Acetylcholine Receptors in the Human Retina

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Evidence for a population of acetylcholine (ACh) receptors in the human retina is presented. The authors have used the irreversible ligand \(^3\)H-propylbenzilylcholine mustard (\(^3\)H-PrBCM) to label muscarinic receptors. \(^3\)H- or \(^125\)I-\(\alpha\)-bungarotoxin (\(\alpha\)-BTx) was used to label putative nicotinic receptors. Muscarinic receptors are apparently present in the inner plexiform layer of the retina. Autoradiographic grain densities are reduced in the presence of saturating concentrations of atropine, quinuclidinyl benzilate or scopolamine; this indicates that \(^3\)H-PrBCM binding is specific for a population of muscarinic receptors in the human retina. Binding sites for radiolabeled \(\alpha\)-BTx are found predominantly in the inner plexiform layer of the retina. Grain densities are reduced in the presence of \(d\)-tubocurarine, indicating that \(\alpha\)-BTx may bind to a pharmacologically relevant nicotinic ACh receptor. The work reported here is consistent with earlier data on cholinergic neurons in the retina of other mammalian species, as well as with previous work on the psychophysical effects of cholinergic agonists on human vision. This study provides evidence for cholinergic neurotransmission in the human retina. Invest Ophthalmol Vis Sci 26:1550–1557, 1985

The identification of functional neurotransmitter receptors is critical to an understanding of the characteristics of intercellular communication in the nervous system. In the case of acetylcholine (ACh), two main types of receptors are known: muscarinic and nicotinic. One method for studying the localization of these receptors is to identify binding sites autoradiographically in fixed, embedded, and sectioned material. Although both receptor types are amenable to in vitro binding studies, identification of receptor location in histologically processed tissue is more difficult.

We now report the localization of putative nicotinic and muscarinic ACh receptors in the human retina using light microscopic autoradiography. We have calculated the density of silver grains over the five well-defined tissue regions of the vertebrate retina. By identifying areas rich in putative ACh receptors, we can infer possible functional roles for cholinergic neurotransmission.

Evidence for ACh as a Neurotransmitter in Mammalian Retina

Rabbit retinas synthesize and release labeled ACh when labeled choline is used as a precursor.\(^1\) Acetylcholinesterase activity is concentrated in the inner plexiform layer of the rabbit retina.\(^3\) Electrophysiological studies indicate that ACh may mediate excitation of rabbit ganglion cells in the “on” retinal pathway.\(^4\) In the cat retina, ganglion cell responses are modified by cholinergic antagonists,\(^5\) and a study using peroxidase-conjugated \(\alpha\)-BTx has shown that amacrine cells are presynaptic to \(\alpha\)-BTx-labeled junctions.\(^7\) Bovine retinal homogenates specifically bind the muscarinic ligand \(^3\)H-quinuclidinyl benzilate.\(^8\)\(^–\)\(^10\) Several comprehensive reviews on the role of ACh in the mammalian retina have been published.\(^11\)\(^–\)\(^13\)

In humans, psychophysical studies have implicated ACh as a neurotransmitter at some point in the visual system. Rengstorff and Royston\(^14\) have reviewed the effects of muscarinic cholinergic agonists and anticholinesterases on the human visual system; many authors report decreases in visual acuity, ability to discriminate a flickering light, and ability to adapt to darkness.

Specificity of Cholinergic Receptor Probes

In the case of nicotinic ACh receptors, the irreversible ligand \(\alpha\)-bungarotoxin (\(\alpha\)-BTx) has been used for nearly two decades for localization of nicotinic ACh receptor sites in the peripheral nervous system.\(^15\)\(^–\)\(^17\) Bound \(\alpha\)-BTx is apparently immobilized by conventional fixatives, and thus can be used for putative nicotinic receptor autoradiography. It should be noted that the specificity of \(\alpha\)-BTx binding in the central nervous system has been called into question by a number of authors.\(^18\) One recent study in goldfish retina found that 84% of the \(\alpha\)-BTx binding sites in this tissue are
located away from morphologically identifiable synapses.\textsuperscript{19}

Since none of the conventional muscarinic agonists or antagonists are retained in tissue during fixation and embedding with the usual reagents, the introduction of the irreversible muscarinic ligand propylbenzilylcholine mustard (PrBCM) by Young et al\textsuperscript{20} provided the first opportunity to study muscarinic ACh receptors autoradiographically in tissue processed by conventional histological methods. The tritiated form of the ligand, \textsuperscript{3}H-PrBCM, has been used to study muscarinic ACh receptors at both the light\textsuperscript{21} and electron microscopic\textsuperscript{22} level in various regions of the rat brain. Subsequently, we have used \textsuperscript{3}H-PrBCM to label salamander retinas\textsuperscript{23} and the human iris sphincter and dilator muscle regions.\textsuperscript{24}

Our observations, reported here, indicate that ACh is a likely neurotransmitter candidate in the human retina.

Materials and Methods

Human eyes were enucleated shortly after death of the donor and transported to the laboratory on ice by technicians from the Lions Eyes of Texas Eye Bank. The time between donor death and receipt of tissue in the laboratory varied from 15 to 60 min, with a mean time of about 35 min. Eleven donors (nine males and two females), ranging in age from 2 to 67 yr of age, were used for these experiments; all decades (except the sixth decade) are represented here. In many cases, corneas were removed by eye bank personnel prior to further dissection of the eye.

Human retinas were isolated according to the following procedure: the globe was hemisected near the ora serrata, the vitreous was removed, and the retina was gently washed with a Ringer's solution and teased away from the sclera with fine forceps. A cut was made around the optic nerve head to release the retina, either as an isolated tissue or as a retina/pigment epithelium/choroid complex. The Ringer's solution used, Ringer's-bicarbonate-pyruvate (RBP) is a modification of the "RBG" medium of Basinger and Hoffman,\textsuperscript{25} as described in an earlier paper.\textsuperscript{24} Retinal "buttons" were cut with a trephine 3 mm in diameter.

Histological Processing and Autoradiography

Buttons were preincubated in either RBP (saturated with 95% O\textsubscript{2}/5% CO\textsubscript{2}) or oxygenated RBP containing 1 to 100 \(\mu\)M inhibitor. Inhibitors used for muscarinic receptor studies were atropine (seven experiments), scopolamine (one experiment) and quinuclidinyl benzilate hydrochloride (QNB, five experiments). QNB-free base was a gracious gift of Dr. Peter Sorter, Hoffman-LaRoche (Nutley, NJ). In four experiments, 1 to 10 \(\mu\)M nicotine was used to further verify the specificity of \textsuperscript{3}H-PrBCM binding (that is, nicotine should have minimal effects on specific labeling). For studies of \(\alpha\)-BTx binding, d-tubocurarine was used in the preincubation solution. Preincubation was carried out for 15 or 30 min at room temperature. Retinal buttons were then labeled with ligand (see below) for 30 min at room temperature, rinsed briefly in fresh RBP, fixed in 2% formaldehyde, 1% glutaraldehyde, 0.1 M sodium phosphate buffer (pH 7.4) for 1 hr, postfixed in phosphate-buffered 1% OsO\textsubscript{4}, dehydrated in a rapid ethanol series and embedded in Epon. Sections (2 \(\mu\)m thick) were floated onto drops of water on a microscope slide and dried onto the slide on a hot plate. Slides were dipped into molten Kodak NTB2 photographic emulsion (diluted 1:1, v:v, with distilled water) and allowed to dry in a cool, dark room. Autoradiograms were exposed from 4 to 22 wk at 4°C. A Zeiss Videoplan system (New York, NY) was used to determine grain counts per unit area. Defined tissue areas were drawn onto a digitizer pad using a camera lucida, and the area of the layer of interest was determined. At least four sections were counted from each protocol in each experiment, and the average grain counts per unit area of tissue were calculated.

Ligand Binding

Retinal buttons were exposed to either \textsuperscript{3}H-PrBCM, \textsuperscript{3}H-\(\alpha\)-BTx or \textsuperscript{125}I-\(\alpha\)-BTx in the presence (controls) or absence of the same inhibitor (at the same concentration) that was used for preincubation. A concentrated ethanolic solution, \textgreek{1}\textsuperscript{3}H-PrBCM was supplied (New England Nuclear; Boston, MA) and was stored in 25 \(\mu\)l (25 \(\mu\)Ci) aliquots in sealed glass tubes at -80°C until used. Each aliquot was diluted with 10 mM phosphate buffer and allowed to stand at room temperature for one hour. This procedure converts the inactive form of \textsuperscript{3}H-PrBCM in this stock solution was 1 \(\mu\)M. After activation of the ligand, it was diluted to its final concentration of 50 nM with either RBP or RBP containing 1 to 100 \(\mu\)M inhibitor, as in the preincubation solution.

\textsuperscript{3}H-propionyl-\(\alpha\)-BTx (\textsuperscript{3}H-\(\alpha\)-BTx, Amersham; Arlington Heights, IL) and \textsuperscript{125}I-\(\alpha\)-BTx (New England Nuclear) were supplied in aqueous solution. The ligand was diluted to a final concentration of 70 nM (0.55 \(\mu\)M/ml) \textsuperscript{3}H-\(\alpha\)-BTx or 10 nM \textsuperscript{125}I-\(\alpha\)-BTx (0.08 \(\mu\)g/ml) in either RBP or RBP containing 100 \(\mu\)M d-tubocurarine.

Lowicryl-Embedded Tissue

Some tissue was labeled with \textsuperscript{3}H-PrBCM in the presence or absence of inhibitors as above, but was embed-
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Fig. 1. Summary histogram of autoradiographic grain counts from ^3^H-PrBCM binding experiments. A, The results of seven experiments using 50 nM ^3^H-PrBCM (with no inhibitor present) in Epon-embedded tissue are summarized here. Since different experiments resulted in various grain densities (eg, due to differences in emulsion thickness, autoradiographic exposure time, etc), data from each experiment were normalized by dividing the crude grain counts per 100 \( \mu \text{m}^2 \) from each tissue compartment (ie, ONL, OPL, INL, IPL or GCL) by the mean grain counts over the entire retina. B, Results from a single experiment using 50 nM ^3^H-PrBCM in Lowicryl-embedded tissue. The IPL is the only layer showing significantly increased binding. Data are expressed as mean ± SEM. Abbreviations and symbols used: ONL (outer nuclear layer); OPL (outer plexiform layer); INL (inner nuclear layer); IPL (inner plexiform layer); GCL (ganglion cell layer); *(significantly different from adjacent bars at \( p < 0.01 \) level); **(significantly different at \( p < 0.005 \) level). Non-tissue background counts are also shown (dashed line).

Results

Muscarinic Acetylcholine Receptors

In all experiments using ^3^H-PrBCM in the human retina, the inner plexiform layer (IPL) showed the highest density of autoradiographic silver grains. Results of seven such experiments are summarized in Figure 1A. The data were normalized by dividing all grain counts (expressed as grains/100 \( \mu \text{m}^2 \)) by the mean grain count over the entire retina in the same experiment. This minimizes differences between experiments which arise from various factors (eg, autoradiographic exposure times or emulsion thickness). As can be seen in Figure 1A, the density of autoradiographic grains over the IPL is almost three times higher than over the cellular layers in Epon-embedded retinas. In Lowicryl-embedded retinas, the IPL grain density was about six times higher than in any other layer. The difference between the IPL and any of the cellular layers (outer nuclear, inner nuclear, ganglion cell) is significant at the \( p < 0.005 \) level (student’s t-test). Grain density over the outer plexiform layer (OPL) in Epon-embedded retinas is also significantly higher than over the cellular layers \( (p < 0.01) \); there is no significant difference between the OPL and the cellular layers in Lowicryl-embedded retinas. This increased autoradiographic silver grain density over the outer plexiform layer presumably reflects a much higher density of ^3^H-PrBCM binding sites in this layer of the retina relative to all other layers.

Figure 2 shows both autoradiographs and histograms of grain density for a typical experiment. In the presence of 1 \( \mu \text{M} \) atropine (Fig. 2B), grain density over all retinal layers is greatly reduced. The presence of similar concentrations of QNB • HC1 also results in a substantial reduction in ^3^H-PrBCM binding to all retinal layers (data not shown). Nicotine (1 to 10 \( \mu \text{M} \)) affects binding in the nuclear layers more strongly than binding in the plexiform layers (Fig. 2C). Note that the total grain density is almost identical to the atropine-resistant (ie, non-specific) counts plus nicotine-resistant (ie, specific) counts, even though the histograms and micrographs represent three separate pieces of tissue. Tissue embed-
Fig. 2. Autoradiograms showing $^3$H-PrBCM binding in the retina. Bright-field (left column) and dark-field (center column) autoradiograms are shown for each of three experimental conditions: 50 nM $^3$H-PrBCM alone (Fig. 2A); $^3$H-PrBCM plus 1 μM atropine (Fig. 2B); and $^3$H-PrBCM plus 1 μM nicotine (Fig. 2C). The right column shows histograms of the grain count data (from this experiment only) for all three conditions, calculated as described in Methods. Histogram data are mean ± SEM; autoradiographic exposure time 15 wk; scale bar 10 μM (all micrographs printed to the same final magnification, X650).
Fig. 3. Autoradiograms of $^{3}$H-PrBCM-labeled, Lowicryl-embedded tissue sections. Bright-field (upper row) and dark-field (bottom row) autoradiograms are shown for each of three different conditions: labeling with 50 nM $^{3}$H-PrBCM (Fig. 3A); $^{3}$H-PrBCM plus 10 μM atropine (Fig. 3B); and $^{3}$H-PrBCM plus 10 μM scopolamine (Fig. 3C). Note the enhanced grain counts in the OPL and IPL in Figure 3A and the substantially reduced grain counts over both plexiform layers in the controls (Fig. 3B and C). The unusual staining pattern of Lowicryl-embedded tissue results in a “negatively-stained” appearance in the bright-field autoradiograms. Sections were stained in 1% acid fuchsin after autoradiographic exposure of 12 wk. Scale bar 10 μm (all micrographs printed to same final magnification, x500).

ded in Lowicryl resin before sectioning is shown in Figure 3. With similar autoradiographic exposure times, the density of grains over the cellular layers is manifestly reduced. However, a high density of labeling of the OPL and IPL can still be seen. The reasons for the improved localization of label in Lowicryl-embedded tissue are not clear. Lowicryl-embedded tissue was not fixed in osmium tetroxide; this fixative may cross-link unbound $^{3}$H-PrBCM remaining in the tissue after the labeling step. DMF or other reagents used in the Lowicryl embedding procedure may be more effective in removing unbound or non-specifically bound $^{3}$H-PrBCM from retinal tissue. Autoradiograms from Lowicryl-embedded retinal buttons preincubated and incubated in the presence of 10 μM atropine or scopolamine are shown in Figures 3B and 3C. Grain counts are markedly reduced in both plexiform layers, demonstrating the specificity of $^{3}$H-PrBCM labeling under these conditions.

In two experiments, a study was made of possible sublamination of $^{3}$H-PrBCM binding sites within the IPL, since it has been suggested that separate physiological pathways are localized to discrete layers of the IPL. We cut oblique sections, and divided the IPL into ten bins of equal width. There may be some increase in muscarinic sites in the center of the IPL relative to the edges, but the difference is slight, if it exists at all (data not shown). The binding capacity of $^{3}$H-PrBCM (expressed as silver grains/unit area) does not vary from foveal to peripheral retina (data not shown).
Nicotinic Acetylcholine Receptors

Autoradiograms of sections from tissue exposed to either 70 nM \( ^3H \alpha-BT x \) (three experiments) or 10 nM \( ^{125}I \alpha-BT x \) (one experiment) gave similar results and will be discussed together in this section. Figure 4 shows a summary histogram of data from these experiments. Again, data were normalized to the mean grain counts over the entire retina from the sections not incubated with d-tubocurarine. Figure 4 shows specific grain distribution (ie, total counts minus non-specific counts). It is apparent that specific grain counts are significantly higher in the IPL than in the cellular layers \((p < 0.005)\); grain counts in the OPL, however, are not significantly different from adjacent layers.

Figure 5 is a pair of autoradiograms from sections of two retinal buttons: one incubated with \( ^3H \alpha-BT x \), and its control labeled with the same ligand in the presence of 100 \( \mu M \) d-tubocurarine. Autoradiographic grain density is reduced throughout the retina in the presence of this nicotinic antagonist (Fig. 5B).

Discussion

The data presented here provide evidence for the presence of muscarinic and nicotinic acetylcholine receptors in the human retina. In this and previous studies with other collaborators,\(^23-24,28\) we have demonstrated the specificity of the ligand \( ^3H \)PrBCM using three disparate methods: (1) binding of \( ^3H \)PrBCM to salamander retinal slices in vitro is saturable and specific; (2) the human iris sphincter muscle, which is a paradigmatic muscarinic cholinergic tissue, has been shown to possess muscarinic receptors using the same techniques described here; and (3) in all of these studies, we have demonstrated pharmacological specificity in controls preincubated and co-incubated with well-characterized muscarinic antagonists (atropine, QNB and scopolamine). The apparently specific grain counts present in the nuclear layers of Epon-embedded retinas reflect either binding sites not associated with receptors and/or binding to non-synaptic receptors (eg, nascent muscarinic receptors). The absence of these “specific” grains over nuclear regions in the Lowicryl-embedded tissue argues for the former interpretation. In an abstract, Zarbin et al\(^29\) described the distribution of \( ^3H \)N-methylscopolamine binding sites in normal human retina; their findings are similar to the distribution of \( ^3H \)PrBCM binding sites shown here (ie, a high degree of binding in the IPL, and a lesser degree in the OPL). At the same meeting, we reported the data discussed in full here.\(^28\) There seems little doubt, then, that the inner plexiform layer of the human retina contains muscarinic receptors for ACh.

The presence of nicotinic receptors is much more difficult to prove using the methods we have described here. The pharmacological specificity (or lack of it) of \( \alpha-BT x \) binding in the CNS has been discussed thoroughly in a number of reviews,\(^18\) and we will not attempt to abstract the arguments pro and con here. At this juncture, it can only be said with certainty that there are binding sites for \( \alpha-BT x \) in the human retina, and that these sites are apparently blocked by d-tubocurarine.

Studies using the rabbit and cat retina have demonstrated quite convincingly that a significant population of cholinergic amacrine and displaced amacrine cells exist in those tissues. Presumably, the cholinceptive cells which we have labeled using our autoradiographic techniques for ACh receptors represent a group of neurons which are postsynaptic to cholinergic amacrine cells. However, the presence of autoreceptors cannot be ruled out, nor can it be assumed that the cholinergic cells, if any, in the human retina are necessarily amacrine cells. In future work, we intend to further characterize components of the cholinergic system in the human retina.
Fig. 5. Autoradiograms showing $^3$H-α-BTx binding in the retina. Bright-field (left column) and dark-field (center column) autoradiograms of sections from retinas incubated in the presence of 70 nM $^3$H-α-BTx alone (Fig. 5A) or 70 nM $^3$H-α-BTx plus 100 μM d-tubocurarine (Fig. 5B) are shown in the micrographs. Note the enrichment of silver grains over the IPL only; silver grains are not enriched in the OPL relative to adjacent layers. These autoradiographic grain densities are summarized (for this experiment only) in the accompanying histograms (right column). In the presence of d-tubocurarine, overall silver grain density is markedly reduced in all tissue layers. However, grains seen over the nuclear layers, the outer plexiform layer, the photoreceptor outer segments, and the interphotoreceptor matrix are not affected by the presence of d-tubocurarine, and presumably represent non-specific binding sites. Abbreviations are the same as in previous figures. Autoradiographic exposure time 22 wk; scale bar 10 μm (all micrographs printed to same final magnification, ×800).
Key words: acetylcholine receptors, α-bungarotoxin, human vision, propylbenzilylcholine mustard, retina

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References