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L-Glutamic acid: A neurotransmitter candidate for cone photoreceptors in human and rat retinas

(aspartate aminotransferase/L-aspartic acid/autoradiography/immunocytochemistry)

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ABSTRACT **We have combined immunocytochemical localization of L-aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1; glutamic-oxaloacetic transaminase) with autoradiographic localization of high-affinity uptake sites for L-glutamate or L-aspartate to identify the neurotransmitters of mammalian photoreceptors. In both human and rat retinas, high aspartate aminotransferase immunoreactivity is found in cones but not in rods; certain putative bipolar and amacrine cells are also heavily stained. In the human retina, and perhaps also in the rat retina, cones possess a high-affinity uptake mechanism for L-glutamate but not L-aspartate, whereas rods and Müller (glial) cells take up both L-glutamate and L-aspartate. Taken together, our results indicate that (*i*) L-glutamate is much more likely than L-aspartate to be the transmitter for human cones, and possibly for cones of other mammalian species as well, and (*ii*) major differences exist between mammalian cones and rods in the transport and metabolism or utilization of L-aspartate and L-glutamate.**

The transmitter substances released by vertebrate photoreceptors onto second-order retinal neurons have not been identified with certainty, but an increasing body of evidence has implicated L-glutamate, L-aspartate, or both as strong candidates for this role, at least in some photoreceptors (1–15). Externally applied aspartate or glutamate causes depolarization and suppression of photoreceptors in horizontal cells of teleost (1, 5, 6, 8), skate (2), and turtle (3) retinas, as well as hyperpolarization of on-center bipolar cells and depolarization of off-center bipolar cells in the carp retina (4). However, high (millimolar) concentrations of aspartate or glutamate are generally required to elicit significant effects in the intact retina, and the effects show little selectivity between glutamate and aspartate. These concerns have been partially resolved by recent observations that isolated or cultured horizontal cells from cyprinid retinas can be depolarized by micromolar concentrations of L-glutamate and its agonists and that L-aspartate as well as D-aspartate and D-glutamate are much less effective at these concentrations (11, 15).

Certain photoreceptors possess high-affinity uptake mechanisms for glutamate, aspartate, or both (7, 9, 10). In the goldfish retina, rods are much more effective in taking up L-glutamate than L-aspartate, whereas red- and green-sensitive cones show similar affinity for aspartate and glutamate (9). In contrast, human cones are much more effective in accumulating L-glutamate than L-aspartate, whereas human rods appear to take up aspartate and glutamate equally well (7, 10). These results show that, unlike acidic amino acid transport systems described in the brain (16–18), certain photoreceptors have uptake systems that clearly distinguish between L-aspartate and L-glutamate.

More recently, an immunohistochemical approach has been used to localize putative glutamate/aspartate neurons. Altschuler *et al.* (19) proposed that the pyridoxal phosphate-dependent enzyme aspartate aminotransferase (AATase; L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1; also called glutamic-oxaloacetic transaminase)

$$\begin{array}{ccc} \text{L-Aspartate} & & \text{L-Glutamate} \\ + & \rightleftharpoons & + \\ \text{2-Oxoglutarate} & & \text{Oxaloacetate} \end{array}$$

is present in high concentrations in aspartate/glutamate neurons. They showed by immunofluorescence that AATase was concentrated in cones and in some putative amacrine cells of the guinea pig retina (13). Unfortunately, as these investigators pointed out, AATase may also be present in neurons that require large amounts of aspartate or glutamate as precursor for the biosynthesis of a transmitter such as γ-aminobutyric acid (GABA).

We felt that the combined use of these two techniques might be particularly useful in the analysis of putative glutamate/aspartate neurons. Accordingly, we have compared the localization of AATase (by using immunocytochemistry) with the localization of high-affinity uptake sites for aspartate and glutamate (by using autoradiography) in human and rat retinas. Results from these combined methods suggest that (*i*) L-glutamate is a much more likely transmitter candidate for mammalian cones than is L-aspartate, and (*ii*) AATase may not be exclusively localized to aspartate/glutamate neurons in the retina.

MATERIALS AND METHODS

A partially purified preparation of AATase (glutamic-oxaloacetic transaminase type 1 from Sigma) was further purified by preparative polyacrylamide gel electrophoresis, emulsified with Freund's adjuvant, and injected into rabbits as described by Altschuler *et al.* (19). After three biweekly injections an antiserum was obtained (Fig. 1) that produced a single precipitin arc when tested by immunodiffusion or immunoelectrophoresis against purified pig heart AATase, a crude rat brain extract, or a mixture of the two.

For the immunocytochemical localization of AATase, isolated retinas from human eyes or male Sprague–Dawley rats were fixed by overnight immersion in 4% (wt/vol) paraformaldehyde in 0.15 M sodium phosphate, pH 7.4, then cut into 50-μm sections with a vibrating microtome, using techniques described in detail previously (20). The sections were incubated,

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Abbreviations: AATase, aspartate aminotransferase; GABA, γ-aminobutyric acid.

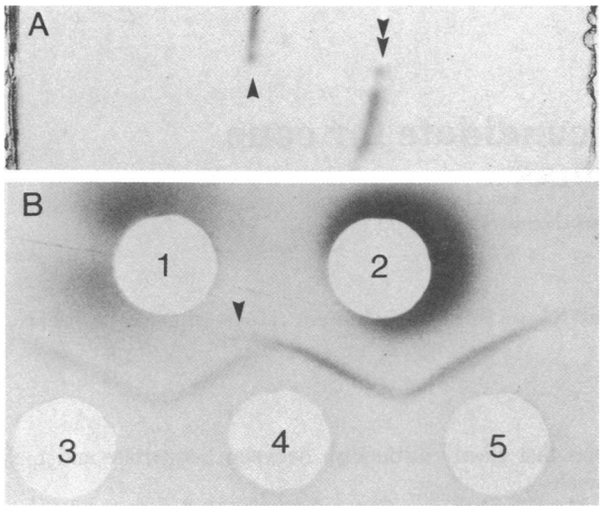

FIG. 1. Preparation of anti-AATase serum. (*A*) $NaDodSO_4$/polyacrylamide gel electrophoresis pattern of 10 μg of electrophoretically purified pig heart AATase (double arrow) and of the principal contaminant in commercial preparations (single arrow). Migration in this 10% gel was from left to right. (*B*) Ouchterlony double diffusion of anti-AATase serum (wells 3–5) against the electrophoretically purified AATase (well 2) and a crude homogenate of rat brain (well 1). The spur (arrow) indicates incomplete immunological crossreactivity between the original immunogen, which was obtained from pig heart, and rat brain AATase.

with intervening washing, in anti-AATase serum (1:2,000 to 1:16,000), goat antiserum to rabbit IgG, and rabbit peroxidase-antiperoxidase, then stained with 0.06% diaminobenzidine and 0.012% hydrogen peroxide. Control sera were made by incubating anti-AATase serum with purified AATase for 24 hr before immersing the sections. Phosphate-buffered saline with 0.2% Triton X-100 was used throughout. After immunocytochemical staining, the sections were treated with osmium tetroxide, dehydrated, and flat-embedded in epoxy resin. Serial 5-μm sections were then cut *en face* through the entire depth of the 50-μm block, using glass knives.

The autoradiographic procedure for studying [^{3}H]glutamate and [^{3}H]aspartate uptake has been described in detail (7, 9, 10, 21). Isolated retinas were incubated at room temperature under ambient illumination with oxygenated mammalian Ringer's solution supplemented with 20% fetal calf serum and 1–10 μM L-[^{3}H]glutamic acid (43.0 Ci/mmol), D-[^{3}H]aspartic acid (20.0 Ci/mmol), or L-[^{3}H]aspartic acid (17.3 Ci/mmol), all from New England Nuclear (1 Ci = 3.7×10^{10} Bq). After a 2-min wash with unlabeled Ringer's solution they were fixed at 4°C overnight with 2% (wt/vol) glutaraldehyde and 2% (wt/vol) formaldehyde in 120 mM sodium phosphate, pH 7.4. The tissues were treated with osmium tetroxide, dehydrated, embedded in epoxy resin, sectioned at 1 to 2 μm thick, and processed for light microscope autoradiography as described (7, 21).

Three human retinas were also used for both immunocytochemical and autoradiographic studies. The human eyes were obtained through the Lions Eye Bank, Cullen Eye Institute, Houston. Soon after death (2–30 min), the eyes were enucleated and the retinas were isolated as described (7, 10).

RESULTS

In the rat retina (Fig. 2), strong AATase immunoreactivity was found in cells with the morphological characteristics of cones, namely, large synaptic terminals, slender axons, and cell bodies located within the distal third of the outer nuclear layer; the inner segments of these cells were also stained. Rods were unstained. Because of the similarity in morphology between rat rods and cones, we cannot exclude the possibility that some cones were not stained for AATase.

In addition to the stained cones, certain neurons morphologically similar to amacrine and bipolar cells also contained immunoreactive AATase (Fig. 2 *A* and *E*). The processes of stained amacrine cells formed several distinct, dense laminae within the inner plexiform layer, and their somas, but not their nuclei, were labeled. Immunoreactive bipolar cells were seen in the most distal portion of the inner nuclear layer, and sent delicate axonal processes vitreally. Sections incubated in AATase-adsorbed sera were devoid of immunocytochemical reaction product (Fig. 2*B*).

The pattern of AATase localization in the human retina (Fig. 3) was very similar to that observed in the rat. Cones were well stained from the inner segment to the synaptic pedicle and were readily distinguished from the unstained rods. Cone outer segments and nuclei were not stained. Again, all staining was blocked by adsorption of the antiserum with AATase (Fig. 3*B*). Occasional presumptive amacrine cell bodies and bipolar cells bodies were darkly stained (Fig. 3 *A* and *C*). Numerous small dense deposits of reaction product were scattered throughout the inner plexiform layer, but they were less clearly laminated than in the rat retina (Fig. 3*A*).

As reported earlier (10), under our experimental conditions all photoreceptors, rods as well as cones, were labeled uniformly after exposure of human retinas to L-[^{3}H]glutamate; no one photoreceptor cell type appeared to be more heavily labeled than any other (Fig. 4*B*). Exposure to L-[^{3}H]aspartate, on the other hand, led to the selective labeling of rods (Fig. 4*A*). Cones appeared to exclude labeled aspartate, to the extent that they were devoid of silver grains even in heavily overexposed autoradiographs (Fig. 4*A*). Additionally, in experiments in which D-[^{3}H]aspartate was used instead of L-[^{3}H]aspartate, a similar uptake pattern was obtained autoradiographically. The high-affinity acidic amino acid uptake systems of cones therefore appear to be capable of distinguishing between glutamate and aspartate. In more proximal retinal layers, radiolabeled glutamate and aspartate were predominantly localized to glial cells, a result in agreement with published reports (7, 10, 22–24).

In the rat retina, occasional unlabeled photoreceptors were apparent in autoradiographs of tissues exposed to L-[^{3}H]aspartate (Fig. 4*C*). We cannot determine whether these unlabeled rat photoreceptors are rods or cones. Clear exclusion of aspartate by cones was difficult to discern in these autoradiographs due to the small number of cones in the rat retina and because of the morphological similarity between rods and cones in this species. As in the human retina, rat photoreceptors were uniformly labeled after exposure to L-[^{3}H]glutamate (Fig. 4*D*). We found generally heavy labeling of photoreceptor cell bodies and inner segments in the outer nuclear layer and heavy labeling of glial processes and cell bodies in other retinal layers, with glutamate as well as aspartate incubations (Fig. 4 *C* and *D*).

DISCUSSION

Our immunocytochemical studies show that, in the human retina, cones contain high levels of AATase-like immunoreactivity (Fig. 3). Our autoradiographic results, on the other hand, indicate that a selective uptake mechanism for L-glutamate is present in all human photoreceptors and glial cells, whereas a similar mechanism for L-aspartate and D-aspartate is found in human rods and glial cells but is not present in cones (Fig. 4 *A* and *B*). Similar conclusions may be made concerning the rat

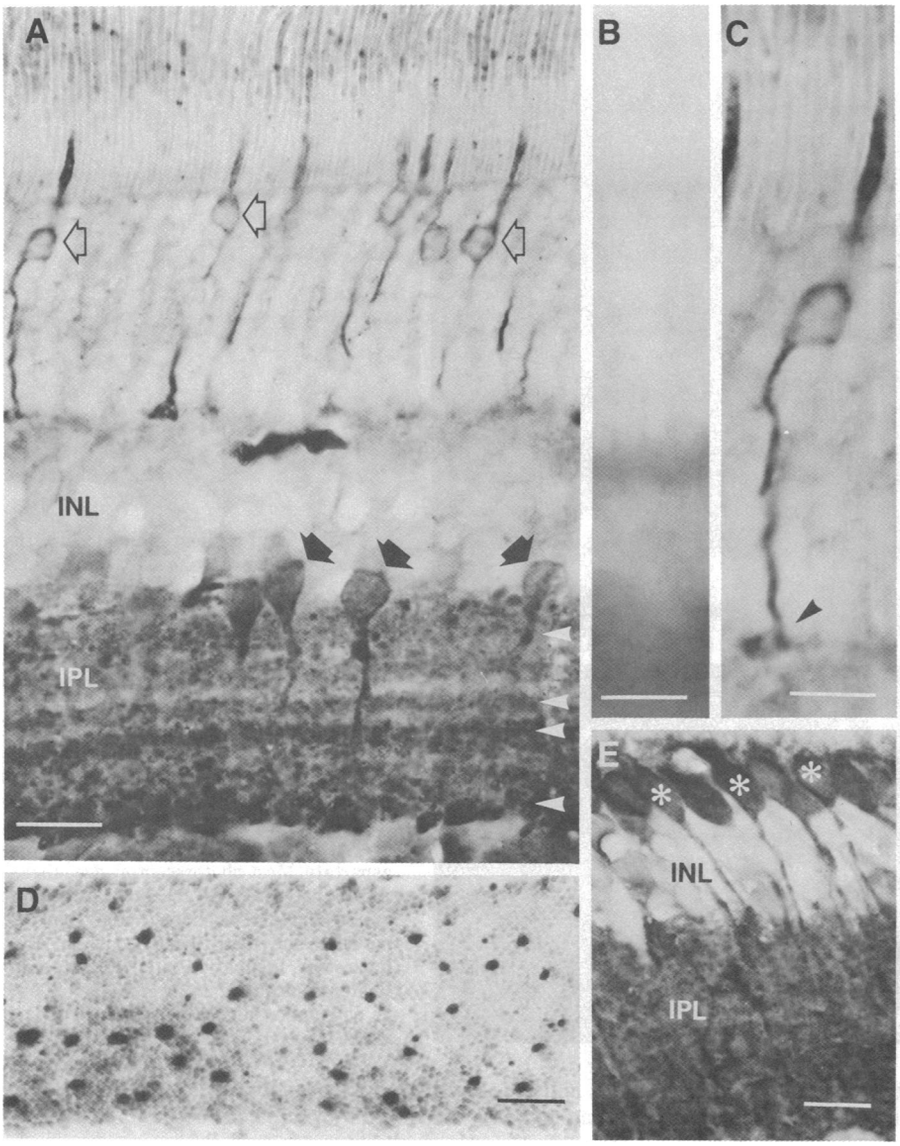

FIG. 2. Immunocytochemical localization of AATase in the rat retina. (*A*) In 5-μm-thick retinal cross sections, AATase immunoreactivity was seen in presumptive cones from inner segment to synaptic terminal (open arrows), and in some amacrine cell bodies (filled arrows). The processes of AATase-containing amacrine cells formed several distinct layers within the inner plexiform layer (IPL) (arrowheads). INL, inner nuclear layer. (*B*) Control section, incubated with anti-AATase serum that had been absorbed with purified AATase, shows complete absence of specific staining in the retina. (*C*) Higher magnification of an AATase-containing cone, showing the characteristically large synaptic pedicle (arrowhead). (*D*) Section cut tangentially through the inner segment layer of the retina, showing the distribution of AATase-positive cones in the photoreceptor mosaic. About 2.3% of the photoreceptors are labeled in this area of the retina. (*E*) Putative AATase-containing bipolar cells (asterisks) are found near the surface of stained sections, only in the distal third of the INL. These stained cells are, however, not observed consistently, especially deeper in the sections. The nature of this variability is not known. [Bars indicate 10 μm (*A, B, D, E*) or 5 μm (*C*).]

retina, although we must stress that we cannot unequivocally identify the occasional rat photoreceptors that fail to accumulate [^{3}H]aspartate as rods or cones.

Additionally, our results clearly indicate that there may not be a one-to-one correspondence between the presence of AATase and the presence of specific glutamate/aspartate uptake sites and that AATase immunoreactivity alone does not imply a neurotransmitter role for glutamate or aspartate. For in-

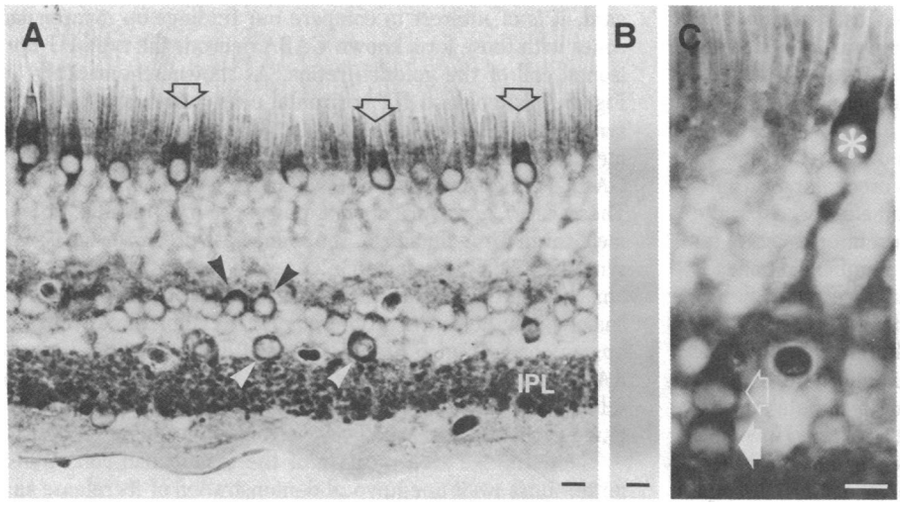

FIG. 3. Immunocytochemical localization of AATase in the human retina. (*A*) In 5-μm-thick cross sections, AATase immunoreactivity is localized to cells clearly identifiable as cones (open arrows), to cell bodies in the distal and proximal thirds of the inner nuclear layer (arrowheads), and to small, dense deposits in the inner plexiform layer (IPL). (*B*) Control section, incubated with anti-AATase serum that had been absorbed with purified AATase, shows very little staining. (*C*) High magnification of a stained cone (asterisk) and two densely stained cell bodies within the inner nuclear layer, one probably amacrine (open arrow) and the other perhaps bipolar (closed arrow). (Bars indicate 10 μm.)

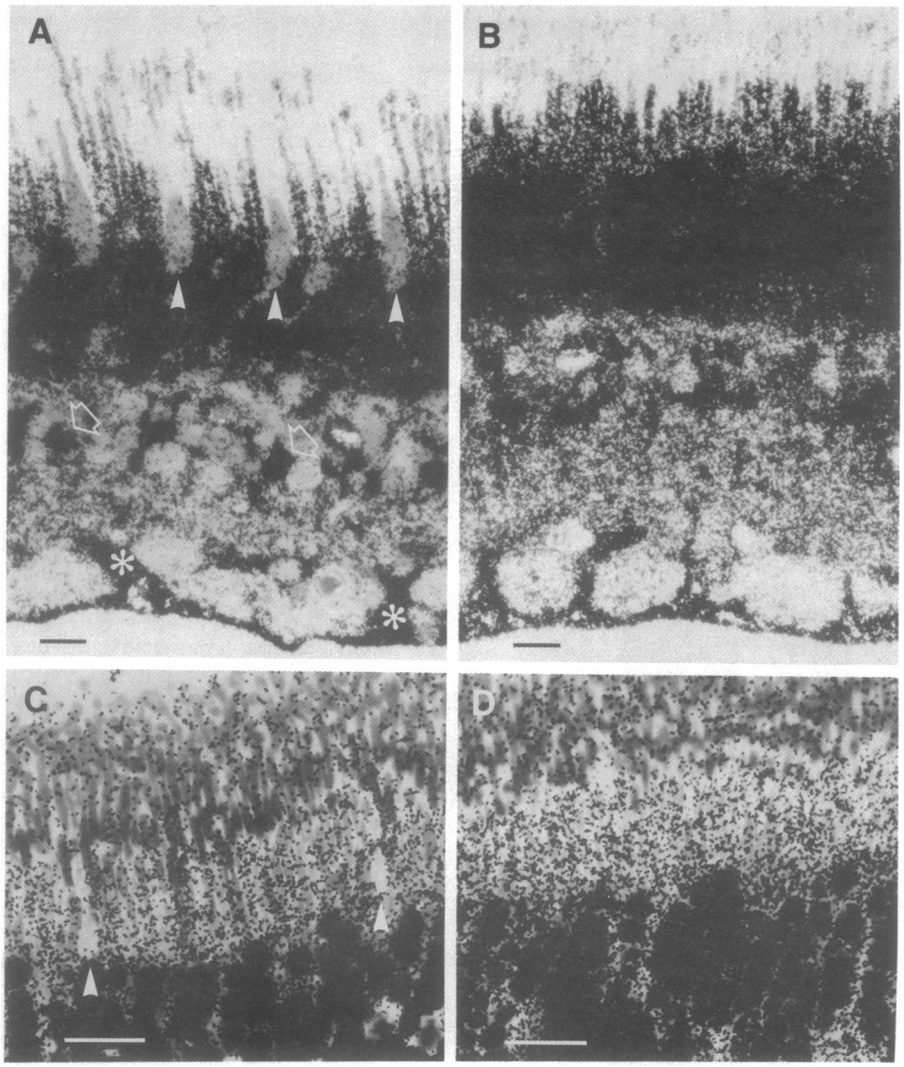

FIG. 4. Autoradiographic localization of high-affinity uptake sites for L-glutamate and L-aspartate. (*A*) Human retina, incubation with 10 μM L-[^{3}H]aspartate. The autoradiograph has been overexposed to emphasize the exclusion of aspartate by cones (arrowheads); other photoreceptors (rods) are densely and uniformly labeled. Müller (glial) cell bodies (open arrows) and their end-feet (asterisks) are also heavily labeled. (*B*) Human retina, incubation with 10 μM L-[^{3}H]glutamate. All photoreceptors (both rods and cones) are uniformly labeled. (*C*) Rat retina, incubation with 10 μM L-[^{3}H]-aspartate. Most photoreceptors are labeled. A few photoreceptors appear to exclude the amino acid (arrowheads), though they cannot be unequivocally identified as cones. (*D*) Rat retina, incubation in L-[^{3}H]glutamate. Photoreceptors are uniformly labeled, with no obvious exclusion of isotope by rods or cones. (Bars indicate 20 μm.)

stance, although it is possible that the putative bipolar and amacrine cells that contain AATase immunoreactivity may use glutamate or aspartate as transmitters, it is equally likely that some of these cells may be GABA neurons, because high AATase levels may be required to maintain the glutamate concentrations necessary for GABA synthesis and because the morphology of certain AATase-immunoreactive amacrine cells resembles that of glutamate decarboxylase-immunoreactive and GABA-accumulating amacrine cells in mammalian retina (20, 25). Identification of the transmitters used by the AATase-immunoreactive bipolar and amacrine cells must therefore await further studies.

In contrast, our combined immunocytochemical and autoradiographic results do shed light on the identity of the cone transmitter. Because GABA and other known metabolites of glutamate and aspartate are unlikely photoreceptor transmitters (7, 9, 10, 19, 21–24), whereas glutamate and aspartate themselves are good candidates, the simplest interpretation for the presence of high levels of AATase in mammalian cones is that glutamate or aspartate are needed in large quantities to serve as transmitter. Because AATase readily interconverts glutamate and aspartate with an apparent equilibrium constant near unity (26), the presence of this enzyme in a neuron provides no information as whether glutamate, aspartate, or a metabolite of these amino acids might be the actual transmitter. However, our demonstration of a selective mechanism for glutamate uptake and the absence of a similar mechanism for aspartate uptake indicates that L-glutamate is much more likely than L-aspartate to be the transmitter for human cones, and possibly for cones of other mammalian species as well.

It is not immediately apparent why a cell should exclude one amino acid but take up another and yet have the metabolic machinery to interconvert the two. Neurons using other amino acids such as GABA or glycine as neurotransmitters usually possess a high-affinity system for the uptake of the transmitter but not for the uptake of its precursor (7, 9, 10, 27–31). In this regard, it is of interest to compare our findings on mammalian cones with those for a known GABA neuron, the type H1 horizontal cell of the goldfish retina. As shown schematically in Fig. 5 *Left*, goldfish H1 horizontal cells, which are known to synthesize and release GABA (15, 31), possess a high-affinity mechanism for GABA uptake (31) and contain high levels of the GABA-synthesizing enzyme L-glutamate decarboxylase (32, 33). These cells, however, do not possess a high-affinity uptake mechanism for L-glutamate, the immediate precursor for GABA synthesis (9). For mammalian cones, if we postulate that glutamate is the transmitter and aspartate the precursor, then an analogy may be drawn to the goldfish H1 horizontal cell: the human cones (Fig. 5 *Right*) possess (*i*) high concentrations of AATase for the synthesis of glutamate from aspartate and (*ii*) a selective uptake mechanism for glutamate but not for its putative precursor aspartate.

The definitive identification of the mammalian cone transmitter must await unequivocal demonstration of its release and

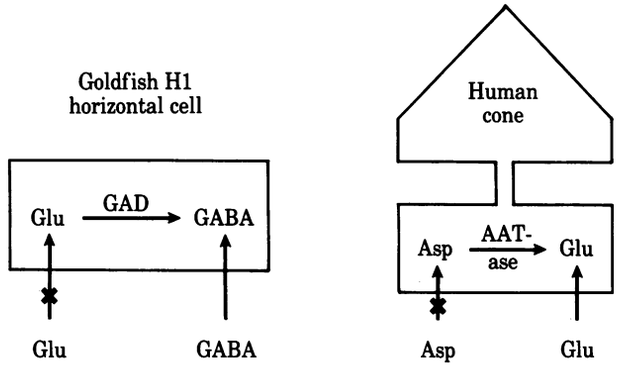

FIG. 5. Diagrams comparing GABA uptake and synthesis in goldfish type H1 horizontal cells (*Left*) with L-glutamate uptake and synthesis in human cone photoreceptors (*Right*). GAD, glutamate decarboxylase.

of the appropriate postsynaptic effects. Also, little is known about the transmitter for mammalian rods, because these cells take up both aspartate and glutamate and apparently contain only low levels of AATase. Nevertheless, our immunocytochemical and autoradiographic results demonstrate major differences between mammalian cones and rods in the transport, metabolism, and utilization of L-aspartate and L-glutamate.

Finally, the combined techniques described in this paper may also be useful for visualizing and distinguishing between putative glutamate and aspartate pathways in other areas of the nervous system.

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1. Murakami, M., Ohtsu, K. & Ohtsuka, T. (1972) *J. Physiol. (London)* **227**, 899–913.
2. Dowling, J. E. & Ripps, H. (1972) *J. Gen. Physiol.* **60**, 698–719.
3. Cervetto, L. & MacNichol, E. F., Jr. (1972) *Science* **178**, 767–768.
4. Murakami, M., Ohtsuka, T. & Shimazaki, H. (1975) *Vision Res.* **15**, 456–458.
5. Wu, S. M. & Dowling, J. E. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 5205–5209.
6. Negishi, K. & Drujan, B. D. (1979) *J. Neurosci. Res.* **4**, 351–363.
7. Lam, D. M.-K. & Hollyfield, J. G. (1980) *Exp. Eye Res.* **31**, 729–732.
8. Ishida, A. T. & Fain, G. L. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 5890–5894.
9. Marc, R. E. & Lam, D. M.-K. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 7185–7189.
10. Lam, D. M.-K., Frederick, J. M. & Hollyfield, J. G. (1982) in *The Structure of the Eye*, ed. Hollyfield, J. G. (Elsevier/North-Holland, Amsterdam), pp. 205–213.
11. Lasater, E. M. & Dowling, J. E. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 936–940.
12. Rowe, J. S. & Ruddock, K. H. (1982) *Neurosci. Lett.* **30**, 257–262.
13. Altschuler, R. A., Mosinger, J. L., Harmison, G. G., Parakkal, M. H. & Wenthold, R. J. (1982) *Nature (London)* **298**, 657–659.
14. Miller, R. E., Slaughter, M. & Massey, S. (1982) *Soc. Neurosci. Abst.* **8**, 131.
15. Lam, D. M.-K. & Ayoub, G. S. (1983) *Vision Res.* **23**, 433–444.
16. Balcar, V. J. & Johnston, G. A. R. (1972) *J. Neurochem.* **19**, 2657–2666.
17. McLennan, H. (1976) *Brain Res.* **115**, 139–144.
18. Wilkin, G. P., Garthwaite, J. & Balazs, R. (1982) *Brain Res.* **224**, 69–80.
19. Altschuler, R. A., Neises, G. R., Harmison, G. G., Wenthold, R. J. & Fex, J. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 6553–6557.
20. Brandon, C., Lam, D. M.-K., Su, Y. Y. T. & Wu, J.-Y. (1980) *Brain Res. Bull.* **5**, 21–29.
21. Lam, D. M.-K. & Steinman, L. (1971) *Proc. Natl. Acad. Sci. USA* **68**, 2777–2781.
22. Ehinger, B. & Falck, B. (1971) *Brain Res.* **33**, 157–172.
23. Bruun, A. & Ehinger, B. (1974) *Exp. Eye Res.* **19**, 435–447.
24. White, R. D. & Neal, M. J. (1976) *Brain Res.* **3**, 79–93.
25. Lam, D. M.-K., Fung, S. C. & Kong, Y. C. (1980) *J. Comp. Neurol.* **193**, 89–102.
26. Braunstein, A. E. (1973) in *The Enzymes*, ed. Boyer, P. D. (Academic, New York), 3rd Ed., Vol. 9, pp. 379–481.
27. Marc, R. E., Stell, W. K., Bok, D. & Lam, D. M.-K. (1978) *J. Comp. Neurol.* **182**, 221–246.
28. Davidson, N. (1976) *Neurotransmitter Amino Acids* (Academic, New York).
29. Chin, C. A. & Lam, D. M.-K. (1980) *J. Physiol. (London)* **308**, 185–195.
30. Marc, R. E. & Lam, D. M.-K. (1981) *J. Neurosci.* **1**, 152–165.
31. Lam, D. M.-K., Su, Y. Y. T., Chin, C. A., Brandon, C., Wu, J.-Y., Marc, R. E. & Lasater, E. M. (1980) *Brain Res. Bull.* **5**, 137–140.
32. Lam, D. M.-K. (1975) *Nature (London)* **254**, 345–347.
33. Lam, D. M.-K., Su, Y. Y. T., Swain, L., Marc, R. E., Brandon, C. & Wu, J.-Y. (1979) *Nature (London)* **278**, 565–567.