Glutamate-Like Immunoreactivity in Retinal Terminals in the Nucleus of the Optic Tract in Rabbits

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ABSTRACT

The ultrastructural organization of retinal terminals within the nucleus of the optic tract of rabbits was investigated with a combination of anterograde tracing and immunocytochemistry. The anterogradely transported WGA-HRP injected in the vitreous of the eye was visualized with the sensitive gold-substituted silver peroxidase (GSSP) method. Glutamate and GABA immunoreactivity were identified with postembedding colloidal gold particles.

Retinal ganglion cell terminals (R-terminals) in the nucleus of the optic tract formed asymmetric synapses and contained spherical vesicles and electron lucent mitochondria. R-terminals were observed in large clusters in the neuropil and in synaptic contact with large initial dendrites and somata. Within the clusters of neuropil the R-terminals formed two types of glomeruluslike arrangements: (1) an R-terminal centrally located and surrounded by small dendritic and axonal profiles and (2) several R-terminals surrounding a single dendrite or a group of dendritic profiles, presumably of interneuronal origin. All R-terminals identified with WGA-HRP as well as those exhibiting similar ultrastructural characteristics showed high levels of glutamate immunoreactivity, but no GABA immunoreactivity. The presence of glutamate and the absence of GABA in R-terminals suggest that glutamate is involved in neurotransmission in the pathway from retina to the nucleus of the optic tract of rabbits.

Key words: pretectum, accessory optic system, anterograde tracing, optokinetic nystagmus, GABA

Comprehensive anatomical data have been published on the connections from the retina to the nucleus of the optic tract (NOT) in rats and rabbits (Giolli and Guthrie, '69; Scalia, '72; Scalia and Arango, '79; Klooster et al., '83). The organization of retinal terminals (R-terminals) in different primary visual centers has been subject to ultrastructural studies, especially in the dorsal part of the lateral geniculate nucleus (LGNd) (Guillery, '69; Lieberman, '73; Hámori et al., '74; Robson and Mason, '79; Rapisardi and Miles, '84; So et al., '85; Hamos et al., '87) and the superior colliculus (SC) (Lund, '69; Vrensen and De Groot, '77; Weber and Harting, '80; Behan, '81; Mize, '83; Schönlitzer and Holländer, '84; Hofbauer and Holländer, '86). The description of R-terminals within the NOT of rabbits is largely based on comparison with R-terminals in other primary visual centers and their assumed similarity in shape and ultrastructural features (Nunes Cardozo and Van der Want, '87, '90; Van der Want and Nunes Cardozo, '88). R-terminals were described as large profiles containing spherical vesicles and electron-lucent mitochondria, which form synaptic contacts with different neuronal elements such as somata, dendrites, dendritic spines, and appendages.

Studies on possible candidates for the neurotransmission in retinal fibers are limited. Putative transmitters so far are glutamate, aspartate, and substance P (Henke et al., '76a,b; Otterson and Storm-Mathisen, '84; Brecha et al., '88; Schmidt, '90). Henke et al. ('76a,b) reported on the possible role of glutamate as neurotransmitter in the optic tectum of the pigeon. Otterson and Storm-Mathisen ('84) described glutamate-like immunoreactivity in neurons and neuropil in different parts of the pretectum, including the NOT. Moreover, pharmacological and physiological data provided evidence that glutamate is involved in transmission of visual information in the NOT (Schmidt, '90). Recent ultrastructural studies (Kageyama and Meyer, '89; Montero and Wenthold, '89; Montero, '90) reported that glutamate-like immunoreactivity was present in the optic tectum of the goldfish and in the dorsal part of the LGN of the cat and macaque monkey. In the goldfish a variety of terminals, among them putative retinal terminals, were

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observed to be glutamate positive. In the macaque LGNd the glutamate immunoreactivity was localized in three types of terminals of different origin. R-terminals, the largest terminals forming asymmetric synaptic contacts, were ultrastructurally characterized in former studies to contain electron-lucent mitochondria and spherical vesicles and glutamate (Montero and Wenthold, '89; Montero, '90).

Although it is generally accepted that distinct classes of retinal ganglion cells convey specific information to distinct areas in the brain, it is still unknown whether the same class of ganglion cells use the same transmitter to the different visual centers. This study was undertaken to define R-terminals in the NOT of the rabbit and furthermore to investigate whether these terminals contain glutamate immunoreactivity like the R-terminals described for the LGNd. To identify R-terminals in the NOT and compare them with those described for the LGNd and SC of different mammals, WGA-HRP was used as an anterograde tracer. The WGA-HRP was visualized with the GSSP method of van den Pol and Görös ('86). Postembedding immunocytochemistry with polyclonal antibodies was used to detect glutamate and GABA immunoreactivity, which were visualized with 15 nm colloidal gold particles. It will be shown that in the NOT anterogradely labeled R-terminals contain electron-lucent mitochondria, spherical vesicles and form asymmetric synaptic membrane specializations. The R-terminals form several types of glomeruluslike arrangements within the neuropil and make synaptic contact with somata or the initial part of dendrites, dendritic profiles, and dendritic spines. Furthermore, it has been found that although all R-terminals show glutamate-like immunoreactivity, they did not show any cross reactivity with a GABA antibody.

MATERIALS AND METHODS

The left eyes of three male Dutch belted rabbits (1,800–2,000 g) were intravitreally pressure injected with 10–15 μ l of 8% WGA-HRP dissolved in double distilled water, after four drops of 2% tetracaine hydrogen chloride were applied to the cornea as a local anaesthetic. After a survival period of 36–48 hours, the animals were deeply anaesthetized with sodium pentobarbital and perfused transcardially with 75 ml of a 0.1 M sodium cacodylate buffer with 0.9% NaCl, pH 7.4 and subsequently with 2,500 ml of a 5% glutaraldehyde solution in 0.08 M sodium cacodylate buffer, pH 7.4 at room temperature.

The brains were removed from the skull and additionally fixed for 20 minutes in a microwave oven, BIORAD Polaron division, at 350 Watt and 45°C in the 5% glutaraldehyde solution. Microwave fixation shortens fixation time without loss of immunoreactivity. Vibratome sections including the pretectum were made in the transverse plane. The slices were washed in tris-buffered saline (TBS) pH 7.6 and incubated in 0.05% 3,3'-diaminobenzidine (DAB) in tris buffer pH 7.6 supplemented with 0.01% hydrogen peroxide for 20 minutes. After rinsing in TBS, the DAB reaction product was intensified with GSSP according to van den Pol and Görös ('86). Subsequently the slices were fixed in a solution containing 1% osmium tetroxide and 1.5% sodium ferrocyanide in 0.1 M sodium cacodylate buffer, pH 7.4 for 20 minutes. The slices were dehydrated and flat embedded in Epon 812® and polymerized. Tissue including the NOT was cut out and mounted on prepolymerized Epon blocks. Series of ultrathin sections were cut and collected on Formvar-coated gold grids.

For the glutamate and GABA immunocytochemistry, polyclonal antibodies obtained from rabbits after immunization with a conjugate of glutamate or GABA glutaraldehyde and thyroglobulin were used (Buijs et al., '87; '89). Alternate serial sections were incubated for glutamate or GABA. The on-grid incubation was as follows: ultrathin sections were etched for 7 minutes in freshly made 1% periodic acid, rinsed three times in double distilled water, etched for 7 minutes 1% sodium metaperiodate (Ottersen, '87), rinsed three times in TBS, and incubated for 120 minutes with the glutamate antibody (1:600) in TBS supplemented with 0.8% BSA, 0.1% gelatin, 2 mM sodium azide, 1% normal goat serum, and 0.05% Triton X100 or overnight with the GABA antibody (1:1,000) in the TBS-enriched buffer. After rinsing six times in TBS, the secondary antibody goat anti rabbit conjugated to colloidal gold particles (GAR 15 nm, Amersham) was used (1:30) in the enriched TBS buffer for 90 minutes to visualize immunoreactive profiles for electron microscopy. Subsequently the grids were rinsed three times in TBS and double distilled water and contrasted with uranyl acetate and lead citrate.

The specificity of the glutamate and GABA antibodies was evaluated as follows: 1 μ l samples with increasing amount of possible cross reacting amino acids were applied in a gelatin-coated nitrocellulose membrane (Van der Sluis et al., '87; '88); thereafter the amino acids were fixed to the gelatin nitrocellulose matrix by using 5% glutaraldehyde, incubated with the glutamate or GABA antibody, and stained with the peroxidase antiperoxidase technique. The peroxidase reaction was performed using 0.05% DAB with 0.2% nickel ammonium sulfate in 0.05 M tris buffer pH 7.6 supplemented with 0.01% hydrogen peroxide for 1 minute. Optical densities of the individual spots were measured on a Kontron/2000 image processing system. This method permits the detection of picogram amount of a distinct peptide or amino acid antigen. (For further details of the procedure, see Buijs et al., '87; '88; Van der Sluis et al., '87; '88.)

RESULTS

The specificity studies of the glutamate antiserum revealed that it also recognized aspartate and GABA when these amino acids had a concentration of 300 ng/ μ l, whereas glutamate was recognized at a concentration of 1.5 ng/ μ l, resulting in a cross reaction ratio of 1:200. If cross absorbed with aspartate or GABA, the staining in the R-terminals remained. The GABA antibody had no detectable cross reactivity with glutamate and cross absorption with glutamate or aspartate did not result in a diminished immunoreactivity. Serial sections alternatively incubated for glutamate and GABA even so demonstrated no cross reaction in retinal fibers and terminals (Fig. 1; see also Figs. 6 and 7).

Two different areas of the NOT were selected for examination: a rostral part with only small patches of neuropil between myelinated fibers and a caudal part with extensive fields of neuropil. At the light microscopic level, the GSSP reaction product showed labeling of elements that was identical to the labeling in tracing studies with TMB as reaction product. At the electron microscopical level, the GSSP reaction product, seen as electron dense precipitates, was observed in myelinated axons and synaptic terminals (Figs. 1, 2). The terminals were large, formed one or more asymmetric synaptic membrane specializations, and contained spherical vesicles and electron-lucent mitochondria. These identified terminals have ultrastructural characteristics for identified or putative terminals of retinal origin

Fig. 1. Adjacent sections of the nucleus of the optic tract. Myelinated fibers immunostained for glutamate (A) and GABA (B). The retinal origin of one of the fibers is indicated by the GSSP precipitate (arrowhead). Note that the axons that are GABA (*) positive show no glutamate immunoreactivity and vice versa. Bar = 0.5 μ m.

Fig. 2. Neuropil of the NOT, illustrating the GSSP precipitates (arrowhead) in large R-terminals (R) characterized by numerous vesicles and electron lucent mitochondria. The R-terminals are without exception densely immunostained for glutamate and make synaptic contact (arrows) with dendritic profiles (den). Bar = 0.5 μ m.

observed in other primary visual centers (Guillery, '69; Lund, '69; Vrensen and De Groot, '77; Campbell and Lieberman, '85). R-terminals were observed throughout the NOT either in clusters or isolated in the neuropil. Within the clusters of neuropil the R-terminals formed two different glomeruluslike arrangements:

1. A single centrally located R-terminal forming synaptic contacts with various profiles. These profiles were either vesicle containing or nonvesicle containing dendrites (Fig. 3A,B),
2. Several dendritic profiles surrounded by R-terminals forming a synaptic contact with the centrally located dendrite or group of dendritic profiles. When the center of the glomerulus consisted of more than one dendritic profile, then one or more of these profiles contained synaptic vesicles (P-terminal). This type of arrangement was largely surrounded by glial processes (Fig. 3C).

In addition to these glomeruluslike arrangements, R-terminals were observed to be in synaptic contact with somata and large initial dendrites (Fig. 4) as well as with smaller dendrites and dendritic spines (Fig. 6). The smaller dendrites or dendritic spines sometimes contained synaptic vesicles (P-terminals). Besides P-terminals, terminals of presumed axonal origin (F-terminal) were observed within or at the periphery of these glomeruluslike arrangements (Fig. 7). Some of these F-terminals were postsynaptic to R-terminals or were in synaptic contact with the dendrites and P-terminals. R-terminals were never observed to be postsynaptic to any neuronal profile. Some R-terminals were observed to be simultaneously in synaptic contact with a P- or F-terminal or a dendrite or a dendritic spine. However, these "triadic arrangements" were rare (Fig. 6).

Immunocytochemistry on serial sections showed that all the WGA-HRP labeled R-terminals or those that were unlabeled but showed a similar ultrastructure showed immunoreactivity to the anti-glutamate antibody (Figs. 2-4, 6, 7). Besides WGA-HRP labeled R-terminals, myelinated axons labeled with WGA-HRP were observed to be glutamate positive (Fig. 1). In addition to the WGA-HRP labeled profiles, a large number of somata, dendrites, axons as well as a number of P- or some F-terminals were observed to have glutamate immunoreactivity (Figs. 5, 7A, 8A). In order to establish whether the observed glutamate-like immunoreactivity represented the presence of the excitatory neurotransmitter and was not related to the synthesis of GABA, adjacent sections were incubated with a GABA antibody. The GABA antibody showed immunoreactivity to certain somata, dendrites, and axons as well as to P- and F-terminals, but was never observed to show any immunoreactivity for R-terminals (Figs. 1, 6, 7). In some cases, double immunoreactivity could be observed in terminals other than R-terminals (Fig. 8).

DISCUSSION

Anterograde tracing studies using WGA-HRP to investigate retinal projections have used TMB as chromogen because DAB is much too insensitive to be detectable under the light microscope. However, the GSSP enhancement of the DAB reaction product gave, at the light microscopical level, labeling of WGA-HRP that was identical to that found in anterograde tracing studies with TMB (van den Pol and Görcs, '86). Moreover, at the electron microscopic level, the GSSP precipitate was restricted to the profile, revealed an

excellent preservation of ultrastructure, and in contrast to TMB no disruption of labeled profiles was observed, possibly because of the limited size of the reaction product.

To exclude the possibility that the observed glutamate immunoreactivity could be related to other metabolic systems, for instance, as a precursor for the inhibitory neurotransmitter GABA, control experiments on alternating serial sections with a GABA and glutamate antibody were performed. Adjacent sections incubated with GABA and glutamate respectively confirmed previous results of Van der Want and Nunes Cardozo ('88), who found no GABA immunoreactivity in R-terminals. Moreover, myelinated axons labeled with WGA-HRP never showed immunoreactivity for the GABA antibody. Colocalization of glutamate and GABA was only infrequently observed and was mainly restricted in dendrites and some F-terminals. It is possible that the metabolic turnover in these profiles is exhibited through the conversion of glutamate to GABA by the action of glutamate decarboxylase. Another possible explanation for the double immunostaining is a high uptake of glutamate in dendrites. Nevertheless, the presence of glutamate in R-terminals cannot be related to the presence of GABA in these terminals. However, double immunostaining of glutamate and GABA corresponds with the observations of Montero ('90), who reported GABAergic F-terminals with higher levels of glutamate in the LGNd that could be derived from several possible origins, including projection neurons.

Irrespective of their targets in the different primary visual areas, R-terminals have ultrastructural characteristics in common. In different species R-terminals are described as the largest terminals within an area having electron-lucent mitochondria, spherical vesicles, and asymmetric synaptic membrane specializations (Gray type I). Notwithstanding these similarities, variations have been mentioned. In the SC of the rat, opaque mitochondria could be discriminated from electron-lucent mitochondria (Mize, '83). Schönitzer and Holländer ('84) observed in the SC of the rabbit significant variations in the size of R-terminals. In the LGNd of the cat, differences exist in the synaptic pattern of R-terminals. Small R-terminals form simple axo-dendritic contacts or are situated on the edge of large glomeruli, whereas large R-terminals participate in large glomeruli (Robson and Mason, '79). Moreover, the position of R-terminals on the dendritic shaft or on dendritic spines was decisive for its organization within the neuropil. R-terminals in synaptic contact with dendritic spines made twice as many synaptic contacts as those on dendritic shafts and were heavily involved in "triadic arrangements" (Rapisardi and Miles, '84).

The R-terminals observed in the NOT of the rabbit exhibited electron-lucent mitochondria, spherical vesicles, and asymmetric synaptic membrane specializations (Gray type I). In this respect the R-terminals observed in the NOT are comparable with those in LGNd, SC, and NOT of the cat and pretectal olivary nucleus (PON) of the rat, as identified with either autoradiography or degeneration methods (Behan, '81; Nakamura et al., '81; Mize, '83; Schönitzer and Holländer, '84; Campbell and Lieberman, '85; Hofbauer and Holländer, '86). However, each primary visual center has its own structural features, which can be exemplified by the termination pattern of retinal fibers and the organization of R-terminals.

In contrast to the data given for certain neurons of the LGNd, SC, and PON, which exhibit numerous spines and

Fig. 3. Small (A and B) and large (C) glomeruluslike arrangements immunostained for glutamate. **A** and **B**. The small glomeruluslike arrangements consists of a central glutamate positive R-terminal surrounded by and forming synaptic contacts with (arrow) dendrites (den) and vesicle containing P-terminals (P). Bar = 0.5 μ m. **C**. A large

glomeruluslike arrangement of GSSP labeled (*) and unlabeled glutamate positive R-terminals (R) surrounding dendritic vesicle containing P-terminals (P). The glomeruluslike arrangement is surrounded by glial processes (arrowheads). Bar = 0.5 μ m.

Fig. 4. Part of a glutamate positive dendrite in synaptic contact with several WGA-HRP and glutamate positive R-terminals (arrowheads). Bar = 0.5 μ m.

Fig. 5. Large glutamate positive F-terminal (F) in synaptic contact with a glutamate positive soma (arrowheads). Bar = 0.5 μ m.

appendages (Guillery, '69; Rapisardi and Miles, '84; Campbell and Lieberman, '85; Hamos et al., '87), the NOT of rabbits consists of a population of neurons that varies in soma size and dendritic architecture but has a relatively low number of spines or dendritic appendages (Gregory, '85;

Fig. 6. Adjacent sections immunostained for glutamate (A) and GABA (B) showing a GABA-positive vesicle containing spine with characteristics of a P-terminal (P). The spinous P-terminal is presynaptic (arrowhead) to a dendrite and postsynaptic (arrowhead) to a glutamate positive R-terminal (R) forming a "triadic arrangement". Bar = 0.5 μ m.

Nunes Cardozo and Van der Want, '87). Moreover, in comparison with published micrographs, the R-terminals in the rabbit NOT tend to be smaller and form fewer synaptic contacts than those described in the LGNd, SC, and PON (Guillery, '69; Rapisardi and Miles, '84; Campbell

Fig. 7. Adjacent sections immunostained for glutamate (A) and GABA (B) of clustered neuropil containing R-, F-, and P-terminals as well as dendrites. Clustered neuropil exhibit glutamate positive R-terminals (R), occasionally labeled with WGA-HRP (arrowhead), glutamate positive F-terminals (F), and glutamate positive dendrites (den), as well as GABA positive F-terminals (F*) and a dendrite in which glutamate and GABA are co-localized (den*). Bar = 0.5 μ m.

and Lieberman, '85; Hamos et al., '87; So et al., '85). The relatively few spines and appendages observed in the rabbit NOT could explain the scarcity of "triadic arrangements" as compared to the LGNd and PON. It is likely that these differences can be ascribed to some extent to differences in afferentation and function of these primary visual centers.

Putative retinal terminals in the NOT were previously described to take part in large clusters that showed complex synaptic patterns (Nunes Cardozo and Van der Want, '87). These clusters consisted of large numbers of terminals with different ultrastructural characteristics and small dendrites and axons surrounded by glial processes. Within

Fig. 8. Adjacent sections immunostained for glutamate (A) and GABA (B) of an GABAergic F-terminal (B) in synaptic contact with a soma (arrowhead) containing flattened vesicles with some immunoreactivity for the glutamate antibody (A). Bar = 0.5 μ m.

these clusters of neuropil, two types of glomeruluslike arrangements were distinguished. These glomeruluslike arrangements resemble those of Robson and Mason ('79). These authors demonstrated that the small glomeruluslike arrangement consisting of a single R-terminal on the edge of a large glomeruluslike arrangement, where a number of R-terminals formed synaptic contacts with a centrally located profile or group of profiles, belonged to the same labeled retinal fiber. The subdivision of glomeruluslike arrangements, as presently observed, could point to different functional properties of retinal fiber organization. The first type of arrangement, where a single R-terminal was surrounded by and in synaptic contact with different neuronal profiles, could be an indication of a divergence of retinal information. The second type of arrangement could indicate a convergence of retinal information. Although anatomical and functional differences for different parts of the NOT are suggested (Robertson, '83; Meakawa et al., '84; Korp et al., '89; Nunes Cardozo and Van der Want, '90; Schiff and Schmidt, '90), both types of arrangements appeared rostrally as well as caudally. Apart from R-terminals observed in glomeruluslike arrangements, R-terminals also formed synaptic contact with large dendrites and somata. These R-terminals were ultrastructurally similar to those integrated in clusters and the glomeruluslike arrangements. The R-terminals in synaptic contact with somata or large dendrites were only infrequently observed to be part of the second type of glomeruluslike arrangement. The appearance of glomeruluslike arrangements in

the rostral part of the NOT was not as striking as in the caudal part. Whether this is due to the orientation of the optic fibers in the brachium of the superior colliculus and without obvious physiological consequences, or is functionally important, remains to be investigated. The frequent appearance of P-terminals in these arrangements as well as the frequency with which these P-terminals are in synaptic contact with R-terminals is noteworthy. P-terminals are often associated with dendrites of interneurons and, as such, associated with inhibition (Van der Want and Nunes Cardozo, '88). The presence of inhibitory interneurons could also imply a further convergence or divergence of the visual stimulus onto the subsequent neuron or group of neurons. R-terminals in the NOT were not the only large terminals. Large terminals with opaque mitochondria and glutamate positive identification were observed. These terminals were often characterized by flattened vesicles and formed synaptic contacts and as such are considered to be F-terminals (Guillery, '69). Although F-terminals often were observed in synaptic contact with dendritic profiles that also were in synaptic contact with R-terminals, they were never observed in the described glomeruluslike arrangements.

A number of possible transmitters have been mentioned for retinal ganglion cells. Among them are glutamate, aspartate, and substance P. Brecha et al. ('87) reported that substance P was observed in 25–35% of all retinal ganglion cells in the rabbit retina. However, after enucleation no difference in substance P reactivity in the NOT was ob-

served, and therefore it was concluded that the substance P containing ganglion cells did not terminate in the NOT. The absence of substance P positive R-terminals was confirmed by light microscopy (Nunes Cardozo, unpublished data). Moreover, in the visual system, glutamate is the putative excitatory neurotransmitter (Lund-Karlson and Fonnum, '78). The development of antibodies against amino acids made it possible to localize glutamate in the retinofugal pathway (Ottersen and Storm-Mathisen, '84) and retina (Kageyama and Meyer, '89). The ultrastructurally observed immunoreactivity for the glutamate antibody in R-terminals is in agreement with observations on the optic tectum in the goldfish (Kageyama and Meyer, '89) and the LGNd in mammals (Montero and Wenthold, '89; Montero, '90; Pasik et al., '90). In these studies, a glutamate antibody showed labeling of large terminals with ultrastructural characteristics of R-terminals. In addition the presence of glutamate is confirmed physiologically. The effects of the glutamate agonist N-methyl-D-aspartate on the optokinetic nystagmus and direction selectivity in the NOT of the cat and rat showed that NOT neurons receive glutamate mediated excitatory input (Schmidt, '90).

The source of the other terminals in the NOT with immunoreactivity to the glutamate antibody can only be speculated about. In the LGNd, besides the R-terminals also C- and F-terminals were described to contain glutamate (Montero and Wenthold, '89; Montero, '90). C-terminals were considered to be cortical afferents, whereas F-terminals analyzed as GABAergic represented a heterogeneous population. For the rabbit, NOT it is still controversial whether this nucleus receives a visual cortical projection (Gioli et al., '78; Müller-Paschinger and Tömböl, '89). Glutamate positive neurons in the pretectal nuclei were mentioned by Beitz ('89) who studied the projection from the periaqueductal gray and deep layers of the superior colliculus of the rat. However, this light microscopical study only mentioned glutamate positive neurons in the posterior and anterior pretectal nucleus and not the NOT.

Although the exhibition of immunoreactivity for glutamate antibody is present in all R-terminals, colocalization with other excitatory neurotransmitters cannot be excluded. Recently, it was reported that N-acetylaspartylglutamate (NAAG) and not glutamate or aspartate could be a possible transmitter in selected areas of the rat visual system, including the NOT (Williamson et al., '89; Pasik et al., '90). Whether NAAG is a major transmitter of retinal terminals in the rabbit NOT or is colocalized with glutamate has to be examined. However, the localization of glutamate in this primary visual system is most likely related to synaptic transmission.

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