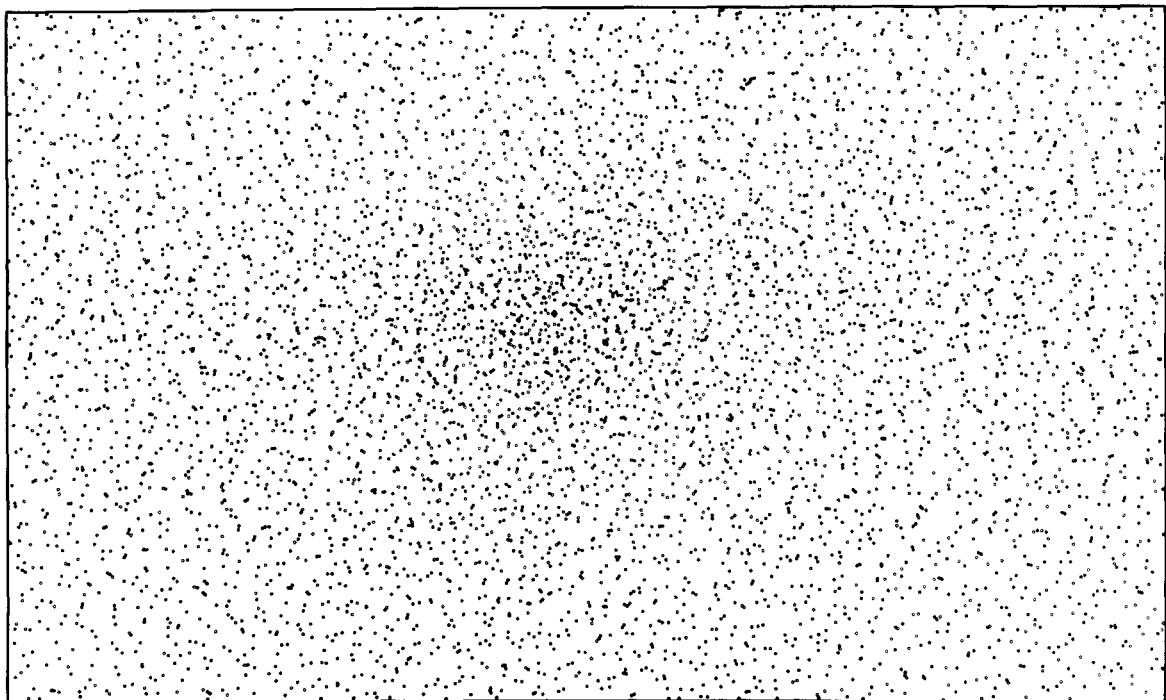


FIG. 4. Distribution of cholinergic amacrines in central cat retina, showing the mapped position of each cell in the inner nuclear (○) and ganglion cell layers (●). The maximum density of  $2,600 \text{ cells/mm}^2$  is located in the region of peak ganglion cell density. Retinal field,  $1.9 \times 3.2 \text{ mm}$ . (D. I. Vaney and G. E. Whitington, unpublished.)

nuclear layer (Schmidt *et al.*, 1985; Pourcho and Osman, 1986a). Displaced cells accounted for 67% of the cholinergic amacrines in the mapped wholemount and 65% in its central area; the proportion of displaced cells was locally variable, however, ranging from 50% to more than 80% at some peripheral sample sites. Moreover, the two mosaics of ChAT-IR cells are much less regular than their counterparts in rabbit retina (Schmidt *et al.*, 1987). A sharp increase in the local density of displaced cells is usually accompanied by a reduced density of inner nuclear cells, and vice versa, such that their combined density increases smoothly towards the area centralis (Fig. 5). Taken together, these findings suggest that the production of cholinergic amacrines is well regulated in cat retina, but that their distribution in the inner nuclear and ganglion cell layers is haphazard.

In local regions of some cat retinae, displaced amacrines may account for more than 90% of the ChAT-IR cells but, in other respects, the

cholinergic system appears normal: the  $\text{Ca}$  plexus is not correspondingly reduced and the combined cell density matches that of surrounding regions. Is it possible that a significant proportion of the displaced cholinergic amacrines branch in sublamina *a*? Although other workers have claimed that the displaced ChAT-IR cells arborize exclusively in sublamina *b*, it is difficult to trace cat cholinergic amacrines for any distance in wholemounts, because their dendrites are both thinner and more tightly fasciculated than the cholinergic dendrites in rabbit retina. The question thus remains open. (Hutchins and Hollyfield (1987) reported that the distribution of cholinergic amacrines in human retina is similar to cat retina, in that two-thirds of the ChAT-IR cells are located in the ganglion cell layer, but their branching level could not be determined.)

Cat amacrine cells with a "starburst-like" morphology have been partially impregnated by Golgi methods (Pourcho and Osman, 1986a; Famiglietti, 1987) or visualized by intracellular

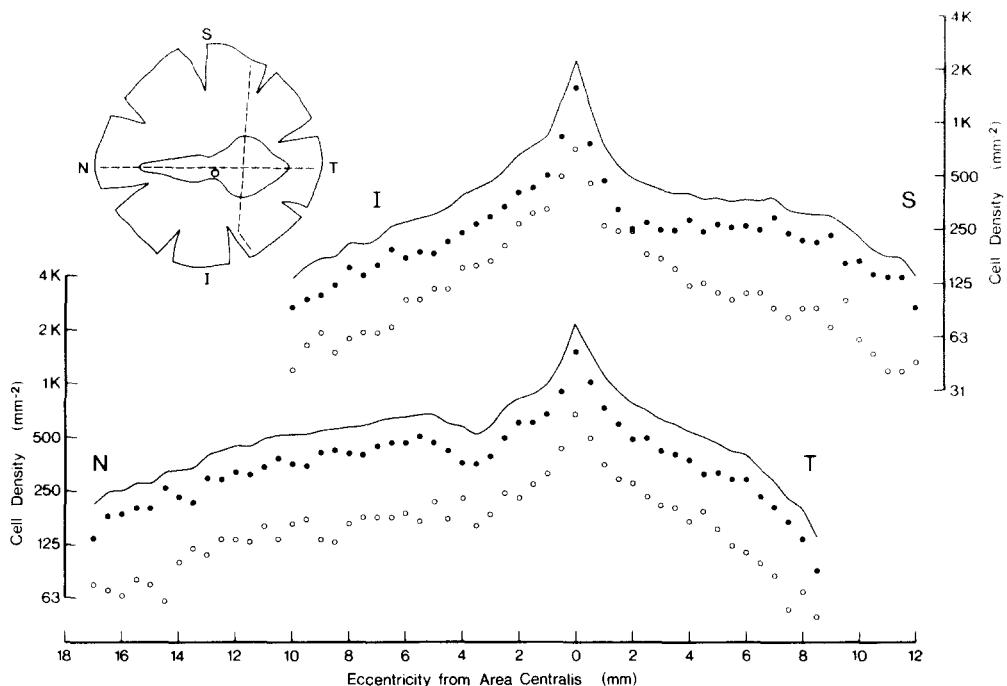


FIG. 5. Cell density gradients of displaced (●), conventionally-located (○) and all cholinergic amacrines (—) in horizontal and vertical transects through the area centralis of cat retina. A sharp increase in the local density of displaced cells is usually accompanied by a reduced density of inner nuclear cells, and vice versa, such that their combined density increases smoothly towards the area centralis. (D. I. Vaney and G. E. Whitington, unpublished.)

injection with Lucifer yellow (Schmidt *et al.*, 1987; D. I. Vaney unpublished). They have a more open branching pattern than their rabbit counterparts, and the lacunae in the fasciculated cholinergic plexus are correspondingly larger (Vaney *et al.*, 1989a). The dendrites of cat "starburst" amacrines are difficult to stain and photograph because they are comparatively fine, but this is not readily apparent in the line drawings that have been published. Figure 6 is an original micrograph of a Lucifer-filled displaced cell from central cat retina: the circular dendritic field contrasts with the oval fields of rabbit *Cb* amacrines at a similar retinal eccentricity. Pourcho and Osman (1986a) argued that the A14 amacrine of Kolb *et al.* (1981) has a "starburst-like" morphology and is thus the Golgi correlate of displaced cholinergic amacrines in cat retina; it is proposed elsewhere, however, that the illustrated A14 cell is a substance P-IR amacrine

and that the cholinergic amacrines may be absent from Kolb's catalogue of Golgi-stained types (Vaney *et al.*, 1989b).

### 2.3. A Third Type of Cholinergic Amacrine?

There are three types of cholinergic amacrines in chicken retina, including two mirror-symmetric populations that appear analogous to the *Ca* and *Cb* amacrines of rabbit retina (Baughman and Bader, 1977; Millar *et al.*, 1985). The cell bodies of the third cholinergic type are located in the middle of the inner nuclear layer, scleral to the *Ca* amacrine cells; their dendrites branch diffusely in strata 1 and 3–5 but are largely overshadowed by the strongly immunoreactive *Ca* and *Cb* strata (Millar *et al.*, 1987). Published micrographs indicate that the type III amacrines account for about 30% of ChAT-IR cells in chicken retina.

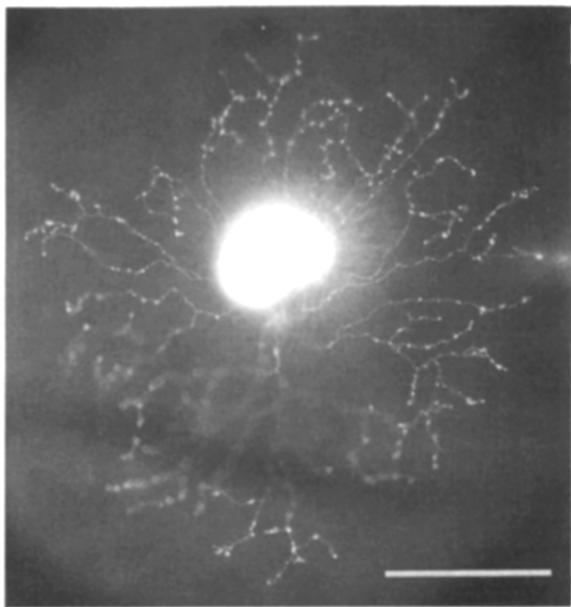


FIG. 6. Displaced "starburst" cell in central cat retina; this presumptive cholinergic amacrine has finer dendrites than the homologous cell type in rabbit retina. Scale bar, 50  $\mu\text{m}$ . (D. I. Vaney, unpublished.)

Among mammals, the tree shrew retina also appears to contain three types of ChAT-IR amacrines (Conley *et al.*, 1986). Two matching populations of large, darkly stained cells in the inner nuclear and ganglion cell layers have similar density distributions, and reportedly branch in strata 2 and 4 respectively. The third ChAT-IR population is also located in the amacrine sublayer, but their cell bodies are smaller and less immunoreactive than those of the *Ca* amacrines: they account for about 60% of ChAT-IR cells in tree shrew retina. In cat retina processed for ChAT immunohistochemistry, Schmidt *et al.* (1985) reported that faint staining could be detected in a sparse population of small amacrines, but this was interpreted as "background labelling". Our wholemount preparations appear similar (Fig. 7), but we conclude that the smaller cells show distinct ChAT-like immunoreactivity and, thus, should be considered as a third type (C3) of presumptive cholinergic amacrine (D. I. Vaney and G. E. Whitington, unpublished). Although the C3 cells are labelled only weakly, they stand out clearly

from the surrounding amacrines when viewed with simple transmitted illumination (the Nomarski optics used by Schmidt *et al.* (1985) masks the C3 cells in their published micrograph). Each cell appears to produce a thin primary dendrite that arises laterally from a restricted cap of cytoplasm. The dendrites could only be traced for about 10  $\mu\text{m}$ , even in well-stained regions of cat retina where the cell mosaic of the three types could be reliably mapped: the C3 amacrines are about twice as numerous as the neighbouring *Ca* amacrines.

Might rabbit retina also contain a third population of cholinergic amacrines that are distinct from the *Ca* and *Cb* amacrines? The specific monoclonal antibody (Eckenstein and Thoenen, 1982) used in the cat and tree shrew studies does not recognize rabbit ChAT, and all of the rabbit studies used a polyvalent antiserum (Johnson and Epstein, 1986) which produces higher background staining. Given that the C3 amacrines in cat retina are only weakly immunoreactive, it seems possible that an analogous population may have gone undetected in rabbit retina. This should be considered when interpreting the effects of cholinergic drugs on retinal ganglion cells that do not branch in either of the principal cholinergic strata (Vaney *et al.*, 1989a). The basic finding that most ChAT in rabbit retina is localized in two populations of mirror-symmetric amacrines stands without further qualification.

#### 2.4. Coexistence of Acetylcholine and GABA

Several laboratories have recently reported that cholinergic amacrine cells in mammalian retinae show intense GABA-like immunoreactivity (rabbit: Vaney and Young, 1988a; Brecha *et al.*, 1988; rat: Kosaka *et al.*, 1988; cat: Vaney *et al.*, 1989a); in addition, the cholinergic amacrines appear to synthesize GABA (Kosaka *et al.*, 1988; Brecha *et al.*, 1988) and to accumulate the transmitter and its analogues (O'Malley and Masland, 1988a; Chun *et al.*, 1988). In rabbit retina, the colocalization of these excitatory and inhibitory transmitters was established by GABA immunohistochemistry of DAPI-labelled wholemounts: all of the fluorescent *Cb* amacrines were

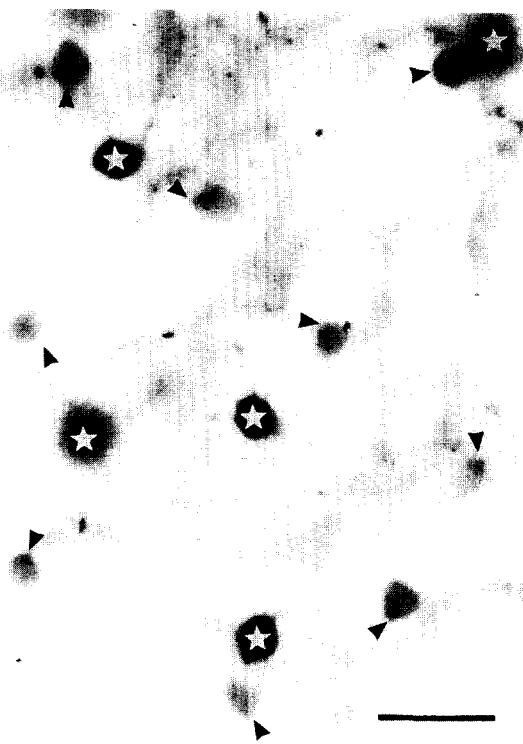


FIG. 7. Choline acetyltransferase immunohistochemistry labels three types of amacrine cells in cat retina, shown here in wholemount with the focus in the inner nuclear layer. The large  $Ca$  amacrines and the out-of-focus  $Cb$  amacrines are intensely labelled (white stars), while the smaller, more numerous  $C3$  amacrines are only weakly labelled (their primary dendrites are marked with arrowheads). Scale bar, 25  $\mu\text{m}$ . (D. I. Vaney and G. E. Whitington, unpublished.)

strongly immunoreactive and they accounted for 65 to 75% of the GABA-IR cells in the ganglion cell layer. In cat retina, the coexistence of cholinergic and GABAergic markers can be shown directly, by using a rat monoclonal antibody against ChAT and a rabbit polyclonal antiserum against GABA (Fig. 8). The colocalization in identified neurons of two "fast-acting" classical transmitters, one excitatory and the other inhibitory, seems to be without precedent (see commentary by Miller, 1988), but may be relatively widespread (Davidoff and Schulze, 1988; Kosaka *et al.*, 1988).

O'Malley and Masland (1988a) subsequently investigated the conditions under which

$[^3\text{H}]$ acetylcholine and  $[^{14}\text{C}]$ GABA are released from the type C cells and from other rabbit amacrines that accumulate GABA alone. They showed that both the acetylcholine and GABA stores could be released by potassium depolarization *in vitro*, but that only the acetylcholine release was blocked under the low calcium conditions which prevent vesicle exocytosis. O'Malley and Masland (1988b) also used antibody-labelled beads to precipitate the synaptic vesicles, showing that they contain much of the stored acetylcholine, but negligible amounts of GABA. They concluded that the labelled acetylcholine is secreted by vesicle exocytosis whereas the GABA release is carrier mediated, as

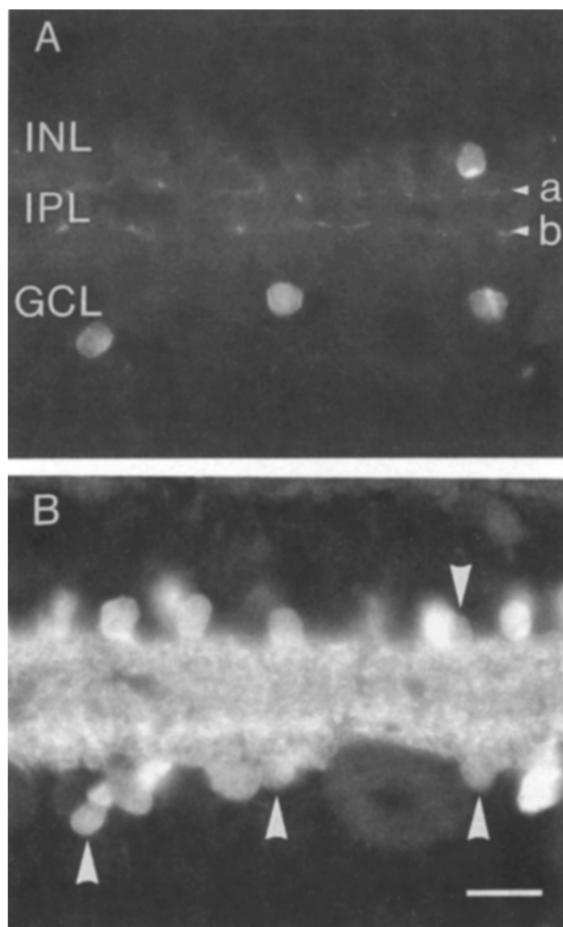


FIG. 8. Colocalization of cholinergic and GABAergic markers in retinal amacrine cells. The frozen transverse section of cat retina was double-labelled with a rat monoclonal antibody against choline acetyltransferase (A) and a rabbit polyclonal antiserum against GABA (B). The Ca amacrine cell in the inner nuclear layer and the three Cb amacrines in the ganglion cell layer all show GABA-like immunoreactivity. Scale bar, 20  $\mu$ m. (From Vaney *et al.*, 1989a.)

demonstrated previously for fish horizontal cells (Schwartz, 1987). Curiously, the basal release of labelled GABA was reportedly unaffected by photic stimulation of any kind, although various flashing or moving lights triggered an increased secretion of acetylcholine in the same preparations (O'Malley and Masland, 1988a). GABA reuptake blockers increased the basal release but did not unmask a light-induced GABA release. These results seem less paradoxical if it is supposed that

a small increment to the basal GABA release, occurring locally into a synaptic cleft or a dendritic fascicle, is subject to intense spatial buffering which becomes saturated during potassium depolarization.

### 3. SEROTONIN-ACCUMULATING AMACRINE CELLS

Ehinger and Florén (1976) identified distinct populations of amacrine cells in rabbit and cat retinae that selectively accumulate exogenous indoleamines and catecholamines, but which differed from the sparse population of endogenous dopaminergic neurons revealed by formaldehyde-induced fluorescence. These amacrine cells could only be visualized if preloaded with serotonin or related indoleamines: neither the Falck – Hillarp method nor immunohistochemistry have shown cellular localization of endogenous serotonin in the retina of any placental mammal, although serotonin-IR processes have been labelled in the inner and outer plexiform layers of a microchiropteran bat (Studholme *et al.*, 1987). By contrast, serotonin-IR amacrine cells have been localized in select retinae from other vertebrate classes (Osborne *et al.*, 1982; Tornqvist *et al.*, 1983), and also in the retinae of marsupial (T. J. Millar, unpublished) and egg-laying mammals (D. I. Vaney, unpublished) (Fig. 9).

Although mammalian retinae contain much less endogenous serotonin than non-mammalian retinae (Florén and Hansson, 1980; Ehinger *et al.*, 1981), the available serotonin appears to be concentrated in the inner retina (Osborne, 1982), and biochemical studies indicate that most of the components required for a functional serotonergic system are present in bovine and rabbit retinae (Osborne, 1980; Mitchell and Redburn, 1985). Do the serotonin-accumulating amacrines in mammalian retinae use serotonin as a transmitter and, if not, might they use another indoleamine? These contentious issues have been addressed at length by other authors, but have yet to be resolved (Ehinger, 1983; Osborne, 1982, 1984; Redburn, 1985). The serotonin-accumulating

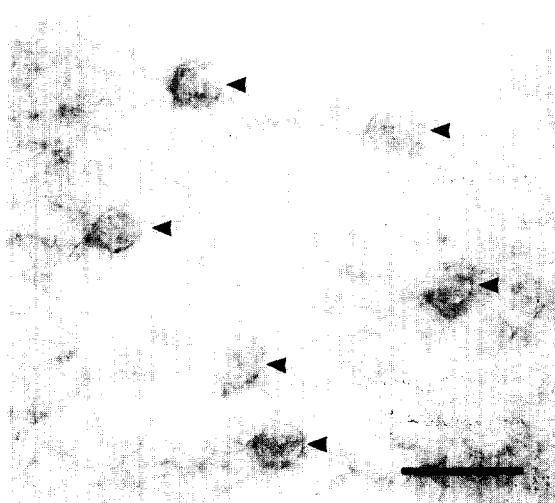


FIG. 9. Immunohistochemical localization of endogenous serotonin in a wholemounted retina of the platypus, *Ornithorhynchus anatinus*, with the focus in the amacrine sublayer of the inner nuclear layer. Scale bar, 20  $\mu\text{m}$ . (D. I. Vaney, unpublished.)

amacrines in cat retina mainly contact rod bipolar terminals, accounting for 75% of reciprocal connections at the rod bipolar dyads (Holmgren-Taylor, 1982). However, more than 90% of the amacrine processes reciprocal to rod bipolars also accumulate GABA (Freed *et al.*, 1987), indicating that the serotonin-accumulating amacrines may be GABAergic (Vaney, 1986b). Colocalization studies have since established that the great majority of serotonin-accumulating amacrines in both rabbit and cat retinae show GABA-like immunoreactivity (Osborne and Beaton, 1986; Wässle and Chun, 1988) (Fig. 10). This does not exclude the possibility that these amacrines use both GABA and serotonin as transmitters, but it can no longer be argued that they are serotonergic by default. Whatever the outcome, serotonin uptake has proved an invaluable marker, enabling several types of amacrines in rabbit and cat retinae to be discriminated and characterized.

### 3.1. Rabbit Retina: S1 and S2 amacrines

In rabbit retina, the serotonin-accumulating cells are normally located at the inner margin of the inner nuclear layer and their dendrites form a

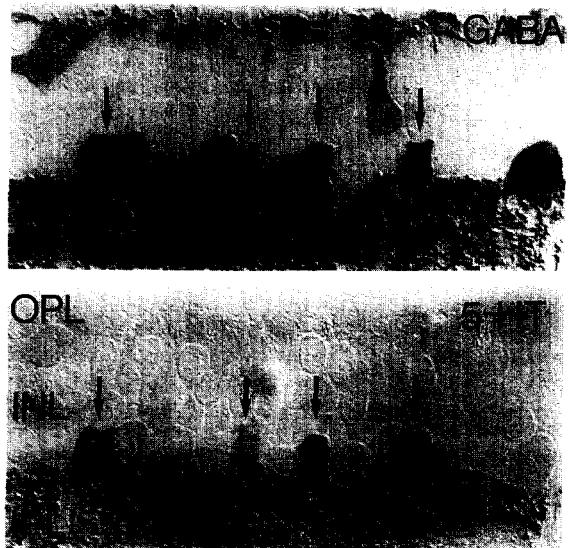


FIG. 10. Consecutive semi-thin sections of cat retina showing localization of GABA-like immunoreactivity in serotonin-accumulating amacrine cells, as viewed with Nomarski optics. Scale bar, 25  $\mu\text{m}$ . (From Wässle and Chun, 1988.)

dense plexus in stratum 5 of the inner plexiform layer, adjacent to the ganglion cells (Ehinger and Flören, 1976). They outnumber the catecholamine-accumulating amacrines by about 15:1 and their density peaks within the visual streak (Ehinger and Åberg, 1981; Negishi *et al.*, 1984). If the retina is labelled *in vivo* or *in vitro* with the cytotoxic serotonin analogs, 5,6-DHT or 5,7-DHT, the mosaic of serotonin-accumulating amacrines can be revealed in isolation simply by fixing the wholemount in paraformaldehyde and coverslipping it with glycerine (Vaney, 1986a) (Fig. 11). The bright formaldehyde-induced fluorescence fades rapidly, however, and more durable preparations can be produced by reacting the wholemount with a monoclonal antibody that recognizes both 5-HT and 5,7-DHT (Wässle *et al.*, 1987b). Although the primary branching pattern can be traced in such material, the labelled processes soon become lost within the lacelike plexus, and it is impossible to map the dendritic trees of individual amacrine cells.

The serotonin analog, 5,7-DHT, is itself moderately fluorescent under ultraviolet excitation, enabling serotonin-accumulating

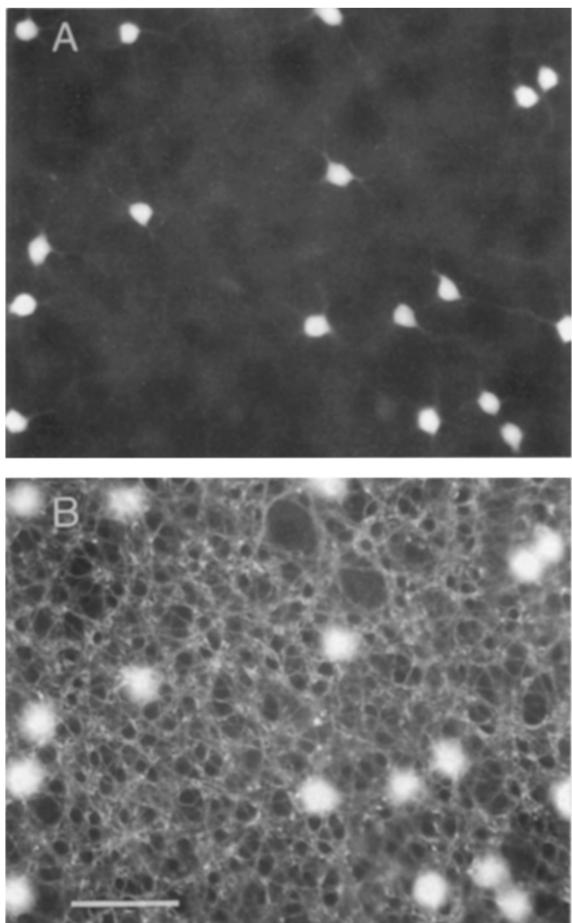


FIG. 11. Serotonin-accumulating amacrine cells in peripheral rabbit retina. The cells were labelled by 5,7-DHT uptake *in vitro*, the retina fixed in paraformaldehyde, flat mounted in phosphate-buffered glycerine, and then viewed under ultraviolet illumination. The fluorescent cell bodies are located in the inner nuclear layer (A) and their dendrites are stratified at the inner margin of the inner plexiform layer (B). The field is overlapped by the dendritic trees of hundreds of serotonin-accumulating amacrices, which form a lace-like plexus around the Müller stalks. Scale bar, 50  $\mu\text{m}$ . (From Vaney, 1986.)

neurons to be visualized in living rabbit retina (Vaney, 1986a): this appears to be the first instance where a transmitter system has been selectively identified in unfixed tissue. Iontophoretic injection of Lucifer yellow into the fluorescent cells under visual control revealed that the serotonin-accumulating amacrices in rabbit retina comprise two morphological types, termed S1 and S2 amacrices (Vaney, 1986a) (Fig. 12).

These results were independently confirmed by Sandell and Masland (1986), using an adaptation of the fluorescence-guided injection method that enables lightly fixed cells to be filled (Tauchi and Masland, 1984). Lucifer yellow does not diffuse so readily in fixed neurons, however, and the formaldehyde-induced fluorescence of the labelled plexus reduces the contrast of Lucifer-filled dendrites, completely masking very thin processes.

Although the S1 and S2 amacrices both branch in stratum 5, they can be readily distinguished by the shape and size of their dendritic trees. The S1 amacrices are characterized by their geometric form and the sparse, leaf-like varicosities in the middle of the arborization; they produce up to 30 radial dendrites that become uniformly thin and which extend for 600 to 1800  $\mu\text{m}$  from the cell body, making this amacrine type one of the largest in rabbit retina (Vaney, 1986a). By contrast, the sinuous S2 dendrites are beaded along their entire length and, proximal to the cell body, they may spread diffusely through the inner plexiform layer. The dendritic field diameter of the S2 amacrices ranges from 300  $\mu\text{m}$  on the visual streak to about 1000  $\mu\text{m}$  in the inferior periphery, and may exceed 2000  $\mu\text{m}$  in the superior periphery (D. I. Vaney, unpublished) (Fig. 13).

There are between 170,000 and 230,000 serotonin-accumulating amacrine cells in total, their density increasing five- to six-fold from peripheral retina to the visual streak. They outnumber the DAPI-labelled Cb amacrices by about 4:3 across the retina, and account for some 4% of the presumptive amacrices in rabbit retina (Negishi *et al.*, 1984; Sandell and Masland, 1986; D. I. Vaney, unpublished). The two types of serotonin-accumulating amacrices can be objectively differentiated in 5,7-DHT-labelled retina by their characteristic bleaching properties (Vaney, 1986a) or pyknotic appearance (Fig. 14); it may also be possible to morphologically distinguish the S1 and S2 amacrices in photo-oxidized or immunoreacted material (Sandell and Masland, 1986). The S1 amacrices account for about 50% of labelled cells in the visual streak and up to 60% peripherally, with the independent overlay of the two populations forming a cell mosaic that is less ordered than either of the constituent arrays. Each point on the retina is

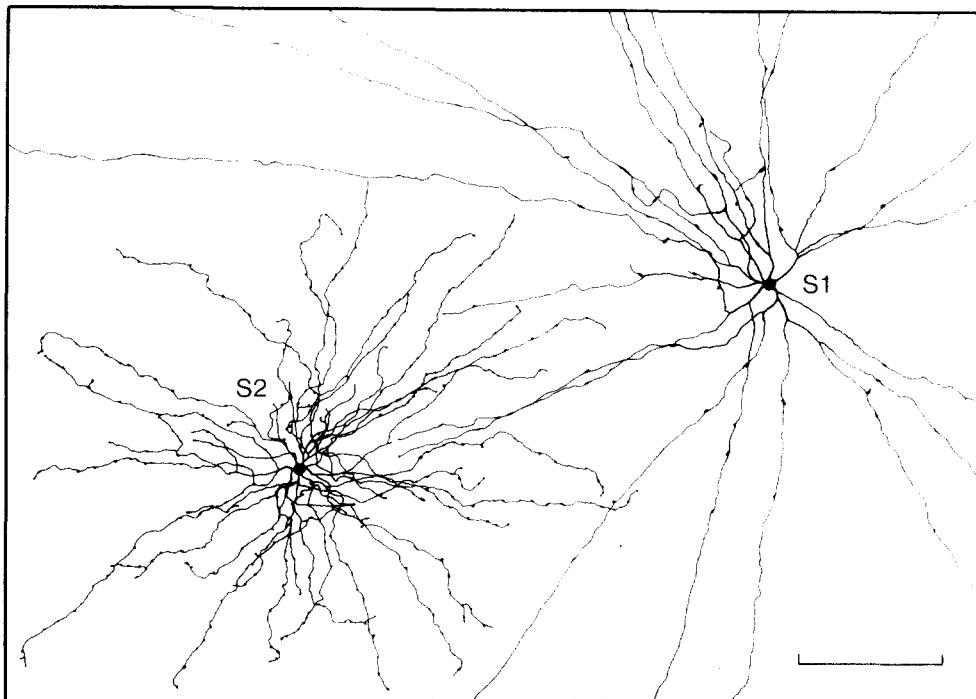


FIG. 12. Dendritic morphology of the two types of serotonin-accumulating amacrine cells in peripheral rabbit retina, as revealed by Lucifer injection of 5,7-DHT labelled cells in isolated living retina. The S1 and S2 amacrine cells can be readily distinguished by their branching pattern, varicosity distribution, and dendritic field size. Scale bar, 200  $\mu\text{m}$ . (From Vaney, 1986.)

overlapped by the dendritic trees of 30 to 60 S2 amacrine cells and 500 to 900 S1 amacrine cells. There are six to eight metres of S1 radial dendrites packed into each square millimetre of retina: if these fine processes were woven as a cloth, the spacing of the warp and weft would be only 0.3  $\mu\text{m}$  (Vaney, 1986a).

Electron microscopy of rabbit retina labelled with 5,6-DHT showed that the serotonin-accumulating amacrine cells mainly form reciprocal synapses with bipolar cell axons (Ehinger and Holmgren, 1979) that have the form and position of rod bipolar terminals (Raviola and Raviola, 1967; Dacheux and Raviola, 1986). This was confirmed directly by Raviola and Dacheux (1987) who filled two S1 amacrine cells with horseradish peroxidase after intracellularly recording their visual responses in an eyecup preparation; these On-centre cells gave a sustained depolarization to a diffuse white flash. Both freeze-fracturing and physiological recording indicated that the output synapses from S1 varicosities to rod bipolar

terminals are inhibitory. The large varicosities reciprocal to the rod bipolar terminals contain numerous synaptic vesicles (Raviola and Raviola, 1967; Ehinger and Holmgren, 1979), perhaps containing either serotonin in small, but functional quantities (see Brunken and Daw, 1988) or the proven store of endogenous GABA (Osborne and Beaton, 1986; Wässle and Chun, 1988; cf. O'Malley and Masland, 1988b).

### 3.2. Displaced and Misplaced Amacrine Cells

It is now accepted that the ganglion cell layer of mammalian retina contains a substantial population of interneurons that do not project into the optic nerve: these "displaced amacrine cells" account for about 35% of the ganglion cell layer neurons in rabbit retina (Vaney, 1980; Hughes and Vaney, 1980; Hayden *et al.*, 1980), 50% in rat retina (Perry, 1981) and 80% in cat retina (Hughes and Wieniawa-Narkiewicz, 1980;

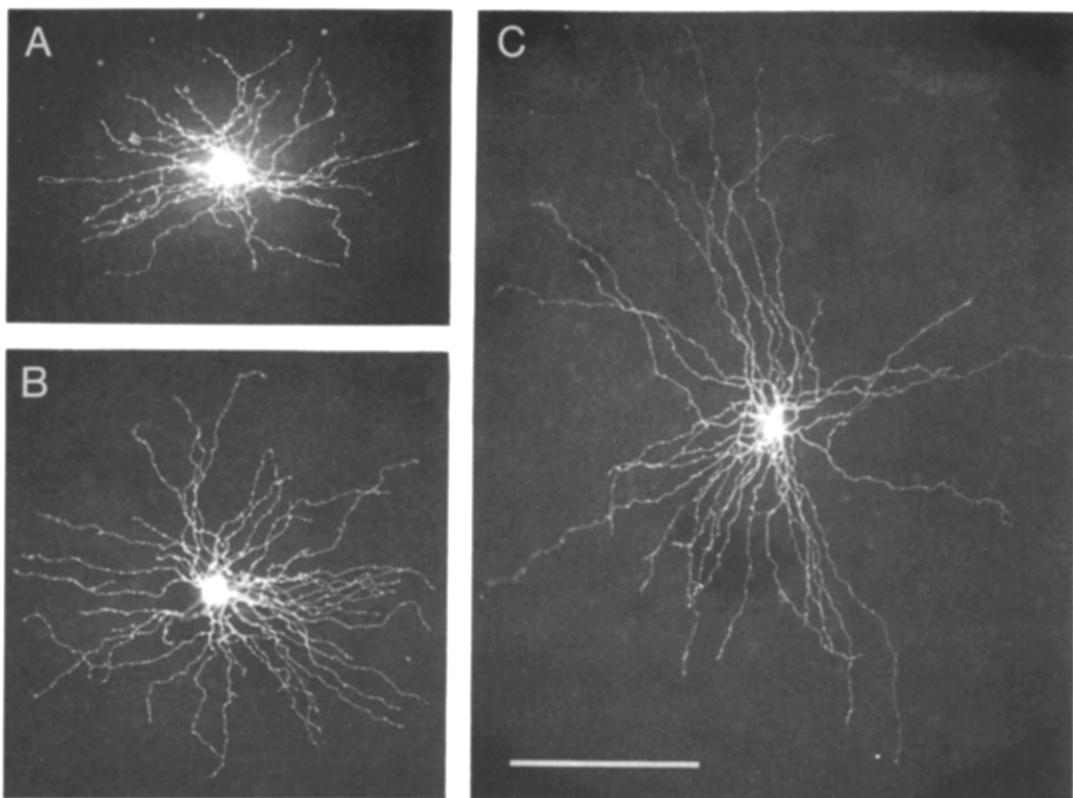


FIG. 13. Lucifer-filled S2 amacrines in rabbit retina located 1mm (A), 3mm (B), and 7mm (C) inferior to the peak visual streak, as photographed in the tissue chamber through a 10 $\times$  objective. Scale bar, 200  $\mu$ m. (D. I. Vaney, unpublished.)

Wong and Hughes, 1987a; Wässle *et al.*, 1987a). However, there is still no convincing rationale why the displaced amacrines are separated from the bulk of interneurons in the inner nuclear layer, although diverse hypotheses have been put forward (Vaney *et al.*, 1981; Famiglietti, 1983b; Hughes, 1985). The present characterization of all these cells as being *displaced* does not distinguish between those amacrines that are *misplaced* from the inner nuclear layer and those that are *normally-placed* in the ganglion cell layer (Wässle *et al.*, 1987a; Vaney *et al.*, 1989b). The former include ectopic representatives of many amacrine types in the retina (Oyster *et al.*, 1985; Vaney, 1985a); the latter include only a few types of interneurons, including the displaced cholinergic amacrines (Masland and Tauchi, 1986), the somatostatin-IR "association" neurons (Sagar, 1987b), and the substance P-IR amacrines in cat

retina (Pourcho and Goebel, 1988b; Vaney *et al.*, 1989b).

In rabbit retina, about 1% of the serotonin-accumulating amacrines are displaced to the ganglion cell layer (Ehinger and Florén, 1976; Vaney, 1986a; Sandell and Masland, 1986); the proportion of displaced cells peaks at the temporal end of the visual streak, where they account for 3 to 6% of the labelled amacrines. Lucifer yellow injection revealed that the displaced cells have either the S1 or S2 dendritic morphology. Sandell and Masland (1986) proposed, however, that each displaced population forms a distinct amacrine type (S1D and S2D) contrasting with the alternative view that these cells are simply misplaced from their normal position in the inner nuclear layer. The arguments for and against their proposal illuminate the criteria necessary for establishing a retinal amacrine type.

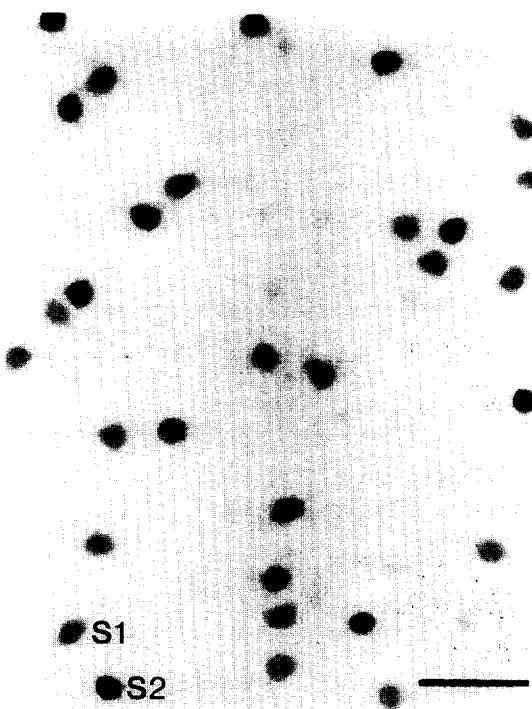


FIG. 14. Negative micrograph of the mosaic of S1 and S2 amacrines in superior rabbit retina, as revealed by their different cytotoxic responses to prolonged labelling with 5,7-DHT, an autofluorescent serotonin analog. The nuclei of the 13 S2 cells fluoresce more strongly than those of the 18 S1 cells, enabling the two populations to be discriminated objectively in unfixated retina. Scale bar, 50  $\mu\text{m}$ . (D. I. Vaney, unpublished.)

Sandell and Masland (1986) argued that the synaptic connectivity of the S1D and S2D amacrines must differ from their inner nuclear counterparts, because the displaced cells send their dendrites directly to stratum 5 and cannot contact processes in strata 1 to 4. However, Ehinger and Holmgren (1979) observed thousands of synapses between 5,6-DHT-labelled dendrites and (rod) bipolar terminals in the inner strata, but only tens of synapses between labelled and unlabelled amacrine dendrites in the outer strata. Moreover, the diffusely spread proximal branches of an S1 or S2 amacrine account for only a small portion of the completely stained dendritic tree. The dendritic field area of the S2 amacrines increases tenfold from the visual streak to peripheral retina, rising monotonically with decreasing cell density; consequently, the dendritic field overlap of the S2 amacrines varies little across the retina (Vaney, 1986a). At each eccentricity, the S2D amacrines have the same dendritic field size as the S2

amacrines, indicating that the displaced cells are not spatially independent (D. I. Vaney, unpublished). Moreover, the S2D amacrines are distributed irregularly, with large gaps in their dendritic coverage in peripheral retina. This is contrary to the expectation that each component of the retinal circuitry should be locally accessible if the processing of visual information is to be equivalent across the retina (Vaney *et al.*, 1988). Similar arguments hold for the S1D amacrines. Finally, the threefold range in the total number of displaced amacrines (Sandell and Masland, 1986) is much greater than the limited variability observed for the combined S1 and S2 populations or for other amacrine types that have been well characterized.

Many of the displaced S2 amacrines produce a sparse second arborization in the overlying nerve fibre layer; these processes are aligned with the fibre bundles and extend in opposite directions from the cell body, usually terminating beyond the

regular dendritic field (D. I. Vaney, unpublished). Displaced cells showing similar processes are consistently found among the neuropeptide-IR amacrinies of larval tiger salamander retina (Yang and Yazulla, 1986). Although such processes could contribute to a "superficial plexus" and thus serve a specific functional role (Hughes, 1985), they may simply result from the ectopic location of the cell body.

In rabbit retina, Sandell and Masland (1986) identified a third type of serotonin-accumulating neuron whose cell body is located at the outer margin of the inner nuclear layer and whose processes branch in both the inner and outer plexiform layers. The total number of type 3 cells is extraordinarily low (about 300 in New Zealand White rabbits) and varies at least fourfold from animal to animal (Sandell and Masland, 1986; Rizzo *et al.*, 1988). They are distributed very irregularly and may be largely absent from the visual streak and superior retina. In 5,7-DHT-labelled retina reacted with a serotonin antibody, each type 3 cell can be securely identified and its outer dendritic field fully mapped (D. I. Vaney, unpublished). In such material, it is apparent that many of the outer processes of these cells dive into the inner plexiform layer where they join the labelled amacrine dendrites in stratum 5. Figure 15 shows the cell body location and outer dendritic fields of all seven type 3 cells in an inferior segment of pigmented rabbit retina. The five complete fields (mean area =  $0.77 \pm 0.27 \text{ mm}^2$ ) and two partial fields totalled  $4.7 \text{ mm}^2$  in a retinal area of  $79 \text{ mm}^2$ , corresponding to a coverage factor of only 0.06: this is considerably less than the minimum 0.43 coverage calculated for type 3 cells in New Zealand White rabbits (Sandell and Masland, 1986). The spatial organization of the type 3 cells resembles that of the S1D and S2D amacrinies in many respects: it is concluded that these sparse populations are probably misplaced varieties of serotonin-accumulating amacrinies and, as such, do not constitute distinct neuronal types.

### 3.3. Cat Retina: Two or Three Types?

In cat retina, serotonin and related indoleamines are accumulated by a heterogeneous

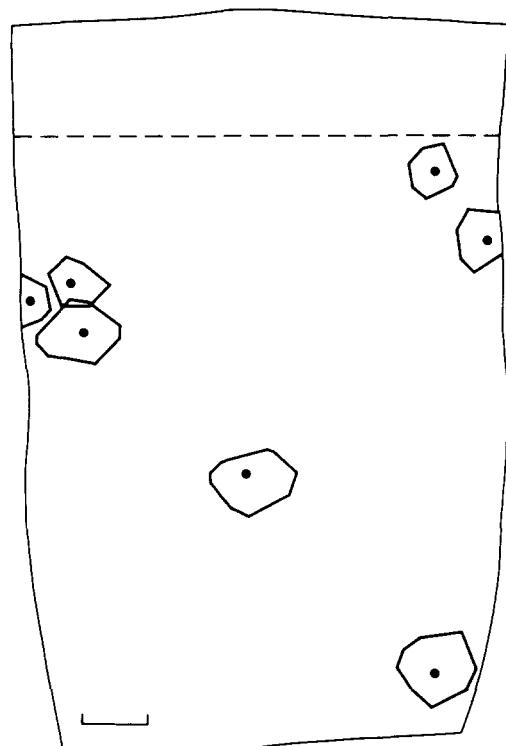


FIG. 15. Mapped location of the cell bodies and outer dendritic fields of all "type 3" serotonin-accumulating neurons in an inferior field of pigmented rabbit retina, as localized by 5,7-DHT uptake and serotonin immunohistochemistry; the visual streak is marked by the dashed line. Scale bar, 1 mm. (D. I. Vaney, unpublished.)

population of amacrine cells that differ in their labelling intensity, cell body size and laminar position (Ehinger and Florén, 1976; Holmgren-Taylor, 1982; Wässle *et al.*, 1987b; Wässle and Chun, 1988) (Fig. 16). Some 23% of the serotonin-accumulating neurons are located in the ganglion cell layer, accounting for 25–30% of the 730–850,000 displaced amacrinies in cat retina (Wässle *et al.*, 1987a). Allowing for some 6,000 serotonin-accumulating ganglion cells, this indicates a total population of between 800,000 and 1,100,000 labelled amacrinies. The lower figure would give a mean density of 1800 cells/ $\text{mm}^2$  in a normal-sized retina, comparable to the mid-peripheral densities measured by Wässle *et*

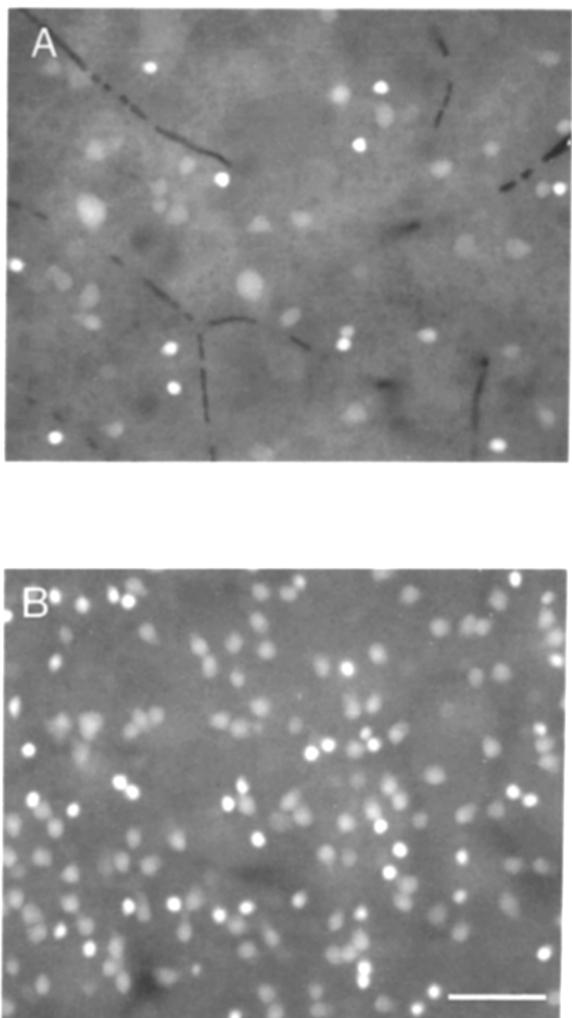


FIG. 16. Serotonin-accumulating neurons in the ganglion cell layer (A) and inner nuclear layer (B) of wholemounted cat retina; the cells were labelled by 5,7-DHT uptake *in vitro* and then visualized by formaldehyde-induced fluorescence. The small, brightly labelled cells correspond to the A20 amacrine of Fig. 17.

Scale bar, 50  $\mu\text{m}$ . (D. I. Vaney, unpublished.)

*et al.* (1987b). Estimates of the proportion of inner nuclear amacrine cells that accumulate serotonin range from 8.3% in wholemounted retina (Wässle *et al.*, 1987b) to 19% in horizontal thin sections (Fig. 3 of Wässle and Chun, 1988). This higher figure may include an amacrine population that was not labelled or counted in the earlier study (see below); alternatively, the density of inner

nuclear amacrines may be over-estimated in wholemount preparations.\*

Wässle *et al.* (1987b) reported that there are two distinct types of serotonin-accumulating amacrines in cat retina, which they characterized by injecting Lucifer yellow into 5,6-DHT-labelled neurons (Fig. 17). The smaller, brightly-labelled cells were identified as "sparsely-branched" amacrines; they give rise to 4 to 8 smooth thin dendrites which extend radially for about 2 mm in stratum 2 or 3, and whose branching level appears independent of cell body position. The medium-sized, moderately-labelled cells were identified as "spiny" amacrines; they have stout primary dendrites and an irregular dendritic tree of 600–800  $\mu\text{m}$  diameter, which may give rise to a few processes that extend for 500  $\mu\text{m}$  or more. The conventionally-located and the displaced "spiny" amacrines branch in outer and inner strata respectively, forming mirror-image populations like the cholinergic amacrines. The "sparsely-branched" type probably corresponds to the A20 amacrine of Kolb *et al.* (1981), while the matching populations of "spiny" amacrines may correspond to Kolb's A19 and A22 amacrines (Wässle *et al.*, 1987b). The "sparsely-branched" type accounted for about 55% of the serotonin-accumulating amacrines in labelled wholemounts.

Vaney (1986c) briefly noted that 5,7-DHT uptake selectively labels several types of amacrine cells and one type of ganglion cell in cat retina, including the "sparsely-branched" amacrines characterized by Wässle *et al.* (1987b). In

\*The total number of cells in the amacrine sublayer of cat retina can be calculated from three different studies, all from Wässle's laboratory. From a wholemount, Schmidt *et al.* (1985) estimated that there were 54 presumptive amacrines for each ChAT-IR cell in mid-peripheral nasal retina; given a ChAT-IR population of 137,000 cells (D. I. Vaney and G. E. Whitington, unpublished), this indicates a total of 7,400,000 inner nuclear amacrines. From horizontal sections, Wässle *et al.* (1986) estimated that there were 15.5 presumptive amacrines for each horizontal cell in mid-peripheral retina; given a combined horizontal population of 282,000 cells (Wässle *et al.*, 1978), this indicates a total of 4,400,000 inner nuclear amacrines. From horizontal sections, Wässle *et al.* (1987a) estimated that there were 6.1 presumptive amacrines for each displaced amacrine at 5 mm eccentricity; given a displaced amacrine population of 850,000 (Wässle *et al.*, 1987a), this indicates a total of 5,200,000 inner nuclear amacrines. Adding the displaced amacrines gives respective grand totals of 8,200,000 or 5,200,000 or 6,100,000 amacrine cells, with the actual number likely to be closer to the lower figures.

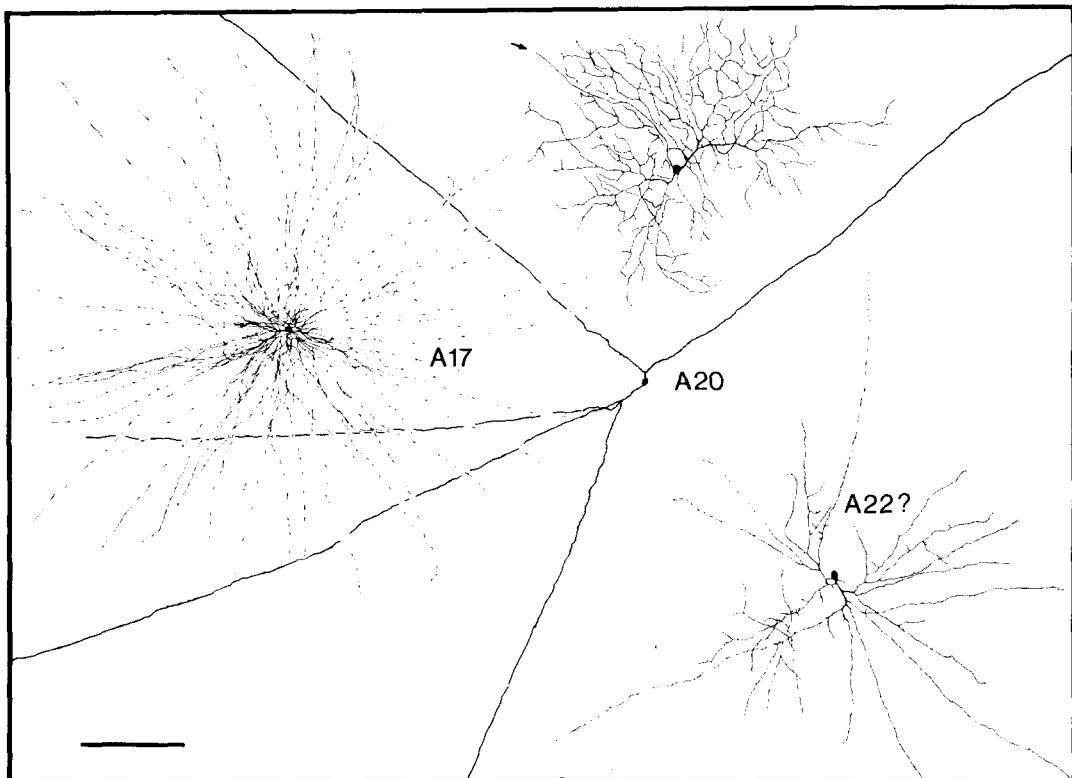


FIG. 17. Dendritic morphology of four types of serotonin-accumulating neurons in cat retina, as revealed by Lucifer injection of 5,6-DHT labelled cells in fixed retina; the three amacrine cells (A17, A20, A22?) and the ganglion cell (arrowed axon) were from mid-peripheral retina and their cell bodies were located in the ganglion cell layer. Scale bar, 200  $\mu$ m.  
(Refigured from Wässle *et al.*, 1987a,b.)

addition, Vaney reported that another type of labelled amacrine had a wide-field radiate morphology characteristic of the A17 amacrine (Kolb *et al.*, 1981; Nelson and Kolb, 1985). Moderately fluorescent cells with this morphology were injected in both the inner nuclear and ganglion cell layers; the displaced variety corresponds to single amacrices illustrated by Boycott and Wässle (1974), Pourcho and Goebel (1983) and Wässle *et al.* (1987a) (Fig. 17), but appears to have been erroneously classified as a ganglion cell (G13) by Kolb *et al.* (1981). Wässle *et al.* (1987b) initially reported that A17 amacrices only showed 5,6-DHT-uptake at concentrations which caused non-specific staining of a variety of neurons, but subsequently subsumed the A17 cells as a third distinct type of serotonin-accumulating amacrine (Wässle and Chun, 1988).

The main synaptic contacts of serotonin-

accumulating processes in cat retina are reciprocal synapses with rod bipolar terminals, accounting for 75% of such connections (Holmgren-Taylor, 1982). Although Wässle *et al.* (1987b) proposed that the displaced "spiny" amacrices provide the substrate for this interaction, the A17 amacrices seem the obvious choice given that they are serotonin-accumulating (Vaney, 1986c). Electron-microscopy of HRP-filled A17 amacrices showed that a labelled varicosity is typically paired with an unlabelled AII dendrite postsynaptic to a rod bipolar dyad (Nelson and Kolb, 1985), identical to the arrangement described for 5,6-DHT-labelled terminals (Holmgren-Taylor, 1982).

The A17 amacrices of cat retina appear homologous to both the S1 and S2 amacrices of rabbit retina with respect to their general morphology, synaptic connectivity, transmitter content and uptake mechanisms. The A17

amacrines more closely resemble the S2 amacrines, however, in that their beaded varicosities are regularly spaced along the full length of their dendrites, whereas the leaf-like varicosities of the S1 amacrines are confined to the proximal dendritic zone (Vaney, 1986a). The A17 amacrines spread more diffusely than the narrowly stratified S1 and S2 amacrines, but this reflects structural differences in the presynaptic, rod bipolar terminals. In cat retina, the rod bipolar axons are typically unbranched and their ribbon synapses are distributed throughout sublamina *b* (Kolb and Famiglietti, 1974; Kolb, 1979; McGuire *et al.*, 1984). In rabbit retina, the rod bipolar axons branch close to stratum 5, giving rise to a few large terminal expansions that cover a field of 10 to 30  $\mu\text{m}$  diameter (Raviola and Raviola, 1967; Dacheux and Raviola, 1986; H. M. Young, unpublished).

#### 3.4. Dendritic Overlap and Spatial Regularity

In rabbit retina, the topographic distribution of the S2 amacrines is comparable to that of the type *a* cholinergic (*Ca*) amacrines: both of these inner nuclear populations have similar maximum densities (500 and 700 cells/ $\text{mm}^2$ , respectively), with each point on the visual streak overlapped by the dendritic fields of 30 S2 amacrines and 30 *Ca* amacrines. However, this overlap may serve quite different functions in the two systems (cf. Masland, 1988), because the spatial regularity of the *Ca* amacrines is significantly greater than that of the S2 amacrines. This is evident at several levels. Firstly, a measure of the regularity of each cell array is provided by the ratio of the mean nearest-neighbour distance to the standard deviation: the higher the ratio, the more regular the array (Wässle and Riemann, 1978). The regularity index of the *Ca* amacrines in peripheral rabbit retina is 5.9 (Vaney *et al.*, 1981), compared with an index of only 2.8 for the S2 amacrines (Sandell and Masland, 1986). Secondly, neighbouring cholinergic amacrines show negligible variation in the size and placement of their dendritic fields: the regularity of the cell arrays accurately reflects the regularity of the dendritic arrays. By contrast, the S2 amacrines show significant variation in the size and shape of

their dendritic fields at each retinal eccentricity, although there is a generalized trade-off between dendritic field size and cell density (Vaney, 1986a). Thus, the S2 dendrites appear to simply blanket the retina, whereas the cholinergic plexus has a microstructure defined by the spatial relationships of neighbouring amacrine cells and their postsynaptic ganglion cells (see Section 9).

There are about 40,000 rod bipolars/ $\text{mm}^2$  in central cat retina, each producing about 30 ribbon synapses, some 75% of which are presynaptic to serotonin-accumulating varicosities (Freed *et al.*, 1987; McGuire *et al.*, 1984; Holmgren-Taylor, 1982). Therefore, the number of A17 varicosities in each square millimetre of retina may total 900,000; this would require an A17 density of at least 900 cells/ $\text{mm}^2$  (cf. Vaney, 1985a), given that each A17 amacrine contains up to 1000 varicosities and has a dendritic field diameter of 650  $\mu\text{m}$  in the area centralis (Nelson and Kolb, 1985). The calculated 300-fold overlap of A17 dendritic fields is thus comparable to the 500 to 900-fold overlap reported for rabbit S1 amacrines (Vaney, 1986a). The dense dendritic overlap of the "reciprocal rod amacrines" (A17, S1 and S2 cells) not only provides the morphological substrate for local feedback at each rod bipolar dyad, but also enables this interaction to be modified by the activity of surrounding rod bipolars. The varicosity distribution within the A17 and S2 dendritic fields ensures that the multiple varicosities postsynaptic to each bipolar arise from different amacrine cells. Thus, focal stimulation of the retina would produce generalized activation in a local region of the A17 plexus, the spread of which appears to be blocked by activity elsewhere in the plexus (Nelson and Kolb, 1985) and perhaps limited by the geometry of the varicosities (Elias and Stevens, 1980).

#### 4. GLYCINERGIC AMACRINE CELLS

The great majority of retinal amacrine cells contain either glycine or GABA, two structurally-related inhibitory transmitters. Although there is general agreement that 40 to 50% of the amacrines in mammalian retina accumulate or contain

glycine, it is not clear how many distinct amacrine types they comprise. Unlike GABA, which coexists with a range of classical transmitters and neuropeptides in diverse amacrine types, glycine has not been colocalized with other neuroactive substances in mammalian retina (cf. Weiler and Ball, 1984; Watt *et al.*, 1985; Yazulla and Yang, 1988). However, the AII amacrines in cat retina, which are the best characterized of the glycinergic amacrines (Famiglietti and Kolb, 1975; Pourcho, 1980), selectively accumulate nuclear stains such as DAPI, thus enabling their topographic distribution and dendritic coverage patterns to be quantitatively defined (Vaney, 1985a). This section examines whether the AII amacrines are representative of glycinergic amacrines in general, by comparison with a second population of narrow-field amacrines. Other aspects of glycinergic function in mammalian retina have been reviewed by Marc (1989).

#### 4.1. Localization of Accumulated and Endogenous Glycine

$[^3\text{H}]$ glycine is accumulated by select types of amacrine cells and cone bipolar cells in rabbit, cat and other mammals (Ehinger and Falck, 1971; Bruun and Ehinger, 1974) (Fig. 18). The labelled amacrines in cat retina account for 43–46% of the cells in the amacrine sublayer, but do not appear to include those amacrines with larger cell bodies (Pourcho, 1980; Wässle *et al.*, 1986) (Fig. 19): the presumptive glycinergic amacrines thus total about 2,000,000 cells at minimum (see footnote on p. 66). Several types of glycine-accumulating amacrines can be distinguished in thin sections, including a moderately-labelled cell with the characteristic cytology of an AII amacrine: their cell bodies protrude into the inner plexiform layer and give rise to a stout process that descends to sublamina *b* (Famiglietti and Kolb, 1975). In Golgi-stained cat retina, Pourcho and Goebel (1985) identified four types of glycine-accumulating amacrines which correspond respectively to the A3, A4, A7 (AII) and A8 cells of Kolb *et al.* (1981). All four types are narrow-field amacrines: the A3 and A4 amacrines are broadly stratified in strata 2 and 3, whereas the

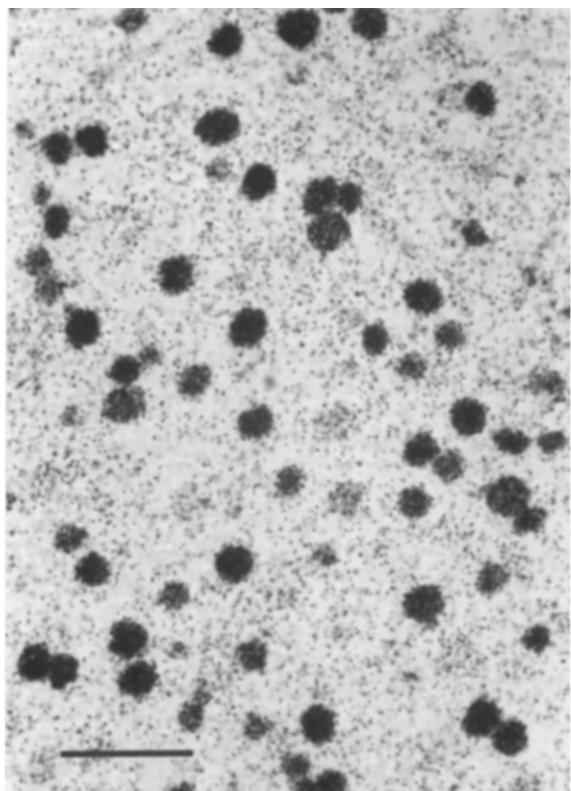


FIG. 18. Glycine-accumulating cells in rabbit retina. Autoradiograph of a semi-thin horizontal section through the amacrine sublayer of a retina that was labelled by a 10 min incubation in 4  $\mu\text{M}$   $[^3\text{H}]$ glycine in tissue culture medium. The grain densities over labelled cells are highly variable within local regions, indicating that several types of amacrine cells accumulate exogenous glycine. Scale bar, 25  $\mu\text{m}$ . (H. M. Young, unpublished.)

AII and A8 types are bilaminar amacrines, producing distinct arborizations in sublaminae *a* and *b* (Fig. 20). The A3 and A4 cells may represent a single type of amacrine (Marc, 1989).

Immunohistochemistry shows that the cellular distribution of endogenous glycine parallels the pattern of exogenous uptake in both cat and monkey retinae (Pourcho and Goebel, 1987; Hendrickson *et al.*, 1988) (Fig. 21). Glycine-IR cells account for about 50% of the presumptive amacrines in cat retina and 40–46% in monkey retina, with many of the glycinergic amacrines in primates showing cytological features that are characteristic of the AII amacrines (Marc and Liu, 1985; Hendrickson *et al.*, 1988). In cat retina,

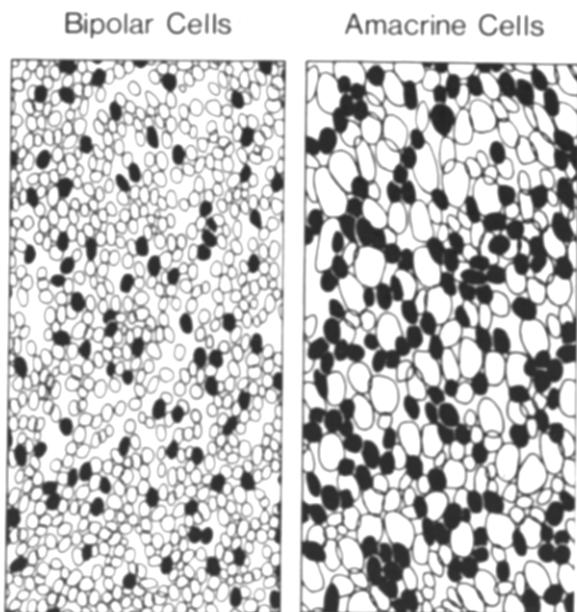


FIG. 19. Glycine-accumulating cells in cat retina. Autoradiographic reconstruction of [<sup>3</sup>H]glycine-accumulating cells (filled profiles) and unlabelled cells (empty profiles) in the bipolar and amacrine sublayers of the inner plexiform layer. Retinal field, 100 µm × 200 µm. (From Wässle *et al.*, 1986.)

both the accumulated and endogenous glycine is concentrated in strata 1 to 3 of the plexiform layer, as are the glycine receptors (Pourcho, 1980; Bolz *et al.*, 1985; Pourcho and Goebel, 1987; Jäger and Wässle, 1987). This labelling pattern probably reflects the superposition of both the A3/A4 dendrites in strata 2–3 and the AII lobular dendrites in strata 1–2, with the A8 amacrices contributing to the weaker labelling in strata 4–5.

#### 4.2. AII Amacrices in Cat and Rabbit

There is a distinctive type of narrow-field bilaminar amacrine cell that has been described in a wide variety of Golgi-stained mammalian retinae including dog, monkey, rat, rabbit and wallaby (Cajal, 1893; Boycott and Dowling, 1969; Perry and Walker, 1980; Dacheux and Raviola, 1986; Wong *et al.*, 1986). In cat retina, these AII amacrine cells have been studied in particular detail (Kolb and Famiglietti, 1974) (Fig. 20), with

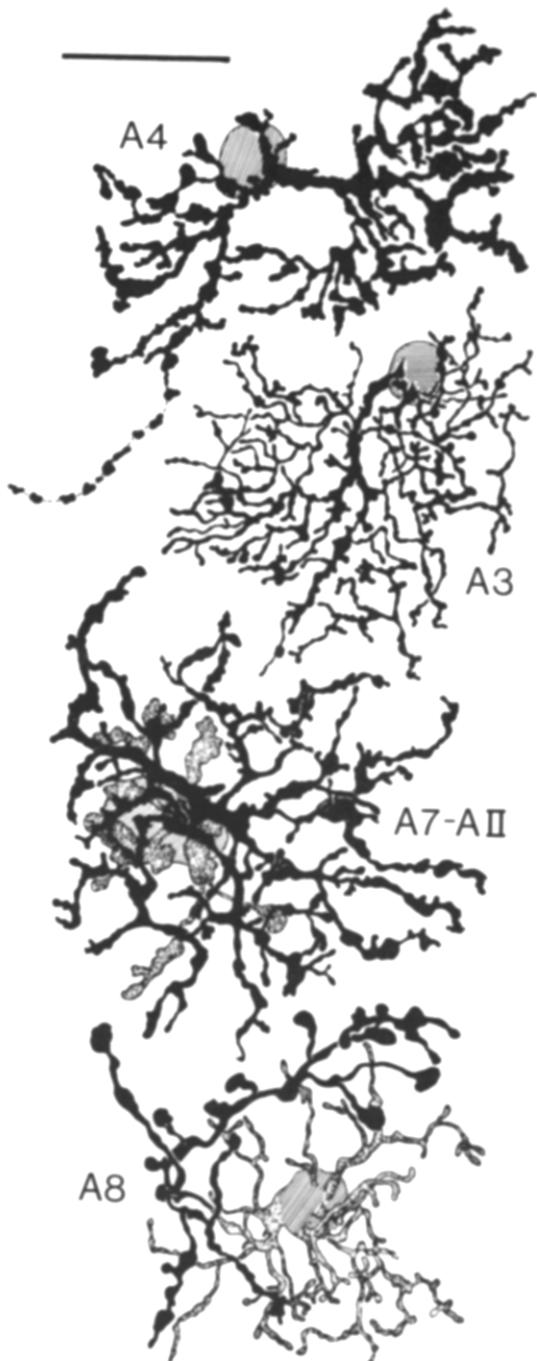


FIG. 20. Drawings of four types of Golgi-impregnated amacrine cells in cat retina that accumulate [<sup>3</sup>H]glycine. All are narrow-field amacrines with broadly stratified (A3,A4) or bilaminar dendritic trees (A7,A8); the sublamina  $\alpha$  dendrites of the A7 (AII) and A8 amacrines are indicated with stippling. Scale bar, 20 µm. (Refigured from Pourcho and Goebel, 1985.)

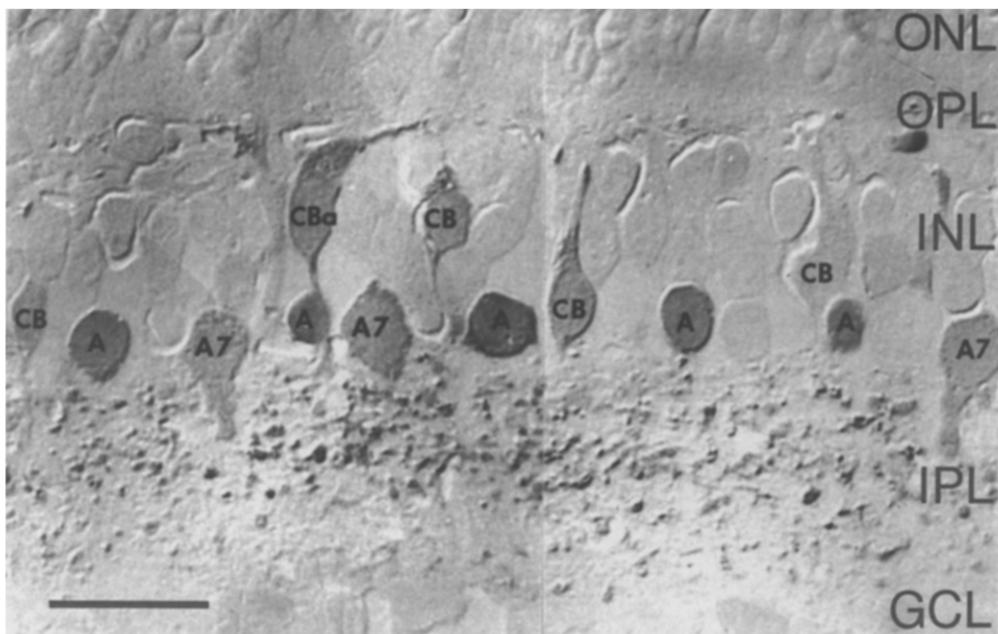


FIG. 21. Immunohistochemical localization of endogenous glycine in a semi-thin vertical section of cat retina, as viewed with Nomarski optics. The glycine-immunoreactive cells in the inner nuclear layer include the moderately-labelled AII (A7) amacrices, other more reactive amacrices (A), and some cone bipolar cells (CB). Scale bar, 20  $\mu\text{m}$ . (From Pourcho and Goebel, 1987.)

emphasis placed on their synaptic connections (Famiglietti and Kolb, 1975; Kolb, 1979; Sterling, 1983; Sterling *et al.*, 1988), physiological responses (Nelson *et al.*, 1976; Nelson, 1982), pharmacological interactions (Pourcho, 1980, 1982; Pourcho and Goebel, 1985; Voigt and Wässle, 1987), and topographic distribution (Vaney, 1985a). They are arguably the best characterized amacrine type in any retina. The bipolar inputs to AII amacrices are segregated, with cone bipolars synapsing on the lobular dendrites in sublamina *a* and rod bipolars synapsing on the arboreal dendrites in sublamina *b* (Famiglietti and Kolb, 1975). Each AII amacrine in the area centralis receives multiple synapses from about 30 rod bipolars (Sterling *et al.*, 1988) and recordings from the cell body indicate that rod-mediated input accounts for 95% of the peak response (Nelson, 1982). Rod bipolars do not contact ganglion cells directly and, therefore, the AII amacrices are third-order neurons in the rod-signal pathway (Sterling, 1983; Kolb and Nelson, 1984).

Cat AII amacrine cells can be selectively labelled *in vitro* with fluorescent nuclear stains such as DAPI, identified in an isolated living preparation, and then injected with Lucifer yellow under visual control (Vaney, 1985a) (Fig. 22). There are some 512,000 AII amacrices in cat retina and their density ranges from 500 cells/ $\text{mm}^2$  in superior periphery to 5,300 cells/ $\text{mm}^2$  in the area centralis; they thus account for less than 10% of the total population of presumptive amacrices, and less than 25% of the glycinergic amacrices (cf. Pourcho, 1980; Cohen and Sterling, 1986). The AII topographic distribution appears kite-shaped at intermediate densities and the visual streak is not strongly developed (Fig. 40). The array of AII amacrine cells has a regularity index of about 5.2 in peripheral retina, identical to the spatial regularity of either the On- or Off-beta ganglion cells in cat retina (Wässle *et al.*, 1981). Occasional AII cells are misplaced to the ganglion cell layer, occupying "holes" in the array of inner nuclear cells. The overlap of the lobular dendritic fields is close to 1 at all eccentricities, whereas the overlap

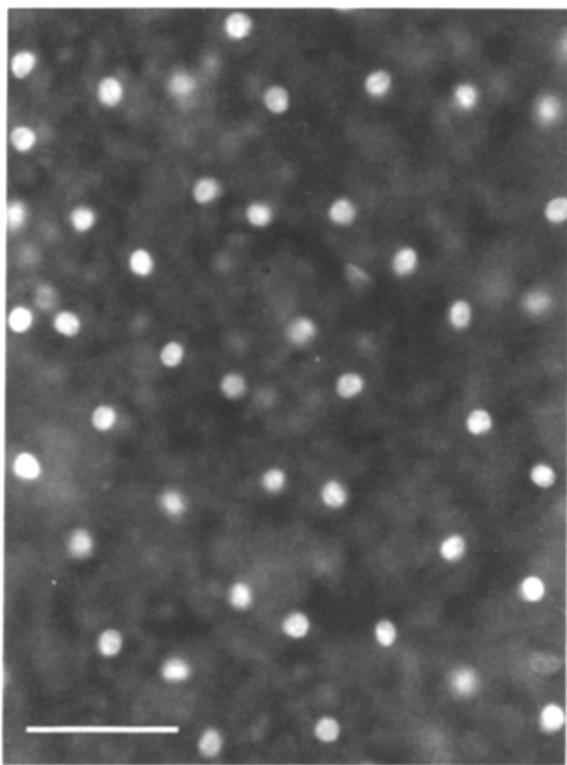


FIG. 22. The array of AII amacrine cells in cat retina, selectively labelled *in vitro* by incubation of the eyecup in the fluorescent nuclear stain, diamidino-phenylindole (DAPI). In peripheral retina, most of the AII cell bodies protrude into the inner plexiform layer and, consequently, their focal plane is above that of the other amacrine cells in the inner nuclear layer. Scale bar, 50  $\mu\text{m}$ . (From Vaney, 1985.)

of the arboreal dendritic fields doubles from 2 to 4 within the first few millimetres and then remains constant throughout the periphery (Vaney, 1985a). This coverage is similar to the 3- to 4-fold dendritic field overlap of A- or B-type horizontal cells and to the 2- to 3-fold overlap of On- or Off-beta ganglion cells, all of which are integrating retinal neurons (Wässle *et al.*, 1978, 1981).

Rabbit AII amacrine cells can be selectively labelled *in vivo* by injecting Nuclear yellow into the posterior chamber two days before enucleation and then incubating the isolated eyecup in tissue culture medium for several hours; the stained retina is strongly metachromatic, with the AII amacrines fluorescing yellow and other inner nuclear amacrines fluorescing blue (D.I. Vaney, unpublished). At each eccentricity, the density of

AII amacrines is about 3.5x that of the overlying *Cb* amacrines, indicating a total population of 525,000 AII amacrines, or some 11% of the presumptive amacrine cells in rabbit retina. The cell array of AII amacrines is less regular than in cat, but their dendritic fields are positioned so as to achieve complete and uniform coverage of the retina (Fig. 23). In the mid-periphery, the dendritic field overlap of the lobular and arboreal dendrites is 1.0 and 2.4 respectively, comparable to the overlap of cat AII amacrines at 1 mm eccentricity. Thus, the AII amacrine cells in cat and rabbit retinae are homologous with respect to their total number, dendritic morphology, spatial coverage, synaptic connections, and physiological responses (Dacheux and Raviola, 1986).

#### 4.3. A New Type of Glycinergic Amacrine

In rabbit retina, DAPI selectively labels the displaced *Cb* amacrines and three types of inner nuclear amacrines, including the matching population of *Ca* amacrines (Masland *et al.*, 1984a; Tauchi and Masland, 1984; Vaney, 1984). The DAPI-labelled cells in the inner nuclear layer outnumber the *Cb* amacrines by 100 to 63 in both central and peripheral retina (Vaney and Young, 1988a), consistent with the finding that only two-thirds of the DAPI-labelled cells in this layer have the cholinergic "starburst" morphology (Vaney, 1984). Most of the additional cells comprise a distinct type of small-field amacrine with a convoluted bilaminar morphology, here termed the "DAPI-3" amacrine. The Lucifer-filled DAPI-3 cell shown in Fig. 24 was located 1.5 mm inferior of the peak visual streak (*Cb* density = 600 cells/mm<sup>2</sup>) and had a dendritic field diameter of 105  $\mu\text{m}$ ; an adjacent *Cb* amacrine was also filled and its dendritic field area was 7.5x that of the DAPI-3 amacrine. It follows therefore that DAPI-3 amacrines have a 2- to 3-fold dendritic field overlap in central rabbit retina (D. I. Vaney, unpublished). This amacrine type has no obvious counterpart in published Golgi catalogues, but is not dissimilar to the A13 amacrine of cat retina (Kolb *et al.*, 1981).

[<sup>3</sup>H]glycine is accumulated *in vitro* by about one-third of the DAPI-labelled cells in the inner

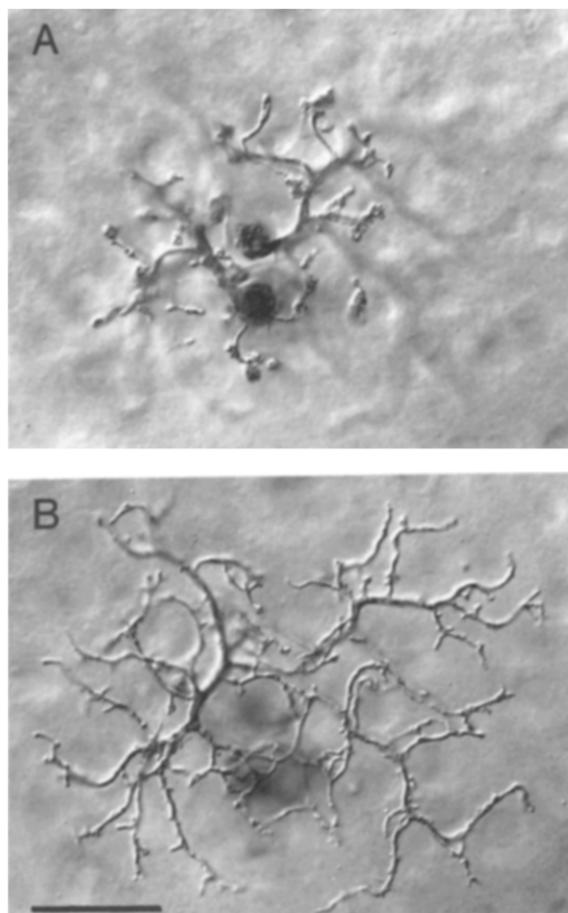


FIG. 23. A pair of Lucifer-filled AII amacrines in rabbit retina, as viewed with Nomarski optics following photoconversion of the dye to an opaque reaction product. AII amacrine cells have a distinctive bilaminar morphology, with lobular appendages in sublamina *a* (A) and arboreal dendrites in sublamina *b* (B). Although the two cell bodies are more closely spaced than would be expected from the local density of AII amacrines, their dendritic fields are positioned asymmetrically in both sublaminae *a* and *b*, thus providing uniform coverage of the retinal surface. Scale bar, 20  $\mu$ m. (D. I. Vaney, unpublished.)

nuclear layer, but none of the DAPI-labelled *Cb* amacrines in the ganglion cell layer show [ $^3$ H]glycine uptake (H. M. Young, unpublished). Conversely, all of the *Cb* amacrines and about two-thirds of the DAPI-labelled inner nuclear amacrines show strong GABA-like immunoreactivity (Vaney and Young, 1988a; cf. Brecha *et al.*, 1988). It is concluded that the DAPI-labelled,

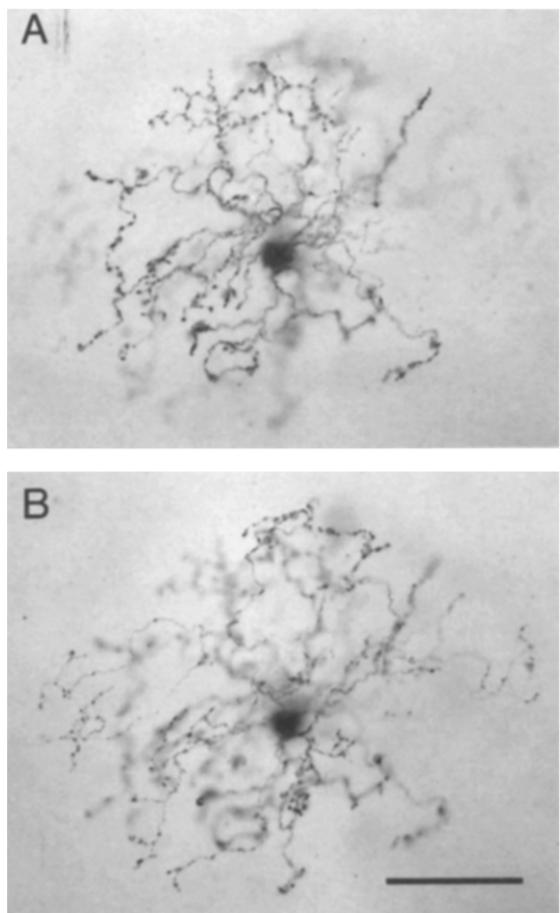


FIG. 24. A photoconverted, Lucifer-filled "DAPI-3" amacrine in central rabbit retina; these cells have a convoluted bilaminar morphology, with most of their thin dendrites narrowly stratified in sublamina *a* (A) or sublamina *b* (B). Scale bar, 50  $\mu$ m. (D. I. Vaney, unpublished.)

[ $^3$ H]glycine-accumulating cells should correspond to the DAPI-3 amacrines. They resemble the glycinergic AII cells in that both are narrow-field amacrines with a bilaminar morphology and a low dendritic field overlap. However, the AII amacrines account for about 11% of the presumptive amacrines in rabbit retina whereas the DAPI-3 amacrines account for less than 2%. Marc (1989) proposed that the 40 to 50% of amacrine cells which are glycinergic might parsimoniously comprise only four or five cell types, each with the population characteristics of AII amacrines. If most types of glycinergic

amacrines were present at the lower frequency of DAPI-3 amacrines, however, many additional types could be accommodated.

## 5. GABAERGIC AMACRINE CELLS

### 5.1. Morphological and Neurochemical Diversity

It outwardly appears that the subdivision of GABAergic amacrine cells into their component types is comparatively well advanced. Distinct populations of GABA-IR amacrines have been identified in mammalian retinae that either accumulate serotonin (Osborne and Beaton, 1986; Wässle and Chun, 1988); show NADPH-diaphorase activity (Müller *et al.*, 1988; Vaney and Young, 1988b), or contain dopamine (Kosaka *et al.*, 1987; Versaux-Botteri *et al.*, 1987; Wässle and Chun, 1988), acetylcholine (Vaney and Young, 1988a; Kosaka *et al.*, 1988; Brecha *et al.*, 1988), or substance P (Pourcho and Goebel, 1988a; Vaney *et al.*, 1989b). However, the homologous populations in rabbit retina account for only 12% of the presumptive amacrines, compared to 40% for all GABA-IR cells (calculated from Mosinger and Yazulla, 1987). Moreover, most of these characterized types are present at low density, some comprising only one amacrine in a thousand (Vaney and Young, 1988b): the unassigned GABA-IR cells could thus accommodate many additional types of amacrines.

Identified GABA-IR amacrines in mammalian retina are typically medium- or wide-field neurons, usually with densely overlapping, stratified dendritic trees; by contrast, the glycinergic amacrines have diffusely branched, narrow-field morphologies. This functional dichotomy does not appear to be rigid however. The A2, A10 and A13 amacrines of cat retina accumulate the GABA agonist [<sup>3</sup>H]muscimol, but are narrow- or small-field neurons with broadly stratified or bilaminar dendritic trees (Pourcho and Goebel, 1983); moreover, the narrow-field A2 amacrine does not accumulate [<sup>3</sup>H]glycine above background levels (Pourcho and Goebel, 1985). A small proportion of amacrine cells in tiger salamander retina show both GABA and glycine

immunoreactivities (Yazulla and Yang, 1988), and such colocalization should not be discounted in mammalian retina.

In cat retina, 30 to 40% of the cells in the amacrine sublayer are labelled with GABAergic markers (Pourcho, 1980, 1981; Mosinger *et al.*, 1986; Wässle *et al.*, 1987b; Wässle and Chun, 1988) and another 43 to 50% are labelled with glycinergic markers (Marc, 1989). These two inhibitory transmitters thus account for 73 to 90% of the presumptive amacrine cells in cat retina. Many of the unlabelled neurons in the amacrine sublayer may be bipolar cells and, thus, it is conceivable that all amacrine cells in mammalian retina contain elevated levels of either glycine or GABA. The following section provides an overview of the distribution and colocalization of various GABAergic markers in rabbit retina. The cellular distribution and dendritic organization of identified types of putative GABAergic amacrines are documented elsewhere in this review, while other aspects of GABAergic function in the vertebrate retina have been reviewed by Yazulla (1986).

### 5.2. Localization of GABAergic Markers

Rabbit retinae show a consistent pattern of GABA-like immunoreactivity in transverse section (Agardh *et al.*, 1986; Mosinger *et al.*, 1986; Osborne *et al.*, 1986; Mosinger and Yazulla, 1987). About 38% of the cells in the amacrine sublayer are labelled and their processes blanket the inner plexiform layer, with greater density in strata 1, 4 and 5 (Fig. 25). About one-sixth as many cells are labelled in the ganglion cell layer: the majority are displaced amacrines but they also include some ganglion cells (Vaney and Young, 1988a; Yu *et al.*, 1988; Brecha *et al.*, 1988). In addition, occasional GABA-IR interplexiform cells branch in the outer plexiform layer (Mosinger *et al.*, 1986), and other labelled processes descend to the optic fibre layer where they divide repeatedly (Vaney and Hughes, 1989). These varicose processes are thus distinct from the optic axons of GABA-IR ganglion cells and may be amacrine dendrites contributing to the superficial plexus (Hughes, 1985). The great density of

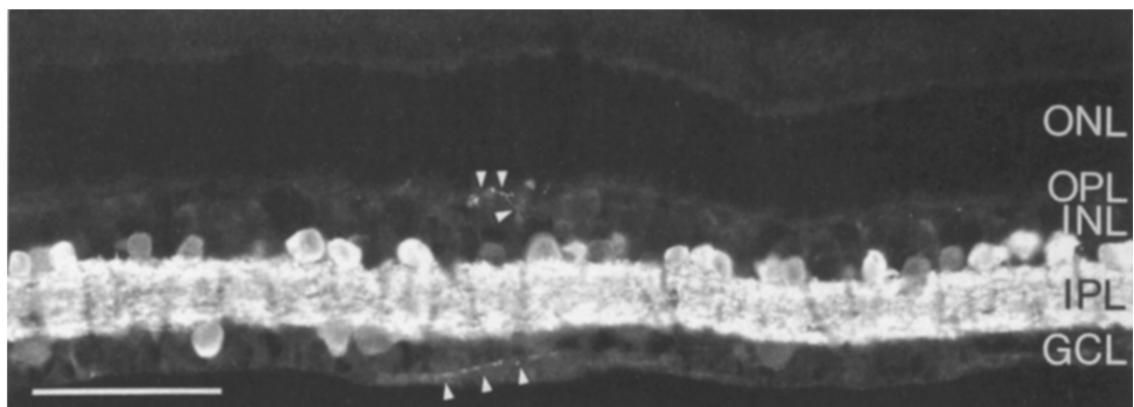


FIG. 25. GABA-like immunoreactivity in a frozen transverse section of rabbit retina. Labelled cells are located at the proximal border of the inner nuclear layer and in the ganglion cell layer; their processes blanket the inner plexiform layer, with greater density in the proximal and distal thirds. In addition, occasional interplexiform processes are GABA-immunoreactive, as are some superficial processes in the nerve fibre layer (arrowheads). Scale bar, 50 µm. (H. M. Young and D. I. Vaney, unpublished.)

labelled amacrices is apparent in a horizontal section through the inner nuclear layer (Fig. 26): it is estimated that rabbit retina contains almost 2,000,000 GABA-IR interneurons, comprising both amacrine cells and a small proportion of interplexiform cells (Nakamura *et al.*, 1980).

In rabbit retina, the cellular distribution of the GABA-synthesizing enzyme (GAD) parallels that of the transmitter; GAD immunohistochemistry does reveal, however, the presence of four labelled strata within the inner plexiform layer that are not apparent with GABA immunohistochemistry (Brandon *et al.*, 1979; Brandon, 1985; Mosinger and Yazulla, 1985). Their banding pattern probably reflects the different stratification levels of distinct types of GABAergic amacrices (cf. Famiglietti and Vaughn, 1981), but the correspondence is not straightforward. Although both the *Ca* and *Cb* cells reportedly show GAD-like immunoreactivity (Brecha *et al.*, 1988), each cholinergic plexus in rabbit retina overlaps a GAD-deficient stratum.

A colocalization study in rabbit retina found that essentially all GAD-IR cells are GABA-positive, but that 21% of the GABA-IR cells are GAD-negative (Mosinger and Yazulla, 1987). A disproportionate number of these neurons were located in the ganglion cell layer and are presumably GABA-IR ganglion cells, because the

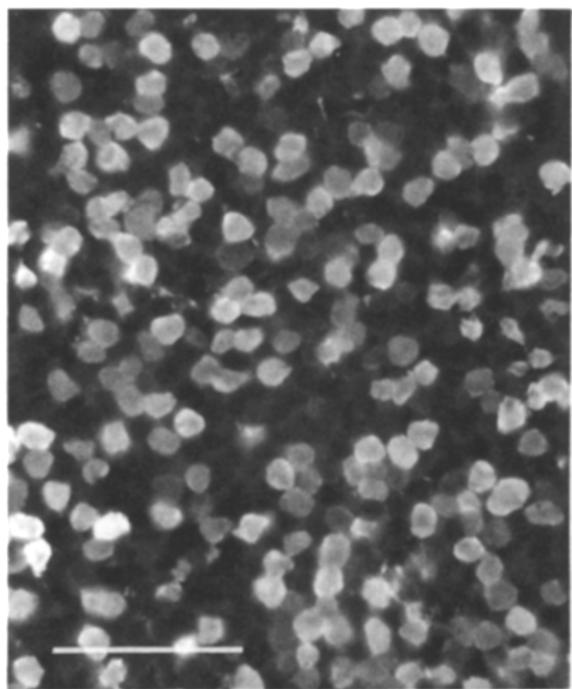


FIG. 26. GABA-like immunoreactivity in a frozen horizontal section through the amacrine sublayer of rabbit retina. Scale bar, 50 µm. (H. M. Young and D. I. Vaney, unpublished.)

GAD-positive *Cb* cells account for most of the GABA-IR displaced amacrine (Vaney and Young, 1988a; Brecha *et al.*, 1988). An earlier study found that 14% of the GAD-IR cells in the amacrine sublayer of rabbit retina do not accumulate [<sup>3</sup>H]GABA (Mosinger and Yazulla, 1985). A similar proportion of the GABA-IR amacrine in monkey and cat retinae are not labelled following [<sup>3</sup>H]muscimol uptake: the GABA-positive/muscimol-negative cells in cat retina include the large dopaminergic amacrine, compatible with the finding in monkey retina that these single-labelled cells are consistently larger than the double-labelled cells (Hendrickson *et al.*, 1985; Wässle and Chun, 1988). It is thus possible that only those GABA-IR amacrine that both synthesize and accumulate GABA are functionally GABAergic.

## 6. PEPTIDE-IMMUNOREACTIVE AMACRINE CELLS

In the ten years since opioid and tachykinin peptides were first localized in amacrine cells of pigeon retina (Brecha *et al.*, 1979; Karten and Brecha, 1980), a wealth of immunohistochemical studies have been published on the cellular distribution of different neuropeptides in vertebrate retinae (reviewed by Brecha *et al.*, 1984a; Stell, 1985). Most of these studies utilized transverse retinal sections which provide valuable information on the dendritic lamination of peptide-immunoreactive amacrine cells (Karten and Brecha, 1983). For technical reasons, these amacrine populations have seldom been labelled in horizontal sections or retinal wholemounts and, thus, information about their topographic distribution and dendritic morphology is often lacking. However, there are some notable exceptions both in non-mammalian retinae (e.g. Eldred and Karten, 1983) and in select mammalian retinae as described below. Regardless of whether or not these neuropeptides function as transmitters or neuromodulators in the retina, their immunohistochemical localization within discrete amacrine types provides a powerful tool for labelling identified populations and, thus, for

further characterizing the neuronal diversity of retinal amacrine cells. Several neuropeptides which appear ubiquitous in non-mammalian retina, such as enkephalin, glucagon and neurotensin, are not detectable in mammalian retina with immunohistochemical methods. It is not clear, however, whether the neuropeptide repertoire of mammalian retina is correspondingly reduced, or whether the available antisera are biased towards a different complement of neuropeptides in non-mammalian retina.

### 6.1. Substance P and VIP

The substance P-immunoreactive (SP-IR) amacrine cells in cat retina are probably the best characterized population of putative peptidergic amacrine in any mammalian retina (Pourcho and Goebel, 1988b; Vaney *et al.*, 1989b). Some 80 to 95% of the SP-IR cells in peripheral retina are displaced to the ganglion cell layer (Brecha *et al.*, 1984a), but this proportion decreases markedly within the area centralis, accounting for 50 to 80% of the labelled cells at maximum density. The SP-IR cells in both the inner nuclear and ganglion cell layers give rise to well-defined varicose dendrites of uniform appearance that stratify in strata 3 and 4 of the inner plexiform layer (Fig. 27). In addition, sparse fine dendrites in stratum 1 can sometimes be traced to inner nuclear cells and occasionally to displaced cells. These medium-field amacrine have a low dendritic field overlap, estimated to be 8-fold in peripheral retina, consistent with the open structure of the SP-IR plexus. The dendritic morphology of the displaced amacrine has been equated with either the A14 or A15 amacrine of Kolb *et al.* (1981) and may correspond to both of these Golgi types. The combined SP-IR cell density in cat retina ranges from less than 50 cells/mm<sup>2</sup> in the far periphery to over 500 cells/mm<sup>2</sup> in the area centralis; the maximum density shows little individual variation despite wide differences in the proportion of displaced cells. The 39,000 SP-IR amacrine in each retina have a triangular topographic distribution, with intermediate isodensity lines extending vertically in superior retina and horizontally along both arms of the visual streak

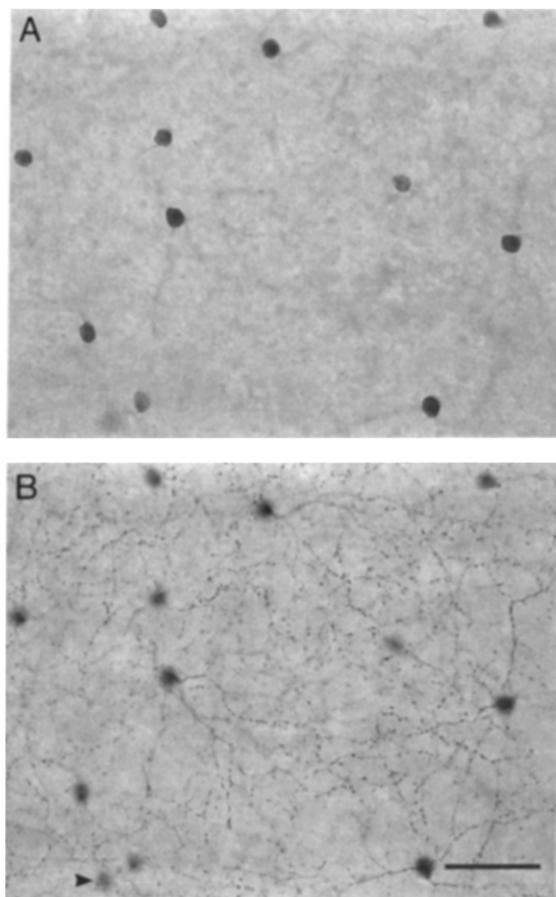


FIG. 27. Substance P-like immunoreactivity in a wholemounted cat retina. In this peripheral field, the cell bodies of 11 labelled amacrines are located in the ganglion cell layer (A) and one other is located in the inner nuclear layer (arrowhead). All of the substance P-immunoreactive amacrines arborize around 60% depth of the inner plexiform layer, producing an open meshwork of varicose dendrites (B). Scale bar, 50  $\mu$ m. (From Vaney *et al.*, 1989b.)

(Vaney *et al.*, 1989b) (Fig. 40). The high proportion of inner nuclear cells in the area centralis suggests that cell body placement in the ganglion cell layer is not a prerequisite for normal function of the SP-IR amacrines in cat retina.

Double immunolabelling experiments established that the SP-IR amacrine cells in cat retina invariably show GABA-like immunoreactivity (Pourcho and Goebel, 1988a; Vaney *et al.*, 1989b) (Fig. 28). This is the first colocalization of a neuropeptide and a classical transmitter in mammalian retina, although a variety of

neuropeptide-IR amacrines in non-mammalian retinae have been shown to accumulate [<sup>3</sup>H]glycine or [<sup>3</sup>H]GABA (Watt *et al.*, 1984, 1985, 1987; Weiler and Ball, 1984; Wu and Lam, 1988). The functional significance of the coexistence of GABA and substance P (or a related tachykinin) in cat amacrine cells remains to be determined.

In monkey and rabbit retinae, the SP-IR amacrines comprise multiple cell types (Brecha *et al.*, 1982, 1987), but it is not clear whether the displaced cells in these retinae are a distinct type of SP-IR amacrine, or whether they include misplaced examples of amacrine types that are more commonly found in the inner nuclear layer. In addition, some 25% of the ganglion cells in rabbit retina show substance P-like immunoreactivity (Fig. 29); these cells degenerate following optic nerve section, leaving a residual population of labelled neurons at densities of 60 to 140 cells/mm<sup>2</sup> (Brecha *et al.*, 1987). SP-IR ganglion cells can also be detected in cat retina, but only following intraocular injection of colchicine (Vaney *et al.*, 1989b).

Although vasoactive intestinal polypeptide-immunoreactive (VIP-IR) amacrine cells are ubiquitous in mammals (Tornqvist *et al.*, 1982; Brecha *et al.*, 1984a), they have been well characterized only in rabbit retina (Sagar, 1987a). Rabbit VIP-IR amacrines resemble cat SP-IR amacrines in that their sparse dendritic trees overlap about 10-fold, producing a comparatively open dendritic plexus (Fig. 30). The VIP-IR amacrine cells, which are conventionally located in the amacrine sublayer, range in density from 15–20 cells/mm<sup>2</sup> in the superior periphery to 40–50 cells/mm<sup>2</sup> in the visual streak. Their dendrites are most numerous in stratum 1 but may also branch in strata 3 and 5, giving an overall tristratified appearance (Sagar, 1987a). This lamination pattern resembles that of rabbit dopaminergic amacrines (Ehinger, 1966b) and there is pharmacological evidence that dopamine and VIP stimulate a common adenylate cyclase in rabbit retina (Pachter and Lam, 1986).

## 6.2. Somatostatin

Somatostatin immunohistochemistry labels about 1000 neurons in the ganglion cell layer of

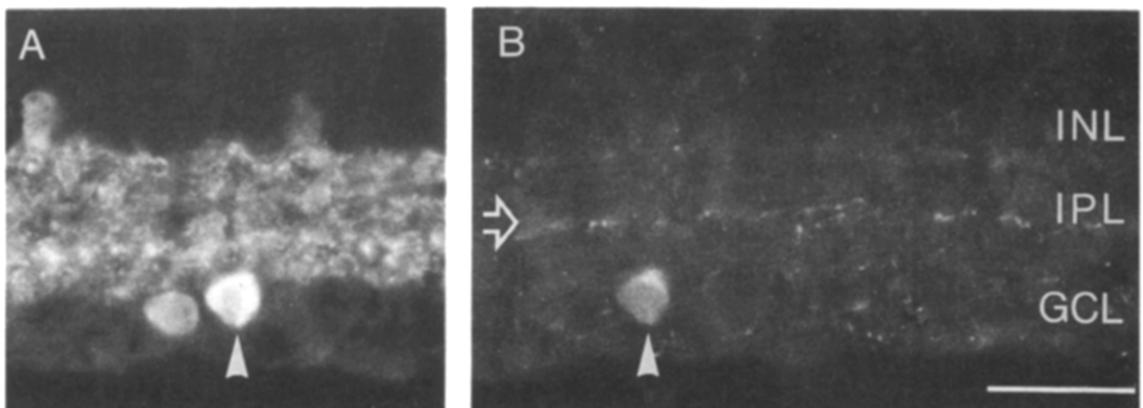


FIG. 28. Localization of GABA-like immunoreactivity in substance P-immunoreactive amacrine cells of cat retina. The frozen transverse section was double-labelled with a rabbit polyclonal antiserum against GABA (A) and a rat monoclonal antibody against substance P (B); the arrowhead marks a double-labelled cell while the open arrow indicates the stratification level of these amacrines. Scale bar, 20  $\mu$ m. (From Vaney *et al.*, 1989b.)

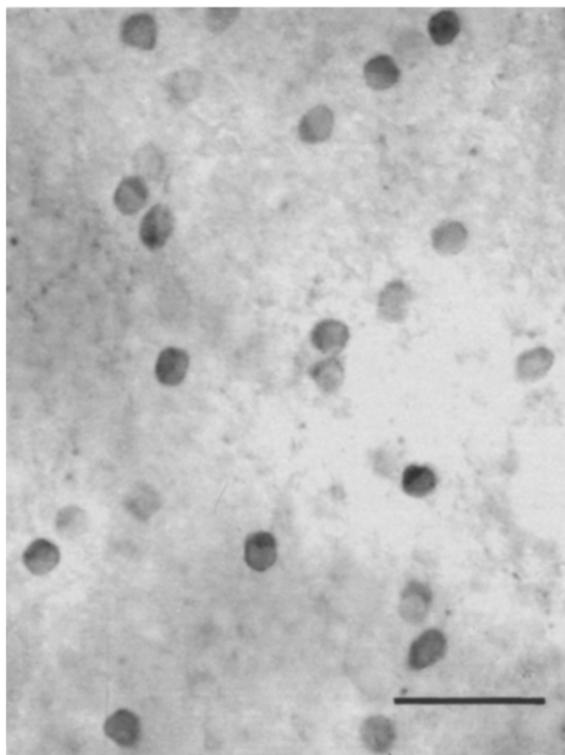


FIG. 29. Substance P-immunoreactive neurons in the ganglion cell layer of a colchicine-treated rabbit retina; Brecha *et al.* (1987) demonstrated that most of the labelled neurons are retinal ganglion cells. Scale bar, 50  $\mu$ m. (H. M. Young, unpublished.)

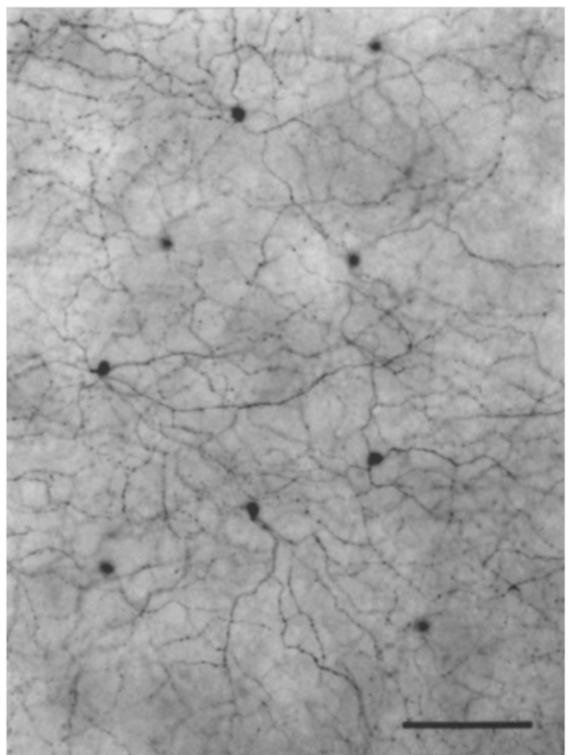


FIG. 30. VIP-immunoreactive amacrine cells in a wholemount preparation of a colchicine-treated rabbit retina; this field from the central visual streak shows the densest packing of labelled cells in the retina. Scale bar, 200  $\mu$ m. (From Sagar, 1987a.)

rabbit retina, most of which are located inferior to the myelinated band (Fig. 31); their tapered dendrites may extend for 500 µm in the inner plexiform layer, branching primarily in strata 1 and 5. In addition, a second somatostatin-IR population forms a circumferential band around the extreme periphery of the retina (Sagar *et al.*, 1986; Sagar, 1987b). Both types of somatostatin-IR neurons give rise to a thin axon-like process that usually runs in stratum 5 and does not enter the optic nerve. The labelled plexus formed by these long varicose fibres covers the entire retina, suggesting that processes from the inferior and circumferential populations extend across superior retina. Somatostatin-IR neurons with a comparable morphology have been labelled in the ganglion cell layer of human retina; they are more numerous in the inferior and their axon-like processes extend for several millimetres at minimum, and can sometimes be traced for 20 mm across the retina (Sagar and Marshall, 1988). In other mammalian retinae, somatostatin-IR cells are labelled in both the inner nuclear and ganglion cell layers (Tornqvist *et al.*, 1982; Sagar *et al.*, 1985); those in guinea pig are located circumferentially and may give rise to the labelled processes observed throughout the retina (Spira *et al.*, 1984).

There are diverse types of neurons in the inner retina which produce axon-like processes that are contained wholly within the inner plexiform layer; these interneurons are thus distinct from retinal ganglion cells or interplexiform cells. They have been variously termed association amacrine cells (Cajal, 1933; Mariani, 1982), associational nerve cells of the ganglion cell layer (Gallego and Cruz, 1965), proprioretinal cells (Catsicas *et al.*, 1987); long-range amacrine cells (Vaney *et al.*, 1988), axon-bearing amacrine cells (Dacey, 1989), or associational ganglion cells, as in the case of the somatostatin-IR neurons (Spira *et al.*, 1984; Sagar, 1987b; Sagar and Marshall, 1988). Although axonal properties have been attributed to the distal processes of these cells, it has yet to be demonstrated that they propagate impulses or serve an efferent function. In this review, the term "amacrine cell" is thus used as a generic label for all interneurons in the inner retina, notwithstanding the contrary argument that

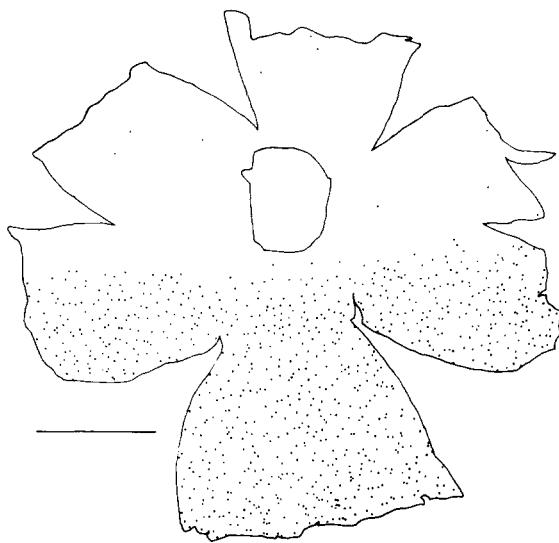


FIG. 31. Mapped distribution of somatostatin-immunoreactive "association" neurons in rabbit retina. Of the 877 labelled cells in the wholemount (excluding those in the circumferential band), only 10 are located in superior retina. Scale bar, 2.5 mm. (From Sagar, 1987b.)

amacrine cells are literally defined as neurons "without a long process". The following section describes other types of low density amacines which give rise to distal processes that are morphologically distinct from the proximal dendrites.

## 7. CATECHOLAMINERGIC AMACRINE CELLS

### 7.1. Rabbit Retina: CA1, CA2 and CA3 Amacines

In rabbit retina, there are several populations of low-density amacines that have similar dendritic morphologies and topographic distributions. They include the putative dopaminergic amacines, which were the first retinal neurons to be neurochemically identified (Häggendal and Malmfors, 1965; Ehinger, 1966b). Although these amacines have been the subject of numerous studies (reviewed by Nguyen-Legros, 1988), there are significant discrepancies in the literature which appear resolvable in the light of recent findings (Masland, 1988).

Immunohistochemical localization of tyrosine hydroxylase (TH), the rate limiting enzyme for dopamine synthesis, reveals a sparse population of wide-field amacrine cells in rabbit retina (Brecha *et al.*, 1984b). Their multipolar cell bodies, which are invariably located at the inner margin of the inner nuclear layer, usually give rise to 2 to 4 stout dendrites that branch in stratum 1 of the inner plexiform layer (Fig. 32). In addition, numerous varicose processes form a dense TH-IR plexus in stratum 1 and a sparse network in strata 2 to 5; these fine processes are difficult to trace and thus their spatial relationship to the proximal dendritic tree cannot be ascertained in immunolabelled wholemounts. The TH-IR amacrines range in density from about 6 cells/mm<sup>2</sup> in the inferior periphery to 20–30 cells/mm<sup>2</sup> in the visual streak (Brecha *et al.*, 1984b; Mitrofanis *et al.*, 1988a; Vaney and Young, 1988b). In mapped wholemounts labelled with the Boehringer monoclonal and viewed from the photoreceptor side, it is apparent that TH-IR amacrines are also present under the myelinated fibre band; new cell counts that include these amacrines indicate a total population of 6,500 to 7,000 TH-IR amacrines (H. M. Young and D. I. Vaney, unpublished). This is still less than the 8,500 TH-IR cells in the retina illustrated by Brecha *et al.* (1984b), but within the range of their unpublished counts. Thus, the TH-IR cells only comprise about 0.1% of the 5,000,000 presumptive amacrines in rabbit retina.

In rabbit retina, there are 15 serotonin-accumulating cells for each catecholamine-accumulating cell (Negishi *et al.*, 1984), indicating a total catecholaminergic population of about 13,000 neurons, double the number of TH-IR amacrines. Nguyen-Legros (1988) proposed that the surplus neurons comprise a population of "noradrenaline-accumulating cells", but new evidence presented below indicates that these cells contain an endogenous catecholamine (D. I. Vaney, unpublished). A rabbit retina was incubated in 5,7-DHT, a serotonin analog that is taken up only by the S1 and S2 amacrines (Vaney, 1986a). Half of the retina was fixed in formaldehyde and glutaraldehyde (FAGLU) to visualize endogenous catecholamines, while the other half was fixed in formaldehyde and then

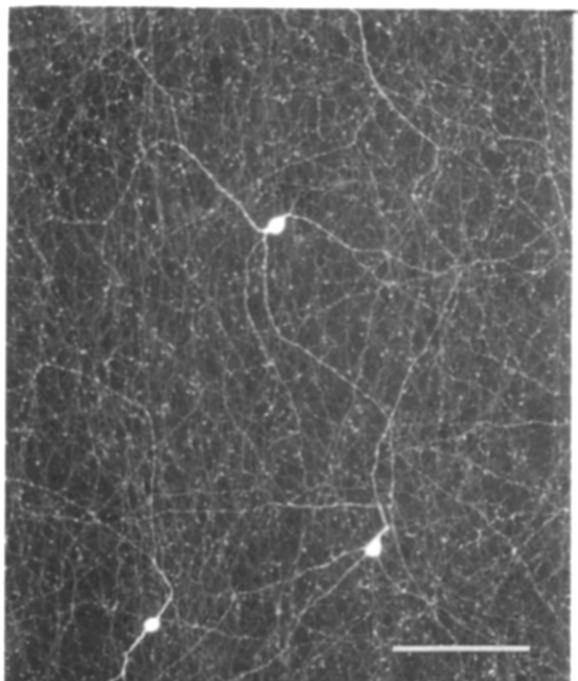


FIG. 32. Tyrosine hydroxylase-immunoreactive amacrine cells in peripheral rabbit retina. Although these cells only account for one amacrine in every thousand, their varicose processes form a dense plexus at the outer margin of the inner plexiform layer. Scale bar, 100 µm.  
(H. M. Young and D. I. Vaney, unpublished.)

processed for TH immunohistochemistry. In both halves, the fluorescent serotonin-accumulating amacrines provided a reference population. In mid-peripheral retina, the serotonin-accumulating cells outnumbered the catecholamine-containing cells by 15:1; in an adjacent field located in the other retinal half, they outnumbered the TH-IR cells by 34:1. Thus, the TH-IR amacrines can only account for about 45% of the catecholaminergic amacrines in rabbit retina. A small proportion of the cells showing endogenous catecholamine fluorescence are located in the ganglion cell layer (Ehinger, 1966b), but none of the TH-IR amacrines have displaced cell bodies.

Lucifer injection into catecholamine-accumulating cells in fixed rabbit retina revealed that these amacrines comprise three morphological types (Tauchi and Masland, 1986; Masland, 1988). The CA1 amacrines have large fusiform cell bodies which give rise to 2 or 3 thick dendrites that

appear to arborize only in stratum 1. The CA2 amacrices have medium cell bodies and their dendrites are bistratified or tristratified in strata 1, 3 and 5. The CA3 amacrices have small round cell bodies which give rise to 1 or 2 vertically-directed dendrites that branch in stratum 3. The morphology of the TH-IR amacrices in rabbit retina corresponds to that of the CA1 amacrices, whereas the TH-negative catecholaminergic cells presumably include both the CA2 and CA3 amacrices. Thus, the trilaminar distribution of catecholaminergic dendrites is more apparent with formaldehyde-induced fluorescence than with TH immunofluorescence (Ehinger, 1966b; Brecha *et al.*, 1984b). The CA2 and CA3 amacrices are alike in many respects (Tauchi and Masland, 1986) and it has yet to be established that they are distinct neuronal types.

## 7.2. Cat Retina: One Type, Several Morphologies

Estimates of the number of TH-IR amacrine cells in cat retina range from 3000 to 5000, the higher figure being obtained with the Boehringer monoclonal antibody (Oyster *et al.*, 1985; Mitrofanis *et al.*, 1988a; Vaney *et al.*, 1989b). The density of labelled cells varies little more than twofold over the retina and reaches an ill-defined peak of about 15 cells/mm<sup>2</sup> in central-superior or temporal-superior retina (Fig. 40). Amacrine cells showing FAGLU-induced fluorescence are present at equivalent densities (D. I. Vaney, unpublished), indicating that the TH-IR amacrices are probably identical to the catecholamine-containing cells identified in earlier studies (Ehinger, 1966a; Törk and Stone, 1979). Although 6% of the TH-IR cells in cat retina are displaced to the ganglion cell layer, all of the labelled amacrices have stellate dendritic trees ramifying in stratum 1 and thus appear to comprise a single neuronal type (Oyster *et al.*, 1985; Voigt and Wässle, 1987; Mitrofanis *et al.*, 1988a). Oyster *et al.* (1985) reported that 5% of the TH-IR cells in cat retina give rise to additional processes that branch sparsely in the outer plexiform layer; they therefore classified these neurons as interplexiform cells. It is likely, however, that all of these TH-IR neurons serve the

same function in the inner retina; moreover, sclerally-directed TH-IR branchlets may be relatively common in cat and other mammals (Wässle and Chun, 1988). It thus seems inappropriate to distinguish the interplexiform population as a separate neuronal type, let alone as a separate class. The cell density, dendritic morphology, synaptic connectivity and putative dopaminergic function of the TH-IR cells in cat retina are clearly homologous to the TH-IR amacrices in rabbit retina: both types are thus termed CA1 amacrices, commensurate with the nomenclature of Mariani and Hokoc (1988).

Voigt and Wässle (1987) injected Lucifer yellow into cat dopaminergic amacrices identified by their characteristic fluorescence in FAGLU-fixed wholemounts. The skeletal dendritic trees revealed by this method accounted for only 10 to 20% of the TH-IR processes in stratum 1, and included few of the varicose processes which dominate the plexus (Fig. 33). More recently, Dacey has injected HRP into the largest cell bodies in the amacrine sublayer of living cat retina and then processed the material for TH-immunohistochemistry. The identified CA1 amacrices give rise to multiple axon-like processes from both the cell body and proximal dendrites; these varicose processes extend for several millimetres, branching sparsely, and skirting around the protruding cell bodies of AII amacrices (D. M. Dacey, unpublished). The AII cells thus appear to be surrounded by dopaminergic "rings" (Tork and Stone, 1979; Oyster *et al.*, 1985), from which they receive substantial synaptic input (Pourcho, 1982; Sterling, 1983; Voigt and Wässle, 1987). TH-IR varicosities are distributed throughout the plexus, however, and there is no evidence that the processes are preferentially aligned with the AII cells. The multiple dopaminergic varicosities presynaptic to each AII cell probably arise from different amacrine cells, analogous to the relationship between rod bipolars and serotonin-accumulating amacrices. (Although CA1 amacrices have a similar morphology to the "dopamine-accumulating" amacrices identified by Dacey (1988), the latter cells are much more numerous and do not show TH-IR (D. M. Dacey, unpublished); they may correspond to the "spiny" serotonin-accumulating amacrices.)

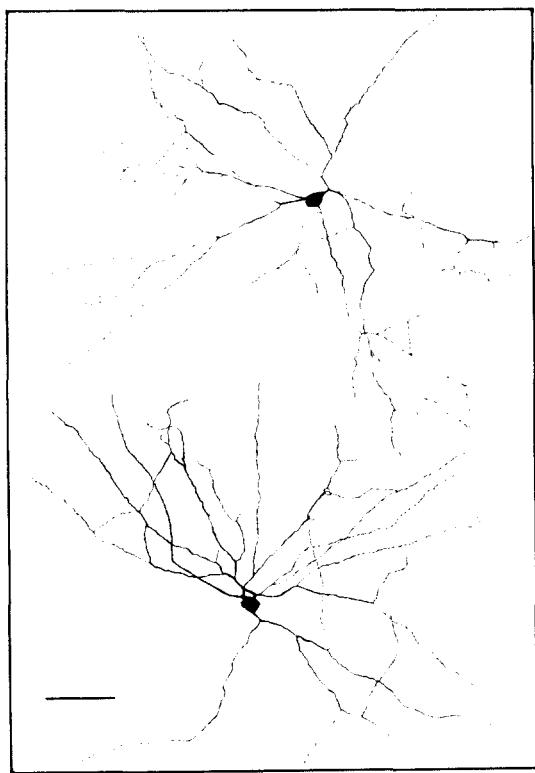


FIG. 33. Dendritic morphology of the proximal dendritic trees of two catecholamine-containing amacrine cells in cat retina, as revealed by Lucifer injection of cells showing FAGLU-induced fluorescence. Scale bar, 100 µm.  
(Refigured from Voigt and Wässle, 1987.)

### 7.3. Monkey Retina: CA1 and CA2 Amacrines

Two types of TH-IR amacrine cells have been identified in rhesus monkey retina (Mariani and Hokoc, 1988). The large brightly-labelled CA1 cells are homologous to their counterparts in rabbit and cat retinae: they branch primarily in stratum 1 of the inner plexiform layer, but also give rise to radially-oriented branchlets in the inner nuclear layer. The smaller weakly-labelled CA2 cells are often located in the inner plexiform or ganglion cell layers and branch in stratum 3; they account for about 60% of the TH-IR cells in rhesus retina. In New World monkeys, a similar dichotomy in cell size and fluorescence intensity has been reported for catecholaminergic neurons identified by formaldehyde-induced fluorescence (Ehinger and Falck, 1969). The topographic distribution of the CA1 amacrines in rhesus retina

follows that of the rod photoreceptors and is maximal 3 mm from the centre of the fovea (Mariani *et al.*, 1984). This correlation is consistent with dopamine modulating rod signal transmission at the level of the AII amacrines, perhaps by regulating the AII gap junctions (Vanney, 1985a). In cat retina, however, the distribution of the dopaminergic amacrines does not follow that of the AII amacrines, which peak within the area centralis.

The CA2 amacrines in rhesus monkey retina resemble the CA2/CA3 amacrines in rabbit retina in several respects including their cell size, stratification level, and relative numbers. In monkey retina, however, differences in the levels of the endogenous catecholamine appear to reflect differences in the TH content of the cells; in rabbit retina, on the other hand, the FAGLU-induced fluorescence of the CA2/CA3 cells is comparable to that of the CA1 cells, but only the CA1 amacrines show TH immunoreactivity. Two types of TH-IR amacrine cells are also labelled in albino rat retina, but their appearance and distribution seem to differ in the Wistar and Sprague-Dawley strains (Nguyen-Legros *et al.*, 1983, 1986; Nguyen-Legros, 1988; Mitrofanis *et al.*, 1988a).

### 7.4. NADPH-Diaphorase Cells: ND1 and ND2 Amacrines

Diaphorase activity is defined histochemically as the ability to reduce tetrazolium salts in the presence of reduced nucleotides (NADH/NADPH), but the physiological enzyme underlying the reaction has not been identified. Nevertheless, the remarkable selectivity and Golgi-like quality of NADPH-diaphorase staining has enabled new amacrine types to be identified and characterized. In rabbit retina, NADPH-diaphorase histochemistry labels two types of amacrine cells, termed ND1 and ND2 amacrines (Sandell, 1985; Sagar, 1986; Vanney and Young, 1988b). The ND1 amacrines have larger and more intensely stained cell bodies than the ND2 amacrines (Fig. 34). Although both types arborize in stratum 3 of the inner plexiform layer, the stout darkly-stained dendrites of the ND1 amacrines can be traced for 500–1000 µm from the cell

body, whereas the ND2 dendrites can be followed for only 50–100  $\mu\text{m}$ . Many thin varicose dendrites are intensely stained in both the plane of primary arborization and other strata of the plexiform layer: they are probably tertiary branches of the ND1 amacrine. On the peak visual streak, the density of ND2 amacrines (130 cells/ $\text{mm}^2$ ) is 6 times that of ND1 amacrines (21 cells/ $\text{mm}^2$ ) while, at the inferior edge, the density of ND2 amacrines (35 cells/ $\text{mm}^2$ ) is 9 times that of ND1 amacrines (4 cells/ $\text{mm}^2$ ). There are about 4,000 ND1 amacrines and 24,000 ND2 amacrines in each rabbit retina (Vaney and Young, 1988b).

Although it has been proposed that NADPH-diaphorase is likely to be a marker for the dopaminergic amacrines (Nguyen-Legros, 1988), TH immunohistochemistry of diaphorase-stained rabbit retina demonstrated that the two labels are localized in separate amacrine populations (Vaney and Young, 1988b). Nevertheless, the ND1 and TH-IR amacrines are strikingly similar in their topographic distribution and dendritic morphology. Although the primary stratification of the TH-IR amacrines is in stratum 1, they also have thin varicose dendrites in strata 3 and 5; the ND1 amacrines are complementary in that their primary stratification in stratum 3 apparently gives rise to varicose processes in strata 1 and 5. It thus seemed possible that the ND1 amacrines might correspond to the TH-negative CA2/CA3 amacrines in rabbit retina. However, a colocalization study involving sequential labelling and mapping of the catecholamine-containing cells and diaphorase-positive cells demonstrated that the two populations do not overlap (D. I. Vaney, unpublished). Other studies have since shown that NADPH-diaphorase amacrines in cat, rabbit and rat retinae are actually GABA-immunoreactive, consistent with their wide-field unistratified morphology (Müller *et al.*, 1988; Vaney and Young, 1988b; Young and Vaney, 1989).

### 7.5. Other Low-Density Amacrine Cells

Neurofibrillar methods selectively stain several types of neurons in rabbit retina, including the *Ca*

and *Cb* amacrines (Cattaneo, 1922; Vaney *et al.*, 1981). A third amacrine population is also labelled (Vaney *et al.*, 1988): their cell bodies may be located in the inner nuclear, inner plexiform or ganglion cell layers and they branch predominantly in stratum 2 of the inner plexiform layer. Characteristically, each cell has two or more stout distal processes which arise from a constricted dendritic segment and extend for 2 to 3 mm beyond a proximal dendritic field of 600 to 800  $\mu\text{m}$  diameter (Fig. 35). In peripheral retina, there are some 31 cholinergic amacrines for each long-range (LR) amacrine, indicating a total population of 9,300 cells or 0.2% of the presumptive amacrines in rabbit retina. Despite their low density, the long-range amacrines achieve effective coverage of the retina by both the proximal and distal dendrites. An homologous population of long-range amacrines has also been identified in neurofibrillar-stained cat retina (Vaney *et al.*, 1988).

In other vertebrate retinae, there are many examples of amacrine cells with a symmetric proximal dendritic field and less regular, distally extended processes. There is the well known "giant amacrine cell" from Golgi-stained lizard retina (Cajal, 1933) which also branches in stratum 2 of the inner plexiform layer; in turtle retina, Kolb (1982) identified a wide-field amacrine cell of comparable morphology. Dye injection in cyprinid fish retinae has consistently revealed amacrine types with robust, sparsely branching dendrites that form a proximal arborization of 400 to 800  $\mu\text{m}$  diameter which, in turn, gives rise to a few thin distal processes that may extend for 3mm (Djamgoz *et al.*, 1984; Teranishi *et al.*, 1987). The cells surveyed above have a common feature in that the straight-running distal processes, which arise from the abrupt tapering of a proximal dendrite, are much thinner distal to the taper than they are proximally; in this respect, they resemble the monoamine-accumulating amacrines which Dacey (1988) injected in cat retina. By contrast, the long-range processes of neurofibrillar amacrines in rabbit retina may be thicker than the tertiary proximal dendrites, and only a 10 to 30  $\mu\text{m}$  segment following the initial taper is constricted in width (Fig. 36).

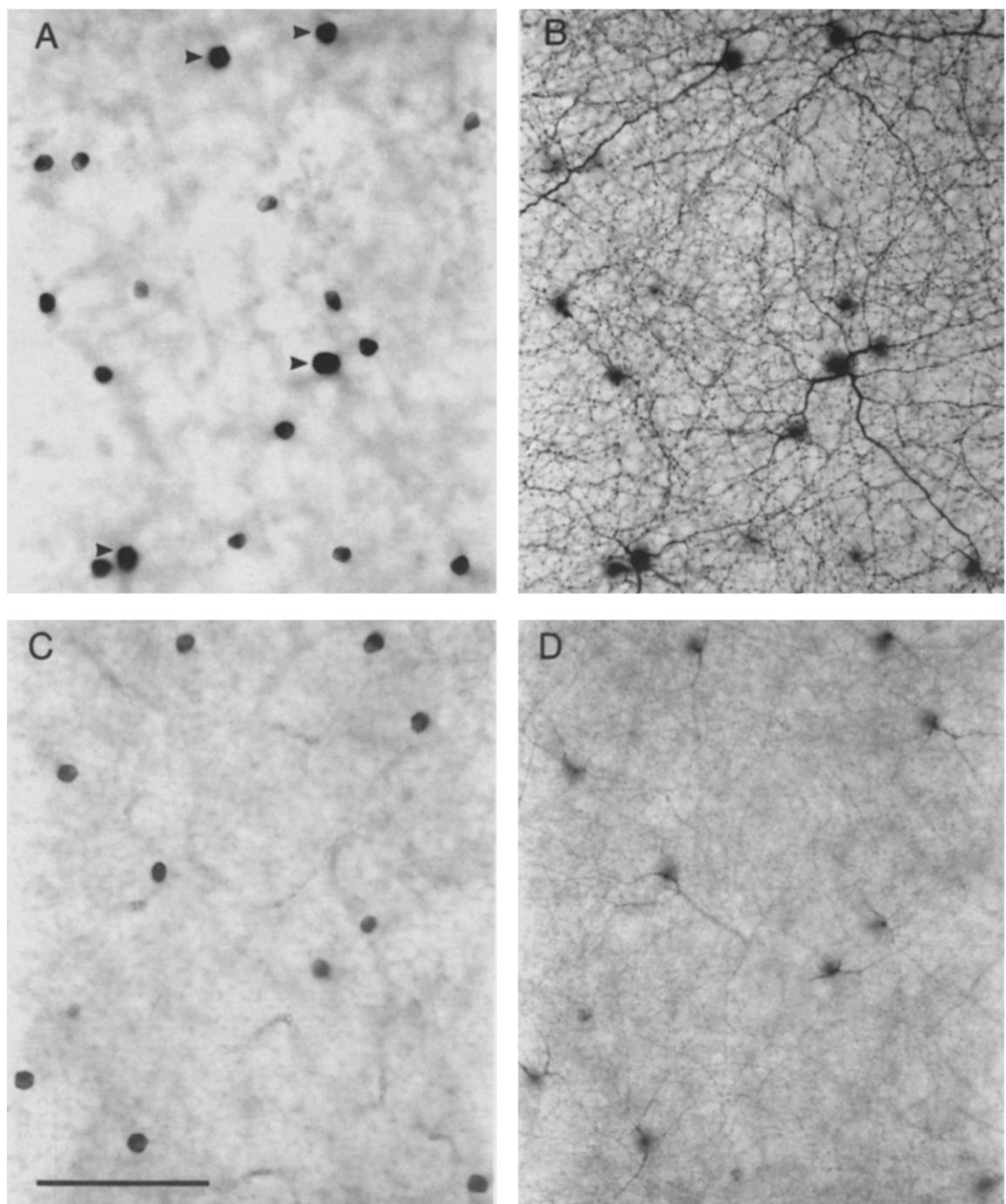


FIG. 34. NADPH-diaphorase histochemical staining of rabbit (A,B) and rat (C,D) retinal wholemounts, with the focus at the inner margin of the inner nuclear layer (A,C) or in the middle of the inner plexiform layer (B,D). In rabbit retina, the ND1 amacrine (arrowheads) have larger and more intensely stained cell bodies than the more numerous ND2 amacrines, and the dendrites of ND1 amacrines can be traced much further within the plexiform layer. The ND amacrines in rat retina comprise a single type of neuron, which may be homologous to the ND1 amacrines of rabbit retina. Scale bar, 100  $\mu$ m. (A and B from Vanney and Young, 1988b.)

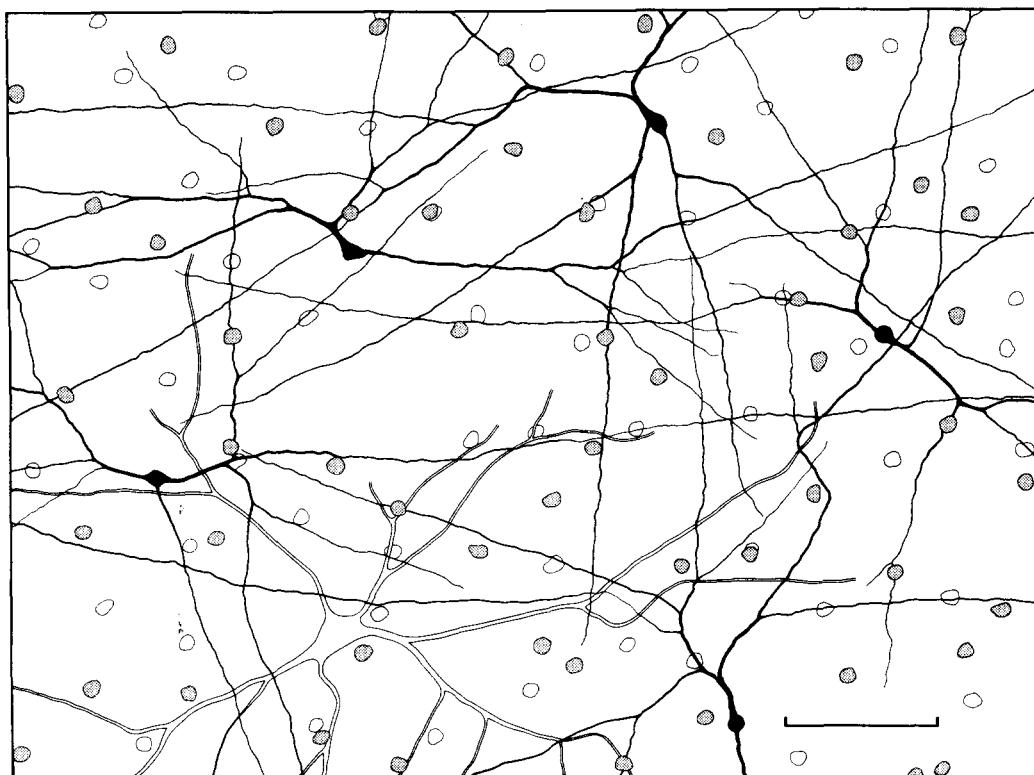


FIG. 35. Drawing of a peripheral field of rabbit retina showing the inner retinal neurons that are selectively stained by neurofibrillar methods. The field includes five long-range amacrines (black), an alpha ganglion cell (outlined), the Ca amacrines (outlined cell bodies) and the Cb amacrines (stippled cell bodies). Scale bar, 100  $\mu\text{m}$ . (From Vaney *et al.*, 1988.)

Dacey (1989) identified a type of interstitial amacrine cell in macaque monkey retina that also has distinct proximal and distal fields. The highly branched, broadly stratified, proximal arborization has a dendritic field diameter of 200 to 600  $\mu\text{m}$ . In addition, 1 to 4 axon-like processes branch in stratum 3, giving rise to thin collaterals that bear distinct boutons along their length; the result is a sparsely branched “axonal field” that is about 10 times wider than the proximal dendritic field (Fig. 37). The diameter of these distal processes increases from 0.5  $\mu\text{m}$  to 1–2  $\mu\text{m}$  as they course beyond the proximal field, which is comparable to the long-range processes identified in neurofibrillar-stained retinae (Vaney *et al.*, 1988).

## 8. THE MOSAIC OF AMACRINE CELLS

### 8.1. How Many Types of Amacrines?

One goal of our research is a full accounting of the morphological types of amacrine cells in rabbit retina, on a population-by-population basis. In order to assess the progress to date, an estimate of the number of distinct amacrine types is required. Using Golgi-stained material, Kolb *et al.* (1981) distinguished 22 types of amacrine cells in cat retina on the basis of their branching pattern, stratification level and dendritic field size. Many of these types were represented by only one or a few stained cells: apart from the problems of characterizing a neuronal type in such samples (cf. Boycott and Wässle, 1974), probability alone

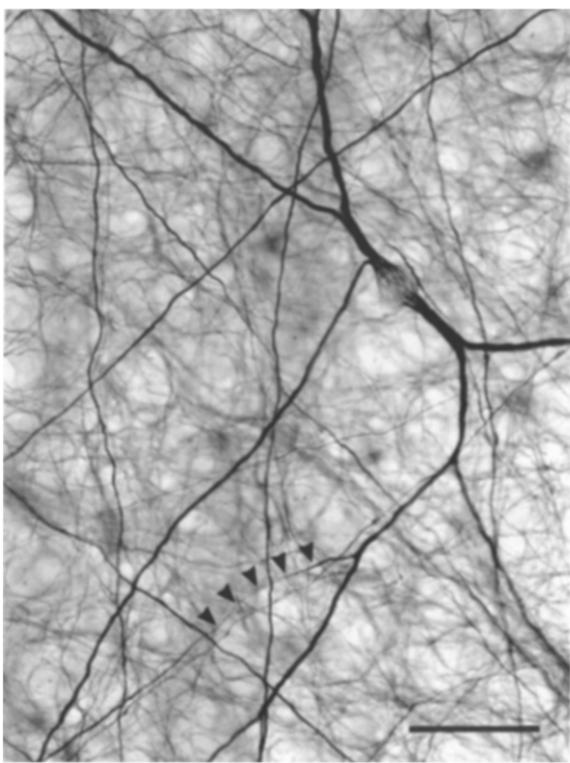


FIG. 36. Neurofibrillar long-range amacrine cell in peripheral rabbit retina, showing the tapered dendritic segment (arrowheads) connecting a long-range process to the proximal dendritic tree. Scale bar, 50  $\mu\text{m}$ . (From Vaney *et al.*, 1988.)

indicates that many more amacrine types must be unrepresented. For example, Kolb's A20 cell has been proposed as the Golgi correlate of either the "sparsely branched" serotonin-accumulating amacrices or the neurofibrillar long-range amacrices, but it cannot be both (Wässle *et al.*, 1987b; Vaney *et al.*, 1988). On the other hand, a single amacrine population whose dendritic field size or stratification level varies continuously over a defined range, may appear dichotomous if those cells with intermediate morphologies are unrepresented in a limited Golgi sample. For example, the A14 and A15 amacrices of Kolb *et al.* (1981) reportedly branch in strata 3–4 and 4–5 respectively, but both cells could be Golgi correlates of a single type of substance P-IR amacrine (Vaney *et al.*, 1989b).

Although retinal amacrine cells are noted for their neurochemical heterogeneity, only a

minority of the amacrine types can be selectively identified with the available histochemical probes. However, it is self evident that amacrine types which are morphologically and functionally distinct must also be biochemically distinct, and rapid progress can be expected as new antibodies and mRNA probes become available. Lam *et al.* (1985) proposed that every type of retinal neuron may be uniquely characterized by the neuroactive substances it contains, but the "signature hypothesis" has proved of limited utility in categorizing amacrine cells in mammalian retina: those GABA-IR amacrices that contain or accumulate another neuroactive substance can be identified by the second substance alone.

An objective estimate of the number of amacrine types in rabbit retina can be obtained, if it is assumed that the 13 populations which have been well characterized are representative of all amacrine types (Vaney and Hughes, 1989). They are listed here in order of decreasing frequency: AII (11%), Cb (3.0%), Ca (2.9%), S1 (2.2%), S2 (1.8%), DAPI-3 and DAPI-4 (1.8%), ND2 (0.5%), VIP (0.3%), LR (0.2%), CA2/CA3 (0.1%), CA1 (0.1%), ND1 (0.1%). These 13 identified populations thus account for only one-quarter of the 5,000,000 presumptive amacrices in rabbit retina, suggesting that there are about 50 amacrine types in total. Moreover, this figure is compatible with Marc's (1989) proposal that the AII amacrices and three other high-density glycinergic populations comprise 40 to 50% of all amacrices in mammalian retina. Those cells in the amacrine sublayer that are not labelled by GABAergic or glycinergic markers may be bipolar cells; if they are excluded from consideration, a revised total of about 40 amacrine types is indicated. This is double the number of Golgi types that Kolb *et al.* (1981) identified in cat retina, but less than the 70 types of amacrices described in Golgi-stained teleost retina (Wagner and Wagner, 1988).

## 8.2. Modularity of the Amacrine Mosaic

In rabbit retina, most amacrine types that have been mapped show a 3- to 5-fold increase in cell density from the inferior periphery to the peak

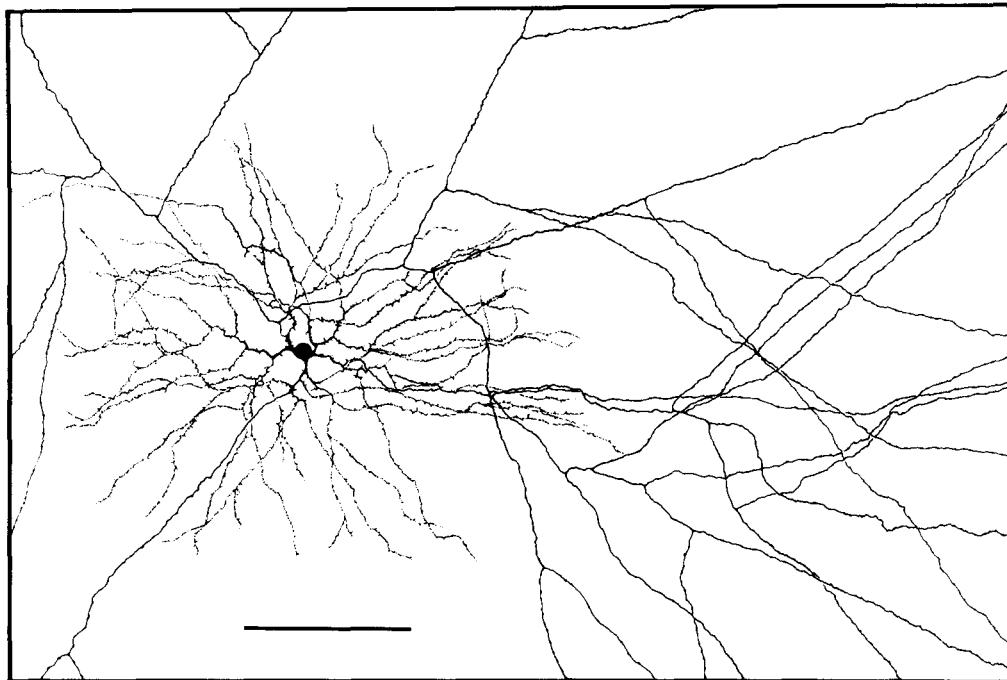


FIG. 37. Drawing of an HRP-filled interstitial amacrine cell in macaque retina; the distal "axonal" field is about 10 times wider than the proximal dendritic field. Scale bar, 100  $\mu\text{m}$ . (From Dacey, 1989.)

visual streak (Fig. 38), the one exception being the displaced SP-IR amacrine which reportedly have a relatively flat distribution (Brecha *et al.*, 1987). Consequently, the different amacrine types are present in similar proportions across the retina. A neuronal module enclosing 1000 presumptive amacrine cells would thus contain all amacrine components of the retinal circuit, the somatostatin-IR association cells notwithstanding. Some 5,000 of these amacrine modules would tile the retina, with each module covering an area of about 225  $\mu\text{m}$  diameter in the visual streak and 550  $\mu\text{m}$  diameter in the superior periphery (Fig. 39). At present, no functional role can be ascribed to such modules, but they are useful for illustrating the differential distribution of amacrine cells either within a retina or between species.

In cat retina, the topographic distributions of different amacrine types appear more varied. Although the AII, cholinergic and substance P-IR amacrine all peak in the area centralis and have elevated densities along the horizontal visual streak, the forms of their isodensity lines are quite

distinctive; this suggests that the weighting function applied to each part of the visual field varies with amacrine type (Fig. 40). The density map of substance P-IR amacrine has a triangular topography, with the density falling steeply in the inferior retina (Vaney *et al.*, 1989b). The cholinergic amacrine have a pronounced visual streak, comparable to both the A-type horizontal cells (Wässle *et al.*, 1978) and retinal ganglion cells (Wong and Hughes, 1987a). The AII amacrine have a kite-shaped distribution similar to that of alpha ganglion cells (Hughes, 1981; Vaney, 1985a). In contrast with these three amacrine populations, the tyrosine hydroxylase-IR amacrine have a relatively flat topography, with higher densities in central-superior (Oyster *et al.*, 1985; Vaney *et al.*, 1989b) or temporal-superior retina (Mitrofanis *et al.*, 1988a). The NADPH-diaphorase cells in cat retina, which appear to comprise several amacrine types, also have a relatively flat distribution with somewhat greater density in the inferior retina (Wässle *et al.*, 1987a).

Although at least 77% of the substance P-IR amacrine and about 67% of the cholinergic

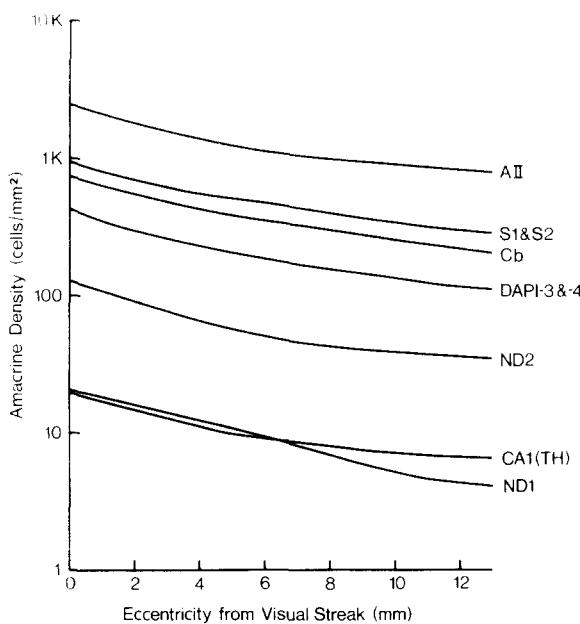


FIG. 38. Cell density gradients of seven populations of identified amacrine cells in rabbit retina, passing from the central visual streak to the inferior edge. The data on the NADPH-diaphorase positive cells (ND1 and ND2), the tyrosine hydroxylase-immunoreactive cells (CA1) and the displaced cholinergic cells (*Cb*) were obtained from a single preparation (Vanney and Young, 1988b). The data on the serotonin-accumulating cells (S1 and S2), the DAPI-3 and DAPI-4 cells, and the AII cells were obtained from separate preparations in which the *Cb* cells were also labelled: their cell densities were normalized to that of the *Cb* amacrine cells in the triple-labelled preparation. On the logarithmic ordinate, constant proportions result in parallel curves. (D. I. Vanney, unpublished.)

amacrines are located in the ganglion cell layer, the distribution map of all displaced amacrines in cat retina resembles that of the AII cells rather than the substance P-IR or cholinergic amacrines (Wong and Hughes, 1987a). These latter two populations account for only 4% and 11–13% respectively of the 730–850,000 displaced amacrines in cat retina (Wong and Hughes, 1987a; Wässle *et al.*, 1987a); they thus contribute little to the cumulative topography of the displaced amacrines, which may be dominated by several types of serotonin-accumulating amacrines (Wässle *et al.*, 1987b). How then are the diverse distribution patterns of displaced amacrines generated? The ganglion cell topography is thought to be largely sculptured by differential

areal growth (Mastronarde *et al.*, 1984; Wong and Hughes, 1987b) and, therefore, it might have been expected that the proportions of other neuronal types in the ganglion cell layer would be constant across the retina. This is not the case for the displaced amacrines, indicating that other factors such as differential cell production and cell death must also play a significant role in amacrine topogenesis (Vanney *et al.*, 1989b).

## 9. DIRECTION SELECTIVITY: AN HYPOTHESIS

Although the apparent simplicity of direction selectivity has fascinated retinal researchers since the process was first characterized by Barlow and Levick (1965), its synaptic mechanism still eludes us. There is substantial cholinergic input to direction-selective (DS) retinal ganglion cells (Masland and Ames, 1976; Ariel and Daw, 1982) but the functions of cholinergic amacrine cells in complex visual processing are poorly understood. Direction selectivity is believed to arise from non-linear interactions between cholinergic and GABAergic inputs, either in the ganglion cell itself or at a presynaptic level. The recent finding that the cholinergic (type C) amacrines also contain, synthesize and accumulate GABA (Vanney and Young, 1988a; Kosaka *et al.*, 1988; Brecha *et al.*, 1988), is compatible with a single type of interneuron mediating both the excitation and inhibition to DS ganglion cells (Dowling, 1970). The input and output synapses of type C amacrines are segregated centrifugally (Famiglietti, 1983b, 1989) and we have proposed that this arrangement could provide the asymmetric inhibition necessary for direction selectivity (Vanney and Young, 1988a). Our hypothesis makes a testable prediction: ganglion cells that respond to different preferred directions should receive synaptic input from different segments of the cholinergic dendritic field (Vanney *et al.*, 1989a).

### 9.1. Cholinergic Fascicles Follow DS Dendrites

DS neurons respond optimally to movement in one “preferred” direction, but show little or no

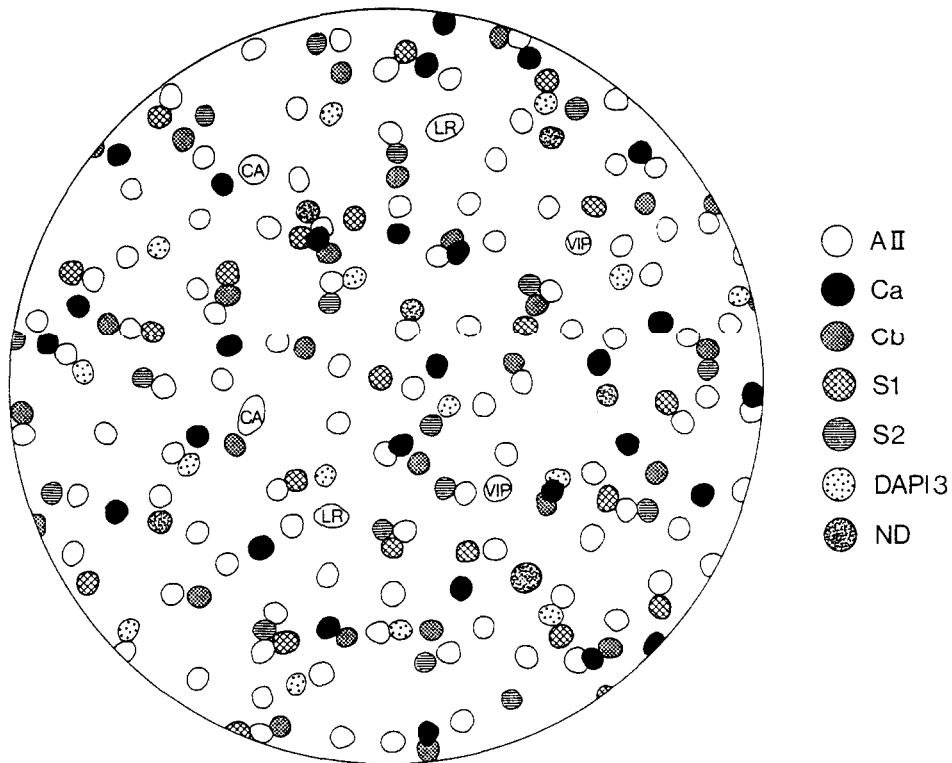


FIG. 39. The mosaic of amacrine cells in rabbit retina, reconstructed for a 300  $\mu\text{m}$  diameter field at 5 mm eccentricity from the visual streak; the mapped cells account for about 25% of the neurons in the amacrine sublayer of the inner nuclear layer. Abbreviations: AII, rod amacrine cells; *Ca* and *Cb*, type *a* and type *b* cholinergic cells; S1 and S2, type 1 and 2 serotonin-accumulating cells; DAPI-3, type 3 DAPI-labelled cells; ND, NADPH diaphorase-positive cells; VIP, vasoactive intestinal polypeptide-immunoreactive cells; LR, neurofibrillar long-range cells; CA, catecholaminergic cells. (D. I. Vaney, unpublished.)

response to movement in the reverse "null" direction. In rabbit retina, there are two distinct types of DS ganglion cells which respond to flashed illumination with On–Off or On-centre responses respectively; the two types also differ in their specificity for stimulus size and speed (Barlow *et al.*, 1964; Oyster, 1968; Wyatt and Daw, 1975). Amthor *et al.* (1984) recorded intracellularly from On–Off DS ganglion cells in an eyecup preparation before injecting them with horseradish peroxidase: the cells have a characteristic bistratified morphology with looping dendrites that cover the field uniformly. Although Famiglietti (1987) reported that these ganglion cells branch in the same strata as the *Ca* and *Cb* amacrices, the spatial relationships of their dendrites were not examined. Does the fasciculated meshwork of the cholinergic

dendrites reflect the looping morphology of the DS ganglion cells (Tauchi and Masland, 1985; Brandon, 1987b), or is it secondary to developmental patterns of growth (Famiglietti and Tumosa, 1987)?

Vaney *et al.* (1989a) answered this question by adapting Tauchi and Masland's method (1985) for visualizing the cholinergic dendritic plexus. A bistratified ganglion cell and 10 of the surrounding *Cb* amacrine cells were injected with Lucifer yellow under direct visual control. Although only two to four filled amacrices overlapped each region of the ganglion cell's dendritic field, compared with a potential 30-fold overlap, it is apparent that the filled dendrites form a fasciculated meshwork (Fig. 41). The inner dendrites of the ganglion cell are stratified in the same plane as the distal dendrites of the *Cb*

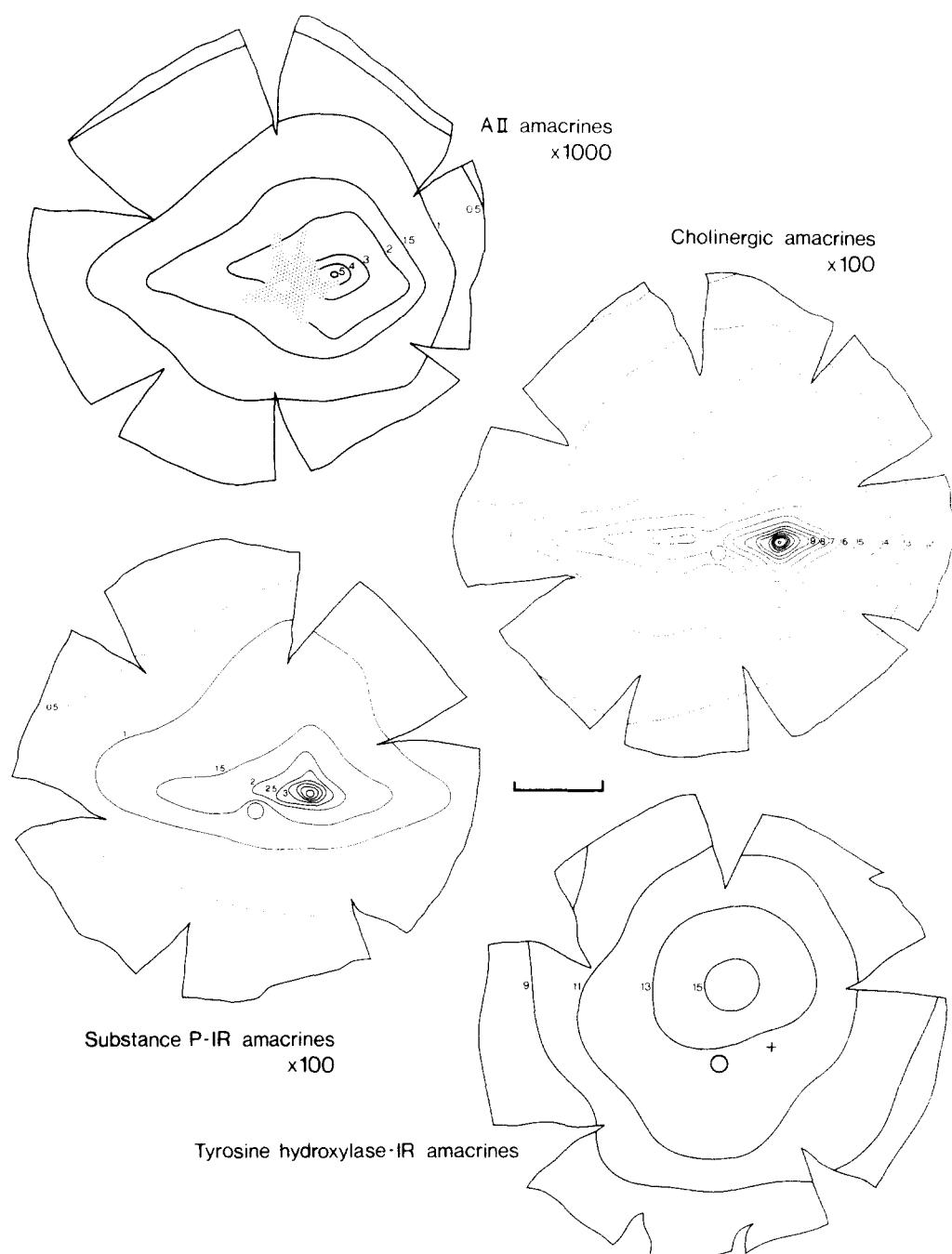


FIG. 40. Topographic distribution maps of four populations of amacrine cells in cat retina, with isodensity lines in cells/mm<sup>2</sup>. For the cholinergic amacrines, the contours between 1000 and 1800 cells/mm<sup>2</sup> are spaced at intervals of 200 cells/mm<sup>2</sup> and, above that, only the peak density of 2,600 cells/mm<sup>2</sup> is marked. Scale bar, 5 mm. (From Vanney *et al.*, 1989b.)

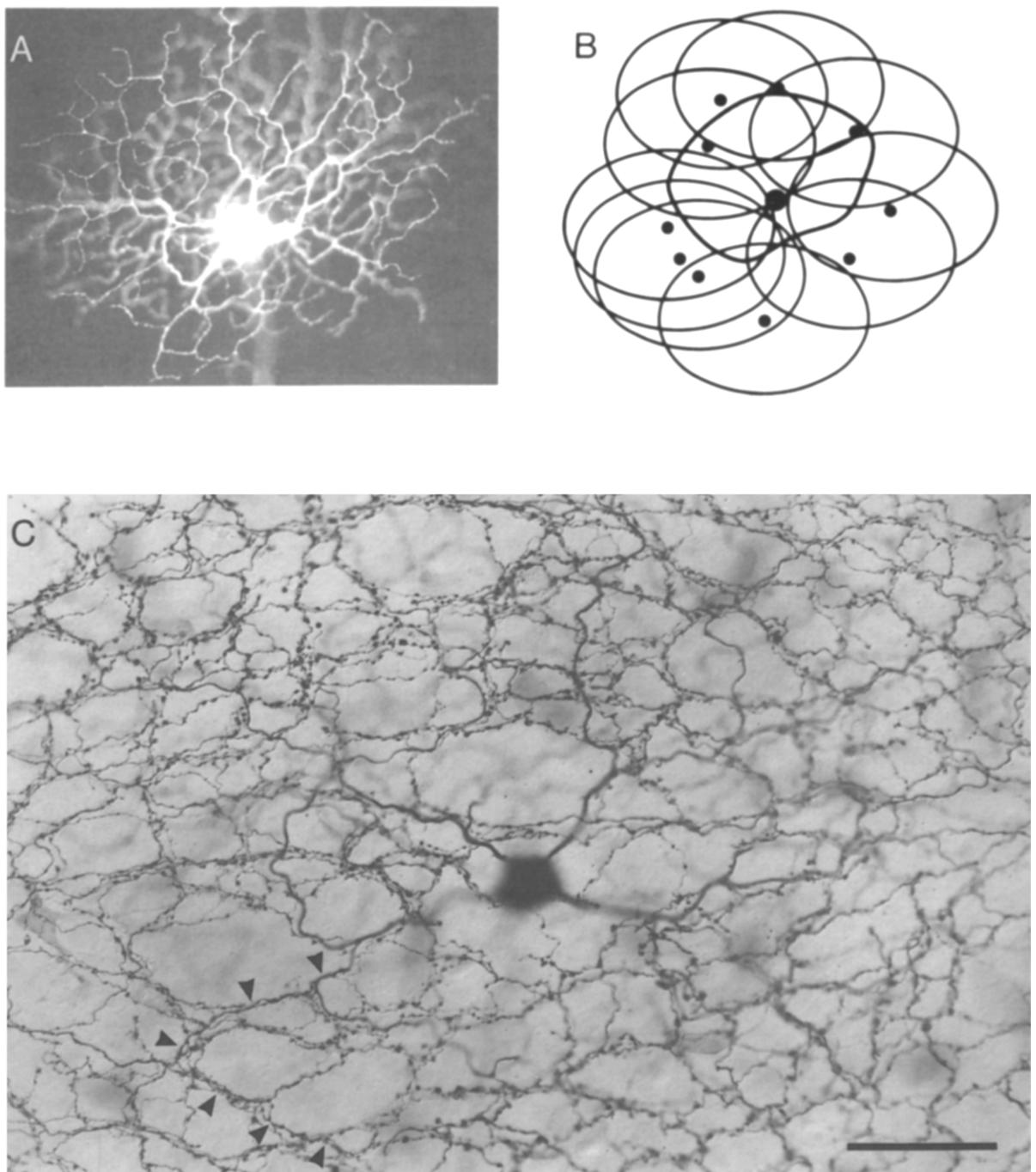


FIG. 41. Dendritic relationships between a direction-selective retinal ganglion cell and overlying cholinergic amacrine cells. A: a Lucifer-filled bistratified ganglion cell in the visual streak of rabbit retina, with the focus on the inner dendritic stratification in sublamina *b*; the cell has a distinctive looping morphology which is characteristic of the On – Off direction-selective ganglion cells. B: schematic diagram showing the cell positions and dendritic field overlap of the bistratified ganglion cell and of 10 surrounding *Cb* amacrines which were subsequently injected with Lucifer yellow. C: central portion of the filled ganglion cell and overlapping *Cb* amacrines, as viewed with Nomarski optics following photoconversion; in many places, the ganglion cell dendrites follow cholinergic fascicles, as indicated by arrowheads. Scale bar for C, 50  $\mu$ m. (From Vaney *et al.*, 1989a.)

amacrines, passing above and below each other. Many of the DS dendrites follow the course of a cholinergic fascicle (or vice versa), and their branch points often correspond to fascicle intersections; this holds for cholinergic amacrines on all sides of the ganglion cell and, thus, the dendritic architecture appears isotropic under the light microscope. The dendritic correlation is most pronounced for the looping terminal dendrites of the ganglion cell, whereas the primary and secondary dendrites follow courses that appear independent of the cholinergic fascicles.

Dendritic mapping of the injected cells showed that the regular terminal dendrites of the bistratified ganglion cell have a coarser periodicity than the cholinergic meshwork, with each terminal loop surrounding several cholinergic lacunae. It seems probable that the interconnecting fascicles follow the dendrites of other ganglion cells, including overlapping On–Off DS cells with preferred directions which complement that of the filled ganglion cell (Oyster and Barlow, 1967). This has intuitive appeal because the apparent irregularity of the cholinergic meshwork would result from the random superposition of four On–Off DS cells with regular arborizations, but it does not explain how the pattern of cholinergic fascicles in sublamina *a* is largely duplicated in sublamina *b* (Brandon, 1987b; Famiglietti and Tumosa, 1987). Cholinergic dendrites skirt around vertical retinal elements such as Müller cell processes, but these local deviations account for only the small cholinergic lacunae.

## 9.2. Cotransmission Model of Direction Selectivity

Two-slit apparent motion experiments on DS ganglion cells indicate that each part of the receptive field (a “subunit”) receives an excitatory input from overlying receptors and an inhibitory input from a larger area that spares the preferred direction; for null-direction movement, interaction between the pathways results in the excitatory input being vetoed by the preceding inhibitory input from the adjacent region (Barlow and Levick, 1965; Wyatt and Daw, 1975). Pharmacological experiments indicate that GABAergic neurons mediate this lateral

inhibition, because GABA antagonists abolish the direction selectivity of both On–Off and On-centre DS cells (Wyatt and Daw, 1976; Caldwell *et al.*, 1978). The type C amacrines appear to provide an appropriate substrate for such lateral interactions: they receive bipolar input over the whole dendritic field but their output synapses to ganglion cells are confined to the distal varicose zone (Famiglietti, 1983b, 1989).

It is proposed that each terminal dendrite of a DS ganglion cell receives cholinergic input only from type C dendrites that are located on the side of the dendritic field which is first stimulated by movement in the null direction; type C dendrites located on other sides of the dendritic field would provide cholinergic input to DS ganglion cells with different preferred directions (Vanney *et al.*, 1989a). It is further proposed that the asymmetric cholinergic input is symmetrically inhibited by all type C terminals; this GABAergic inhibition could act either presynaptically on the afferent cholinergic dendrite or postsynaptically at the ganglion cell membrane (Dowling, 1970; Torre and Poggio, 1978; Koch *et al.*, 1982). Although it is postulated that one amacrine system mediates both the excitation and inhibition, the spatial extent of the GABAergic inhibition would be asymmetric to, and larger than that of the cholinergic excitation (Fig. 42), in agreement with Ariel and Daw's findings (1982).

The light-evoked release of acetylcholine is potentiated by GABAergic antagonists (Massey and Redburn, 1982), suggesting that the inhibition occurs presynaptically; this is consistent with recent reports that synapses between type C dendrites are frequent (Millar and Morgan, 1987; Mariani and Hersh, 1988). Other evidence indicates that the inhibitory interactions occur at the ganglion cell level: when the cholinergic input is blocked with nicotinic antagonists, leaving intact the direct bipolar input to the ganglion cell, the light response is halved but the ganglion cell retains its direction selectivity (Ariel and Daw, 1982). However, it does not necessarily follow that these amacrines have two types of output synapses, one cholinergic and the other GABAergic: the GABA release may be carrier-mediated, resulting in generalized inhibition of both amacrine and ganglion cell dendrites within the

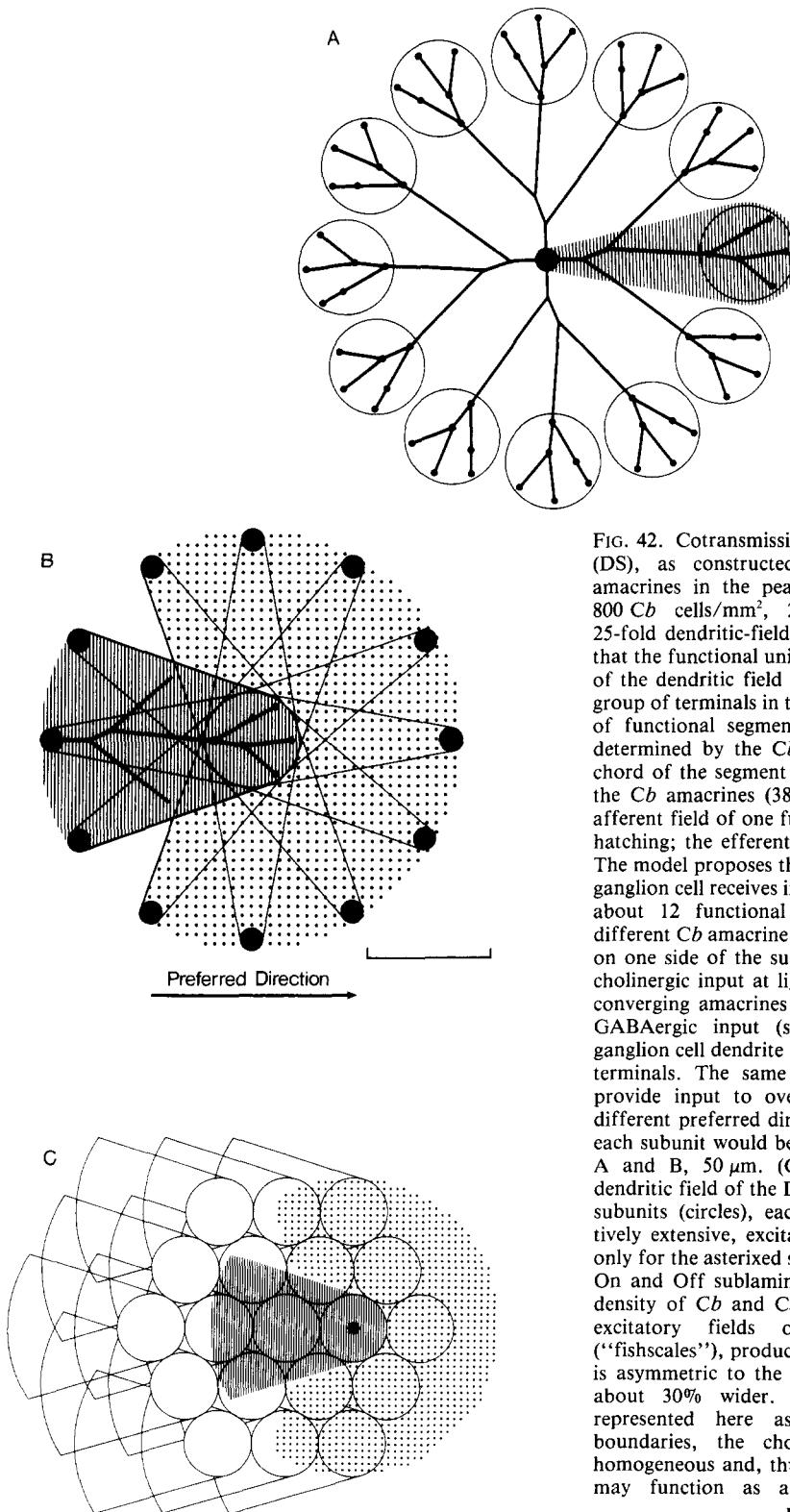


FIG. 42. Cotransmission model of direction selectivity (DS), as constructed for the On cholinergic (*Cb*) amacrine cells in the peak visual streak of rabbit retina: 800 *Cb* cells/mm<sup>2</sup>, 200  $\mu\text{m}$  dendritic-field diameter, 25-fold dendritic-field overlap. (A) The model proposes that the functional unit of cholinergic action is a segment of the dendritic field extending from the cell body to a group of terminals in the varicose distal zone; the number of functional segments in a dendritic field would be determined by the *Cb* amacrine density, such that the chord of the segment equals the intercellular spacing of the *Cb* amacrines (38  $\mu\text{m}$  for hexagonal packing). The afferent field of one functional segment is indicated with hatching; the efferent fields are outlined by circles. (B) The model proposes that a local region (subunit) of a DS ganglion cell receives input from the terminal dendrites of about 12 functional segments, each arising from a different *Cb* amacrine. At least three of the *Cb* amacrines on one side of the subunit would provide an excitatory cholinergic input at light On (hatching), while the other converging amacrines would only provide an inhibitory GABAergic input (stippling), either directly on the ganglion cell dendrite or presynaptically on the excitatory terminals. The same set of *Cb* segments would also provide input to overlapping DS ganglion cells with different preferred directions, but the excitatory field of each subunit would be shifted accordingly. Scale bar for A and B, 50  $\mu\text{m}$ . (C) The model proposes that the dendritic field of the DS ganglion cell encompasses many subunits (circles), each receiving input from comparatively extensive, excitatory and inhibitory fields (shown only for the asterisked subunit). The subunit density in the On and Off sublaminae would correspond to the local density of *Cb* and *Ca* amacrine cells respectively. The excitatory fields of the DS subunits overlap ("fishscales"), producing a combined receptive field that is asymmetric to the ganglion cell's dendritic field and about 30% wider. Although the DS subunits are represented here as discrete elements with fixed boundaries, the cholinergic substrate is effectively homogeneous and, thus, any point in the dendritic field may function as a subunit centre. (D. I. Vaney, unpublished.)

cholinergic fascicle (O'Malley and Masland, 1988a,b). Temporal differences in the onset and duration of the cholinergic excitation and GABAergic inhibition might then result from the different modes of transmitter release and inactivation.

If the inhibitory interactions that underlie direction selectivity occur presynaptically within the type C dendrites, then the terminal arborization arising from each primary dendrite needs to be isolated from the rest of the dendritic tree, consistent with Miller and Bloomfield's calculations (1983). Otherwise, the inhibition induced by centripetal movement into the amacrine field would spread throughout the dendritic tree, preventing the selective release of acetylcholine to centrifugal movement. In support of this, two-slit experiments indicate that the receptive-field diameter of On–Off DS cells is 2.5 to 3 times greater than the extent of asymmetric inhibition (Barlow and Levick, 1965; Wyatt and Daw, 1975); given that the DS receptive field is about 1.25 times larger than the dendritic field of a *Cb* amacrine at each eccentricity (M. Tauchi and R. H. Masland, unpublished), it is calculated that the inhibition extends for only half the width of a type C amacrine (Vaney *et al.*, 1989a).

In the cotransmission model, the cholinergic synapses are located on one side of the excitatory amacrine field, suggesting that facilitatory interactions in the preferred direction may contribute to the mechanism of direction selectivity; moreover, such facilitation would function over the same range as null-direction inhibition, but show tighter directional tuning. Grzywacz and Amthor (1988) recently examined the responses of On–Off DS cells to two-slit apparent motion; they concluded that direction selectivity involves both inhibitory and facilitatory mechanisms that are spatially asymmetric, and which extend over much of the receptive field centre. Barlow and Levick's (1965) original experiments also indicated that preferred-direction apparent motion gives rise to limited facilitation, but over a shorter range than was apparent for null-direction inhibition.

Barlow and Levick (1965) proposed that the complete mechanism for direction selectivity is contained within a subunit of the receptive field

extending about 0.25° in the preferred-null axis, corresponding to 42 µm on the retina. However, this figure was based on the minimum spatial window for directional discrimination of edge motion and more correctly represents the distance between DS subunits rather than their spatial extent (cf. Grzywacz and Amthor, 1988). The cotransmission model proposes that each DS subunit comprises a broad dendritic assemblage as represented in Fig. 42B, with the retinal distribution of the subunits defined by the cell mosaic of type C amacrines. This would give a subunit spacing of 38 to 47 µm within 3 mm of the peak visual streak, assuming hexagonal packing of the *Cb* amacrines. (Some authors have argued that retinal interneurons with a single asymmetric process may underlie null-direction inhibition (e.g. Mariani, 1982), but 6,000 processes/mm<sup>2</sup> would be needed to serve the On–Off DS cells in central rabbit retina, requiring one-quarter of the amacrine cells. By comparison, the cotransmission model appears remarkably parsimonious in its cellular requirements).

The model rationalizes the 25- to 70-fold overlap of *Cb* dendritic fields in two ways (Vaney, 1985b; Vaney *et al.*, 1989a). Firstly, the overlap is not redundant because each terminal field that provides input to a local region of retina has a different vectorial component. Secondly, only the varicose distal zone on one side of each amacrine would provide cholinergic input to an overlying DS ganglion cell: in the visual streak, therefore, a DS subunit would receive excitatory input from as few as three or four *Cb* amacrines. Wide amacrine fields that are regularly spaced at narrow intervals across the retina would ensure that the DS subunit was responsive to both small and large displacements, corresponding to slow and fast movements of the visual stimulus. The concept that pronounced functional asymmetries may be derived from the most symmetrical neurons in the retina is not without irony.

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