Choline acetyltransferase and acetylcholine esterase activities in normal and biologically fractionated mouse retinas

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Radiometric biochemical assays were carried out for choline acetyltransferase (ChAc) and acetylcholine esterase (AChE) in retinas isolated from 90-day-old pigmented mice. The retinas were either normal [(C57Bl/6 and rd/le controls), or genetically receptorless (rd/le and C3H), or had markedly reduced inner layers due to postnatal treatment of C57Bl/6 mice with monosodium glutamate. The receptorless rd/le retinas were obtained from an inbred hybrid stock that provided littermate controls with normal retinas. Glutamate-damaged retinas were compared to retinas from untreated control animals. Receptorless C3H retinas were compared to both sources of normal retinas. Control retinas had specific activities (millimoles per kilogram dry weight per hour) of 36.5 to 38.5 for ChAc and 2,180 to 2,350 for AChE and total activities per retina (nanomoles per hour) of 23 to 26 for ChAc and 1,360 to 1,160 for AChE, with the higher total values coming from slightly heavier rd/le controls. Receptorless retinas had 44 to 48 times the ChAc specific activity of those from glutamate-treated animals and had 2.4 to 2.5 times that of retinas from control animals. They had five to six times the AChE specific activity of retinas of glutamate-treated animals and 2.4 to 2.5 times that of controls. On a total activity per retina basis, receptorless rd/le retinas had 81 per cent of the ChAc activity and 66 per cent of the AChE activity of those from littermate controls and receptorless C3H retinas had 97 per cent and 98 per cent of the total ChAc and AChE activity of the C57Bl/6 controls, but only 91 per cent and 82 per cent of the same total activities in the heavier rd/le controls. The data are consistent with the view that both enzymes are normally more concentrated in the inner than outer retina, with the disparity being more marked for ChAc.

Key words: choline acetyltransferase, acetylcholine esterase, retina, receptor dystrophy, glutamate damage, histochemistry, photoreceptors, mouse.

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Except for the classical work of Hebb and Silver,¹ who assayed the choline acetyltransferase (ChAc) activity in a number of vertebrate retinas, most reports on the

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cholinergic transmitter-related enzymes in retina have been histochemical studies of the distribution of acetylcholinesterase (AChE). Many such studies have been made in an effort to determine the localization of cholinergic retinal neurons.\textsuperscript{2-8}

These studies have consistently given positive results for processes and cells in the inner retina, but positive findings in the outer retina have been relatively few\textsuperscript{2,7} and unimpressive. However, Lam\textsuperscript{9} has developed an elegant technique for isolating individual turtle cones and has assayed small groups of these for choline acetyltransferase (ChAc). His studies demonstrated that these photoreceptors did possess some ChAc activity and lacked certain enzymes involved in the syntheses of some other transmitters. He has therefore suggested that these cells might use acetylcholine as a transmitter. On the other hand, using chemical procedures similar to those employed by Lam in direct chemical assays on layers of frozen-dried rat, mouse, and monkey retinas, Ross and McDougal\textsuperscript{10} have recently reported activities of choline acetyltransferase in the outer plexiform layer which are less than one-hundredth of those in the inner plexiform layer. They interpreted the low ChAc activity in the outer plexiform layer as evidence against receptors being cholinergic. The present studies were undertaken to determine the effect of genetic and chemical dissection upon the activities of retinal ChAc and AChE. Inbred races of mice with retinas which lack photoreceptors as a result of a genetic dystrophy were used. We also used mice whose retinas possessed photoreceptors but had undergone a massive reduction of the inner retinal layers consequent to postnatal treatment with monosodium glutamate.

Direct analytic data exist for the distribution of ChAc in the frozen-dried mouse retina.\textsuperscript{11,12} We can, therefore, ask whether at three months following either receptor dystrophy or early glutamate damage is altered to where it is not consistent with its original activities and distribution in the normal retina.

Detailed descriptions of mouse retinas with "rd" receptor dystrophy or following glutamate damage are both available in the literature.\textsuperscript{12,13} Normal eyes and those with receptor dystrophy (rd) have diameters of 3.0 mm., while those of glutamate-treated mice are but 2.8 mm. in diameter. The "rd" dystrophic retinas show no sign whatsoever of outer segments and only rare receptor somata and terminals. Nonetheless, "rd" mice have been shown to respond behaviorally to light. The response has a high threshold and it is abolished by bilateral enucleation.\textsuperscript{14} In addition to the loss of photoreceptors, Grafstein, Murray, and Ingoglia\textsuperscript{15} found a 20 per cent loss in ganglion cells in their rd/le mice, and Cohen, McDaniel, and Orr\textsuperscript{16} noted an approximate 10 per cent loss in the thickness of the inner plexiform layer of retinas of three-month-old C3H mice, which also carry homozygous "rd." Retinas of our rd/le mice seemed similar to those of C3H mice in this respect. A persistent a-wave, said to be a sign of electrical activity in photoreceptors, can be elicited from eyes of glutamate-treated mice,\textsuperscript{17} and they have normal concentrations of these cells; but this should not be taken to mean that the photoreceptors are entirely normal. These "glutamate" retinas exhibit a marked although less than total loss of ganglion cells and amacrine cells and much less loss of bipolar and horizontal cells. The combined thickness of inner nuclear, inner plexiform, ganglion cell, and optic fiber layers amounts to 40 per cent of that of control retinas.

Methods

Isolation of whole mouse retinas. Retinas were removed from light- or dark-adapted eyes of three-month-old mice and placed on the inner wall of a glass test tube which was then immersed in liquid nitrogen for quick freezing. The methods of dissection are described in detail in Cohen, McDaniel, and Orr.\textsuperscript{16} In the present study, retinas lacking photoreceptors were obtained from both
C3H/HeJ mice and also from offspring of heterozygous mice of the C57Bl (6j) le/rd strain (Jackson Memorial Laboratory, Bar Harbor, Me.). Because of the close linkage of the recessive le and rd genes, offspring homozygous for receptor dystrophy (rd) also possess light ears (le) and are readily identified. Their dark-eared littermates (which include heterozygotes and normal animals in a 2:1 ratio) possess normal retinas and act as controls with the same genetic background apart from the rd and le genes. Other C57Bl (6j) mice also served as control animals and glutamate-damaged retinas were prepared from animals of this stock by postnatal treatment with glutamate according to the schedule of Potts, Modrell, and Kingsbury, but limited to 10 days.

Although the rd/le stock is based upon an introduction of these genes into the same C57Bl (6) line we used to prepare glutamate-damaged retinas, backcrossing to the C57 line has not proceeded to the point where the two lines can be considered co-isogenic except for rd/le. Chemical methods. Frozen retinas were dried under vacuum at -40° C. for three days and weighed on a quartz fiber "fish-pole" balance. All retinas, regardless of weight (from 0.27 mg. to 0.6 mg. dry), were homogenized in 36 µl of 50 mM potassium phosphate, pH 7.4, containing 0.08 per cent Triton X-100 and 0.05 per cent bovine serum albumin. Homogenates were stored at -80° C.

Acetylcholinesterase. Aliquots of each homogenate were preincubated in 3 µM tetraisopropyl pyrophosphoramide (Sigma Chemical Co., St. Louis, Mo.) for 30 minutes at 25° C. to inhibit nonspecific cholinesterase activity. AChE activity was determined at 25° C. in 10 µl incubation medium containing 650 mM L-14C-acetylcholine (4.5 mCi. per millimole, New England Nuclear, Boston, Mass.), 0.05 per cent bovine serum albumin, 0.1 per cent Triton X-100, and 50 mM phosphate buffer, pH 7.8. The reaction was stopped after 30 minutes by adding HCl to a final concentration of 0.4 N. The rate of acid hydrolysis of acetylcholine was retarded by placing the tubes in an ice bath. After acidification, 14 C-acetic acid was extracted by adding 72 µl ethylacetate to each tube and mixing. Both the incubation and extraction procedures for each sample were done in the same 400 µl plastic microtube (Hruden Laboratory Products). The blank for the reaction, 1,200 c.p.m. at 26° C. (1,900 c.p.m. at 38° C.), represented almost 3 per cent of the total counts in the reaction medium, correcting for aliquot volumes and recovery (91 per cent). The number of counts produced by the enzyme activity in the tissue of dystrophic and control animals was more than three times the blank. In the case of glutamate-treated animals, it was usually more than twice the blank. The disadvantage of the relatively high blank of this method, compared to others, is offset by the procedure being rapid and simple. The method is easily adaptable for use with smaller incubation volumes.

Choline acetyltransferase. ChAc activity was determined at 37° C. L-14C-Acetylcholine, formed from 1-14C-acetyl CoA (45 to 54 mCi. per millimole, New England Nuclear) and choline, was extracted using sodium tetraphenylboron in 3-heptanone.

Results

In a previous study, retinas of three-month-old mice with receptor dystrophy (C3H) were found to contain 0.14 mg. of protein and to have a volume of 2.8 mm.2; glutamate-treated retinas (C57Bl [6]) contained 0.18 mg. protein and had a volume of 3.2 mm.2; normal (C57Bl [6]) retinas contained 0.32 mg. of protein and had a volume of 4.7 mm.2. In the present study, freeze-dried control retinas (C57Bl [6]) weighed 0.61 ± 0.01 mg.; and retinas from glutamate-treated mice weighed 0.41 ± 0.02 mg. Control C57Bl/6J retinas (C57Bl [6] litters) of rd/le mice weighed 0.67 ± 0.04 mg. and dystrophic retinas (C3H or rd/le) weighed 0.23 ± 0.005 mg.

In glutamate-treated animals, the specific activity of ChAc was 5 per cent of the control values while that of AChE was 40 per cent (Table I). The control littermates of rd/le mice had similar ChAc and AChE specific activities to the C57Bl (6) control stock, but greater total activity for AChE, partly because the former retinas averaged somewhat heavier. However, as noncoisogenic stocks, part of the difference may be genetic. The retinas of homozygous rd/le animals possessed 200 to 250 per cent of the specific activities of those of their normal littermates for both ChAc and AChE, but on a per retina basis, rd/le retinas possessed about 20 per cent less ChAc and 33 per cent less AChE than their littermate controls (significant at < 0.001 level). The receptorless C3H mice also had similarly high ChAc and AChE specific activities and total activities constituting about 97 to 98 per cent of those of the C57Bl (6) control animals. However, as there is no isogenic nondystrophic C3H
**Table I**

<table>
<thead>
<tr>
<th></th>
<th>ChAc (specific activity)</th>
<th>Per cent control</th>
<th>AChE (specific activity)</th>
<th>Per cent control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control “A”</td>
<td>37.6 ± 0.8 (9)</td>
<td>100</td>
<td>2,180 ± 80 (12)</td>
<td>100</td>
</tr>
<tr>
<td>Glutamate damaged “A”</td>
<td>2.0 ± 0.5 (11)</td>
<td>5</td>
<td>900 ± 50 (13)</td>
<td>41</td>
</tr>
<tr>
<td>Control “B”</td>
<td>37.6 ± 0.8 (10)</td>
<td>100</td>
<td>2,350 ± 60 (11)</td>
<td>100</td>
</tr>
<tr>
<td>rd/le dystrophic “B”</td>
<td>95.0 ± 4.0 (14)</td>
<td>253</td>
<td>4,820 ± 10 (10)</td>
<td>202</td>
</tr>
<tr>
<td>C3H dystrophic</td>
<td>88.9 ± 3.2 (15)</td>
<td>—</td>
<td>5,140 ± 250 (13)</td>
<td>—</td>
</tr>
<tr>
<td>ChAc (total per retina)</td>
<td></td>
<td>Per cent control</td>
<td>AChE (total per retina)</td>
<td>Per cent control</td>
</tr>
<tr>
<td>Control “A”</td>
<td>23.6 ± 0.7 (9)</td>
<td>100</td>
<td>1,360 ± 50 (12)</td>
<td>100</td>
</tr>
<tr>
<td>Glutamate damaged “A”</td>
<td>0.8 ± 0.2 (11)</td>
<td>3</td>
<td>360 ± 20 (13)</td>
<td>26</td>
</tr>
<tr>
<td>Control “B”</td>
<td>25.1 ± 0.8 (10)</td>
<td>100</td>
<td>1,610 ± 50 (11)</td>
<td>100</td>
</tr>
<tr>
<td>rd/le dystrophic “B”</td>
<td>20.4 ± 0.8 (14)</td>
<td>81</td>
<td>1,070 ± 40 (10)</td>
<td>66</td>
</tr>
<tr>
<td>C3H dystrophic</td>
<td>22.9 ± 1.0 (15)</td>
<td>—</td>
<td>1,330 ± 80 (13)</td>
<td>—</td>
</tr>
</tbody>
</table>

Specific activity (above) and total activity per retina (below) of choline acetyltransferase at 37° C. and acetylcholinesterase at 26° C. in (above) millimoles per kilogram of dry weight per hour ± S.E.M. and (below) nanomoles per hour ± S.E.M. Numbers of retinas in parentheses.

Although dark- and light-adapted groups were run, differences between them were neither systematic in direction nor statistically significant. Accordingly, data from dark- and light-adapted groups are pooled.

"A" retinas were from mice of the C57Bl (6J) strain; "B" retinas were from mice of the C57Bl (6J) le/rd strain (see Methods).

control, conclusions drawn from quantitative comparisons are more precarious.

**Discussion**

Chemical comparisons of biologically fractionated retinas and normal retinas are useful in extending the description of the abnormal retinas and may also suggest possible molecular distributions in the normal retina. However, the surviving tissue in the abnormal retinas may have been modified, either by the mechanisms creating the lesions or by the loss of normal synaptic or glial associations. Moreover, normal and abnormal retinas are in different physiologic states. Yet, in previous studies, uniformities or disparities (in either direction) between the inner and outer retina in the concentrations of glycine, GABA, glutamate, and taurine, as suggested by comparisons of retinas of normal, C3H, and glutamate-treated mice, have now been confirmed by direct analyses of known layers of frozen-dried retinas of a number of species.

The current data strongly suggest that a much higher activity of ChAc and a higher activity of AChE are to be found in the inner than outer retina. The current findings for ChAc are consistent with the direct assays by Ross and McDougal of layers of frozen-dried retinas in the rat, mouse, and monkey and also with their similar findings in as yet unpublished studies of other species from five classes of vertebrates to the effect that the specific activity of ChAc in the inner plexiform layer exceeds that in the outer layer by not less than two orders of magnitude. On the other hand, the current data are not inconsistent with Lam's findings of low levels of choline acetyltransferase in turtle cones. Indeed, Lam also found much higher levels of ChAc activity by "dissociated retinal cells" which included mixed cell types from all retinal levels. In evaluating low levels of ACh synthesis one must consider possible ACh synthesis by carnitine acetyltransferase or arylamine N-acetyltransferase and the recent demonstration of ACh synthesis by a neuron which also contains high levels of serotonin. There is also the possibility of contamination of hand-dissected preparations by ChAc.

At least three nonexclusive explanations suggest themselves for the fact that receptorless rd/le retinas contain 10 to 20 per cent less total ChAc than do their controls. First, some ChAc may be lost with the receptors. However, the level of ChAc in the receptor layer by direct assay is extremely low, and would not account for the amount lost in the rd/le retina. Second, in the absence of normal physiologic ac-
tivity, the level of ChAc activity in the remaining retina may not be fully maintained. Finally, some ChAc containing neurons of the inner retina may have been lost at three months in a transneuronal degeneration. This last view is strengthened by the small but significant reduction in the thickness of the inner retina in these animals.

The current study does not bear directly on the use by photoreceptors of acetylcholine as a transmitter or for other metabolic purposes. The ChAc activity in the abnormal retinas seems consistent with its activity in corresponding portions of frozen-dried retinas. Taken together with other work, the current study raises the question of the meaning of the marked activity disparity for both ChAc and AChE in the inner as opposed to the outer retina. Is this due to a greater concentration of cholinergic cells in the inner retina, and/or more terminals per cholinergic cell due to branching, and/or a greater enzyme activity per terminal?

Suppose one assumes that all photoreceptors possess ChAc activity. There are about 1,935 photoreceptors per 10,000 μm² of retina of normal three-month-old mice and their terminals are distributed in depth over a similar area of the outer third of an outer plexiform layer whose total thickness is 12 μm. The terminals consist of frequent spherules of 2.3 μm diameter and much fewer, flat-based, multisynaptic pedicles of 8 μm diameter. An accurate ratio of the two terminal varieties is not known. Assuming spherules to be spheres and pedicles hemispheres and pedicles to constitute 2 per cent of the total number of terminals, the volume of terminals is not more than 15 per cent of the full volume of the outer plexiform layer. However, not all the ChAc may be located in the terminal. Lam's data on turtle cones suggest that cone terminals may contain only 50 per cent of a cone's ChAc activity. Terminals probably constitute a greater percentage of the mass and volume of the inner retina than do receptor terminals a percentage of the mass and volume of the outer retina, but terminals of the inner plexiform layer represent a variety of cells and presumably of transmitter types, whereas all the photoreceptors may contain the same transmitter. We are aware of no data giving the volume per cent of terminal cytoplasm in the inner plexiform region of any retina. This layer is 40 μm thick in the mouse. Again, as in the outer retina, transmitter-related enzymes are probably concentrated in but not confined to the terminal volumes. However, one can say the following. If photoreceptors are assumed to contain ChAc and if terminals and preterminal processes containing the same concentrations of ChAc as those of photoreceptors occupied 45 per cent of the total volume of the inner plexiform layer, i.e., three times the volume per cent occupied by the receptor terminals in the outer plexiform layer, it would not account for the 100-fold ChAc activity disparity shown by the direct assay of the mouse retina by Ross and McDougal or for the 40- to 50-fold disparity for ChAc activity suggested by a comparison of the receptorless retinas and retinas with glutamate-damaged inner layers as reported here.

*ChAc activity (25° C.) in frozen-dried samples dissected from mouse inner plexiform layer was 40.2 ± 0.7 and from outer plexiform layer less than 0.36 mmoles per kilogram of dry weight per hour. ChAc activity at 25° C. was about one-third of that at 38° C.*

REFERENCES

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