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EFFECTS OF GLUTAMATERGIC, CHOLINERGIC AND GABAERGIC ANTAGONISTS ON TECTAL CELLS IN TOADS

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Abstract—The present paper using microiontophoresis analysis describes transmitters and their receptor subtypes used in retinotectal and isthmotectal transmission, and suggests several modes converging retinotectal and isthmotectal afferents on tectal neurons in toads (*Bufo bufo gargarizans*). Neuronal responses of tectal cells were extracellularly recorded to both visual stimulation and electrical stimulation of the nucleus isthmi, and effects of glutamatergic, cholinergic, GABAergic and glycinergic antagonists on these responses examined. Visual responses in 80% of tectal cells were reversibly blocked by the *N*-methyl-D-aspartate antagonist 3-RS-2-carboxypiperazin-4-yl-propyl-1-phosphonic acid, and those of the remaining 20% of cells by the muscarinic antagonist atropine, suggesting that there may be at least two kinds of retinotectal synapse that use glutamate and *N*-methyl-D-aspartate receptors, and acetylcholine and muscarinic receptors, respectively. Electrical stimulation of the nucleus isthmi elicited excitatory responses in 67% of tectal cells, excitatory–inhibitory responses in 16% of cells, and inhibitory responses in 17% of cells examined. The excitatory responses were reversibly abolished by atropine, but not affected by either 3-RS-2-carboxypiperazin-4-yl-propyl-1-phosphonic acid or the α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate antagonist 6-cyano-7-nitroquinoxaline-2,3-dione, whereas the inhibitory responses were released by the GABA receptor A antagonist bicuculline, but not influenced by the GABA receptor B antagonist 2-hydroxysaclofen and glycinergic antagonist strychnine. Excitatory and inhibitory components in the excitatory–inhibitory responses were blocked by atropine and bicuculline, respectively.

It appears that glutamatergic and cholinergic afferents from the retina, and cholinergic and GABAergic afferents from the nucleus isthmi may converge on tectal neurons in at least five modes of synaptic connections, in agreement with the heterogeneous populations of tectal cells in amphibians. © 1999 IBRO. Published by Elsevier Science Ltd.

Key words: antagonist, nucleus isthmi, optic tectum, transmitter, receptor, toad.

The optic tectum is the principal destination of optic axons in lower vertebrates such as frogs and toads. The neuroanatomy and visual electrophysiology of the anuran tectum have been extensively studied.[16,34] However, until now little was known about the chemical nature of neurotransmitters and their receptors in retinotectal projection. Several studies on amphibians have indicated that acetylcholine (ACh) may be involved in retinotectal transmission.[9,13,25] This is supported by the findings that there exists in tectum a high density of both nicotinic and muscarinic cholinergic receptors,[2,9,10,19] and that ACh acts as an excitatory neurotransmitter in the frog tectum, with predominant mediation of muscarinic receptors, suggesting that the ACh involved may be released from retinotectal and/or isthmotectal afferents.[8] However, several lines of evidence have been accumulating that anuran retinotectal transmission appears to be mediated by glutamate. For example, *N*-methyl-D-aspartate (NMDA)- and quisqualate-sensitive glutamate binding sites are localized in the superficial layers of the frog tectum.[3] The non-NMDA antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) reduces both monosynaptic and polysynaptic responses of tectal cells, while a NMDA receptor antagonist strongly reduces polysynaptic responses in frog tadpoles.[17] In toads, most retinotectal terminals are glutamate-like immunoreactive.[11] It is therefore postulated that glutamate may be an excitatory transmitter in the anuran retinotectal pathway.[3,11,17,24] These discrepant findings may be attributable, in part, to different involvements of transmitters in retinotectal, pretecto-tectal and/or isthmotectal pathways.

The anuran tectum has reciprocal connections with the nucleus isthmi (NI) located in the dorsolateral tegmentum. This nucleus has been electrophysiologically proven to be a visual center.[15,42,43] Its neurons are stained for acetylcholinesterase[40] and choline acetyltransferase,[22] and it is the principal source of cholinergic input to the tectum,[4,28,36] suggesting the cholinergy of the isthmotectal pathway. In fact, it has been shown that tectal cells in

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Abbreviations: ACh, acetylcholine; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; CPP, 3-RS-2-carboxypiperazin-4-yl-propyl-1-phosphonic acid; NI, nucleus isthmi; NMDA, *N*-methyl-D-aspartate.

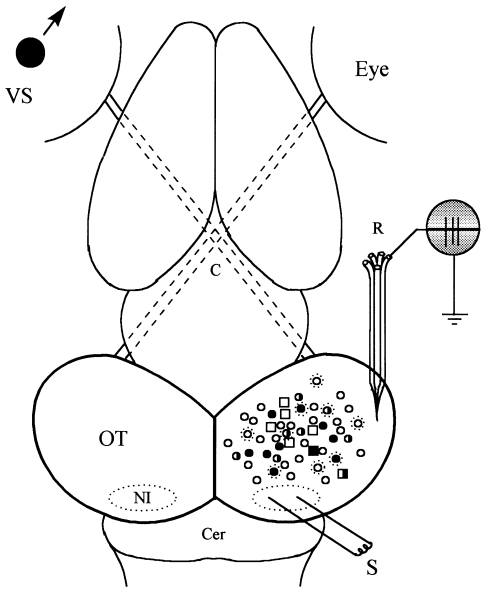

Fig. 1. Experimental arrangement and distribution of 57 recording sites on the dorsal surface of the optic tectum (OT). Open, half-filled and filled circles represent sites where excitation, excitation–inhibition sequence and inhibition were evoked following nucleus isthmi (NI) stimulation, respectively. Circles with dots symbolize recording sites marked by cobalt sulfide. Open, half-filled and filled squares signify two recording sites in the same tracks, corresponding to excitatory, excitatory–inhibitory or inhibitory responses, respectively. C, optic chiasm; Cer, cerebellum; R, five-barrel micropipette; S, stimulating bipolar electrode; VS, visual stimulation. Arrow indicates motion.

frogs respond to iontophoretically applied ACh in an excitatory manner, and ACh might be released from isthmotectal and/or from retinotectal afferents.[8] Alternatively, electrical stimulation of NI could elicit inhibitory responses in 70% of and excitatory responses in 30% of tectal cells.[38] This isthmotectal inhibition might be, in part, explained by a recent immunohistochemical finding that there exists a population of GABA-immunoreactive cells within the frog NI (Pollák *et al.*, unpublished observations), and these isthmic cells may project to the ipsilateral tectum to exert an inhibitory influence on tectal cells.

Our previous studies[7,12,41] have reported that in pigeons the isthmotectal pathway from the NI pars parvocellularis to tectum is primarily GABAergic, while that from the NI pars magnocellularis to the tectum is both glutamatergic and cholinergic. Tectal cells receive glutamatergic afferents from the retina, with the mediation of α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) receptors.[18,26] With these factors in mind, the present study was undertaken in an attempt to elucidate the functional organization of the retina–optic tectum–NI circuitry in terms of functional connections, neurotransmitters and their related receptors, by using microiontophoresis, visual and electrical stimulation techniques.

EXPERIMENTAL PROCEDURES

The experiments were performed in the winter on 31 adult toads (*Bufo bufo gargarizans*; wild-caught in the suburbs of Beijing and bred in the authors' laboratory) with body lengths of 7–9 cm from snout to vent, following the Policy on the Use of Animals in Neuroscience Research approved by the Society for Neuroscience in 1995. The toad was immobilized with an injection of gallamine triethiodide (5%, initial dose of 0.5–1.0 ml). The head wounds and fixed sites were locally infiltrated with procaine hydrochloride (2%). The skull was opened with a dental drill to expose the optic tectum and rostral cerebellum on the right side, and the dura mater overlying them was excised. The nictitating membrane of the left eye was cut to keep the eye open, and the right eye covered with an occluder. The toad's body was covered with a wet gauze to facilitate breathing. The animal was then placed in a stereotaxic apparatus.

The right NI was explored and its largest extents were measured using its stereotaxic coordinates[43] and visual responses recorded with a micropipette. The micropipette was then replaced with a tungsten bipolar electrode. For electrical stimulation of NI, rectangular pulses of 100–500 μA in intensity and 50–100 μs in duration were delivered. The visual stimulus was an 8° black disc that was 25–35 cm distant from the viewing eye and moved at 9–17°/s by an electrical motor. The luminance in the vicinity of visual stimulus was 15–30 lux and the stimulus was about 2.0 cd/m^2. Extracellular recordings of action potentials were obtained using one barrel of a five-barrel micropipette (3–5 μm diameter, 5–15 MΩ impedance) filled with 2 M sodium chloride (NaCl) and 100 mM cobalt chloride ($CoCl_2$). The other barrels contained the following chemical compounds that could be iontophoretically ejected by appropriate currents: CPP (3-RS-2-carboxypiperazin-4-yl-propyl-1-phosphonic acid; RBI; 0.01 M, pH 7.5), CNQX (Tocris Neuramin, 0.01 M, pH 8.3), atropine sulphate (Sigma, 0.01 M, pH 5.7), bicuculline methiodide (RBI, 0.02 M, pH 3.5), 2-hydroxysaclofen (RBI, 0.02 M, pH 3.0) or strychnine hydrochloride (Sigma, 2 mM in 165 mM NaCl) (Fig. 1). In some experiments, one barrel filled with 165 mM NaCl was used for minimizing current effects by current neutralization[29] or drug control. No apparent current effects were observed at the current intensities used in the present study.

Neuronal firings were preamplified and fed into an oscilloscope and a tape recorder. The data were then analysed off-line with a computer. At the end of some experiments, recording sites were marked iontophoretically with cobalt chloride (positive current pulses of 5–10 mA intensity, 0.2–0.5 s duration, 10–15 min). The brain was then removed from the skull and immersed in saline containing 10% ammonium sulfide for 10–15 min to form black precipitate of cobalt sulfide. The brain was fixed in 4% glutaraldehyde for 6 h, and then soaked in 30% sucrose overnight. Frozen sections were cut at 80 μm in thickness, mounted, counterstained with Cresyl Violet and covered for microscopic observations. In each of the animals, a constant current of 30–35 μA was applied through the stimulating electrode for 10 s to verify the stimulated sites by conventional histological procedures.

RESULTS

Electrophysiological responses of 57 tectal neurons were extracellularly recorded to a visual stimulus moving through their receptive fields and to electrical stimulation of NI, and effects of glutamatergic, cholinergic, GABAergic and glycinergic antagonists on their responsiveness to both stimulations were examined. These cells were distributed

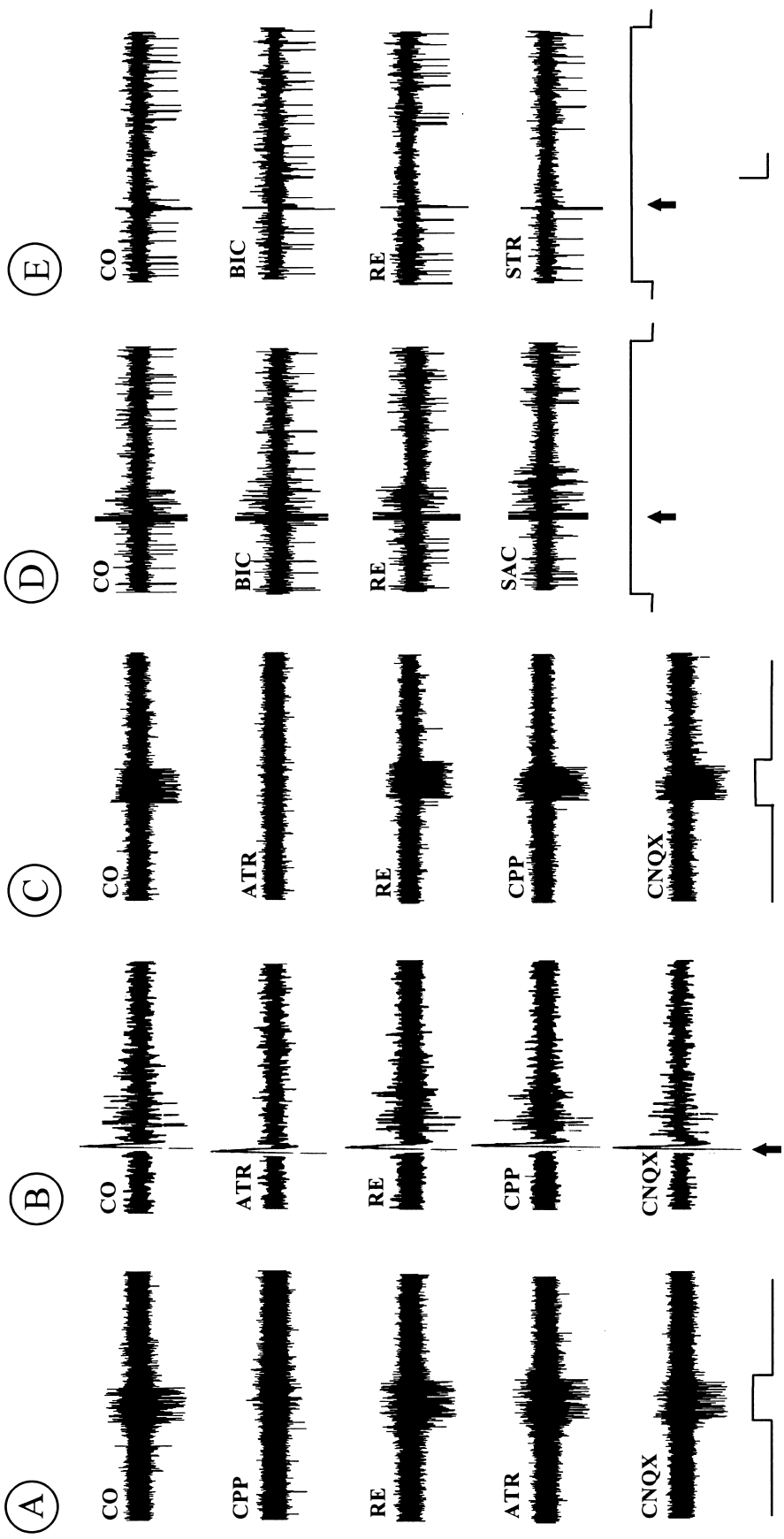

Fig. 2. Neuronal responses showing effects of glutamatergic (CPP, CNQX), cholinergic (ATR, atropine), GABAergic (BIC, bicuculline; SAC, 2-hydroxysaclofen), and glycinergic (STR, strychnine) antagonists on visual responses (A, C) and on responses evoked by electrical stimulation of the NI (B, D, E) in four tectal cells (responses in A and B were recorded from the same tectal cell). Visual responses in A were blocked by CPP but not by either atropine or CNQX, whereas those in cell C were selectively blocked by atropine, and by neither CPP nor CNQX. Excitatory responses of cell A–B to isthmic stimulations were selectively blocked by atropine, and not influenced by CPP and CNQX. Excitatory responses of E-I type cell D were selectively blocked by atropine (not shown), and its inhibitory responses were released by bicuculline but not by hydroxysaclofen. In I-type cell E, visual responses were inhibited by isthmic stimulations, and this inhibition was released by bicuculline but not affected by strychnine. Three sweeps were superimposed. CO, control; RE, recovery. Arrows point to electrical stimulation artifacts. Upward deflection of bottom traces represents visual stimulation using an 8° black disc. Scale bars: 20 mV, 500 ms in A and C, 10 ms in B, and 20 ms in D and E.

throughout the dorsal tectum (Fig. 1), with the recording depths ranging from 30 to 530 μm. Nine recording sites were marked with cobalt sulfide stainings, and they were located in tectal layers 6 (two cells), 8 (four cells), and 9 (three cells). Electrolytic markings histologically confirmed that all stimulating electrode tips in 31 toads were localized within NI, with eight sites being in the rostral, 14 in middle and nine in caudal nucleus. No apparent correlation between the stimulation sites within NI and the response properties of tectal cells was observed in the present study.

Tectal cells produced vigorous responses to an 8° black disc moving through their receptive fields at velocities of 9–17°/s. These visual responses usually lasted for 0.5–1.5 s, and ceased after terminating visual stimulation. The responses in 37 of 47 cells (79%) were completely blocked or significantly reduced by NMDA receptor antagonist CPP at current intensities of 50–300 nA, but not by either the AMPA antagonist CNQX or muscarinic antagonist atropine applied at higher current intensities of up to 400 nA, and this blockage was recovered 3–10 min after stopping CPP application (Fig. 2A). In contrast, visual responses of 10 others (21%) were abolished by atropine at currents of 20–80 nA, but not affected by either CNQX or CPP applied at much higher current intensities of 400 nA (Fig. 2C). Atropine-induced blockage was replaced by the normal responsiveness in 1.5–10 min after terminating drug application. Therefore, it appeared that there might exist at least two kinds of retinotectal synapse, i.e. glutamatergic and cholinergic synapses, that are mediated by NMDA receptors and muscarinic cholinergic receptors, respectively.

Among the 57 cells examined, 38 cells (67%) responded to isthmic stimulation in an excitatory manner (E-type), with a firing rate of 0.8–8 spikes per stimulation and an average latency of 8.0 ± 2.5 ms (mean ± S.D., $n = 38$), ranging from 4 to 12 ms. Neuronal responses evoked by isthmic stimulation in all E-type cells were eliminated by atropine (20–80 nA) and recovered 2–5 min after stopping drug application. They were not influenced by either CPP or CNQX applied at currents of up to 400 nA (Fig. 2B). Meanwhile, retinotectal transmission in eight of these cells (21%) was blocked by atropine, and that in 30 others (79%) blocked by CPP. Nine tectal neurons (16%) produced excitation followed by inhibition (E-I type) after isthmic stimulations (Fig. 2D). The average latency of the excitations was 6.9 ± 1.8 ms ($n = 9$), ranging from 4 to 10 ms, and that of the following inhibitions was 25–40 ms, with a mean of 32 ± 4.9 ms ($n = 9$). The excitatory responses were of cholinergic origin, characterized by atropine blockage (20–50 nA), whereas the inhibitory ones lasting for 50–80 ms were GABAergic, because they were completely blocked by the $GABA_A$ antagonist bicuculline (10–20 nA), but not by the glycinergic antagonist strychnine (20 nA). They were not affected by the $GABA_B$ antagonist 2-hydroxysaclofen (20 nA), either. It seemed likely that these long-latency inhibitions were elicited disynaptically or polysynaptically by isthmic stimulations. Retinotectal responses in two of these cells (22%) were blocked by atropine, and those in seven others blocked by CPP. Ten cells (17%) responded to isthmic stimulations only in an inhibitory manner (I-type), characterized by suppression of visual responses maintained only for examining inhibitory effects. These inhibitory responses lasted for 30–80 ms and had an average latency of 28 ± 16 ms ($n = 10$), ranging from 3.0 to 60 ms. It was obvious that tectal cells with short latencies might contact isthmic cells monosynaptically, while those with long latencies had disynaptic or polysynaptic contacts with NI cells. The inhibition was blocked by bicuculline at current intensities of 10–20 nA, and reappeared 2–4 min after ceasing antagonist application when isthmic stimulation occurred. Neither 2-hydroxysaclofen (10–20 nA) nor strychnine (10–20 nA) could block these inhibitions (Fig. 2E). In these cells, retinotectal transmission was blocked by CPP. It appears that there may exist at least two kinds of isthmotectal synapse, including cholinergic and GABAergic synapses, which could form three types of connection: a tectal cell could receive (i) cholinergic, (ii) both direct cholinergic and indirect GABAergic, or (iii) GABAergic afferents from the NI.

Micromanipulator readings corrected by cobalt sulfide markings of nine recording sites assigned most tectal cells examined to tectal layers 6, 8 and 9 (Fig. 3). Responsiveness of tectal cells to isthmic stimulations seemed to be somewhat related to their recording depths. The percentage of E-type cells increased from 57% in layer 9 to 75% in layer 6, whereas that of I-type cells decreased from 43% in layer 9 to none in layer 6; E-I type cells occupied about 20% of tectal cells recorded in layers 6 and 8. Therefore, E-type cells tended to be localized in the deep layers, whereas I-type cells were found in the superficial layers, and E-I type cells mainly within an intermediate region between them. However, comparisons of cobalt sulfide markings in tectum and electrolytic lesions in NI did not show apparent correlation between responsive types of tectal cells and stimulating sites within the nucleus.

DISCUSSION

The present study indicates that visual responses in most tectal cells in toads can be blocked by CPP, a specific NMDA antagonist, showing that retinotectal neurotransmission is mediated by glutamatergic synapses. This result is supported by several immunohistochemical and *in vitro* electrophysiological studies indicating that: (i) most optic axon terminals in toads are immunoreactive for glutamate;[11] and (ii) the dominant excitatory transmitter in the frog

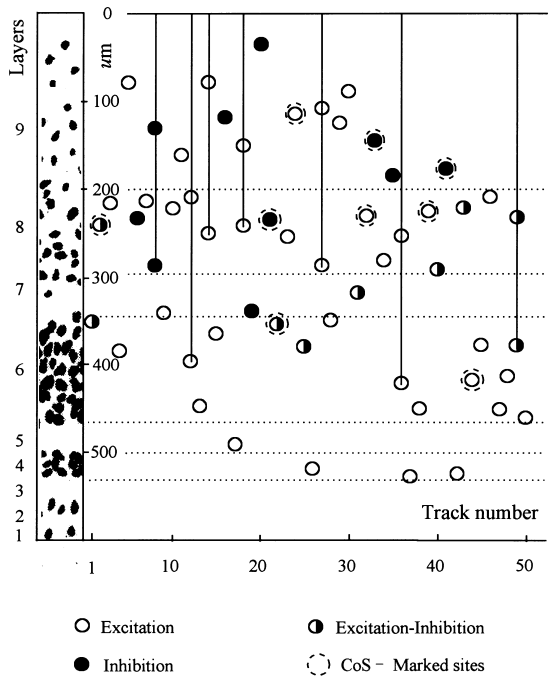

Fig. 3. Laminar distribution of recording sites in 50 electrode tracks in tectum. Recording sites of nine out of 57 cells were marked with cobalt sulfide (circles surrounded by dots), and other sites were approximately assigned to tectal layers according to their micromanipulator readings corrected with cobalt sulfide markings. Symbols representing different types of responses correspond to those in Fig. 1. Vertical lines signify tracks with multiple recordings, symbolized by squares in Fig. 1. On the left is a drawing of a cross-section of toad tectum, numbering tectal layers (1–9) and scaling depths in μm.

tectum appears to be glutamate.[17,24] Glutamate is also used as the principal excitatory transmitter in retinotectal pathways of teleosts,[20] reptiles[27] and birds.[5,18,37] The blockage of visual responses in tectal cells by CPP confirms the mediation of NMDA receptors, in agreement with the finding that NMDA and quisqualate receptor binding sites are localized on postsynaptic dendrites of tectal cells in frogs.[3] However, our *in vivo* study is discrepant with some *in vitro* studies showing that retinotectal transmission may be mediated by AMPA receptors, although bath-applied NMDA could elicit transient enhancement in excitatory field potentials recorded from the frog tectum *in vitro* preparations,[24] and NMDA receptor antagonist could strongly reduce polysynaptic responses to electrical stimulation of the optic nerve.[17] Recently, Wu *et al.*[45] have indicated that in clawed toads glutamatergic retinotectal transmission is mediated by NMDA receptors first, and then by both NMDA and AMPA receptors in maturational process of tectal cells. These discrepancies may be due to differences in species, preparations, drug applications and recording techniques. The seasonal variation in the anuran visual system should also be considered. For example, toads prefer white prey objects moving against a black background in the summer, while their contrast-preference is reversed during the autumn and winter.[6]

This study also shows that a small percentage of optic axons in toads may use ACh as an excitatory transmitter in retinotectal transmission. These axons might belong to only two classes of retinal ganglion cells.[9] This notion is supported by several histochemical and electrophysiological findings showing that retinotectal terminal layers contain high levels of acetylcholinesterase,[21,31] that the tectum possesses a high density of both muscarinic and nicotinic cholinergic receptors,[2,9,10] and that ACh could enhance visual responses of tectal cells in frogs, predominantly through a muscarinic mode of action at postsynaptic levels.[8] Recently, Gernert and Ewert[13] have reported that visually evoked field potentials in the toad tectum could be increased by ACh and decreased by tubocurarine, suggesting the involvement of nicotinic receptors at postsynaptic dendritic arborizations. However, field potentials represent the sum activity of neuronal populations in the vicinity of the recording electrode, and ACh here may be involved in various cholinergic systems.[13] It is probable that activation of a small proportion of cholinergic optic fibers cannot contribute much to the field potentials.

The second finding of the present study is that about 80% of isthmic cells exert excitatory or excitatory–inhibitory actions on tectal cells, mediated by ACh and muscarinic receptors, and 20% of cells produce "pure" inhibitions in tectal cells through GABAergic monosynaptic, disynaptic or polysynaptic synapses mediated by $GABA_A$ receptors. The cholinergic isthmotectal transmission has been evidenced by several histochemical studies suggesting that NI in lower vertebrates and its mammalian homologue, the parabigeminal nucleus, are the primary extrinsic source of cholinergic input to the tectum or superior colliculus.[4,23,28,36,47] Our results indicate that muscarinic cholinergic receptors are involved in this transmission, in agreement with cholinergic transmission from the NI pars magnocellularis to the tectum in pigeons.[41] Several biochemical, immunohistochemical and pharmacological studies have shown that there exist GABA and GABAergic receptors in the amphibian tectum, particularly in the superficial layers.[1,11,32,33,35,46] There is also a population of GABA-immunoreactive neurons within the NI, which may project to the ipsilateral tectum (Pollák *et al.*, unpublished observations). These isthmotectal axons terminate in tectal layers 8 and 9 and on dendrites of tectal cells.[14] Therefore, tectal cells could produce excitatory, excitatory–inhibitory or inhibitory responses to isthmic stimulation, somewhat depending on their recording depths. Generally speaking, more E-type cells are located in the deep layers, with more I-type cells in the superficial layers, and between is a transition region where E-I type cells are frequently found. This is supported by intracellular recordings

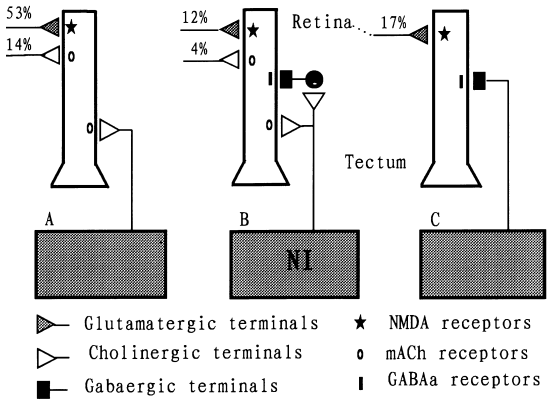

Fig. 4. Convergence of retinal and isthmic afferents on tectal neurons in toads. (A) Tectal cells with cholinergic isthmotectal synapses receive either glutamatergic or cholinergic inputs from the retina. (B) Tectal cells receiving both cholinergic and indirect GABAergic afferents make either glutamatergic or cholinergic synapses with retinal afferents. (C) Tectal cells make both GABAergic synapses with isthmotectal terminals and glutamatergic synapses with retinotectal terminals. Triangles and solid rectangles with bars represent nerve fiber terminals, beneath which are related receptor subtypes marked by various symbols; solid circles with bars represent inhibitory interneurons. The percentages on the retinotectal terminals indicate the proportion of each connection mode in the total population of tectal neurons examined pharmacologically in this study.

from tectal cells in frogs following isthmic stimulation.[38]

CONCLUSIONS

The present study provides neuropharmacological evidence indicating that there exist at least five combinations of retinal and isthmic afferents to tectal cells in toads: (i) glutamatergic inputs from the retina and cholinergic inputs from NI to tectal cells; (ii) both retinal and isthmic inputs to tectal cells are cholinergic; (iii) tectal cells receive glutamatergic afferents from the retina, cholinergic afferents directly and GABAergic afferents indirectly from NI; (iv) tectal cells receive cholinergic inputs from both the retina and NI, and indirect GABAergic inputs from the NI as well; and (v) glutamatergic afferents from the retina and GABAergic afferents from the NI converge on tectal cells (Fig. 4). These arrangements appear to be in agreement with the heterogeneous populations of tectal cells. Retinal afferents activate visual responses from tectal cells in toads, whereas NI exerts both positive and negative controls over tectal cells that are mediated by muscarinic ACh receptors and $GABA_A$ receptors, respectively. When amphibians evolved through reptiles to birds, the positive and negative actions of the NI on tectal cells were shared by two subdivisions of this nucleus, the magnocellular and parvocellular parts, respectively.[39] There exist two populations of magnocellular cells: (i) excitatory glutamatergic input mediated by both NMDA and AMPA receptors; and (ii) excitatory cholinergic input mediated by muscarinic receptors.[41] Following electrical stimulation of the parvocellular part, tectal cells respond in an inhibitory or an excitatory–inhibitory manner, with the mediation of $GABA_A$ receptors in all inhibitory responses and muscarinic receptors in excitatory responses.[7,12] This functional dichotomy of NI in birds may imply that this nucleus plays an important role in the discrimination and selection of visual targets by a "winner-takes-all" neuronal network.[30,39,44]

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