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Pharmacology, Distribution and Development of Muscarinic Acetylcholine Receptor Subtypes in the Optic Tectum of *Rana Pipiens*

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Abstract

Visually evoked behaviors mediated by the frog optic tectum require cholinergic activity, but the receptor subtypes through which acetylcholine acts are not yet identified. Using quantitative autoradiography and scintillation spectrometry, we examined the binding of [³H]pirenzepine and [³H]AF-DX 384 in the laminated optic tectum of the frog. In mammalian systems, these substances bind excitatory (m1 and m3 subtypes) and inhibitory (m2 and m4 subtypes) muscarinic acetylcholine receptors, respectively. Pharmacological analyses, including the use of specific muscarinic toxins, confirmed the subtype selectivity of the radioligands in the frog brain. Binding sites for [³H]pirenzepine were distinct from those for [³H]AF-DX 384. In the adult tectum, [³H]pirenzepine demonstrated specific binding in tectal layers 5–9. [³H]Pirenzepine binding was also present in tadpoles as young as stage V, but all sampled stages of tadpole tectum had significantly less binding when compared to adults. Lesioning of the optic nerve had no effect on [³H]pirenzepine binding. Specific [³H]AF-DX 384 binding was found in all layers of the adult tectum. All sampled tadpole stages exhibited binding sites for [³H]AF-DX 384, but the densities of these sites were also significantly higher in adults than they were in developing stages. Short-term lesions of the optic nerve reduced [³H]AF-DX 384 binding in all tectal layers of the deafferented lobe when compared to the afferented one. Long-term lesions decreased [³H]AF-DX 384 sites in both lobes.

These results indicate that multiple muscarinic acetylcholine receptor binding sites reside in the frog optic tectum at all stages of development, and their pharmacology resembles that of mammalian m1/m3, m2 and m4 subtypes. Our data indicate that few, if any, of these receptors are likely to be located on retinal ganglion cell terminals. Furthermore, the expression of inhibitory muscarinic subtypes seems to be regulated by different mechanisms than that for excitatory subtypes.

Keywords

cholinergic; visual plasticity; autoradiography; amphibia

The optic tectum is the primary visual area of non-mammalian vertebrates and serves as a useful model for studying mechanisms of visual processing and the structural organization of topographic maps.^{14,15,18,25,84} Many studies in the brain have shown the importance of neuronal activity in maintaining the order of the visual map.^{17,50,63,74} One of the factors affecting this activity is the presence of modulatory neurotransmitters within the system.

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Although neuromodulators have been shown to change visual activity in the tectum, little is known about the receptors that mediate the observed alterations.^{29,58}

Acetylcholine (ACh) is one such modulator of visual signals. The majority of ACh released in the tectum is supplied by the nucleus isthmi, another mesencephalic structure.^{23,59,86} The facilitatory and necessary effects of ACh on visual signal processing are demonstrated by the fact that destruction of the nucleus isthmi produces a visual scotoma, although retinal input remains intact.³⁶ Furthermore, stimulation of the nucleus isthmi or application of ACh to the tectum enhances the response to visual stimulation,^{29,47} and interfering with cholinergic receptor activity disrupts the topography of the activity-dependent retinotectal map.^{75,76,82}

ACh has similar effects on the processing and organizing of visual signals in the mammalian brain.⁶⁴ The visual cortex is innervated by the nucleus basalis magnocellularis, a cholinergic center in the basal fore-brain.⁷² The ACh released from this projection is thought to influence the excitability^{68,71} and receptive field size³³ of cortical neurons that respond to visual stimuli. ACh also plays a role in the developmental plasticity of this brain structure by affecting ocular dominance column plasticity,⁴³ visual map formation^{4,38,39} and the connectivity of inputs from the lateral geniculate.⁵⁶ However, the mechanisms behind the effects of ACh in the visual system are still unclear, because our understanding of the many cholinergic receptor subtypes, their properties and their functional roles is incomplete. Previous work in our laboratory has characterized the binding of nicotinic ACh receptors in the visual system of the frog.¹⁰ This report focuses on muscarinic ACh receptors.

Muscarinic cholinergic receptors typically produce long-lasting responses that are due to the activation of G-protein cascades; therefore, these receptors could be potent modulators of visual activity. Cloning studies have revealed five genes that encode distinct muscarinic ACh receptor (mAChR) subtypes (m1–m5) in mammals. Native studies and ectopic expression systems have described the general intracellular effects of these different mAChR subtypes when activated. The pharmacologically defined M1-like subtypes (m1, m3 and m5) mediate neuronal excitation via activation of phospholipases and tyrosine kinases, an increase in intracellular calcium, and membrane depolarization. All M1-like receptors are bound by [³H]pirenzepine, but this radioligand has a particularly high affinity for the m1 subtype.^{7,24} The M2-like receptors (m2 and m4) are selectively bound by [³H]AF-DX 384,^{24,61} and their activity results in neuronal inhibition due to membrane hyperpolarization and decreases in cyclic nucleotide production.²⁷

Although mAChRs are present in the frog optic tectum,^{5,29,81} little is known about their subtype classification. In this study, we have used [³H]pirenzepine and [³H]AF-DX 384 to examine the pharmacology, distribution and development of M1-like and M2-like mAChRs, respectively, in the frog brain. Some aspects of this work have appeared previously in abstract form.^{8,9}

Experimental Procedures

Animals

Adult *Rana pipiens* were purchased from Charles Sullivan (Nashville, TN, USA). These adult animals were kept in 10-gallon glass tanks and fed mealworms. Tadpoles were produced from fertilized eggs and raised in the laboratory in 4-gallon plastic tubs. They were fed boiled nettle until they were large enough to eat boiled Romaine lettuce. The developmental stage of the tadpoles was determined by the morphological characteristics described by Taylor and Kollros.⁷⁹ Every effort was made to minimize the suffering and number of animals used. All animal care, dissection and surgical procedures were approved by the Institutional Animal Care and Use Committee at the University of Kentucky.

Materials

All radioligands were purchased from New England Nuclear (Boston, MA, USA). Radioactive standards and autoradiography film were purchased from the Amersham Corporation (Arlington Heights, IL, USA). Unlabeled AF-DX 384 methanesulfonate was a gift from Dr Stephen Müller at Boehringer-Ingelheim GmbH (Biberach, Germany). Muscarinic toxin-1 (MT1) and muscarinic toxin-3 (MT3) were purchased from Calbiochem (La Jolla, CA, USA). Atropine sulfate, unlabeled pirenzepine and all buffer reagents were purchased from Sigma (St Louis, MO, USA).

Tissue preparation and data analysis

Determination of radioligand binding properties—Pharmacological analyses were used to determine the selectivity of the radioligands for muscarinic binding sites. Each experiment was carried out in triplicate ($N = 4$ or 5). Saturation and competition analyses of muscarinic radioligand binding were performed according to an adaptation of the methods of Birdsall *et al.*,⁵ Fiedler *et al.*,²⁸ Leslie *et al.*,⁵² and Jerusalinsky *et al.*⁴⁴

Tissue homogenates were made from the whole brains of two adult animals that had been anesthetized with an intraperitoneal injection of 2% ethyl *m*-aminobenzoate (MS222; 100 μ l/15 g animal weight) prior to decapitation. Fresh brains were rapidly removed and placed in ice-cold (4°C) buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.4). The dura mater and pia mater were removed, and the brains were weighed. The tissue was pooled and homogenized in 10 volumes of ice-cold buffer. Membrane homogenates were prepared through the method of Romano and Goldstein.⁶⁹ No detergent was added nor were any attempts made to purify the membrane proteins. The protein content of the membrane preparations was determined by the Bradford method,⁶ and the preparations were diluted to a final protein concentration of 300 μ g/ml at incubation. All incubations were conducted in 12 mm $\times$ 75 mm polypropylene tubes, and the final incubation volume was 200 μ l. Incubations were performed to equilibrium with [³H]*N*-methylscopolamine ([³H]NMS; 82.0 Ci/mmol; 5 nM), [³H]pirenzepine (85.6 Ci/mmol; 0.4–111.2 nM) or [³H]AF-DX 384 (133.1 Ci/mmol; 0.2–55.8 nM) and lasted for 90 min at 30°C (adapted from Birdsall *et al.*⁵). Non-specific binding was determined with the addition of atropine sulfate (10 μ M final concentration). The reaction was terminated by immediate vacuum filtration onto glass fiber filters (Schleicher & Schuell, Keene, NH, USA), which were presoaked in ice-cold 0.5% polyethylenimine, and four washes with ice-cold buffer.

Competition analyses of [³H]NMS (5 nM), [³H]pirenzepine (10 nM) and [³H]AF-DX 384 (5 nM) binding were performed through the filtration method described above. [³H]NMS binding was competed against 10 concentrations of unlabeled pirenzepine or unlabeled AF-DX 384 (1 nM to 1 mM). [³H]Pirenzepine binding was competed against 10 concentrations of unlabeled AF-DX 384 (1 nM to 10 μ M), 11 concentrations of MT1 (500 pM to 1 μ M) and 11 concentrations of MT3 (300 pM to 300 nM). [³H]AF-DX 384 binding was competed against 10 concentrations of unlabeled pirenzepine (1 nM to 1 mM) and the same concentrations of MT1 and MT3 mentioned above. All experiments using the toxins had bovine serum albumin (1 mg/ml final concentration) added to the incubation solutions in order to protect the toxins from proteolysis. Non-specific binding was again accounted for by adding excess atropine sulfate (10 μ M) to some samples during the radioligand incubation. The MT3 experiments in these whole-brain preparations were performed only twice because the toxin did not significantly inhibit radioligand binding.

The amount of radioactivity bound to the glass-fiber filters was determined by liquid scintillation spectrometry in 3a70B scintillation cocktail (RPI, Mt Prospect, IL, USA). The counting efficiency was 40%. The amount of binding (decays/minute) was converted to fmol/mg protein based upon the specific activity of the radioligand being used, the volume of the

reaction mixture and the amount of brain protein in the reaction. Specific binding was calculated as the difference between total binding and non-specific binding. The results were then plotted in terms of specific binding and analyzed with GraphPad Prism 3.0 (GraphPad Software, San Diego, CA, USA). To decide whether to use one-site versus two-site fits to the data, the fits were compared with an *F*-test ($P < 0.05$). Significant differences in the calculated affinity values of the various radioligands were determined by taking the negative logarithm of those values and comparing them with an unpaired *t*-test ($P < 0.05$).

Binding site localization in the optic tectum—In order to localize the binding to specific tectal layers, autoradiographic experiments were performed on sagittal sections of the frog brain with [³H]pirenzepine (10 nM) and [³H]AF-DX 384 (5 nM). Tissue was collected from adult frogs that had been anesthetized as described above. The chest cavity was opened and the animals were perfused through the conus arteriosus with ice-cold buffer. The brains were extracted, the pia mater was removed and the tissue was frozen in isopentane that had been chilled with dry ice. The frozen brains were then mounted on a cryostat and cut along the sagittal or frontal plane into 20-μm sections. The sections were thaw-mounted on Superfrost Plus slides (VWR Scientific Products, Cincinnati, OH, USA) and dried overnight in a vacuum desiccator at 4°C. The mounted sections were stored at -80°C until use or prepared for radioligand binding the next day.¹⁰

Receptor autoradiography was performed through standard methods.⁵² Briefly, the tissue was slowly brought to room temperature, preincubated in buffer for 15 min, then incubated in a buffer solution containing radioligand. Non-specific binding was determined on adjacent tissue sections by the addition of excess atropine sulfate (10 μM) to the radioligand solutions. Incubations lasted for 90 min at room temperature and were terminated by a 5-min wash in fresh buffer solution, a 5-min wash in 10% buffer and a 10-s dip in deionized water. All washes were performed at 4°C (adapted from Aubert *et al.*³). After washing, the tissue was dried under a gentle stream of ambient air and placed in a vacuum desiccator overnight at room temperature. The following day, quantitative standards (Amersham, Arlington Heights, IL, USA) and the treated tissue were set together against ³H-Hyperfilm (Amersham). The film was exposed for an appropriate length of time (generally nine to 12 weeks for [³H]pirenzepine and three to five weeks for [³H]AF-DX 384), and the resulting autoradiograms were captured with a digital camera (Dage CCD100, Michigan City, IN, USA) connected to an Apple PowerPC running NIH Image 1.61 (<http://www.nih.gov>). The treated tissue was then Nissl stained with Thionin after being defatted with xylene and ethanol.¹³ Bright-field images of the stained tectal tissue were then superimposed over their respective autoradiograms using Adobe Photoshop 5.0. Staining procedures did not cause appreciable shrinkage of the tissue because it had already been dried during its preparation for autoradiography. Consequently, the alignment of the two types of images was precise.¹⁰

The density of binding was determined by comparing the optical densities of the tissue autoradiograms to those made by the radioactive standards, which contained a known amount of radioactivity and were the same thickness as the tissue sections. The gray-scale values of the standards were converted from nCi/mg wet tissue weight to fmol/mg wet tissue weight, based upon the specific activity of the radioligand being used. A standard curve, constructed from a third-degree polynomial equation, interpolated the gray-scale value into the binding density of radioligand in the tissue. For each radioligand, a minimum of three sections from at least five animals were used in these experiments.

Developmental studies—Tissue from adults and tadpoles (stages V, X, XV, XX and XXV)⁷⁹ was prepared for autoradiography as described above. Tadpole tissue was prepared in a slightly different fashion than that of adults. The tadpoles were anesthetized by immersion in 0.1% MS222, decapitated and the whole heads were then frozen in isopentane that had been

chilled with dry ice. To avoid differences in radioligand concentration between experiments, the binding runs were performed simultaneously on all stages with the same incubation solutions. The treated tissue from all stages was then exposed to the same piece of ^3H -Hyperfilm in order to avoid possible sensitivity differences in the film.

The optic tectum matures along rostrocaudal and lateromedial gradients. The tectal layers and their thickness are first established in rostralateral regions, and they extend more caudomedially as the animal matures.⁶⁷ To examine whether differences in binding density correlated with the anatomical differences in the tectum during development, we measured binding in three distinct areas of the superficial layers in sagittal sections: midway at the highest point of the tectum, anterior at approximately 45° from the midpoint and posterior at approximately 45° from the midpoint (Fig. 1). The sampling area of these measurements was $25\ \mu\text{m} \times 25\ \mu\text{m}$ ($625\ \mu\text{m}^2$). The purpose of this constraint was to keep our sampling within the superficial tectal layers, which exhibited the most robust binding. The chosen sampling area was based upon our observations that the superficial layers of the posterolateral tectum in the youngest animals we examined (stage V) was $95 \pm 6\ \mu\text{m}$ thick ($N = 4$) when sliced in the sagittal plane.¹⁰ Although specific binding of both radioligands was present in the deeper tectal layers of all sampled stages, it was not quantitatively analyzed because the lack of differentiated tissue in the younger animals produced a high degree of variability of binding in these layers. Significance between stages and between tectal areas of the same age was determined with one-way ANOVA followed by Tukey's multiple comparison test. These studies compared sections from four animals per stage with at least three sections from each animal. In all experiments, specific binding was calculated as the difference between total and non-specific binding.

Pharmacology of muscarinic binding in the optic tectum—Autoradiographic competition experiments allowed for the determination of binding values in the optic tectum. These competition experiments used [^3H]pirenzepine (10 nM) against eight concentrations (1 nM to 1 mM) of unlabeled AF-DX 384 or [^3H]AF-DX 384 (5 nM) against unlabeled pirenzepine (1 nM to 1 mM). Non-specific binding was determined on adjacent tissue sections by the addition of excess atropine sulfate (10 μM) to the radioligand solutions (adapted from Happe *et al.*⁴⁰ and Butt *et al.*¹⁰).

Displacement experiments using the muscarinic toxins against [^3H]pirenzepine or [^3H]AF-DX 384 binding were also performed using autoradiography. In these experiments, the sliced tissue was preincubated with muscarinic toxin (MT1: 50, 250 or 500 nM; MT3: 5, 75 or 150 nM) for 30 min prior to the application of radioligand. The same concentrations of muscarinic toxins were also utilized during the radioligand applications.

In this adult tissue, data were gathered from three groups of layers because of the distribution of binding seen in the localization studies. All of layers 7–9, layer 6 and layers 1–5 throughout the entire tectal section were quantified, and the groupings were analyzed separately for the competition and displacement experiments. [^3H]Pirenzepine binding could not be detected in layers 1–4, so only binding in layer 5 was analyzed for this particular radioligand. Such analysis was possible because, unlike in the developing tissue, all cells along the rostrocaudal dimension of the tectum were mature. This, along with the greater size and layer definition of the adult tissue, allowed us to quantify the entire area in which specific muscarinic binding was present with an increased level of detail. A minimum of three sections from each animal ($N = 5$) for each concentration were used. Graphical results were plotted and analyzed as described above. Significant differences in the displacement experiments were determined by Student's paired, two-tailed *t*-test.

Optic nerve lesions—Optic nerve lesions were performed to help determine if radioligand binding sites were present on the terminals of retinal ganglion cells or on other tectal elements. Animals were anesthetized by injection of MS222. The optic nerve was severed approximately 1 mm from the posterior aspect of the right eye through an incision in the roof of the mouth.^{10,55} The cut ends of the nerve were cauterized to prevent regeneration and the surgical site was closed with Vetbond (3M, St Paul, MN, USA). Feeding resumed within three days after surgery, which was viewed as an indication of an animal's recovery. Short-term lesioned animals were allowed to survive for six weeks, while long-term animals were maintained for six months. Earlier studies have shown that most retinal fibers in the tectum have degenerated after short-term lesions, but long-term lesions allow for the degeneration of all presynaptic retinal fibers.^{49,60,70} Behavioral tests were conducted during the survival period to demonstrate that animals responded only to prey seen through the unlesioned left eye. At the end of the survival period, the frogs were anesthetized, the optic nerve lesion was confirmed and the brain was prepared for autoradiography as described above.

To evaluate the results of these experiments, as in the tectal pharmacology experiments, entire layers were analyzed. Binding densities in layers 7–9, layer 6 and layers 1–5 were compared between lesioned and unlesioned tectal lobes. Again, with [³H]pirenzepine binding the third data group consisted of layer 5 only. A minimum of three sections from each animal ($N = 4$ or 5) for each treatment were used in the analysis. Significant differences between the two tectal lobes were determined by Student's paired, two-tailed, t -test.

Results

Pharmacology of [³H]pirenzepine and [³H]AF-DX 384 binding in whole frog brain

The mAChR subtype selectivity of the radioligands that we employed was first determined in mammalian systems. Therefore, it was important to confirm that these radioligands had similar binding characteristics in the frog brain. This was achieved by performing saturation and competition analyses of radioligand binding in whole-brain homogenates.

[³H]Pirenzepine, a compound selective for M1-like mAChRs, bound to whole frog brain with saturable kinetics (Fig. 2A). Scatchard linearization (Fig. 2B) produced essentially the same binding constants as those determined by the non-linear regressions: the dissociation constant (K_d) of [³H]pirenzepine in the frog brain was approximately 50 nM, with a maximum binding capacity (B_{max}) near 450 fmol/mg protein. However, the high degree of scatter seen in the data for these saturation equilibrium experiments and the likelihood that higher radioligand concentrations skewed the derived K_d towards lower affinity sites reduced the reliability of this value. Competition experiments of unlabeled pirenzepine against [³H]NMS (5 nM), a high-affinity antagonist at all muscarinic receptor subtypes, provided a more detailed view of pirenzepine's affinity for muscarinic binding sites (Fig. 2C). Use of the Cheng–Prusoff equation¹² and the K_d of [³H]NMS in the *Rana* brain (6.9 nM)⁵ yielded two inhibition constants (K_i ; $K_{i1} \sim 1$ nM and $K_{i2} \sim 1$ μ M). The Hill slope (n) of this competition curve was greater than -1.0 , a value consistent with the assignment of more than one binding site for pirenzepine. The stronger curve fitting to the data of the [³H]NMS inhibition studies and their high-affinity K_i value for pirenzepine that was significantly different from the K_d found in the [³H]pirenzepine saturation studies suggested that the K_i value was more reliable. Therefore, that value was used as the apparent affinity constant for later experiments conducted with [³H]pirenzepine.

The results of similar experiments with [³H]AF-DX 384, which selectively labels M2-like mAChRs in mammals, are shown in Fig. 2D–F. [³H]AF-DX 384 binding in the frog brain had saturable kinetics, a K_d near 15 nM, which was significantly different from that of [³H]pirenzepine, and a B_{max} that was equivalent to that of [³H]pirenzepine (~ 450 fmol/mg protein;

Fig. 2D). Scatchard analysis (Fig. 2E) once again yielded binding constants similar to those seen in the non-linear analysis. Unlabeled AF-DX 384 also competed with [^3H]NMS (5 nM) binding at two sites (5 and 164 nM; Fig. 2F), but these K_i values were significantly different from the analogous values in Fig. 2C–E. As with pirenzepine inhibition, the better fit of the data for AF-DX 384 inhibition of [^3H]NMS binding at equilibrium encouraged us to use the high-affinity K_i (5.10 nM) as the apparent affinity constant in later studies rather than the K_d of saturable [^3H]AF-DX 384 binding at equilibrium.

The binding parameters shown in Fig. 2 suggest that [^3H]pirenzepine and [^3H]AF-DX 384 bound to distinct receptors in the frog brain. This is best seen by comparing Fig. 2C and F. The first fractions of [^3H]NMS binding competition, which correspond to the high-affinity binding sites of the inhibitors, show that the proportion of radioligand binding inhibited at the high-affinity pirenzepine site (~10%) is considerably different than that at the high-affinity AF-DX 384 site (~60%). In mammalian systems, the difference in rank order potency of these compounds at purified mAChR subtypes imparts their selectivity. However, the two compounds are known to share some binding sites, resulting in the loss of their subtype selectivity at high concentrations.^{7,24,61} To determine whether the concentrations of the radioligands used were appropriate for the frog brain, we performed competition analysis with the two compounds and with muscarinic toxins that have higher subtype selectivity than the radioligands.

Unlabeled AF-DX 384 competed at a single [^3H]pirenzepine (10 nM) binding site with a K_i of approximately 2.66 nM (Fig. 3A). The m1/m3-selective MT1⁴⁴ competed at two [^3H]pirenzepine sites ($K_{i1} \sim 5.40$ nM; $K_{i2} \sim 472$ nM; Fig. 3B). The fraction of specific [^3H]pirenzepine binding inhibited by the high-affinity MT1 site was $28.4 \pm 8.52\%$, but total inhibition of [^3H]pirenzepine binding by MT1 was not achieved. MT3, which is selective for m4 mAChRs,^{45,54} had no effect on [^3H]pirenzepine binding in whole-brain homogenates (Fig. 3C).

Similar experiments with [^3H]AF-DX 384 (5 nM) competing against unlabeled pirenzepine and MT1 produced completely different K_i values. Experiments with unlabeled pirenzepine indicated that this compound competed at two [^3H]AF-DX 384 sites, with K_i values near 69.4 and 991 nM (Fig. 3D). MT1 competition also demonstrated a two-site fit to the data, with $K_{i1} \sim 0.13$ nM and $K_{i2} \sim 2.35$ μM (Fig. 3E). Again, MT1 did not fully inhibit radioligand binding, but the fraction of specific binding inhibited by the high-affinity site was $6.76 \pm 3.51\%$. MT3 could not inhibit [^3H]AF-DX 384 binding in whole-brain homogenates (Fig. 3F). These experiments indicated that, although some of the signal associated with [^3H]pirenzepine and [^3H]AF-DX 384 is shared, the radioligands are indeed capable of discriminating between different mAChR subtypes at the concentrations used (see Discussion).

Localization of binding sites in the optic tectum

We next wanted to determine whether [^3H]pirenzepine and [^3H]AF-DX 384 would bind specifically to the optic tectum and to identify the laminar location of those binding sites. This task was aided by digital imaging, which allowed the results of standard receptor autoradiography and tissue staining to be superimposed.

The lamination pattern of the tectum reflects its structural organization.⁷⁸ The widest and most superficial of these laminae is designated layer 9. The majority of tectal afferents come into this layer, where they synapse with tectal dendrites.³⁴ Layer 8 contains both tectal neurons and afferents,⁴² while layer 7 is the principal efferent layer of the tectum.^{35,73} The main cellular layer of the tectum is layer 6. The dendrites of its cells intersect afferent input as they ascend to layer 9.⁴² The deep tectal layers, 2–5, alternate between thin cellular and plexiform layers.³⁴

Specific binding sites for [^3H]pirenzepine were found in most of the layers of the adult optic tectum (Fig. 4A, C). The highest density of specific binding achieved had only intermediate values, and was present in a broad, diffuse band that consisted of tectal layers 7, 8 and the deeper half of layer 9. Lower binding densities were seen in layer 5 and the superficial half of layer 9. Layer 6 contained very low densities of binding, and specific binding could not be detected in layers 1–4. The distribution and density of the receptors did not change along the lateromedial or rostrocaudal axes of the tectum. Non-specific binding, as determined by competition with atropine (10 μM), ranged between 15% and 30% of the total binding (Fig. 4D). Higher concentrations of atropine did not reduce the non-specific binding any further, nor did micromolar concentrations of unlabeled pirenzepine.

The laminar distribution of specific binding sites for [^3H]AF-DX 384 (5 nM) in the adult optic tectum was different from that of [^3H]pirenzepine. High-density [^3H]AF-DX 384 binding was found in two bands: a thin one in tectal layer 5 and a broad one that encompassed layers 7–9 (Fig. 4E, G). Intermediate binding densities were also found in two bands, but were located in layers 4 and 6. Layers 1–3 had low levels of [^3H]AF-DX 384 binding sites. Changes in the distribution of binding sites were not evident between lateral and medial sections, nor between anterior and posterior sections. Non-specific binding, as determined by the addition of excess atropine (10 μM), was not detected when [^3H]AF-DX 384 was used at a concentration of 5 nM (Fig. 4H). However, increasing the radioligand concentration above 10 nM resulted in a non-specific binding value that was 5–10% of the total binding (data not shown).

Development of [^3H]pirenzepine and [^3H]AF-DX 384 binding sites in the tectum

We were interested in ascertaining whether the different muscarinic binding sites present in the adult system were also found in the developing tadpole tectum. To determine this, we examined the patterns and density of tectal binding in early (V), middle (X, XV) and late (XX, XXV) tadpole stages, and compared them to that in the adult.

The development of the optic tectum proceeds along an anterolateral to posteromedial gradient that correlates with the growth of retinal ganglion cell terminals into this brain structure. New cells are continually being born throughout the tectum during its maturation, but the rate of cell birth decreases significantly in areas that have been innervated by retinal ganglion cell terminals.⁴⁸ Differentiation and lamination of the tectum occurs in these innervated areas.^{21,67} Thus, a developing tectum is characterized by its thick anterolateral regions, where differentiated tectal cells receive input from innervating afferents, and its thin posteromedial regions, where tectal cells are undifferentiated and dividing and inputs have not yet invaded the superficial neuropil.

Specific [^3H]pirenzepine binding was detected in every tadpole stage that was sampled (Fig. 5A). The binding patterns in tadpoles (not shown) were similar to those in the adult, but for every tectal region binding densities were significantly higher in adults than in any tadpole stage. Within each tectal region, binding tended to increase with development, but these increases were not significant between tadpole stages. For any given stage of development, there was a tendency for the binding to be less dense in posterior regions than in anterior ones, but these differences were not significant except in stage X animals, which demonstrated significantly less binding in the central and posterior regions when compared to the anterior region.

Specific [^3H]AF-DX 384 binding was also present in the tectum at every stage examined (Fig. 5B). The distribution of [^3H]AF-DX 384 binding in all stages of the developing tectum was also similar to that seen in adults (data not shown), but adult binding was again significantly higher than that of any tadpole stage in all tectal regions. Within a given region, binding site increases with tectal maturity were slight and not significant between the five tadpole stages

sampled. In every developmental stage, binding in the tectum was generally less dense in central and posterior regions when compared to that in anterior regions. Early stage tadpoles had significantly less binding in the central (stage V) and posterior (stages V and X) tectal regions compared to the anterior region. Binding in the posterior tectal region of stage XV animals was significantly less dense than that in both the anterior and central regions. However, in stage XX and XXV tadpoles and in adults, these differences were not significant. At all stages and within all regions, the absolute density of [^3H]AF-DX 384 binding was approximately 10 times greater than that of [^3H]pirenzepine binding (compare the scales of Fig. 5A and B).

Pharmacology of muscarinic binding in the optic tectum

In order to examine the possibility that the relative proportions of the different mAChR subtypes in the optic tectum may be different from those in the whole brain, we performed competition and displacement experiments on brain slices with autoradiography. This method allowed the analyses to focus on binding parameters in the optic tectum. Although the data presented in Fig. 6 are from layers 7–9 only, this layer grouping was also representative of tectal layers 1–5. Toxin displacement in layer 6 suggested that this layer had a slightly greater proportion of m1/m3 receptors and slightly less m4 receptors than the other layers of the tectum. This was true when [^3H]pirenzepine was displaced by MT1 and when [^3H]AF-DX 384 was displaced by MT3. However, this result was not evident when [^3H]pirenzepine was displaced by MT3 (data not shown).

Competition analysis of unlabeled AF-DX 384 against [^3H]pirenzepine binding in the tectum again produced a curve that fit best to a single-site equation (Fig. 6A). However, the K_i value (~ 9.25 nM) of this competition was of lower affinity and higher variability than that seen in whole-brain preparations. Layer-specific analysis showed that the binding parameters in any of the deeper layers were not significantly different from those in layers 7–9 (data not shown).

Displacement of tectal [^3H]pirenzepine binding by MT1 and MT3 was also different from that obtained in the whole brain. Although MT1 significantly displaced specific [^3H]pirenzepine binding at all concentrations used, it was also capable of virtually eliminating the binding of this radioligand in the tectum (Fig. 6B). Fifty nanomolar MT1 reduced [^3H]pirenzepine binding by $53.7 \pm 4.2\%$ and 250 nM MT1 displaced the binding by $68.8 \pm 6.7\%$. The highest concentration of MT1 (500 nM) displaced specific [^3H]pirenzepine binding to $2.3 \pm 1.2\%$ of its total density. However, it is unlikely that MT1 is subtype specific at this concentration (see Discussion). MT3 (5–150 nM) significantly displaced [^3H]pirenzepine binding by an average of $53.2 \pm 2.3\%$ in the tectum (Fig. 6C), although it had no effect in whole-brain preparations.

Competition of unlabeled pirenzepine against tectal [^3H]AF-DX 384 binding produced similar results to those in the whole brain (Fig. 6D). Curve fitting of the data again showed that two binding sites existed, and the K_i value of the high-affinity site was indistinguishable from that determined in homogenates. The second K_i value was of somewhat lower affinity in the tectum when compared to that in the whole brain.

The effects of MT1 and MT3 on [^3H]AF-DX 384 binding in the tectum were not only different from those seen in homogenates, but were also distinct from the effects of the toxins on tectal [^3H]pirenzepine binding. Low concentrations of MT1 (50 and 250 nM) had no effect on [^3H]AF-DX binding. Only the highest, and least selective, concentration of MT1 (500 nM) significantly displaced binding to $54.3 \pm 11.7\%$ of the total signal (Fig. 6E). All concentrations of MT3 significantly blocked an average of $47.5 \pm 9.3\%$ of [^3H]AF-DX 384 binding in the tectum. Increasing MT3 concentrations seemed to have greater effects on binding, but none of these decreases were significantly different from those obtained with the lowest dose (Fig. 6F).

Effect of optic nerve lesions on [³H]pirenzepine and [³H]AF-DX 384 binding in the tectum

To gain some understanding of whether [³H]pirenzepine and [³H]AF-DX 384 binding sites in the tectum were associated with tectal cells or with retinal afferents, we severed the right optic nerve and allowed the animals to survive for six weeks (short-term treatments) or six months (long-term treatments). Optic nerve lesions cause the degeneration of retinal ganglion cell terminals in the tectum and the disappearance of elements associated with them.^{49,60,70}

Optic nerve lesions had no significant effect on specific [³H]pirenzepine binding in the tectum (Fig. 7A, B). There was no qualitative difference between any layer of lesioned and unlesioned tectal lobes regardless of treatment period.

In contrast, significant changes in the optic tectum were detected with [³H]AF-DX 384 after the loss of one optic nerve (Fig. 7C, D). Short-term lesions reduced [³H]AF-DX 384 binding in all layers of the lesioned lobe when compared to that in the unlesioned lobe (Fig. 7C). Long-term lesions appeared to affect both tectal lobes (Fig. 7D). No clear difference existed between the two tectal lobes of long-term animals. However, [³H]AF-DX 384 binding in both lobes of the tectum treated for six months was markedly less than that in the tectum treated for six weeks. This conclusion was reached because binding in the tegmental region beneath the tectum was qualitatively similar between the two treatment groups.

Quantitative analysis of muscarinic binding in the tectum after optic nerve lesions confirmed our qualitative impressions. No significant change could be detected in [³H]pirenzepine binding after optic nerve lesions regardless of the tectal layer analyzed or survival period (Fig. 8A, B). However, short-term lesions significantly reduced [³H]AF-DX 384 binding in all layers of the lesioned tectal lobe when compared to the unlesioned lobe (Fig. 8C). In layers 7–9, [³H]AF-DX 384 binding was reduced $30.5 \pm 8.0\%$ during short survival periods. Likewise, binding in layer 6 decreased $22.3 \pm 8.2\%$ and that in layers 1–5 dropped $40.3 \pm 5.9\%$. There was no difference in [³H]AF-DX 384 binding between lesioned and unlesioned lobes after long-term survival periods (Fig. 8D). Changes in [³H]AF-DX 384 binding between short- and long-term survival periods could not be verified quantitatively because the experiments were performed at different times, the treatment groups were exposed to different pieces of film, and no accurate method of data normalization was available. Although a direct comparison cannot truly be made, the absolute value (fmol/mg wet tissue weight) of [³H]AF-DX 384 binding after long-term lesions was 30–50% of that calculated in short-term experiments (data not shown).

The high selectivity of the muscarinic toxins allowed us to determine whether any changes in the relative proportions of the different mAChR subtypes occurred after optic nerve lesions. In these experiments, we compared the amount of binding in lesioned and unlesioned lobes after toxin displacement. We displaced [³H]pirenzepine binding from optic nerve-lesioned tissue with MT1 (500 nM) and MT3 (150 nM). Since MT1 had no effect on [³H]AF-DX 384 binding except at non-specific concentrations (see Fig. 6E), only MT3 (150 nM) was used against this radioligand.

The quantitative results of [³H]pirenzepine binding in optic nerve-lesioned tissue after toxin displacement are shown in Fig. 9A–D. No significant difference in [³H]pirenzepine binding between lesioned and unlesioned lobes could be detected regardless of the layers analyzed, toxin treatment or survival period. The results were the same as those seen with non-competed [³H]pirenzepine binding in lesioned tissue. They indicate that optic nerve lesions did not change the relative proportions of mAChRs within the subset of receptors labeled by [³H]pirenzepine.

In contrast to [^3H]pirenzepine binding, there was a differential displacement of [^3H]AF-DX 384 binding by MT3 (150 nM) after short-term optic nerve lesions. Comparison of layers 7–9 of the lesioned and unlesioned lobes showed that the lesioned lobe had significantly less [^3H]AF-DX 384 binding remaining ($39.7 \pm 5.1\%$ decrease) after the toxin blocked binding to m4 receptors (Fig. 10A). There was no effect on binding in layers 1–6 after this treatment. Interestingly, no such difference could be detected in the long-term lesioned tissue (Fig. 10B).

Discussion

We have shown that the muscarinic radioligands [^3H]pirenzepine and [^3H]AF-DX 384 bind to distinct receptor sites in the brain of the frog, *Rana pipiens*. Although binding sites for both radioligands were present in the superficial layers of the optic tectum, the distribution of these sites in the deeper tectal layers, their density, their expression during development and their pharmacology differed. Furthermore, the loss of one optic nerve had no effect on [^3H]pirenzepine binding, but did change the binding of [^3H]AF-DX 384.

Efficacy and selectivity of [^3H]pirenzepine and [^3H]AF-DX 384 for muscarinic acetylcholine receptor subtypes in the frog brain

The purpose of our kinetic studies was to determine whether [^3H]pirenzepine and [^3H]AF-DX 384 bound to the frog brain with affinities and selectivities for mAChR-like subtypes that were similar to those observed in the mammalian brain. Although the genetic diversity of mAChR subtypes in the frog is unknown, our results indicate that the radioligands used appear to have the same mAChR pharmacology in the frog optic tectum as they do in mammalian systems. However, the relative proportions of possible mAChR subtypes in the tectum seem to be different from those in the entire frog brain.

Table 1 shows that the affinities of [^3H]pirenzepine and [^3H]AF-DX 384 binding in the whole frog brain were within an order of magnitude of those demonstrated in the rat cerebral cortex, ^{1,2,57} which is known to express genes for all five mAChR subtypes.^{46,85} The relatively small differences in the affinity values could be attributed to differences in tissue preparation, experimental protocols, species or the prevalence of a particular mAChR subtype.

Also shown in Table 1 is the affinity of muscarinic agents at purified populations of human mAChR subtypes. The m5 mAChR is not considered here because it constitutes a very low percentage of all the mAChRs in the mammalian brain.^{46,85} The similarity of the affinity values for m1, m2, m3 and m4 mAChRs makes it difficult to assign the binding of currently available muscarinic radioligands in mixed mAChR populations to a single subtype. The rank order potency of pirenzepine at pure populations of mAChR subtypes is $m1 > m4 > m3 > m2$,^{7,24} and that of AF-DX 384 is $m2 \geq m4 > m3 > m1$.^{24,61} Consequently, our demonstration that [^3H]pirenzepine (10 nM) and [^3H]AF-DX 384 (5 nM) share binding sites in the frog (Fig. 3A, D) is not surprising when the radioligands' affinity profiles are considered. The cross-selectivity of muscarinic radioligands at the different mAChRs has normally been addressed, as we have done, by using rigorous arrays of competition analyses and careful selection of radioligand concentration.^{2,32,56}

[^3H]Pirenzepine appears to bind to the frog brain with the same mAChR subtype selectivity shown in mammals. The first indication of this was the two-site kinetics of pirenzepine competition at sites labeled by [^3H]NMS (Fig. 2C). The results of these experiments demonstrate that the concentration of the radiolabeled pirenzepine (10 nM) we chose for autoradiography was appropriate because the unlabeled pirenzepine maintained its selectivity for its high-affinity binding sites at concentrations up to 50 nM. These experiments also explain why the autoradiographic binding was relatively weak. The high-affinity pirenzepine site constituted only ~10% of all muscarinic sites in the frog brain, as shown by the extent of

competition at [^3H]NMS sites when pirenzepine concentrations were less than 100 nM. The preponderance of low-affinity sites is probably what produced the high K_d demonstrated in the saturation studies. In mammalian systems, pirenzepine has its highest affinity for the m1 mAChR subtype (Table 1). However, in the frog, the high saturation K_d , coupled (Fig. 2A) with the two K_i values in the [^3H]NMS competitions, suggest that [^3H]pirenzepine (10 nM) binds to more than one mAChR subtype.

In addition to m1-like subtypes, [^3H]pirenzepine (10 nM) might also bind m3 or m4 mAChRs. Unlabeled AF-DX 384 inhibits [^3H]pirenzepine (10 nM) binding at a single site whose K_i value is close to the K_d of AF-DX 384 at the mammalian m3 and m4 mAChRs (Fig. 3A). The m1/m3-selective MT1 competes with [^3H]pirenzepine (10 nM) in the frog brain with a high-affinity K_i that is within an order of magnitude of that shown in the mammalian brain for m1/m3 receptors (Fig. 3B).⁴⁴ Although the m4-selective MT3 had no significant effect on [^3H]pirenzepine binding in whole-brain homogenates (Fig. 3C), [^3H]pirenzepine binding to m4 receptors cannot be disregarded because pirenzepine has a higher affinity for m4 receptors over m3 receptors, and total inhibition of [^3H]pirenzepine binding by MT1 did not occur. Therefore, in whole-brain homogenates, [^3H]pirenzepine (10 nM) probably binds to a mix of m1/m3 mAChRs, as well as a small population of m4 receptors, but it should be noted that the true selectivity of the muscarinic toxins in the frog brain is unknown.

[^3H]Pirenzepine binding in the optic tectum had a slightly different profile that seemed to be due to the presence of higher concentrations of m1/m3 and m4 receptors. The first indication that tectal binding was different from that in the whole brain came from the competition studies with unlabeled AF-DX 384 (Fig. 6A). The competition parameters from these experiments were of lower affinity and higher variability than those seen in the whole brain. Although we do not have the tools to appropriately discriminate between m1 and m3 receptors, a larger proportion of m1 receptors would explain the lowered affinity of AF-DX 384 competition (see Table 1). This interpretation is supported by the increased effectiveness of MT1 at [^3H]pirenzepine binding sites in the tectum (Fig. 6B) over that seen in the whole brain (Fig. 3B). The results also indicate that a larger proportion of putative m4 receptors are labeled by [^3H]pirenzepine in the tectum. These m4 receptors would contribute to the higher variability of the Hill slope and K_i of AF-DX 384 competition that was observed, and their presence is strongly supported by the fact that low concentrations of MT3 significantly decreased [^3H]pirenzepine binding.

[^3H]AF-DX 384 (5 nM) binding in the whole frog brain also appeared to have the same selectivity for muscarinic subtypes as that shown in mammalian systems. However, this radioligand bound to a different subset of mAChRs than [^3H]pirenzepine. This was indicated by the K_i values, Hill slope and shape of the competition curve for AF-DX 384 at [^3H]NMS sites (Fig. 2F), all of which were significantly different from the parameters of pirenzepine competition at [^3H]NMS sites (Fig. 2C). The K_i of high-affinity AF-DX 384 competition at [^3H]NMS sites (5.1 ± 2.5 nM) in the whole brain was virtually identical to the concentration chosen for [^3H]AF-DX 384 autoradiography (5 nM), indicating that our choice of radioligand and concentration was appropriate for high-affinity [^3H]AF-DX 384 binding.

Competition with unlabeled pirenzepine and the muscarinic toxins indicated that a small amount of [^3H]AF-DX 384 binding in whole-brain homogenates was due to non-M2-like receptors. The first K_i of pirenzepine at [^3H]AF-DX 384 sites (69.4 ± 30.1 nM; Fig. 3D) was close to that of pirenzepine's affinity for pure m3 and m4 receptors (Table 1), but the moderate value and variability of this K_i indicates that [^3H]AF-DX 384 primarily binds to M2-like mAChRs.²⁴ MT1 competed with very high affinity at a small population of [^3H]AF-DX 384 sites (Fig. 3E). This non-M2-like binding could most likely be caused by [^3H]AF-DX 384's low affinity for m3 receptors, which is only approximately three times greater than the

radioligand concentration we used (Table 1). The low affinity of [³H]AF-DX 384 for these non-M2-like receptors explains why MT1 was able to compete with a K_i of such high affinity (0.13 ± 0.03 nM). The MT1 competition also demonstrated the loss of MT1's selectivity for M1-like sites at concentrations above 100 nM. Interestingly, competition of MT3 with [³H]AF-DX 384 also demonstrated a very low proportion of putative m4 receptors in whole-brain preparations. We conclude that [³H]AF-DX 384 (5 nM) binding in the whole frog brain likely consists primarily of m2-like receptors with some binding (<5%) at m4- and m3-like mAChR subtypes.

The binding parameters of [³H]AF-DX 384 in the tectum were similar to those in the whole frog brain, with one exception: [³H]AF-DX 384 binding was significantly displaced by MT3 in the tectum, indicating the possibility of m4 receptors. The fact that a substantial amount of [³H]AF-DX 384 binding remains after displacement with MT3 suggests that putative m2 receptors are also present in the tectum, as well as the entire brain. The high-affinity pirenzepine competition site was equivalent to that shown in the whole brain, suggesting the presence of m3-like receptors in the tectum. MT1 did not significantly displace [³H]AF-DX 384 binding until 500 nM (Fig. 6E), a concentration beyond MT1's selectivity range (Fig. 3E), was used. However, m3-like receptors in the tectum should not be discounted because the error in this tectal analysis was greater than the 5% reduction in [³H]AF-DX 384 binding that was demonstrated after treatment with low concentrations of MT1 in whole-brain homogenates. Furthermore, the presence of m3 receptors in the tectum is supported by studies that demonstrate m3 mAChRs in the superficial layer of the superior colliculus.^{32,53}

In summary, both [³H]pirenzepine and [³H]AF-DX 384 have the same apparent pharmacological selectivities for putative mAChR subtypes in the frog brain that have been established in mammalian brain (Table 1). The m1/m3-like and m4-like subtypes probably have higher expression levels in the tectum than they do in the whole brain, and the presence of m2-like mAChRs throughout the frog brain can be inferred from the pharmacological analyses that have been conducted in this study.

Laminar distribution of distinct muscarinic binding sites in the optic tectum

The use of [³H]NMS, [³H]3-quinuclidinylbenzilate (QNB) or monoclonal antibody M-35, agents which recognize all mAChRs, has shown muscarinic binding in the optic tecta of *Rana*,⁵ *Xenopus laevis*,⁸¹ goldfish³⁰ and chicks.⁶⁶ Although the laminar distribution of mAChR binding sites was not assessed in these studies, the presence of mAChRs in the tectum is consistent with our findings. Furthermore, our previous studies with [³H]NMS and [³H]QNB show that the tectal distribution patterns of these sites match closely with those of [³H]pirenzepine and perfectly with those of [³H]AF-DX 384.^{8,9,82} It is therefore evident that mAChRs labeled by [³H]pirenzepine and [³H]AF-DX 384 comprise respective subsets of the total muscarinic binding seen with [³H]NMS.

mAChRs have also been found in the superior colliculus, the mammalian structural homologue of the optic tectum. Autoradiographic studies with [³H]NMS or [³H]QNB have indicated mAChR binding sites throughout the superior colliculi of humans,²⁰ rats,⁸⁷ mice^{62,65} and cats.²² Furthermore, studies using subtype-selective compounds have shown a higher proportion of M2-like sites over M1-like sites in the superior colliculus.^{22,41,77} Our data also show a preponderance of [³H]AF-DX 384 sites over [³H]pirenzepine sites and directly parallel the earlier findings in the superior colliculus.

Developmental expression of [³H]pirenzepine and [³H]AF-DX 384 binding sites

Specific binding sites for both [³H]pirenzepine and [³H]AF-DX 384 were present in developing tissue. Both radioligands seemed to bind to sites throughout the tectal layers. However, the

lack of differentiation in this developing tissue precluded the assignment of binding sites to specific tectal layers. Binding was not as strong in the posterior tectal regions as it was in anterior ones, a pattern that is consistent with the normal maturation of the tectum. The absolute density of [^3H]AF-DX 384 binding sites was clearly higher than that of [^3H]pirenzepine in both the developing and adult tectum.

Quantitative analysis of the density of [^3H]pirenzepine sites in the superficial layers during maturation demonstrated that these sites are expressed in the tectum at every stage. We also observed that binding increased dramatically during the juvenile period between stage XXV and full adulthood. In the rat cerebral cortex, the development of [^3H]pirenzepine binding can be detected as early as embryonic day 20. This binding increases sharply for the first two to three weeks after birth and then asymptotes around postnatal day 20, a peak period of synaptogenesis. The density of [^3H]pirenzepine sites is then maintained at a relatively constant level from day 20 into adulthood.^{3,26}

[^3H]AF-DX 384 binding was more robust than [^3H]pirenzepine binding and was also present in all stages of the developing optic tectum. However, adults had significantly more binding than any tadpole stage. Again, a dramatic increase seemed to occur sometime within the juvenile period. Our results are similar to those seen in the rat cerebral cortex in that M2-like binding is first detected at low levels in prenatal individuals, and adults have the highest binding densities.^{3,80} During the development of the frog optic tectum, we only observed steady, small increases in binding density up to stage XXV, the earliest point in post-metamorphic life.

It is difficult to determine exactly what causes the observed up-regulation of [^3H]AF-DX 384 or [^3H]pirenzepine binding during the juvenile period. In frogs, both the tectum and retina undergo extensive transformations due to growth during the juvenile period.^{37,83} The increased receptor density that we observe could be a product of the increased complexity in tectal cell morphology that occurs during development.⁵¹ However, there is also the possibility that the innervation of the tectum by new afferent projections during and after metamorphosis^{16,83} could initiate a change in the tectal cells that causes more mAChRs to be expressed.

Location of muscarinic subtypes within the tectum

Muscarinic receptors in the frog optic tectum have been demonstrated both physiologically²⁹ and pharmacologically.⁵ Their location on specific tectal elements is, however, not known. We attempted to assess whether mAChR subtypes were associated with retinal ganglion cell terminals by lesioning the optic nerve.

Specific [^3H]pirenzepine binding was not affected by lesions of the optic nerve. No change in the binding density was observed in either short- or long-term lesioned animals. Furthermore, displacement of [^3H]pirenzepine by MT1 or MT3 resulted in the same amount of binding in both the lesioned and unlesioned lobes, indicating that the relative proportions of the receptors bound by [^3H]pirenzepine had not been affected by the manipulation. Our findings indicate that putative m1/m3 and m4 mAChRs are associated with the tectum, not with retinal ganglion cell terminals. This conclusion is consistent with results demonstrated in the optic tecta of the chick⁶⁶ and goldfish,³⁰ and the superior colliculus of mammals.^{11,31} These earlier studies used either [^3H]QNB or [^3H]NMS, both non-selective muscarinic agents, and showed no change in the density of radioligand binding in tectal regions after the loss of one optic nerve.

In contrast to the results with [^3H]pirenzepine, optic nerve lesions had marked effects on [^3H]AF-DX 384 binding in the optic tectum. Short-term lesions decreased the proportion of [^3H]AF-DX 384-labeled receptors in all layers of the lesioned lobe to those in the unlesioned lobe. Because retinal ganglion cell terminals are found only in superficial layers 8 and 9,⁴² this

decrease in binding in all tectal layers likely indicates a compensatory reaction by the tectum to this loss of input. Different subtypes of mAChRs are, however, lost preferentially in the different layers. The [³H]AF-DX 384 binding that remains after displacement with MT3 most likely represents m2 mAChRs alone, because m3-like receptors comprise less than 5% of all [³H]AF-DX 384 binding. After short-term lesions, there is a decrease in this MT3-competed binding in layers 7–9, but not in layers 1–6. Since total [³H]AF-DX 384 binding in these layers decreases, the MT3-competed binding further suggests that the loss of total [³H]AF-DX 384 binding in layers 1–6 after short-term lesions is due to decreases in m4-like mAChR expression. This finding was likely not detected by the MT1-displaced [³H]pirenzepine binding because of the low signal-to-noise ratio of [³H]pirenzepine (10 nM) binding.

The results of our long-term experiments indicate that optic nerve lesions decrease M2-like mAChR expression not only in the lesioned but also in the unlesioned lobe. Although no differences in [³H]AF-DX 384 binding could be detected between the lesioned and unlesioned lobes in any layer, binding in both lobes was proportionately less than that in the midbrain tegmentum, a result that did not occur in the short-term experiments. Trans-synaptic effects have been reported in the tectum: ablation of the optic nerve is known to reduce the volume of the nucleus isthmi contralateral to the surgical site¹⁹ and decrease the expression of substance P in the ipsilateral tectal lobe through a cholinergic mechanism.⁸² It appears from our experiments that both tectal lobes react to the loss of input by reducing their expression of binding sites that are consistent with the presence of M2-like mAChRs.

The m2 mAChRs have been regarded classically as presynaptic autoreceptors on cholinergic projections in the mammalian cortex and hippocampus. In fact, destruction of cholinergic afferents to the hippocampus within the fimbria–fornix results in small decreases in m2 expression and no change in m1, m3 or m4 expression.⁸⁵ Our observations in the tectum are similar to those in the hippocampus, as some putative m2 receptors may reside on the cholinergic terminals of nucleus isthmi projections. Loss of these binding sites in the afferented lobes of long-term lesioned animals prevents the effective arguing that any are on retinal ganglion cell terminals, though this may be the case. However, the presence of both m2-like and m4-like mAChRs in the deeper cellular layers of the tectum strongly suggests that they can be found at least on tectal cells. Definitive evidence regarding the synaptic location of these receptors will not be possible until subtype-specific antibodies that recognize epitopes in the frog are available.

Functional muscarinic-like receptors have been demonstrated physiologically in the *Rana* tectum. The enhancement of visually stimulated responses by ACh can be blocked by atropine, a non-selective mAChR antagonist.²⁹ Atropine is also known to disrupt the organization of the retinotopic map created at the tectum.⁸² These physiological data suggest that at least an excitatory mAChR exists in the tectum and are supported by the demonstration of [³H]pirenzepine binding sites in the present study. Although there are no published accounts of an inhibitory muscarinic response in the tectum, our [³H]AF-DX 384 binding and muscarinic toxin studies clearly indicate the presence of distinct M2-like receptors.

Conclusions

The results of the binding studies reported here support the conclusion that at least four different subtypes of mAChRs exist in the optic tectum of *Rana pipiens*. Binding sites for [³H]pirenzepine, a radioligand selective for the M1-like receptors, exist at moderate to low densities in tectal layers 5–9. [³H]Pirenzepine is likely binding to m1/m3 and m4 receptors in the frog tectum, presuming that the genetic diversity and pharmacological profiles of mAChRs in the frog are the same as those in mammals. Binding sites for [³H]AF-DX 384 are found throughout the tectum, and they are most likely comprised of putative m2 and m4 (M2-like) mAChRs.

Both M1- and M2-like binding sites are present in the optic tectum throughout development, with their highest densities at adulthood. Optic nerve lesions produced no evidence that mAChRs were on retinal ganglion cell terminals but did demonstrate, in the case of M2-like receptors, both ipsi- and contralateral trans-synaptic regulation. This identification and localization of the different mAChR subtypes should assist in the design of specific physiological experiments that will better evaluate the modulatory role of ACh in the frog visual system.

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Abbreviations

ACh	acetylcholine
mAChR	muscarinic acetylcholine receptor
M1-like	m1, m3 and m5 muscarinic acetylcholine receptors
M2-like	m2 and m4 muscarinic acetylcholine receptors
MS222	ethyl <i>m</i> -aminobenzoate
MT1	muscarinic toxin-1
MT3	muscarinic toxin-3
NMS	<i>N</i> -methylscopolamine
QNB	3-quinuclidinylbenzilate

Fig. 1.

Illustration of a sagittal section of the adult optic tectum showing its general structure. The neurons in the main cellular layer (layer 6; 6) send their dendrites up and into layer 9 (9), where they synapse with the terminals of retinal ganglion cell axons and other tectal afferents. The boxes in the anterior, middle and posterior tectum indicate the areas sampled for the developmental studies. Optical density readings were taken from each of these 625- μm^2 areas located in the superficial layers. Scale bar = 500 μm .

Fig. 2.

Pharmacological analysis of pirenzepine and AF-DX 384 binding in homogenates of the adult frog brain. (A) Saturation binding analysis of [^3H]pirenzepine binding. The saturable nature of the fitted data indicated binding to a receptor complex. (B) Scatchard analysis of the same data as in A yielded similar binding constants. (C) Competition binding analysis of unlabeled pirenzepine against [^3H]NMS (5 nM) binding. A two-site competition equation with a Hill slope (n) of approximately -0.85 fit best to the data. (D) Saturation analysis of [^3H]AF-DX 384 binding. These data also indicate that a receptor is bound by the radioligand. (E) Scatchard analysis of the data in D produced binding values that were very similar to those calculated in D. (F) Competition of unlabeled AF-DX 384 against [^3H]NMS (5 nM) binding. This competition curve also fit to a two-site equation, but the binding values were markedly different from those in C. Error bars represent the mean $\pm$ S.E.M. ($N = 4$ or 5).

Fig. 3.

Clarification of [³H]pirenzepine and [³H]AF-DX 384 binding sites in the whole frog brain with the highly subtype-selective muscarinic toxins. (A) Unlabeled AF-DX 384 competed at a single [³H]pirenzepine (10 nM) site. (B) Competition of MT1 against [³H]pirenzepine suggested that this radioligand bound at more than one site. (C) MT3, a selective antagonist at mammalian m4 sites, had no effect on [³H]pirenzepine binding. (D) Unlabeled pirenzepine bound to two [³H]AF-DX 384 (5 nM) sites with K_i values different from those in A. (E) MT1 competition at [³H]AF-DX 384 sites indicated that the radioligand has some affinity for M1-like sites. (F) MT3 had no effect on [³H]AF-DX 384 binding in whole-brain homogenates. In A, B, D and E, all points represent the mean $\pm$ S.E.M. ($N = 4$). The points and error bars in C and F represent the mean $\pm$ S.E.M. of the replicates in each of the two trials for the MT3 competition experiments.

Fig. 4. Localization of $[^3\text{H}]$ pirenzepine (A, C) and $[^3\text{H}]$ AF-DX 384 (E, G) binding sites in the adult optic tectum. The binding scales above each column provide a respective index of binding density (fmol/mg wet tissue weight) for each radioligand (see Experimental procedures for details). (A) Total $[^3\text{H}]$ pirenzepine (10 nM) binding in the optic tectum. Intermediate binding densities (yellow/green) were seen in layers 7, 8 and the deeper half of 9. Layer 5 and the upper half of layer 9 contained low binding densities (green/light blue), while layer 6 had very low densities (light blue/blue). Binding in layers 1-4 was non-existent after subtraction of non-specific binding (D). Tectal layers, indicated with numbers, were assigned using the superimposed image seen in C. (B) Bright-field image of the same tissue section that produced

the autoradiogram in A. The optic tectum is a laminated structure consisting of alternating cellular and plexiform layers. (C) Enlargement of a portion of images A and B after superimposition accurately localizes the binding to specific, tectal layers. (D) Non-specific binding, as determined by the addition of atropine (10 μ M) to the incubation solution, constituted 15–30% of the total [3 H]pirenzepine signal. (E) Specific [3 H]AF-DX 384 binding (5 nM) in the optic tectum. The densest binding (red/orange) was seen in layers 5 and 7–9. Intermediate binding densities were present in layers 4 and 6, and low binding was seen in layers 1–3. (F) Bright-field image of the section that produced the autoradiogram in E. (G) Superimposition of E and F localizes the [3 H]AF-DX 384 binding to the tectal layers in the same way as described for C. (H) Non-specific binding, as determined by the addition of excess atropine (10 μ M), did not exist when [3 H]AF-DX 384 was used at 5 nM. Anterior is to the right. Scale bar = 500 μ m.

Fig. 5.

Quantitative analysis of [^3H]pirenzepine (10 nM) and [^3H]AF-DX 384 (5 nM) binding in the superficial layer of the tectum during development. (A) Histograms displaying specific [^3H]pirenzepine binding (fmol/mg wet tissue weight) in the anterior, middle and posterior regions of the optic tectum in tadpoles and adult frogs. Within any given tadpole stage, comparison of binding in central and posterior regions to anterior regions indicated a significant difference only for stage X animals ($\dagger P < 0.05$). There were no significant differences between any of the tadpole stages for any given region. Binding densities in every region of the adult tectum were equivalent, but significantly higher than those found in any tadpole stage ($*P < 0.02$). (B) Histograms of [^3H]AF-DX 384 binding densities during development. [^3H]AF-DX 384 binding was generally greatest in the anterior regions of tadpole tecta, but all tectal regions had equivalent levels of binding in the adult. Stage V animals had significantly less binding in central and posterior regions of the tectum, while in stage X animals only posterior regions were less dense than anterior ones ($\dagger$). The posterior regions of stage XV animals had significantly less binding than both central and anterior regions ($\dagger P < 0.05$). Within any given region there were no significant differences in binding when tadpole stages were compared, but binding was significantly greater in adults than in any tadpole stage ($*P < 0.0001$). Error bars represent the S.E.M. ($N = 4$).

Fig. 6. The pharmacological profiles of $[^3\text{H}]$ pirenzepine (10 nM) and $[^3\text{H}]$ AF-DX 384 (5 nM) binding in the optic tectum were different from those seen in whole frog brain homogenates. (A) Unlabeled AF-DX 384 competed at $[^3\text{H}]$ pirenzepine sites, but the binding values had a large degree of variability. (B) $[^3\text{H}]$ Pirenzepine was significantly displaced by lower concentrations of MT1 (50 and 250 nM), but a high concentration of MT1 (500 nM) with reduced subtype selectivity blocked nearly all of the specific $[^3\text{H}]$ pirenzepine binding. (C) All concentrations of MT3 significantly displaced $[^3\text{H}]$ pirenzepine binding. (D) Unlabeled pirenzepine competed with moderate and low affinity at two $[^3\text{H}]$ AF-DX 384 binding sites (K_{i1} and K_{i2} , respectively). (E) Only the highest concentration of MT1 (500 nM) was able to significantly displace $[^3\text{H}]$ AF-DX 384 binding in the optic tectum. (F) Similar to the results in C, all concentrations of MT3 were also able to significantly displace $[^3\text{H}]$ AF-DX 384 binding. All bar graphs represent the mean $\pm$ S.E.M. (* $P < 0.05$; ** $P < 0.01$; $N = 5$).

Fig. 7.

Optic nerve lesions did not affect specific [³H]pirenzepine (10 nM) binding but reduced specific [³H]AF-DX 384 (5 nM) binding in the optic tectum. (A, B) No difference in [³H]pirenzepine binding was apparent after short-term (A) or long-term (B) lesions of the optic nerve when lesioned and unlesioned lobes were compared. (C) Short-term optic nerve lesions reduced [³H]AF-DX 384 binding in all layers of the lesioned tectal lobe when compared to the unlesioned lobe. (D) No difference between the lesioned and unlesioned lobes could be seen after long-term lesions of the optic nerve. These representative pictures should not be directly compared because the separate treatments underwent radioligand binding at different times and were exposed to different sheets of film.

Fig. 8.

Quantitative analysis of [^3H]pirenzepine (10 nM) and [^3H]AF-DX 384 (5 nM) binding in the optic tectum after short-term (A, C) and long-term (B, D) lesions of the optic nerve. Neither short- nor long-term lesions significantly affected specific [^3H]pirenzepine binding (A, B). Short-term lesions significantly reduced specific [^3H]AF-DX 384 binding in all layers of the lesioned lobe when compared to the unlesioned lobe (C), but no differences were seen between the two lobes after long-term lesions (D). Histograms represent the mean $\pm$ S.E.M. ($N = 4$ or 5 ; * $P < 0.05$).

Fig. 9.

Quantitative analysis of specific $[^3\text{H}]$ pirenzepine (10 nM) binding after optic nerve lesions and displacement by muscarinic toxins. (A) Quantitative comparison of $[^3\text{H}]$ pirenzepine binding in the lesioned and unlesioned tectal lobes after short-term lesions and displacement by MT1 (500 nM). No significant differences in binding existed between the two lobes. (B) $[^3\text{H}]$ Pirenzepine binding after short-term lesions and displacement by MT3 (150 nM). Lesioned and unlesioned lobes demonstrated equivalent levels of binding. (C) MT1-competed $[^3\text{H}]$ pirenzepine binding after long-term lesions showed no significant difference between the two lobes. (D) No significant differences in MT3-competed $[^3\text{H}]$ pirenzepine binding could be detected between the two lobes after long-term optic nerve lesions. Histograms represent the mean $\pm$ S.E.M. ($N = 4$ or 5).

Fig. 10.

Quantitative analysis of specific [^3H]AF-DX 384 (5 nM) binding after optic nerve lesions and displacement by MT3. (A) Short-term lesions were able to significantly reduce [^3H]AF-DX 384 binding only in layers 7–9 of the lesioned lobe when radioligand binding was displaced with MT3. (B) Quantitative differences in [^3H]AF-DX 384 binding after long-term lesions were not detected in any tectal layer, although radioligand binding was displaced with MT3. Histograms represent the mean $\pm$ S.E.M. ($N = 4$ or 5 ; $*P < 0.05$).

Comparison of the apparent affinity constants (nM; $\pm$ S.E.M. if available) of muscarinic ligands in the frog brain with those found in adult rat cerebral cortex and at purified human muscarinic receptors

Muscarinic ligand	Frog	Rat	Human			
			m1	m2	m3	m4
[³ H]Pirenzepine	0.91 $\pm$ 0.47	16.3 ⁵⁷	6.30 ²⁴	224 ²⁴	138 ²⁴	37.2 ²⁴
[³ H]AF-DX 384	5.10 $\pm$ 2.52	0.28 $\pm$ 0.08 ²	54.7 ⁶¹	1.00 ⁶¹	14.7 ⁶¹	2.20 ⁶¹
MT1	164 $\pm$ 36.9	28.0 $\pm$ 5.00	45.0 ⁴⁴	4000 $\pm$ 44	72.0 ⁴⁴	19,000 $\pm$ 44
MT3	Not determined	180 ⁴⁴	250 ⁴⁵	1000 $\pm$ 45	1000 $\pm$ 45	1.20 ⁴⁵