Localized binding of [^3H]muscimol to synapses in chicken retina

(electron microscopic autoradiography/inner plexiform layer/GABAergic synapses/amacrine synapses/outer plexiform layer)

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ABSTRACT Binding sites for [^3H]muscimol, an analogue of γ -aminobutyric acid (GABA) were localized in the synaptic layers of chicken retina by light microscopic and electron microscopic autoradiography. Light microscopic autoradiography of cryostat sections incubated in [^3H]muscimol or [^3H]GABA revealed identical binding patterns: a band over the inner plexiform layer (IPL) and a band over the outer plexiform layer (OPL). This binding pattern differed from the uptake pattern for [^3H]GABA: labeling over horizontal, amacrine, and ganglion cell bodies as well as very intense labeling over lamina 5 in the proximal IPL. Statistical analysis of electron microscopic autoradiography data from the IPL indicated that only amacrine synapses bind [^3H]muscimol (i.e., make GABAergic synapses). Processes of amacrine, bipolar, or ganglion cells can be postsynaptic to these amacrine synapses. The highest concentration of synapses binding [^3H]muscimol occurred in laminae 2 and 4 of the IPL and not in lamina 5 as might be expected from the density of [^3H]GABA uptake. In the OPL, [^3H]muscimol binding occurred over specialized junctions proximal to photoreceptor terminals. In cone receptor terminals, [^3H]muscimol binding was suspected near horizontal cell dendrite/receptor terminal membranes lateral to the synaptic ribbon, supporting the hypothesis that horizontal cells are involved in a GABAergic feedback loop with cone terminals. We conclude that the synaptic binding pattern provides a more accurate concept of GABAergic synaptic interaction than does the uptake pattern for [^3H]GABA because the two patterns in the IPL are not related.

Identification of neurons in the vertebrate retina that may use γ -aminobutyric acid (GABA) as their neurotransmitter has relied mainly on the results of studies of [^3H]GABA uptake (see ref. 1 for review) or the localization of glutamate decarboxylase (L-glutamate 1-carboxy-lyase, EC 4.1.1.15) (2). Such studies, however, may not provide sufficient information regarding the density and distribution of GABAergic synapses. Biochemical and histological studies have used the GABA analogue [^3H]muscimol as a specific probe to investigate the properties of GABA synaptic receptors in the vertebrate central nervous system (3–8). However, the usefulness of [^3H]muscimol has been questioned because of demonstrations of appreciable transport of [^3H]muscimol into central nervous system tissue (6, 7). In a previous study (9) we found specific and saturable binding of [^3H]muscimol to retinal membranes of goldfish and chickens. However, the localization of this binding to membranes in intact retina by light microscopic (LM) autoradiography was not determined because a large amount of uptake labeling occurred under our incubation conditions. In the study reported here we have localized GABAergic synaptic receptors by dry-process LM and electron microscopic (EM) autoradiography in both the outer plexiform layer (OPL) and inner plexiform layer (IPL) of chicken retina.

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MATERIALS AND METHODS

Experimental animals were adult chickens (*Gallus domesticus*). [^3H]Muscimol (side-chain labeled, 12.9 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) and [^3H]GABA (54 Ci/mmol) were obtained from New England Nuclear. The purity of [^3H]muscimol after *in vitro* incubation in retinal tissue is presented elsewhere (9).

Light Microscopic Autoradiography. Chickens were decapitated, the eyes were enucleated and hemisected, and the vitreous fluid was drained off. The posterior portion of the eyecup was immersed in 0.1% formalin/0.1 M sodium phosphate pH 7.4, at 20°C for 90 min and cryoprotected in 30% (wt/vol) sucrose/phosphate buffer for 6–12 hr at 4°C. Frozen sections 10 μm thick were picked up on acid-cleaned gelatin-coated slides, brought to room temperature, and washed for 5 min in Ringer's solution to remove the cryoform support medium. After drying, sections were covered with 20 μl of 0.29 μM [^3H]muscimol or 0.8 μM [^3H]GABA dissolved in Ringer's solution for 15 min. Slides were then rinsed for 10 sec in two successive changes in distilled water and dried in less than 30 sec. LM autoradiographs were produced according to the method of Young and Kuhar (10).

EM Autoradiography. Pieces of isolated chicken retina were incubated in 200 μl of 0.29 μM [^3H]muscimol dissolved in Ringer's solution for 30 min at 20°C under ordinary room light conditions. The subsequent processing through Epon for electron microscopy is described elsewhere (9). Silver and gold sections were collected on 200-mesh high-transmission grids. Grids were coated with a monolayer of Ilford L4 emulsion (purple interference color) by a fixed-loop technique and stored in desiccated boxes at 4°C for 1–12 weeks. Grids were developed in freshly prepared filtered D-19 for 1 min at 14°C, fixed in 30% sodium thiosulfate for 1 min, washed in distilled water, rinsed in 0.1% sodium hydroxide for 20 min to clear the emulsion, and double stained in uranyl acetate and F.H.S. lead citrate. Under these conditions, the half-distance for silver grains from a radioactive source is 1450 Å (11).

Previously, we found (9) that 1 mM GABA abolished specific [^3H]muscimol labeling in living chicken retina. The grain density of LM autoradiographs of such controls is at about the same level as that over photoreceptors of retina treated with [^3H]muscimol alone. The grain density over photoreceptors in our EM autoradiographs was <1 grain per 250 μm^2 and is indicative of the low density of GABA-insensitive nonspecific labeling. Because this value is low compared to the grain density in the plexiform layers, the background grain activity was ignored in the data analysis.

Abbreviations: GABA, γ -aminobutyric acid; LM, light microscope(ic); EM, electron microscope(ic); IPL, inner plexiform layer; OPL, outer plexiform layer; HD, half distance.

FIG. 1. LM autoradiographs of chicken retina incubated in $0.8 \mu\text{M}$ [^3H]muscimol. (a) Dry-processed (dark field) $10\text{-}\mu\text{m}$ -thick cryostat section; exposure, 3 weeks. (b) Bright-field micrograph of section shown in a, illustrating histological integrity of the tissue and location of the plexiform layers. (c) Bright-field autoradiograph of $1.5\text{-}\mu\text{m}$ Epon section from isolated retina incubated in [^3H]muscimol; exposure, 11 weeks. (d) Dry-processed (dark field) $10\text{-}\mu\text{m}$ -thick cryostat section incubated in [^3H]muscimol in the presence of 1 mM GABA; exposure, 6 weeks. Calibration bar equals $25 \mu\text{m}$.

RESULTS

Light Microscopy. The labeling pattern of [^3H]muscimol in frozen retinal sections showed two bands of label, one over the OPL and a broader band over the IPL (Fig. 1a). There was no indication of somatic labeling. A bright-field micrograph of the same retinal area illustrated the histological integrity of the cryostat sections (Fig. 1b). The pattern in Fig. 1a is in contrast to the [^3H]muscimol labeling pattern observed in retinal tissue conventionally processed through Epon (Fig. 1c) in which there is significant labeling over horizontal cell and amacrine cell bodies (9). Because uptake mechanisms should be inactive in aldehyde-fixed and cryostat-sectioned tissue, we conclude that the labeling pattern in Fig. 1a represents membrane binding of [^3H]muscimol. This binding could be blocked by 1 mM GABA (Fig. 1d).

Fig. 2a shows that the binding pattern of [^3H]GABA on cryostat sections was qualitatively identical to the binding pattern of [^3H]muscimol (Fig. 1a) and very different from the uptake pattern of [^3H]GABA (9, 12) (Fig. 2b). Muscimol at 1 mM inhibited the binding of [^3H]GABA to cryostat sections but did not inhibit the uptake of [^3H]GABA in living retinal tissue (9).

EM of IPL. After *in vitro* incubation in [^3H]muscimol, most of the label observed in $1.5\text{-}\mu\text{m}$ Epon sections is due to uptake (9). However, evidence for [^3H]muscimol binding from biochemical assay studies (9) and dry-process autoradiographs

FIG. 2. LM autoradiographs of chicken retina incubated in $0.29 \mu\text{M}$ [^3H]GABA. (a) Dry-processed (dark field) $10\text{-}\mu\text{m}$ -thick cryostat section; exposure, 3 months. (b) Bright-field autoradiograph of $1.5\text{-}\mu\text{m}$ Epon section obtained from isolated retina incubated in [^3H]GABA; exposure, 11 weeks. Calibration bar equals $25 \mu\text{m}$.

(Fig. 1a) plus the fact that muscimol is stabilized by aldehyde fixation suggests that some membrane binding should be expected in Epon-processed tissue. Synaptic membrane binding may not have been detected in LM autoradiographs of such tissue because thick sections introduce a sampling bias in favor of a uniform concentration of label within cells and against a lower number of concentrated sources such as postsynaptic membranes. Ultrathin sections reverse this bias because a large proportion of a postsynaptic membrane $2000 \text{ \AA}$ long is contained in a tissue section $1000 \text{ \AA}$ thick. With this in mind, EM autoradiographs of Epon-processed retina were obtained and analyzed.

A survey of EM autoradiographs of the IPL indicated that about 25% of the silver grains were in contact with, or within $725 \text{ \AA}$ of, the synaptic membrane of conventional synapses (Fig. 3) in which amacrine cells are always the presynaptic process (13). Many of the postsynaptic elements could be identified as amacrine cell processes by their cytoplasmic appearance or the presence of conventional synapses. A smaller percentage of bipolar processes, identified by their dark cytoplasm, dense aggregate of synaptic vesicles, and occasional synaptic ribbon, were postsynaptic elements as well. Other unidentified postsynaptic processes could belong to ganglion cell dendrites. No grains were ever observed overlying a ribbon (bipolar) synaptic complex. Silver/gold sections ($1000 \text{ \AA}$) exposed for 20 days were subjected to statistical analysis to determine whether amacrine synapses were a major source of the silver grains. In the area of the IPL investigated ($1600 \mu\text{m}^2$), there were 441 amacrine synapses and 97 silver grains. The distance from the center of each grain was measured to the center of the nearest amacrine synaptic membrane. This measurement treats the amacrine synapse as an integrated point source. The largest frequency of grains occurred within $725 \text{ \AA}$ of an amacrine synapse, with progressively fewer as distance increased, although there was evi-

FIG. 3. EM autoradiographs of amacrine synapses binding [^3H]muscimol; exposure, 20 days. Calibration bar equals $0.5 \mu\text{m}$.

FIG. 4. Frequency histogram of the IPL showing the number of silver grains as a function of their distance from the nearest amacrine synapse measured in units of half distance (HD) (1450 Å). (Inset) Cumulative frequency histogram of these data (○) in comparison to theoretical functions for an integrated point source (—) of radioactivity (11) and a random distribution (---) of silver grains within this population of amacrine synapses.

dence for a minor peak between 2.5 and 5.5 HD (Fig. 4). The observed cumulative frequency histogram of these data is not significantly different from a theoretical function for an integrated point source (11) when tested by the Kolmogorov-Smirnov test ($P = 0.2$), suggesting that, on statistical grounds, the large majority of silver grains were produced by a radioactive source ($[^3\text{H}]$ muscimol) bound to the synaptic membrane of amacrine synapses.

The discrepancy in the two curves between 2.5 and 5.5 HD is probably due to uptake. The relative contribution of uptake to the grain density could vary throughout the IPL. Therefore, the laminar distribution of silver grains in the IPL was analyzed from two photomontages obtained from EM autoradiographs exposed for 3 months. The montages covered $2900\ \mu\text{m}^2$ with 605 amacrine synapses and 464 silver grains. The IPL was divided into five equal layers from the inner nuclear layer to the ganglion cell layer. The percentages of grains in each layer within 1 and 3 HD of an amacrine synapse are plotted in Fig. 5.

FIG. 5. Percentage of grains within each layer of the IPL which occur within 1 HD (squares) and 3 HD (circles) of the nearest amacrine synapse. The expected frequencies at these distances is 24% at 1 HD and 74% at 3 HD (dashed lines). INL, inner nuclear layer; GC, ganglion cell layer.

The IPL was not uniform with respect to the distribution of grains around amacrine synapses. The data depart differentially from the frequencies expected for an integrated point source at 1 HD and 3 HD (broken lines). In lamina 2, the expected and observed frequencies were very close, suggesting that specific amacrine synapses are behaving as integrated point sources. This implies that a large majority of the grains are due to $[^3\text{H}]$ muscimol binding at amacrine synapses and that very little of the labeling is due to cellular uptake. For laminae 4, 5, 3, and 1, respectively, the observed frequencies increasingly deviate from the expected values, suggesting a proportionate decrease in synaptic binding relative to uptake labeling.

The variable of greater interest, however, is the density of

Table 1. Density of amacrine synapses binding $[^3\text{H}]$ muscimol in each layer of chicken IPL

IPL layers	Synapses/ $10\ \mu\text{m}^2$	% Grains <3 HD	Observed/ expected*	Synapses <3 HD†	Max. synapses‡	Synapses binding $[^3\text{H}]$ muscimol§	$[^3\text{H}]$ Muscimol synapses/ $10\ \mu\text{m}^2$
1	1.6	32	0.43	6	8	3	0.09
2	2.7	73	0.99	27	36	36	1.06
3	1.8	44	0.59	17	23	14	0.41
4	2.0	60	0.81	32	43	35	1.03
5	1.4	51	0.69	24	32	22	0.65

* Ratio of the observed frequency <3 HD and the expected frequency (74%) at 3 HD.

† Number of synapses within 3 HD of a silver grain; situations in which more than one grain was associated with a single synapse are not counted.

‡ Maximal number of synapses expected to be associated with silver grains; this was obtained by dividing the number observed at 3 HD by the fraction expected at 3 HD (0.74).

§ Number of synapses calculated to bind $[^3\text{H}]$ muscimol in our sample, obtained by multiplying the maximal number of synapses by the ratio of observed to expected. This was done because the value for maximal number of synapses assumes that all of the grains were derived from amacrine synapses behaving as integrated point sources. However, each layer only approximates this assumption by an amount shown as the ratio.

FIG. 6. Laminar distribution, within the IPL, of the density of all amacrine synapses (○) and those amacrine synapses binding [^3H]muscimol (□). INL and GC, as in Fig. 5.

synapses that bind [^3H]muscimol in each layer of the IPL. To calculate this value we assumed that each synapse binding [^3H]muscimol has produced at least one silver grain and that the ratio of the observed to the expected frequency at 3 HD provides a direct estimate of the ratio of [^3H]muscimol binding to total labeling (binding plus uptake). The manner in which the density values were calculated is presented in Table 1. The density of [^3H]muscimol synapses is shown graphically in Fig. 6. There was a higher concentration of [^3H]muscimol synapses in layers 2 and 4 and little [^3H]muscimol binding in layer 1.

EM of OPL. A 2010- μm^2 area of the OPL was analyzed with respect to the distribution of 150 silver grains which fell into three groups. In the first group, there were 29 cone terminals associated with 34 silver grains. Of these, 31 grains were localized over the lateral process of a ribbon synaptic arrangement (Fig. 7a). These grains rarely appeared over the synaptic ribbon itself but tended to be over the membranes of the horizontal cell dendrite and the cone terminal, suggesting that the source was membrane bound rather than uptake by the horizontal cell. Statistical treatment of the distribution of these grains was not fea-

FIG. 7. EM autoradiographs of [^3H]muscimol labeling in chicken OPL; exposure, 20 days. Calibration bar equals 0.5 μm . (a) Cone receptor terminal with grain over the horizontal cell/cone terminal membrane lateral to a synaptic ribbon. (b) Specialized junctions in the proximal OPL (arrowheads) with an adjacent silver grain.

FIG. 8. Frequency histogram for the specialized junctions in the proximal OPL, showing the number of silver grains as a function of their distance from the nearest specialized junction. (Inset) Cumulative frequency histogram of these data (○) in comparison to a theoretical function for an integrated point source (—) of radioactivity (11).

sible because there is no obvious postsynaptic specialization on the cone terminal to serve as a target for measurement. However, we believe that these grains represent membrane binding and not uptake of [^3H]muscimol because horizontal cell dendrites that were not lateral to a synaptic ribbon but still were within the cone invagination rarely contained a silver grain (2 of 34 grains). No grains were located over rod photoreceptor terminals.

In the second group, 45 grains were associated with specialized membrane junctions proximal to photoreceptor terminals (Fig. 7b). These junctions were characterized by a regular spacing between the two processes and the appearance of a dense fuzz on the cytoplasmic side of the plasma membrane of one of the processes. Neither process contained synaptic vesicles. The number of grains plotted as a function of the center-to-center distance to the nearest junction is shown in Fig. 8. Most grains occurred at 1–2 HD, with successively fewer grains at larger HD. A cumulative frequency histogram of these data adequately fits the theoretical function for an integrated point source (Fig. 8 Inset). Therefore, it is likely that these junctions are sites of [^3H]muscimol binding. The remaining 71 grains in the proximal OPL were more than 8 HD from any identifiable membrane specialization and appeared to be confined to horizontal cells and their dendrites.

DISCUSSION

These data show that [^3H]muscimol binds to amacrine synapses in the IPL and to structures in the OPL which may include cone terminals and specialized junctions in the proximal OPL. In the chicken retina, GABA inhibits the binding of [^3H]muscimol in biochemical (9) and histological studies (ref. 9; Fig. 1d). The GABA-sensitive binding of [^3H]muscimol is localized to amacrine synapses and in the OPL. Furthermore, the binding pattern of [^3H]GABA shown by dry LM autoradiography is identical to that for [^3H]muscimol, and this binding is inhibited by

muscimol. This is important because muscimol does not inhibit the uptake of [^3H]GABA in chicken or goldfish retina (9). This is consistent with the specificity of [^3H]muscimol for GABA synaptic binding sites rather than uptake in other parts of the central nervous system (4–6) although, in rat brain, [^3H]muscimol may bind to sites that are not affected by low concentrations of GABA (6). We thus conclude that binding sites for [^3H]muscimol in chicken retina represent GABAergic synapses.

The localization of synaptic binding in the IPL shows that only certain classes of amacrine cells make GABAergic synapses that can include ganglion cells, bipolar cells, and other amacrine cells as the postsynaptic element. This is consistent with electrophysiological studies in other vertebrates which demonstrate the involvement of amacrine cells in GABAergic transmission (14, 15). The uptake of [^3H]GABA by amacrine cells would appear to support our findings except that the laminar distributions of [^3H]GABA uptake and GABAergic synapses are different. This suggests that amacrine cells making GABAergic synapses either do not take up [^3H]GABA or do so at a rate that is not related to the density of their synaptic contacts. This is most evident for laminae 2, 4, and 5 in which the relative density of [^3H]GABA uptake and synaptic binding are inversely related. We therefore conclude that the synaptic binding pattern and not the uptake pattern provides a more accurate description of the synaptic organization of GABAergic synapses in the chicken IPL.

The presence of [^3H]muscimol binding in the OPL provides new evidence supporting the role of GABA as a neurotransmitter in the outer retina. Recent evidence in some fishes suggests that certain horizontal cells chemically inhibit photoreceptor terminals via a GABAergic mechanism (16, 17); in bovine retina, [^3H]GABA binding was observed in a particulate fraction enriched in photoreceptor terminals (18). Thus, the suggestion of [^3H]muscimol binding over the lateral processes of cone triads may represent GABA-releasing horizontal cell dendrites. Another class of probable binding sites for [^3H]muscimol was observed among specialized junctions in the proximal OPL. The identity of these junctions is not clear although it is likely that one of the elements is a horizontal cell dendrite whereas the second process could belong to a neurite of a horizontal cell, bipolar cell, or photoreceptor. It is possible that horizontal cells exert a direct inhibitory effect on bipolar cells at these junctions, complementing an indirect effect through a feedback synapse at the cone terminal.

These findings raise serious questions concerning the use of amino acid uptake patterns as indicators of synaptic integration not only in the retina but in other central nervous system tissues as well. We have shown in the chicken IPL that the distribution

of GABAergic synapses differs markedly from the density of [^3H]GABA uptake. Although this may not be the case for all preparations, one should exercise extreme caution in making statements about synaptic function based on uptake studies alone. For example, in chicken retina, classes of both amacrine cells and ganglion cells take up GABA. Ganglion cells, however, are not presynaptic neurons in the retina. Therefore, their uptake of GABA is either as a postsynaptic element or unrelated to GABAergic transmission. How then can one decide whether the uptake of [^3H]GABA by amacrine cells reflects their activity as presynaptic or postsynaptic processes? In addition, the redistribution of [^3H]GABA throughout the neuron during the incubation period does not permit identification of uptake sites, which may or may not be related to synaptic receptor sites. Thus, to specify neuronal integration, the distribution of synaptic binding appears more appropriate than the distribution of uptake.

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