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Microiontophoresis and Single-Unit Analysis of Cholinergic Drugs in the Optic Tectum of Frog

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Abstract. Microiontophoresis of acetylcholine (ACh), atropine, or curare was accompanied by single-unit analysis of visual neuronal responses in the optic tectum of *Rana nigromaculata*. In 71% of the units tested, ACh enhanced responses to visual stimuli, while atropine suppressed visual responses. Twenty-two percent of the remainder showed no ACh- or atrophine-induced effects and were recorded within 125 μm of the pial surface. The majority of ACh-enhanced, atropine-suppressed units were recorded from visually responsive units localized in the postsynaptic cellular layer 8. The iontophoretic effects of curare were considerably more varied, with approximately equal numbers of units showing an increase (33%), a decrease (35%) or no change (32%) in visually activated responses. The specific effects of curare were also related to the depth from which unit recordings were obtained. These findings indicate that ACh functions as a modulatory neurotransmitter in the frog optic tectum, with a predominantly muscarinic mode of action at postsynaptic levels.

Introduction

The optic tectum of vertebrates contains a wealth of cholinergic circuits, and a marked similarity in the distribution of cholinergic markers has been reported in a variety of nonmammalian species [Oswald and Freeman, 1980; Freeman and Norden, 1984]. In amphibians, several studies have provided evidence that acetylcholine (ACh) is an excitatory optic-nerve neurotransmitter; furthermore, the optic tectum contains a high density of both nicotinic and muscarinic ACh receptors [Krnjevic, 1974; Freeman, 1977; Birdsall et al., 1980; Freeman et al., 1980].

Following the topical application of cholinergic substances to the surface of the frog optic tectum, Stevens [1973] reported that ACh and nicotine inhibited neuronal responses to visual stimulation, while *d*-tubocurarine produced response enhancement and often induced spontaneous bursting as well. Stevens concluded that

the optic tectum in frogs contains an inhibitory, nicotinic cholinergic system that is mediated by ACh. In a subsequent behavioral study, Hock [1983] also used topical application of curare, atropine, and strychnine to the tectal surface and reported a 'dramatic disinhibition' of predatory responses to visual stimuli. Hock also concluded that ACh functions as an inhibitory neurotransmitter in the optic tectum of anurans. However, Freeman and Norden [1984] have recently suggested that these results could be accounted for by the very high concentrations of cholinergic substances used in both studies. Further, they have suggested that while curare in the micromolar range appears to block retinotectal synaptic transmission, millimolar concentrations may have a direct excitatory effect on the optic tectum.

Microiontophoresis of known and presumptive neurotransmitters in amounts that approximate physiological concentrations can provide a powerful and sensitive method for testing their effectiveness in vivo. However, relatively few studies have utilized a simultaneous, microiontophoretic and neurophysiological analysis of cholinergic drug effects in the vertebrate optic tectum. The present study has combined both techniques, using a multibarrelled micropipette assembly to investigate the effects of ACh, atropine and curare upon visual neuronal responses in the optic tectum of a ranid frog.

Method

Forty-four, adult *Rana nigromaculata*, 4–7 cm in body length, were either immobilized with an intramuscular injection of 0.15–0.25 ml of 2% gallamine triethiodide (30 animals) or anesthetized by immersion in MS222 (1:1,000 concentration, 14 animals). The entire dorsal surface of the optic tectum was surgically exposed, and a local anesthetic (procaine) was periodically applied to the transected skin and muscle surrounding the surgical area during surgery and throughout the recording sessions which lasted 2–4 h [see also Wang et al., 1981; Wang et al., 1982].

A multibarrelled micropipette assembly was constructed of 4 closely adjacent micropipettes containing a recording microelectrode (1–2 μm tip diameter) filled with 3 *M* NaCl and 50 m*M* cobalt chloride (5–15 MΩ resistance). This pipette was used both for extracellular recording and for iontophoretic marking of recording sites. Three other micropipettes in the same assembly contained, respectively, ACh (0.5 *M*, pH 3.5), atropine sulphate (10 m*M*, pH 5.7) and *d*-tubocurarine chloride (10 m*M*, pH 5.7), all in aqueous solution. The micropipette assembly was advanced in increments of 2 μm, beginning at the pial surface. The entire extent of the dorsal tectum was explored during the course of the study. Response characteristics of each isolated single unit were evaluated with regard to (a) presence or absence of spontaneous activity, (b) location and approximate size of receptive field, (c) on-off responses to transient illumination, (d) monocular versus binocular responsiveness tested with opaque occluders [see Fite, 1969], and (e) responses to black and white discs subtending 3 and 8° at a distance of 33 cm, moved across the receptive field in horizontal, vertical and oblique trajectories. Any alterations in response characteristics that occurred following iontophoresis were evaluated with reference to the normal response profile previously obtained for each individual unit.

Action potentials were amplified and displayed on a Tektronix 5115 storage oscilloscope and photographed on polaroid film. The low signal-to-noise ratios which characterized the majority of recorded units did not permit a quantitative spike frequency analysis of unit responsiveness in most cases. However, a detailed assessment was made of each unit using a relatively slow oscilloscope time base in the oscilloscope storage mode. For each well-isolated unit, following a determination of the unit's normal response characteristics, the visual stimulus judged to be most effective was presented 3–6 times and a photographic record was obtained. ACh was then ejected (100–400 nA for 2 min and the unit's responsiveness to visual stimulation was reevaluated. If no change in visual responsiveness occurred, successively higher

Table I. Effects of cholinergic drugs on visual responsiveness of single units in the optic tectum

Response	ACh	Atropine	Curare
Increase	41/58 (71)	4/55 (7)	16/48 (33)
Decrease	4/58 (7)	39/55 (71)	17/48 (35)
No effect	13/58 (22)	12/55 (22)	15/48 (32)

Numbers in parentheses represent percentage.

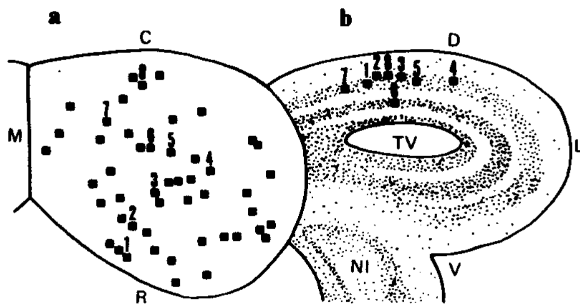

Fig. 1. **a** Localization (squares) of 49 recording sites at the dorsal surface of the left tectum. **b** Cross-section taken through mesencephalon at the level of nucleus isthmi (NI). Arabic numerals 1–8 in both **a** and **b** indicate the rostrocaudal location of 8 units whose recording sites were marked with cobalt sulfide. C = Caudal; D = dorsal; L = lateral; M = medial; V = ventral; TV = tectal ventricle, R = rostral.

currents (500–800 nA for 2 min) were applied, followed by a retest of the unit's visual responsiveness. A minimum recovery period of 3 min was allowed between successive current applications. For units which showed altered responsiveness following iontophoresis, this recovery period was extended until normal response levels returned. Atropine and curare were then tested in successive order, using comparable procedures. Ejection currents for atropine were 200–700 nA (1–2 min), and for curare were 300–800 nA (1–3 min). In each case, photographic records of normal, preiontophoretic responses were obtained and compared with those obtained after iontophoresis of each cholinergic substance.

Recording sites were marked iontophoretically with cobalt chloride (anodal current pulses, 2 μA, 0.5 s duration for 10 min). The brain was then removed and immersed in saline containing ammonium sulfide for 45 min. Brains were processed for paraffin embedding and sectioning. Serial sections were saved through the optic tectum and were examined with a light microscope in order to identify and localize individual recording sites.

Results

Fifty-eight single units were tested following iontophoresis of ACh; 55 of these were also tested with atropine, and 48 were held for a period of time sufficient to permit an analysis of the effects of curare. Preiontophoretically, the majority of units responded well to an 8° black disc moved across the receptive field. Fifty-two percent showed spontaneous (nonevoked) activity, and 43% adapted rapidly to repeated stimulus presentations. Most units showed either off or on-off responses to transient changes in room illumination. Receptive-field sizes ranges from 25° to 60°, and approximately half of the units recorded from the rostral tectum could be activated binocularly.

Three quarters of the units showed enhanced responsiveness to visual stimulation following ACh iontophoresis, which included an increase in the number of action potentials and reduced adaptation to a repeated visual stimulus. These ACh-induced effects often required as long as 30–90 s to develop. These same ACh-enhanced units showed a decrease or loss of visual responsiveness following iontophoresis of atropine (table I, fig. 1). Similar effects were observed upon spontaneous (nonevoked) levels of activity in approximately 50% of these units. An increase in the size of the receptive field was

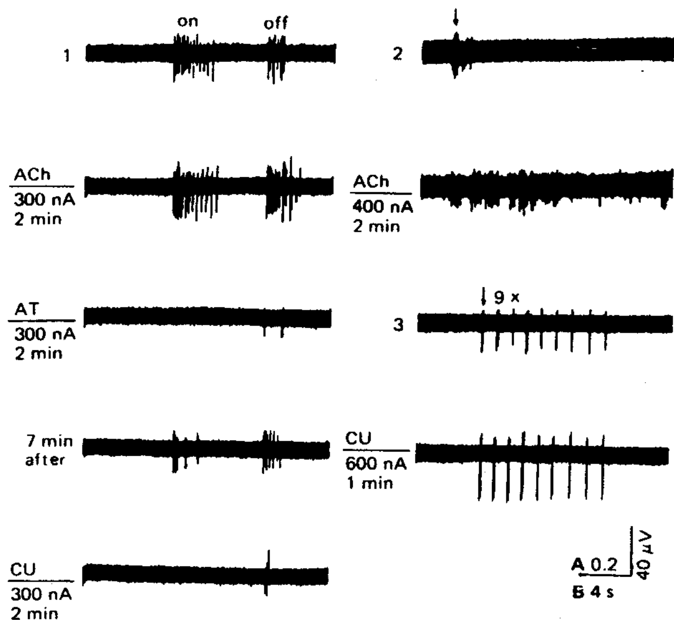

Fig. 2. The effects of iontophoretically applied ACh and its antagonists, atropine (AT) and curare (CU) on the action potentials of 3 neurons. **a** Responses of unit 1 to on- and offset of background illumination were enhanced by ACh, but diminished by AT and CU. **b** Unit 2 responses to visual stimulation (arrow), ejection of ACh-induced sustained discharge. Responses of unit 3 to a moving black disc; 9 repetitions of stimulus (9×) were enhanced by CU. Underlying numerals denote iontophoretic current intensities (nA) and time period of iontophoresis.

often observed following ACh ejection; and in binocular units, an increase in the effectiveness of ipsilateral stimulation often occurred as well. The average recorded depth of ACh-facilitated, atropine-suppressed units was 230 μm (SD ± 110 μm). Localization of recording sites marked with cobalt sulfide indicated that the majority of recordings were obtained from layer 8, and only rarely from layer 6 of the superficial tectal neuropil (fig. 1). Approximately 10% of ACh-enhanced units showed a short-latency (1–3 s) burst of action potentials beginning shortly after the onset of ACh iontophoresis (fig. 2). These drug-induced responses continued throughout the period of ACh iontophoresis, but ceased shortly after termination of current flow. Both ACh enhancement and atropine suppression effects were readily reversible, even following repeated drug applications, with normal response patterns being restored within 3–5 min. ACh current intensities ranging from 100 to 400 nA typically produced an enhancement of visual responses. In some cases, higher current intensities (600–800 nA) caused response suppression which was often irreversible.

The iontophoretic effects of curare were considerably more varied across the population of units tested. Fifteen of 48 units (31%) showed no change in responsiveness to visual stimuli following curare ejection, even with the highest current intensities used. The average recorded depth of these unaffected units was 125 μm (SD ± 75 μm). A comparable number of units, 16/48 (33%) showed an increase in visual responsiveness (average depth = 200 μm, SD ± 94 μm), and 17/48 (35%) of the total showed a decrease in activity (average

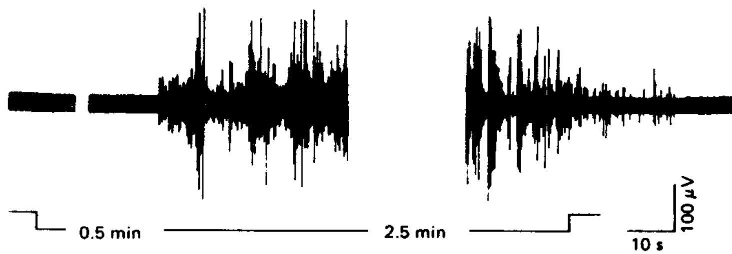

Fig. 3. Curare-induced discharges accompanied by spasmodic muscular contractions of hindlegs in tectal unit with caudally located receptive field. Ejection current of 600 nA lasted 3 min. At 0.5 min, responses began (intervening responses between onset and offset are omitted). Lower trace indicates total time period of iontophoresis.

depth = 250 μm, SD ± 143 μm) following iontophoresis of curare (table I). Of 41 units which showed both ACh-enhanced, and atropine-suppressed visual responses, 11/44 (25%) showed increased visual responsiveness following curare ejection, while 12/41 (29%) showed decreased responsiveness. Twenty percent (8/41) showed no effects of curare.

In several cases, curare induced a sudden, tetanic bursting of action potentials within 0.5–2 min of current onset (fig. 3), which was often accompanied by spasmodic muscular contractions in the hindlimbs, forelimbs or flanks and appeared to be correlated with the location of the unit's receptive field. These reactions quickly subsided within 1–2 min of current withdrawal, although initial baseline levels of unit responsiveness were rarely recovered. Five units of this kind were localized in layer 8 of the tectum, based upon cobalt sulfide markings. The pattern of results obtained from animals immobilized with gallamine triethiodide did not differ in any way from those obtained from MS222-anesthetized animals.

Discussion

Microiontophoresis of ACh and atropine consistently altered the visual responsiveness of tectal units in an ACh-enhanced, atropine-suppressed pattern. The reversibility and replicability of such results indicate that the observed neuropharmacological effects were not due to neurotoxicity or other abnormal alterations in neuronal cytologic properties. Both the recorded depths and sites marked with cobalt sulfide revealed that the majority of cholinergically altered unit responses were obtained, extracellularly, from tectal neurons located in layer 8. This layer contains a variety of cell types, including large ganglionic and small pyriform neurons which are among the efferent neurons of the optic tectum. These neurons are postsynaptic to retinal axons and afferents from other sources [i.e. nucleus isthmi, pretectum] which terminate in the superficial tectal laminae [Wang et al., 1982; Wilczynski and Northcutt, 1977; Lazar, 1984; Montgomery et al., 1985; Wang et al., in press]. By comparison, units iso-

lated in the more superficial laminae showed brisk reponses to visual stimulation and response characteristics typical of primary optic afferents, but showed little or no change in responsiveness following iontophoresis of ACh, atropine or curare. These findings suggest that ACh functions as an excitatory, modulatory neurotransmitter in the optic tectum of anurans, with primary effects occurring at postsynaptic levels. Sillito and Kemp [1983] have described quite similar effects in cat visual cortex, suggesting that ACh serves a neuromodulatory role in neocortex and mediates some of the effects of arousal as well.

Cholinergic neurotransmission is mediated by muscarinic and/or nicotinic receptors in the central nervous system, and the specific functional role of ACh appears to depend upon which type of receptor is activated. McGeer et al. [1978] proposed that nicotinic receptor actions are 'ionotropic', while muscarinic actions are 'metabotropic'. The latter effects are characterized by slow onset and long-lasting effects [Krnjevic, 1974; Bernardi et al., 1976; Misgeld et al., 1980]. In mammalian neostriatal neurons, Bernardi et al. [1976] have shown that ACh induces a slow depolarization which is mediated by muscarinic receptors and can be antagonized by atropine. The results of the present study are consistent with known muscarinic and metabotropic effects of ACh action, since the observed facilitatory effects of ACh were relatively slow at onset and could be blocked by atropine.

The general absence of direct excitatory effects of ACh upon tectal units further suggests that nicotinic effects of ACh are rare and, thus, do not represent a major mode of ACh action in the frog optic tectum. The highest concentration of muscarinic receptors in the brain of one ranid species *(Rana temporaria)* has been found in the optic tectum [Birdsall et al., 1980]. High densities of muscarinic receptors also occur in the superficial layers of the mammalian superior colliculus [Rotter et al., 1979; Wamsley et al., 1981], and in the avian optic tectum as well [Burkhalter and Henke, 1981; Por and Bondy, 1982]. Thus, muscarinic receptors may be involved in neuromodulatory functions associated with the initiation and guidance of visuomotor behaviors mediated through the optic tectum in a wide range of species. Indeed, Ciani et al. [1978] have suggested that cholinergic mechanisms are primarily related to intrinsic neural circuits of the optic tectum. More recent studies have shown that tectal postsynaptic cholinergic sites may be largely muscarinic [Francis et al., 1980; Por and Bondy, 1982].

As with ACh and atropine, the effects of curare were related to the depth from which individual unit recordings were obtained. No effects of curare were seen in units recorded from the most superficial layers of the afferent neuropil (layer 9, laminae A–E). However, recordings obtained at greater depths yielded either curare enhancement or suppression effects in those units showing an ACh-enhanced, atropine-suppressed pattern of visual-response modulation. Since curare is an antinicotinic agent, the suppressive effects may indicate the presence of nicotinic receptor sites. Alternatively, the excitatory effects of curare may be mediated via a nonnicotinic, cholinergic, inhibitory system or may represent a general membrane effect that induces twitching in some mus-

cles via the tectospinal pathway. Several investigators [Banerjee et al., 1970; Dasheiff, 1985] have reported epileptiform discharges and seizures following microinjection of curare during acute experiments. Dasheiff [1985] has further demonstrated that curare may act as a selective neurotoxin and suggested that a nicotinic mechanism may underlie both the epileptiform and neurotoxic actions of curare.

ACh involved in tectal neurotransmission may originate both from isthmotectal and from retinotectal afferents. Felix et al. [1985] have recently provided evidence that ACh is an excitatory neurotransmitter in nucleus isthmi of toad since isthmic cells are strongly excited by relatively low current ejections of ACh. Those same responses were blocked by atropine, which also decreased the cells' responsiveness to visual stimulation. Neurons most sensitive to ACh were also the most sensitive to atropine-blocking actions, as was also observed in the present study. Ricciuti and Gruberg [1985] have recently assessed the relative contributions of retinal afferents and nucleus isthmi fibers to the total tectal cholinergic input in *Rana pipiens.* Their findings indicate that nucleus isthmi is the principal source of cholinergic input to the optic tectum, since choline acetyltransferase activity is reduced by 94% following bilateral ablation of nucleus isthmi. Caine and Gruberg [1985] have further demonstrated that ablation of nucleus isthmi abolishes all visually guided responses to prey and eliminates threat avoidance responses as well. Thus, ACh may be released by axons from nucleus isthmi that terminate in tectal layers 8 and 9 [Wang et al., 1982; Wang et al., in press], with modulatory (and perhaps arousal) effects upon tectal neurons and circuits which mediate prey-catching and threat-avoidance behaviors in anurans. ACh has also been described as a neurotransmitter in the tecto-nucleus isthmi (pars parvocellularis) pathway in pigeon [Hunt and Brecha, 1984]. Wang et al. [in press] have recently demonstrated that microiontophoretically applied ACh produced a slow-onset, excitatory modulation in three quarters of visually activated units in the pigeon optic tectum, while atropine diminished or suppressed visually evoked responses. Their findings are remarkably similar to those of the present study, and further support our hypothesis that ACh has a predominantly muscarinic mode of action in the optic tectum.

In summary, the present study does not support the earlier conclusions of Stevens [1973] and Hock [1983] concerning the mode of action of cholinergic drugs in the frog optic tectum. The discrepancy between studies may be explained, at least in part, by the different drug concentrations that result from topical versus microiontophoretic applications. Our findings indicate that ACh, when applied microiontophoretically, produces effects upon tectal visual neurons which are predominantly excitatory and neuromodulatory in nature, and are consistent with a muscarinic mode of cholinergic action. However, the specific effects of ACh originating from different extratectal sources remain to be clarified, particularly in terms of site-specific versus neuromodulatory effects. The optic tectum offers a valuable opportunity to further understand the neuropharmacological and neurophysiological correlates of cholinergic action in the vertebrate nervous system.

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