Influence of Excitatory Amino Acids and Ischemia on Rat Retinal Choline Acetyltransferase-Containing Cells

N. N. Osborne, A. Larsen, and N. L. Barnett

Purpose. To compare the effects of glutamate agonists and different types of ischemic insult on choline acetyltransferase (ChAT) immunoreactivity in the rat retina.

Methods. Rat retinas were exposed to different glutamate agonists in vivo or in vitro for specific periods of time, and the retinas were then fixed and processed for the localization of ChAT immunoreactivity. In other experiments, rats were administered an ischemic insult either by ligaturing the carotids (two-vessel occlusion [2-VO] procedure), cannulating the anterior chamber, and raising the intraocular pressure (high intraocular pressure [HIOP] procedure) or placing a ligature around the optic nerve sufficiently tightly to prevent blood flow through the central retinal artery (vascular ligation [VL] procedure). The electroretinogram was recorded, and, after a specific period of time, reperfusion was allowed to occur. Thirty to 36 hours after reperfusion, the retinas were dissected and processed for the localization of ChAT, as well as for parvalbumin, Thy-1, and αPKC immunoreactivities.

Results. Of the glutamate agonists tested, only kainate reduced ChAT immunoreactivity significantly in vivo and in vitro. This effect of kainate could be counteracted by the antagonist CNQX (6-cyano-2,3-dihydroxy-7-nitroquinoxaline-2,3-dione). The ChAT immunoreactivity was unaffected in retinas in which ischemia was induced by the 2-VO procedure. In contrast, ChAT immunoreactivity was obliterated in retinas in which the HIOP was used and drastically reduced when the VL procedure was used. Interestingly, neither αPKC nor Thy-1 immunoreactivities were affected in retinas subjected to HIOP or VL methods. However, parvalbumin immunoreactivity was reduced in the HIOP model but only slightly altered in the VL model.

Conclusions. The current results suggest that kainate receptors are associated with the cholinergic retinal neurones in the rat retina. Activation of these receptors by kainate causes a reduction in the neurones’ ChAT content. This effect can be mimicked by subjecting the retina to a sufficiently harsh ischemic insult, as occurs in the VL and HIOP procedures. When the ischemic insult is mild, as in the 2-VO procedure, no obvious change in ChAT immunoreactivity is apparent. The HIOP procedure for inducing an ischemic insult was found to be the most severe of the three procedures used, because ChAT immunoreactivity was obliterated and clear changes in the parvalbumin immunoreactivity also were recorded. Interestingly, neither the HIOP nor the VL procedures caused a change in the Thy-1 and αPKC immunoreactivities.
FIGURE 1. The in vitro effect of various glutamate agonists on retinal choline acetyltransferase (ChAT) immunoreactivity. Retinas were incubated either in physiological solution alone (A) or in solution containing kainate (50 μM) (B), kainate plus CNQX (75 μM) (C), kainate plus MK-801 (5 μM) (D), NMDA (200 μM) (E) or ACPD (200 μM) (F) for 45 minutes. It can be seen (A) that ChAT immunoreactivity in the control retina was associated with amacrine cells on either side of the inner plexiform layer (small arrows) and dendrites, forming two clearly defined layers (large arrows) within the inner plexiform layer. The effect of kainate (B) was to reduce drastically ChAT immunoreactivity, resulting in a few weakly "stained" amacrine cells (small arrows) with the intensity of the two layers in the inner plexiform layer drastically reduced (large arrows). This effect of kainate was completely counteracted when CNQX was present (C) but not by MK-801 (D). Neither NMDA (E) nor ACPD (F) influenced ChAT immunoreactivity. Scale bars = 30 μm.

rapid cellular efflux of potassium and an influx of sodium, calcium, and chloride with osmotically obligated water. The neuronal damage is probably secondary to the influx and mobilization of internal stores of calcium and/or activation of enzymes, such as protein kinase C, the proteases, and lipases. 

Although it is unproven that neurones containing glutamate receptors are the first neurone type to be affected critically by ischemia, the consensus is that overactivation of glutamatergic receptors (particularly ionotropic subtypes), such as occur in ischemia, will lead to cell destruction. The retina contains metabotropic (ACPD and APB) and ionotropic (NMDA, kainate–AMPA) glutamatergic receptors, so it may be predicted that those retinal neurones containing ionotropic glutamate receptors are the most susceptible to ischemia. Because evidence from a number of laboratories suggests that the cholinergic amacrine cells have kainate–AMPA-type receptors, the idea can be tested.

Our recent studies have shown how immunocytochemistry can be used to provide a means for deducing whether ischemia influences a particular retinal cell type. Acetylcholine-containing amacrine cells in
mammalian species, such as the rat, rabbit, and pig, have similar morphologies; their cell bodies are distributed in the inner nuclear and ganglion cell layers, and their dendrites branch into two sublamina in the inner plexiform layer. Because the acetylcholine-containing amacrine cells can be immunocytochemically identified by “staining” for the presence of the enzyme choline acetyltransferase (ChAT), experiments were carried out to see how retinal ChAT-immunoreactivity was affected by excitatory amino acid agonists and ischemia.

It is debatable which is the preferable procedure for inducing experimental ischemia to the retina. Some procedures are clearly more effective than others, but whether this is caused solely by the intensity of the ischemic insult, which can range from mild (oligemia), acute (hypoxia), and chronic (true ischemia), or to additional causes, such as breakdown of the blood–ocular barriers, remains to be clarified. In the experiments to be described, ischemia was induced for comparative purposes by three different procedures in which the b-wave of the electroretinogram was reduced or eliminated. Certain antigens, such as ChAT, were then examined by immunohistochemistry at different reperfusion times.

METHODS

All investigations involving animals conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Wistar rats (200 to 250 g), bred in our laboratory and kept on a 12-hour light–12-hour dark cycle were anesthetized with sodium pentobarbital (60 mg/kg, intraperitoneally).

AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), NMDA (N-methyl-D-aspartate), kainate, ACPD (1S,3R-1-aminocyclopentane-1,3-dicarboxylic acid), and CNQX (6-cyano-2,3-dihydroxy-7-nitroquinoxaline-2,3-dione) were obtained from Tocris Neuramin (Essex, UK). MK-801 (dizocilpine maleate) was obtained from Cambridge Research Biochemicals (Cambridge, UK).

Induction of Ischemia on Anesthetized Rats

Three procedures were used: (1) The common carotid arteries were exposed and occluded for 45 minutes (two-vessel occlusion [2-VO procedure]). (2) The anterior chamber of the eye was cannulated with a 30-gauge infusion needle and connected to a normal saline reservoir. The intraocular pressure was raised to approximately 120 mm Hg (normal range, 84 to
134 mm Hg) for 60 minutes by elevating the saline chamber (high intraocular pressure [HIOP] procedure). (3) A ligature was placed around the optic nerve and tightened sufficiently to prevent blood flow through the central retina and ciliary arteries for 60 minutes (vascular ligation [VL] procedure).

After ischemic insult for a defined period, animals were allowed to recover for specific reperfusion times. The rats were anesthetized, and the blood system was perfused with prewarmed Locke’s solution and then by 2% paraformaldehyde in phosphate buffer. The retinas were dissected and placed in a solution of 2% paraformaldehyde—phosphate buffer for 30 minutes.

In Vitro Studies
Anesthetized rats were rapidly perfused with prewarmed Locke’s solution and decapitated, and their retinas were dissected and placed in Locke’s solution (magnesium free) containing (in mmol/l): 154 Na⁺, 5.6 K⁺, 2.3 Ca²⁺, 164.2 Cl⁻, 3.6 HCO₃⁻, 5.6 glucose, and 5.6 HEPES. The Locke’s solution was equilibrated before use with O₂/CO₂ (95%/5%), pH 7.2, at 37°C.

Whole retinas in Locke’s solution (volume, 2 ml) were incubated either without or with the presence of different concentrations of excitotoxins (NMDA, kainate, ACPD) for 45 minutes at 37°C and then fixed for 30 minutes in 2% paraformaldehyde—phosphate buffer. When antagonists were used, tissues were preincubated with the antagonist for 10 minutes before the addition of the agonist for another 45 minutes.

In Vivo Studies
Injection of drugs into the eye was performed on anesthetized rats. A specific volume (2 μl) of a drug solution was injected into the vitreous humor of one eye from the rat while the other eye (injected with 2 μl of saline) served as a control.

Immunohistochemistry
Retinas fixed in 2% paraformaldehyde (0.1 M phosphate buffer) for 30 minutes at room temperature were cryopreserved in 30% sucrose for 6 to 12 hours. Ten micrometer-thick sections were produced at −20°C and recovered on gelatin-coated glass slides. The sections were incubated overnight at 4°C with rabbit anti-parvalbumin (diluted 1:200; Sigma, St. Louis, MO), mouse anti-αPKC monoclonal antibody (clone MC-5, 1:100; Amersham, UK), mouse anti Thy-1 monoclonal antibody (clone OX-7, 1:10), or mouse anti-ChAT monoclonal antibody (clone 1.B3.9B3, 1:10; Boehringer, Lewis, UK) and developed with goat anti-rabbit immunoglobulin G or rabbit anti-mouse immunoglobulin G conjugated with fluorescein isothiocyanate (1:60; Sigma) when the primary antibodies were, respectively, polyclonal or monoclonal. The sections were mounted in glycerol—phosphate buffer and viewed with epifluorescence optics.

Electroretinography
Rats were dark adapted for at least 30 minutes, their pupils were dilated with 1% tropicamide, and their body temperatures were maintained at 37°C with a heated water jacket. A platinum recording electrode was placed on the cornea, a reference electrode was connected to the tongue, and a ground electrode was connected to the ear. A strobe placed 0.5 m in front of the animal presented the stimulus at 0.5 Hz. Fifteen consecutive responses were amplified and averaged using a 1902 Signal Conditioner—1401 Laboratory Interface (CED; Cambridge, UK). The b-wave amplitudes of electroretinograms were measured from the troughs of the a-waves to the peaks of the b-waves.

RESULTS
In Vitro Studies
In these experiments, the localization of ChAT immunoreactivity was studied, and no attempt was made to determine the influence of glutamate agonists on the overall thickness or morphology of the retina. ChAT immunoreactivity in the rat retina was associated with amacrine perikarya in the ganglion cell and inner nuclear layers and two laminae of processes in the inner plexiform layer (Fig. 1A). No changes were observed in ChAT immunoreactivity in retinal tissues fixed immediately after dissection or incubated in vitro in physiological Locke’s solution, which lacks magnesium ions or retinas perfusion fixed in vivo. The effects of adding kainate (100 μM), NMDA (200 μM), or ACPD (200 μM) to the Locke’s solution for an in vitro incubation period of 45 minutes on ChAT immunoreactivity are shown in Figure 1. Only kainate of the substances tested reduced the overall intensity of ChAT immunoreactivity and the number of immunopositive cell bodies (Fig. 1B). This kainate effect was antagonized when CNQX (75 μM) was present in the incubation medium at the same time as kainate (Fig. 1C). However, when MK-801 (5 μM) was added to the medium, the kainate effect was unchanged (Fig. 1D).

In Vivo Studies
Twenty-four hours after an injection of kainate (calculated to be equivalent to 75 μM) into the eye, ChAT immunoreactivity was almost completely eliminated (Fig. 2B). The ChAT immunoreactivity in the untreated eye was unaffected (Fig. 2A). Injection of CNQX and kainate (equivalent to 50 μM and 75 μM, respectively) into the eye only partially reduced the retinal ChAT immunoreactivity (Fig. 2D) when compared with the staining in the control eye (Fig. 2C). When only CNQX was injected into the eye, ChAT immunoreactivity was unaffected (Fig. 2F) compared with the staining in the retina of the control eye (Fig. 2E). The injection of NMDA or ACPD (equivalent to
FIGURE 3. Comparative reduction of the b-wave of the electroretinogram at the end of an ischemic insult delivered either by the vascular ligation (VL) (n = 7), two-vessel occlusion (2-VO) (n = 6), or high intraocular pressure (HIOP) (n = 8) procedures (see Methods) relative to the b-wave before (Pre.) the insult. The amplitude of the b-wave was completely reduced at the end of a VL or HIOP procedures, whereas in the 2-VO procedure, the b-wave was only reduced by approximately 50%. Results are mean values ± SEM.


FIGURE 4. Effect of the two-vessel occlusion procedure on choline acetyltransferase (ChAT) immunoreactivity. It can be seen that 36 hours after reperfusion, ChAT immunoreactivity was no different (B) from control sham-operated animals (A).
FIGURE 5. In these experiments, one eye (A,C,E,G) served as a control, whereas the other eye (B,D,F,H) received high intraocular pressure (HIOP) as described in Methods. In this model, the insult caused an obliteration of ChAT immunoreactivity (B) when compared with the control eye (A). However, the insult did not appear to influence the αPKC associated with bipolar cell bodies (open arrows) and terminals (arrowheads) (D) or the Thy-1 antigen associated with ganglion cell dendrites (curved arrow) (F) because the result was identical with the control retina (Figs. C,E, respectively). In contrast, the parvalbumin immunoreactivity located to amacrine cells (arrows) and their terminals (G) was almost completely reduced by the HIOP procedure (H). Scale bars = 30 μm.

does not exclude the possibility that the visible effects of kainate on the ChAT-containing neurones is an indirect one. Although all the evidence suggests that this is unlikely, experiments, perhaps on retinal cultures, are necessary to exclude this as a possibility.

The drastic reduction in retinal ChAT immunoreactivity caused by 75 mM kainate in vitro suggested that the enzyme was either "released" or affected in some way within 45 minutes. If the enzyme was released, the "release process" could have been caused by a consequence of membrane disruption due to overactivation of kainate receptors or to exocytosis associated with the synaptic release of acetylcholine. It is known that ChAT is present in vesicles and in the cytoplasm, so both processes could have occurred when the cell was overactivated and in a compromised state. It might be revealing in future experiments to measure ChAT activity in the extracellular fluid after incubation of retinas with kainate. Because ChAT immunoreactivity in the rat retina appeared unaltered after exposure of the retina in vitro to 50 mM KCl for 45 minutes (Osborne, unpublished, 1995), conditions known to cause the release of neurotransmitters, it appeared that the changes seen in ChAT immunoreactivity after exposure to kainate were not caused only by synaptic release.

Another possibility is that kainate and ischemia induced a modification in the enzyme ChAT, perhaps
by phosphorylation, and so made it unrecognizable by the antibody. What was clear was that a sustained activation of kainate receptors, such as occurred in the in vitro experiment, was needed to cause a reduction in the retinal ChAT immunoreactivity within 45 minutes, but when the receptors were activated less drastically, as in retinal ischemia, similar changes in ChAT immunoreactivity occurred after only 36 hours.

As indicated in the introduction, biochemical, pathophysiological, and pharmacologic studies of the events that precede ischemia-related cell death have suggested the overstimulation of excitatory amino acid receptors.\textsuperscript{3-5} Retinal tissues, therefore, were given an ischemic insult in vivo by three different procedures, ranging from mild to chronic; after a reperfusion time of 30 to 36 hours, the retinas were examined for changes in ChAT immunoreactivity. The least drastic of the three methods used to induce an ischemic insult is the 2-VO model in which the blood supply to the retina was reduced partially, whereas in the other two procedures (VL and HIOP models) it was predicted that the retinal blood supply would be suppressed. This was substantiated by the degree of reduction in the b-wave of the electroretinogram at the end of the insult (see Fig. 3), which was least affected in the 2-VO model. The finding that retinal ChAT immunoreactivity was unaffected in the 2-VO model but was either drastically (VL procedure) or completely (HIOP) reduced in the other two models is consistent with this view. The overall findings suggested that when ischemia is of sufficient severity (VL and HIOP models), kainate-type receptors associated with cholinergic neurons are overstimulated to cause a change in ChAT immunoreactivity; when the insult is mild (2-
VO procedure), the released glutamate insufficient to cause a detectable effect on ChAT immunoreactivity. Previous studies\textsuperscript{9,27} on the 2-VO model revealed that the ischemic insult caused an expression of GFAP in the Müller cells, but no histologic changes or alterations in either the GABA, αPKC, or calretinin reactivities could be detected. Thus, all the data on the 2-VO model showed that the ischemic insult to the retina (for a period of 45 minutes) was mild and insufficient to record long-term detrimental effects.

The finding that retinal ischemia, sufficient to reduce more or less completely the b-wave of the electroretinogram (as in the VL and HIOP models), causes ChAT immunoreactivity to be affected is consistent with the findings of Moroni et al.\textsuperscript{28} These authors photochemically induced lesions to the rat retina using rose bengal and found that the activity of ChAT was reduced by approximately 50% (depending on the time used to coagulate the rose bengal) after 36 hours.\textsuperscript{28} The fact that retinal ChAT immunoreactivity was reduced completely in the HIOP model but only partially in the VL model suggested that the "ischemic insult" was more pronounced in the HIOP model. This was supported by the finding that the parvalbumin immunoreactivity was drastically reduced in the HIOP model but only slightly in the VL model. In the HIOP model, the insult should have induced a complete reduction in the ocular blood flow to the retina, whereas in the VL model, it is possible that part of the blood flow remained intact. It was also likely that in the HIOP procedure, part of the insult was caused by a hydrostatic pressure effect, inflammation, or both, and that either of these may have contributed to the greater apparent ischemic insult than occurred in the VL procedure.

The glutamate receptors associated with the on-bipolar cells, which contain αPKC, have been characterized and shown to be of the APB-type.\textsuperscript{6,29} Activation of APB-type receptors is associated with an increase in the hydrolysis of cyclic guanosine 3,5'-monophosphate\textsuperscript{30} and not a direct influx of calcium. Thus, one would expect the on-bipolar cells to be relatively resistant to an ischemic insult. Such a prediction was strengthened by the current study in which the αPKC immunoreactivities in the HIOP and VL models were unaffected by the same insults that reduced ChAT immunoreactivity. However, in the case of Thy-1 immunoreactivity, which was also unaffected in the two models, the result was surprising because the Thy-1 antigen was associated with ganglion cells\textsuperscript{30} and these neurons have been shown to contain various types of glutamate receptors, including the NMDA-type.\textsuperscript{31,32}

Because activation of NMDA receptors leads to a massive influx of calcium, it has been predicted that neurons containing NMDA receptors would be particularly susceptible to an ischemic insult.\textsuperscript{3-5} The finding that the ganglion cells, indexed by their Thy-1 immunoactivity, were unaffected by ischemia was inconsistent with this view. However, caution in interpretation is necessary because the Thy-1 antigen is associated with membranes,\textsuperscript{30} and any changes may have been evident only at advanced stages of cell destruction, that is, more than 36 hours after the ischemic insult. This is not true of αPKC because this enzyme is involved in transduction processes and is activated by calcium\textsuperscript{33,34}; therefore, it would have been affected rapidly by the cascade of events associated with an ischemic insult.

**Key Words**

choline-acetyltransferase, immunoreactivity, ischemia, kainate, retina

**Acknowledgments**

The authors thank Nigel Swietalski for his expert technical assistance.

**References**


12. Eckenstein F, Theonen H. Production of specific anti-


