Reports

Morphological Identification of Serotonin-Accumulating Neurons in the Living Retina

DAVID I. VANEY

Neurons that accumulate the transmitter serotonin have been identified in the living retina by being labeled with 5,7-dihydroxytryptamine (5,7-HT), an autofluorescent serotonin analog. Iontophoretic injection of Lucifer yellow into the labeled cells under microscopic control revealed that the serotonin-accumulating neurons in rabbit retina constitute two morphological types of amacrine cells, termed S1 and S2, whose distal dendrites are stratified at the inner margin of the inner plexiform layer. The dendritic overlap of the S1 type is extraordinarily large: each point on the retina is covered by the fields of 550 to 900 S1 amacrines, and 6 to 8 meters of their dendrites are packed into each square millimeter of retina. Such a pervasive neuropil may provide an effective substrate for diffuse transmitter release, as proposed for serotonergic fibers elsewhere in the central nervous system.

NEURONS THAT CONTAIN OR ACCUMULATE serotonin (5-hydroxytryptamine, 5-HT) can be identified by formaldehyde-induced fluorescence, immunohistochemistry, or uptake autoradiography. In addition, the cytotoxic transmitter analogs—5,6-dihydroxytryptamine (5,6-HT) and 5,7-dihydroxytryptamine (5,7-HT)—are also accumulated by serotonergic neurons and form a bright fluorophore with formaldehyde (*1*). Because all these methods require fixation of the tissue, until now there has been no way to identify serotonergic neurons in living material. However, the 5,7-HT is itself moderately fluorescent and can be visualized in living neurons under ultraviolet excitation.

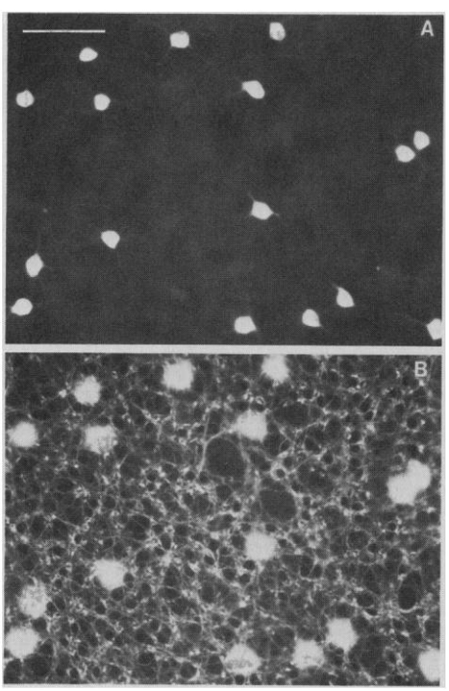

Fig. 1. Micrographs of a peripheral field of a formaldehyde-fixed rabbit retina labeled with 5,7-HT flat-mounted and viewed from the vitreal side. The fluorescent cell bodies are located in the amacrine sublayer of the inner nuclear layer (A), and their dendrites are stratified at the inner margin of the inner plexiform layer (B). The field is overlapped by the dendritic trees of hundreds of these amacrine cells, which form a rich plexus that blankets the retina. Excitation filter, 355 to 425 nm. Barrier filter, long pass (LP) 460 nm. Scale bar, 50 μm.

Retinal interneurons containing endogenous serotonin have been localized in fish, amphibians, reptiles, and birds, and many mammalian retinas also contain a subset of neurons that actively accumulate serotonin and related indoleamines (*2*). The distribution of their cell bodies and the arrangement of their processes in the inner retina have been described in detail, but the dendritic morphologies of these putative serotonergic neurons have not been identified.

In the rabbit retina, about 99 percent of the serotonin-accumulating cells are located in the amacrine sublayer, and their dendrites are concentrated in the inner third of the inner plexiform layer (*3*). The lacelike dendrites are readily apparent in wet-mounted retina that has been incubated in the 5,7-HT and then fixed with formaldehyde (*4*): the fluorescent dendrites blanket the retina, skirting around the stalks of Müller's cells and the terminals of rod bipolar cells (Fig. 1). Although the branching pattern of the primary dendrites can be traced in such material, the labeled processes soon become lost within the dense plexus, and it is impossible to map the dendritic trees of individual cells.

To establish the dendritic morphology of the serotonin-accumulating neurons, we mounted the living retina in a tissue chamber on a fixed-stage microscope and superfused with an oxygenated medium; the blue-fluorescing cell bodies of the neurons labeled with the 5,7-HT were impaled with a micropipette under a ×40 water-immersion objective, and their dendrites were filled with Lucifer yellow CH by iontophoresis (Fig. 2). This technique has been used to probe the cholinergic amacrine cells in rabbit retina and the rod amacrine cells in cat retina (*5*). The ability to combine whole-population and single-cell studies in the same tissue, coupled with the great efficiency provided by visual control of the injection pipette, unlocks these amacrine systems for focused investigation of their structure and function.

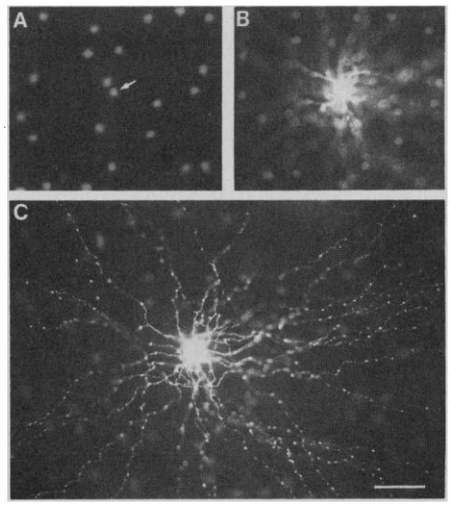

Fig. 2. (A) The labeled amacrines seen in isolated living retina under ultraviolet excitation. (B) The cell body indicated by the arrow was impaled with a micropipette and injected with Lucifer yellow. (C) Raising the focus to the inner margin of the plexiform layer reveals the full dendritic morphology of this S2 amacrine cell. Excitation filters: (A) 340 to 380 nm, (B and C) 400 to 440 nm. Barrier filters: (A) LP 430 nm, (B and C) LP 520 nm. Scale bar, 50 μm.

Injecting dye into more than 300 labeled cells revealed that the serotonin-accumulating neurons in the rabbit retina are a heterogeneous population made up of two discrete types of amacrine cells (S1 and S2) that can be distinguished by the shape and size of their dendritic trees (Fig. 3). The S1 amacrines are characterized by their geometric form and the sparse, leaf-like varicosities in the middle of the arborization; five to eight primary dendrites usually branch close to the cell body to form 19 to 30 radial dendrites that become stratified at the inner margin of the inner plexiform layer. The fine distal dendrites are uniformly thin and can

National Vision Research Institute of Australia, 386 Cardigan Street, Carlton, Victoria 3053, Australia.

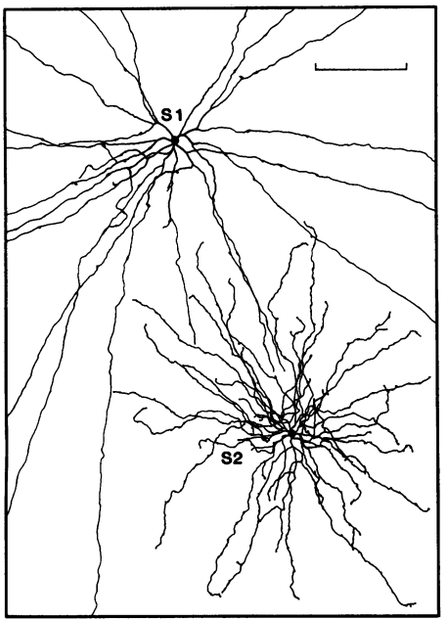

Fig. 3. Serotonin-accumulating neurons in the rabbit retina. The two discrete types of amacrine cells, termed S1 and S2, can be distinguished by the shape and size of their dendritic trees. Representative cells from a retinal field in the inferior periphery are illustrated. Scale bar, 200 μm.

be mapped only when viewed at high numerical aperture, with the field diaphragm closed down; these dendrites end abruptly, neither branching nor forming terminal varicosities. The dendritic field diameter of S1 amacrines ranges from 1200 μm in the central retina to about 3500 μm in the periphery, making this cell type one of the largest in the rabbit retina.

The S2 amacrines have a structure similar to that of the S1 amacrines, and the distal dendrites of both types are stratified in the same plane. However, the dendrites of the S2 amacrines follow a more sinuous course and have beaded varicosities along their length. At each eccentricity the dendritic field diameter of the S2 amacrines is one-quarter that of the S1 amacrines, ranging from 300 μm in the central retina to more than 1000 μm in the periphery. None of the fluorescent cells injected with Lucifer yellow had a dendritic morphology resembling that of the dopaminergic amacrines, although it has been reported that these cells also accumulate indoleamines (*6*).

Cells labeled with the fluorescent analog in the superfused rabbit retina fade under ultraviolet excitation; this fading is correlated with a shift in the emission spectrum from blue to yellow and an increased yield to violet excitation. The fluorescence shift is not synchronous: a subset of the cells fade 2 to 10 seconds before the others, so there is a temporal window during which 50 to 60 percent of the labeled amacrines appear blue and the remainder yellow. If the bleaching is halted within this period, the color difference can be protected against further fading simply by cover-slipping the unfixed retina with incubation medium. Intracellular injection of Lucifer yellow into the labeled neurons consistently showed that the early-fading (yellow) cells are the S2 amacrines and the late-fading (blue) cells are the S1 amacrines.

In such material, the two types of amacrines can be mapped in retinal fields at a range of eccentricities and their spatial distribution assessed quantitatively (Fig. 4). The cell bodies of each type form a regular array, comparable to that of the ON-cholinergic amacrines in the rabbit retina (*7*). The independent overlay of the two populations forms a mosaic of fluorescent cells that is less ordered than either of the constituent arrays. Although the density of labeled cells increases sixfold from the peripheral retina (150 cells per square millimeter) to the central visual streak (900 cells per square millimeter), the ratio of the S1 and S2 types is uniform throughout the retina.

Each point on the retina is overlapped by the dendritic trees of many labeled amacrines. The coverage (cell density multiplied by dendritic field area) of the S2 amacrines ranges from 30 in the central retina to about 60 in the periphery; this is similar to the overlap of the cholinergic amacrines in the rabbit retina, but is an order of magnitude greater than that of rod amacrine cells and retinal ganglion cells in the cat retina (*5, 8*). The dendritic field coverage of the S1 amacrines is 20 times that of the S2 amacrines, ranging from 550 in the central retina to 900 in the periphery. In each square millimeter of retina, the radial dendrites of S1 amacrines total 6 to 8 m; if these fine processes were woven as a cloth, the spacing of the warp and weft would be only 0.3 μm.

Electron microscopy of the rabbit retina labeled with the 5,6-HT indicates that the processes of serotonin-accumulating amacrines are both pre- and postsynaptic to bipolar cell axons (*9*) that have the form and position of rod bipolars (*10*). Each axon terminal forms several lobes whose ribbon structures are presynaptic to a pair of processes, usually the varicosity of a labeled amacrine and the unlabeled dendrite of a rod amacrine cell. The varicosities resemble the regular beaded dendrites of S2 amacrines rather than the thin uniform dendrites of S1 amacrines. The terminal spread of a rod bipolar is less than the varicosity spacing in the dendritic tree of an S2 amacrine, which suggests that the multiple varicosities postsynaptic to each bipolar arise from different amacrine cells. This may explain the 30- to 60-fold overlap of the S2 amacrines.

Although the intervaricose segments of the labeled processes have structures resembling pre- and postsynaptic specializations, the described circuitry of serotonin-accumulating amacrines is based on the identified connections of varicosities and proximal dendrites (*9*). The fine distal dendrites of S1 amacrines, which dominate the labeled plexus, seem to have no place in that scheme. However, it may not be necessary for the binding and release of transmitters to occur at discrete synapses on S1 dendrites, because their blanket coverage provides an appropriate substrate for diffuse interactions with neighboring processes. Serotonergic terminals elsewhere in the brain are also characterized by a paucity of synaptic connections and, for these neurons, diffuse release of transmitter seems consistent with a neuromodulatory function (*11*).

Now that serotonergic neurons can be labeled in living tissue and injected with diffusible markers, the synaptic connections of single identified cells can be elucidated. The serotonin-accumulating amacrines are

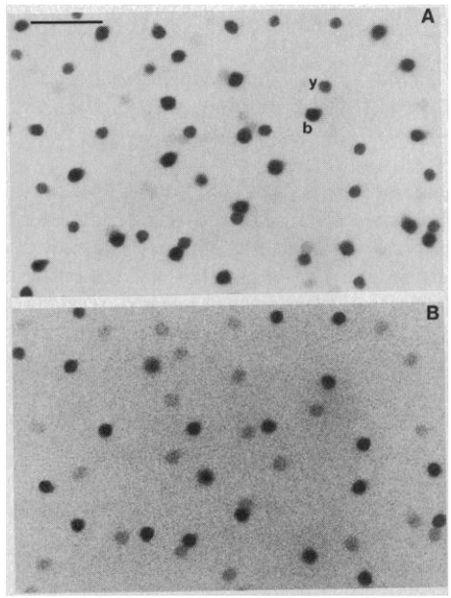

Fig. 4. Differential fading of labeled cells in superfused rabbit retina revealing the cellular arrays of the S1 and S2 amacrines. These negative micrographs were printed from color transparencies. (A) Under ultraviolet excitation, the S1 cells fluoresce blue (b) and the faded S2 cells fluoresce blue-yellow (y). (B) When the same field is viewed under violet excitation, the yellow fluorescence of the S2 cells appears much stronger than that of the S1 cells. Adjacent S1 and S2 cells often seem to form pairs but this spurious order arises simply from the overlay of two regular arrays. A third population of amacrines with weakly labeled cell bodies is apparent under ultraviolet, but not violet, excitation; their morphological and fluorescent properties are distinct from those of S1 and S2 amacrines (*13*). Excitation filters: (A) 340 to 380 nm, (B) 400 to 440 nm. Barrier filters: (A) LP 430 nm, (B) LP 520 nm. Scale bar, 50 μm.

particularly accessible because the retina is a thin sheet of tissue that can be readily isolated from the eye. It should also be feasible to use these techniques on brain-slice preparations to study serotonergic neurons in the raphe nuclei.

Note added in proof: Sandell and Masland (*12*) have identified two morphological types of indoleamine-accumulating amacrines in the rabbit retina by injecting Lucifer yellow into cells labeled with 5,7-HT in retina fixed in formaldehyde. Although their type 1 and type 2 cells seem to correspond to the S1 and S2 amacrines of this study, Sandell and Masland reported that the dendritic field diameter of type 1 cells averaged only 376 μm, giving a coverage factor of 16 in the peripheral retina and 50 in the visual streak. In fixed tissue, Lucifer yellow may not diffuse along very thin processes, and the strong fluorescence of the plexus labeled with the 5,7-HT would mask any Lucifer yellow fluorescence in the S1 radial dendrites.

REFERENCES AND NOTES

1. H. G. Baumgarten and A. Björklund, *Annu. Rev. Pharmacol. Toxicol.* **16**, 101 (1976).
2. B. Ehinger, *Vision Res.* **23**, 1281 (1983); N. N. Osborne, *Prog. Retinal Res.* **3**, 61 (1983).
3. B. Ehinger and I. Florén, *Cell Tissue Res.* **175**, 37 (1976); K. Negishi, T. Teranishi, S. Kato, *Neurosci. Lett.* **45**, 27 (1984).
4. To label the serotonin-accumulating neurons, we injected 40 μg of 5,7-HT into the vitreous 60 to 90 minutes before enucleation, or the eyecup was incubated at 37°C in an oxygenated medium [A. Ames and F. B. Nesbett, *J. Neurochem.* **37**, 867 (1981)] containing the 5,7-HT (10 mg/liter, $2.5 \times 10^{-5} M$) for 40 to 60 minutes. Both populations of amacrines accumulated the 5,7-HT in vitro at $10^{-6} M$, as detected by formaldehyde-induced fluorescence.
5. D. I. Vaney, *Proc. R. Soc. London Ser. B* **220**, 501 (1984); M. Tauchi and R. H. Masland, *ibid.* **223**, 101 (1984); D. I. Vaney, *ibid.* **224**, 475 (1985).
6. B. Ehinger and I. Florén, *Exp. Eye Res.* **26**, 1 (1978); N. C. Brecha, C. W. Oyster, E. S. Takahashi, *Invest. Ophthalmol.* **25**, 66 (1984).
7. D. I. Vaney, L. Peichl, B. B. Boycott, *J. Comp. Neurol.* **199**, 373 (1981); R. H. Masland, J. W. Mills, S. A. Hayden, *Proc. R. Soc. London Ser. B* **223**, 79 (1984).
8. H. Wässle, L. Peichl, B. B. Boycott, *Proc. R. Soc. London Ser. B* **212**, 157 (1981); H. Wässle, B. B. Boycott, R.-B. Illing, *ibid.*, p. 177.
9. B. Ehinger and I. Holmgren, *Cell Tissue Res.* **197**, 175 (1979).
10. G. Raviola and E. Raviola, *Am. J. Anat.* **120**, 403 (1967); H. Kolb and E. V. Famiglietti, *Science* **186**, 47 (1974).
11. R. K. Dismukes, *Nature (London)* **269**, 557 (1977); A. Beaudet and L. Descarries, *Neuroscience* **3**, 851 (1978).
12. J. H. Sandell and R. H. Masland, *Soc. Neurosci. Abstr.* **11**, 337 (1985).
13. In the peripheral retina, the cell bodies of the third population of 5,7-HT–accumulating neurons were only faintly labeled and smaller than the S1 and S2 amacrines. The contrast in fluorescence intensity and soma size was less pronounced for cells in the visual streak, but discrimination is a problem only with retinal tissue that has been strongly fixed after incubation periods of 2 hours or longer. Injecting dye into these weakly labeled cells revealed that they are medium-field amacrines that branch in the middle of the inner plexiform layer. When the superfused retina is bleached under ultraviolet excitation, these amacrine cells show a violet fluorescence that contrasts with the blue-yellow fluorescence of the faded S1 and S2 amacrines.

14. I thank P. M. Whitington, A. Hughes, and H. Wässle for comments on the manuscript; P. D. Larsen for animal care; and G. E. Whitington for help with the illustrations. The gift of a microscope camera from the Thomas and Lockyer Charitable Trust is gratefully acknowledged. Supported by National Health and Medical Research Council grants to A. Hughes and D.I.V. The NVRI is an Affiliate Institution of the University of Melbourne.

18 October 1985; accepted 25 February 1986

A Budget for Continental Growth and Denudation

DAVID G. HOWELL AND RICHARD W. MURRAY

Oceanic crustal material on a global scale is re-created every 110 million years. From the data presented it is inferred that potential sialic material is formed at a rate of about 1.35 cubic kilometers per year, including hemipelagic volcanic sediments that accumulate at a rate of about 0.05 cubic kilometer per year. It is estimated that the influx of 1.65 cubic kilometers per year of terrigenous and biogenic sediment is deposited on the deep ocean, and this represents continental denudation. Because all this material is brought into a subduction zone, continental accretion rates, which could include all this material, may be as high as 3.0 cubic kilometers per year with a potential net growth for continents of 1.35 cubic kilometers per year.

ALTHOUGH THE CURRENT VOLUME of continental crust is a relatively well-defined value of 7.6×10^9 km^3 (*1*), the amount and rates of continental growth are controversial (*2–5*). From magnetic lineations, we know that the average age of ocean crust is 55 million years (*6*). Applying this age to the volumes and global budgets of oceanic sediments, ocean crust, volcanic island arcs, seamounts, and circum-Pacific accreted terranes, we can model (i) continental denudation rates, (ii) the growth rate of new crystalline material, specifically volcanic island arcs and seamounts, and (iii) the mix and percentage of rock types available for continental accretion. Comparison of these data to the area, volume, and composition of circum-Pacific terranes provides a qualitative test for the hypothesis of continental growth outlined below. These relations support the contention of some geologists and geochemists (*5*, *7*, *8*) that only a small percentage of sediment is subducted and recycled into the mantle. Thus, most sediment, particularly the clastic-terrigenous component, is accreted to continental margins along with newly formed volcanic rock. In this way, continental material is recycled and the accretion of newly formed volcanic rock results in the net growth of continents.

We used isopach maps (Fig. 1) to determine the total volume of oceanic sediment. Sediment shoreward from the base of the continental rise is not included in our calculations as such material is still part of the continental mass. Major divisional isopachs were plotted on equal area projections at a common scale of 1:10,000,000. The volume between each isopach is described by volume (cubic kilometers) = area (square kilometers) × thickness (kilometers).

Summation of these volumes yields a total sediment volume for each ocean basin (Table 1). This volume is made up of five ingredients: terrigenous debris, volcanic sediment, biogenic silica, biogenic carbonate, and porosity. We did not account for diagenetic products as a separate component.

The determination of the volume of terrigenous plus volcanic material (the nonbiogenic constituents) follows a process that requires as a first step biogenic sedimentation data.

The amount of biogenic silica can be calculated from published estimates of silica accumulation rates based on studies of Deep Sea Drilling Project (DSDP) cores (*9*): silica thickness (kilometers) = average accumulation rate (grams per cubic centimeter per year) × duration of core (years) × 1/density (1/2.30 g/cm^3) × unit conversion (1 km/100,000 cm).

Only 37% of the DSDP sites used in our calculations penetrated the entire thickness of sediment above the basaltic ocean floor. For the remaining 63% of the cores, we assume that the average biogenic silica value continues below the base of the drilled interval down to the basalt floor. The calculated thickness of biogenic silica must also be expanded to account for in situ porosity. The porosity at a depth equal to half the total sediment thickness at the DSDP site

D. G. Howell, U.S. Geological Survey, Menlo Park, CA 94025.
R. W. Murray, U.S. Geological Survey, Menlo Park, and University of California, Berkeley, CA 94720.