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Cholinergic Modulation of non-NMDA Glutamatergic Transmission in the Chick Ventral Lateral Geniculate Nucleus

Jian-Zhong Guo, Eva M. Sorenson, and Vincent A. Chiappinelli*

Department of Pharmacology & Physiology School of Medicine and Health Sciences The George Washington University Washington, D.C. 20037, USA

Abstract

Neurotransmission between glutamatergic terminals of retinal ganglion cells and principal neurons of the ventral lateral geniculate nucleus (LGNv) was examined with patch clamp recordings in chick brain slices during electrical stimulation of the optic tract. Since muscarinic and nicotinic receptors are present in high densities in LGNv, the present study examined possible roles of both receptors in modulating retinogeniculate transmission. During whole-cell recordings from LGNv neurons, acetylcholine (ACh, 100 μ M) caused an initial increase in amplitudes of optic tract-evoked non-NMDA glutamatergic postsynaptic currents (PSCs). This increase was unchanged when 1 μ M atropine was present, indicating that this initial enhancement of PSCs was due entirely to activation of nicotinic receptors. However, during washout of ACh the amplitudes of evoked PSCs became significantly decreased by $40.4 \pm 5.0\%$ for several minutes before recovering to their original amplitudes, an effect blocked by 1 μ M atropine. Exogenously applied muscarine (10 μ M) markedly depressed optic tract-evoked PSCs, and this decrease in amplitude was blocked by atropine. In a second set of experiments, we examined effects of releasing endogenous ACh prior to optic tract stimulation. This was accomplished by stimulation of the lateral portion of LGNv via a separate conditioning electrode. Following a brief train of low intensity conditioning stimuli, non-NMDA glutamatergic PSCs evoked by optic tract stimulation were potentiated. However, at higher conditioning stimulus intensities the PSCs were markedly decreased compared with control, and this decrease was partially blocked by atropine (1 μ M). Neither ACh nor muscarine altered amplitudes of PSCs elicited by exogenously applied glutamate. Muscarine significantly reduced the frequency but not the amplitudes of miniature PSCs, consistent with a presynaptic location for muscarinic receptors mediating these effects. Thus while activation of nicotinic receptors potentiates retinogeniculate transmission, activation of muscarinic receptors mediates depression of transmission, demonstrating a complex cholinergic modulation of sensory information in LGNv.

Keywords

muscarinic receptors; nicotinic receptors; presynaptic modulation

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*Corresponding author. Tel: 1-202-994-3541; fax: 1-202-994-2870. phmvac@gwumc.edu (V.A. Chiappinelli) Mailing address: Dept. of Pharmacology & Physiology, The George Washington University Medical Center, Suite 640, 2300 Eye St NW, Washington, DC 20037 USA .

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The avian ventral lateral geniculate nucleus (LGNv) is a retinorecipient thalamic nucleus that plays an important role in processing information in both the thalamofugal and tectofugal visual pathways, which are the two primary visual pathways in birds, reptiles and mammals (Shimizu and Karten, 1993). Specific roles for the LGNv include color vision and visual-motor control (Maturana and Varela, 1982; Gioanni et al., 1991). The axons of retinal ganglion cells traverse the distance between the retina and the central visual nuclei in the optic tract, and form synapses on nearly all neurons within the LGNv (Guiloff, 1991). This sensory information is processed along with afferent information from other visual and motor centers, and then transmitted out of the LGNv by projection neurons located in the lamina interna.

A prominent feature of the LGNv is the presence of high levels of the synthetic enzyme and receptors for ACh. Choline acetyltransferase immunohistochemistry reveals a dense network of cholinergic fibers throughout the LGNv (Sorenson et al., 1989). Autoradiography using selective radioligands demonstrates that there are high densities of muscarinic (Ball et al., 1990) and nicotinic (Sorenson and Chiappinelli, 1992) receptors in the LGNv. Functional studies have examined the possible role of cholinergic transmission in the LGNv. Patch clamp recordings from LGNv neurons in chick brain slices have shown that nicotinic agonists increase the frequencies of spontaneous glutamatergic and GABAergic PSCs by activation of several subtypes of presynaptic nicotinic receptors (McMahon et al., 1994; Guo et al., 1998; Guo and Chiappinelli, 2002). Because the optic tract is well-delineated in fresh brain slices, it is possible to selectively stimulate the retinal ganglion cell axons. Optic tract stimulation elicits monosynaptic glutamatergic PSCs in LGNv principal neurons that are mediated almost entirely by non-NMDA receptors, and these evoked responses are increased by exogenous nicotinic agonists (Guo et al., 2005). There is also more direct evidence that the cholinergic fibers within the LGNv modulate retinogeniculate transmission. Electrical stimulation of the lateral portion of the LGNv prior to optic-tract stimulation results in a marked enhancement of optic tract-evoked glutamatergic PSCs recorded in principal LGNv neurons (Guo et al., 2005). This enhanced response is completely blocked by nicotinic antagonists, demonstrating that it is due to the stimulated release of endogenous ACh from cholinergic fibers in the LGNv activating nicotinic receptors. Since neither ACh nor nicotine have any effect on postsynaptic responses to exogenously applied glutamate, the nicotinic receptors mediating enhanced neurotransmission in the LGNv appear to be located on the presynaptic nerve terminals of the retinal ganglion cells (Guo et al., 2005). In these previous functional studies, 1 μ M atropine was present throughout the experiments to prevent activation of muscarinic receptors. The goal of the present study was to more fully characterize the cholinergic modulation of retinogeniculate neurotransmission by examining the contributions of both muscarinic and nicotinic receptors.

EXPERIMENTAL PROCEDURES

Brain Slice Preparation

White Leghorn chick embryos between 18 and 20 days of incubation were rapidly decapitated and their brains removed and immediately placed in 4°C Mg²⁺-enriched-artificial cerebrospinal fluid (in mM: 126 NaCl, 2.5 KCl, 1.24 NaH₂PO₄, 10.0 MgSO₄, 2.4 CaCl₂, 26 NaHCO₃, 10 D-glucose, pH 7.3 when bubbled with 95% O₂/5% CO₂). The brains were blocked and attached to the stage of a vibrating tissue slicer. Coronal slices (350–400 μ m) containing the LGNv were placed in fresh normal artificial cerebrospinal fluid (ACSF; in mM: 126 NaCl, 2.5 KCl, 1.24 NaH₂PO₄, 1.3 MgSO₄, 2.4 CaCl₂, 26 NaHCO₃, 10 D-glucose, pH 7.3 when bubbled with 95% O₂/5% CO₂) at room temperature for at least one hour prior to use in experiments. Slices were then placed in the center of a submerged recording chamber (Warner Instruments, Hamden, CT) and stabilized with a U-shaped platinum holder. Slices were continuously superfused (2–3 ml/min) at room temperature in ACSF containing bicuculline

(10 μ M) and D(-)-2-amino-5-phosphonopentanoic acid (AP5; 50 μ M) to block GABA_A and NMDA receptors, respectively. All efforts were made to minimize both the suffering and the number of animals used in these experiments. All experiments conformed to the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the IACUC at George Washington University.

Electrophysiological Methods

Whole-cell patch-clamp recordings in brain slices were made from neurons in the lamina interna of the LGNv, visualized with Nomarski optics as described in Guo et al. 1998. Patch pipettes were fabricated from borosilicate glass with a Flaming-Brown Micropipette Puller (P-87) to produce a tip opening of 1-2 μ m with a resistance of 5-8 megaohms (M Ω). The pipette solution contained, (in mM), 150 K-gluconate, 2 MgCl₂, 2 EGTA, 2 Mg-ATP, 10 HEPES, adjusted to pH 7.3 with 1.0 N KOH. Electrical stimulation of retinal ganglion cell afferents to the LGNv was accomplished with a concentric bipolar electrode (50 μ m tip; FHC Corp) placed onto the surface of the optic tract about 1 mm ventral to the LGNv (Fig. 1). Stimulation pulses (5-200 μ A intensity, 200-300 μ s duration) were triggered at a frequency of 0.008-0.05 Hz and evoked PSCs were recorded in LGNv neurons. In experiments examining the effects of endogenously released ACh, a second stimulating electrode was placed onto the surface of the lateral portion of the LGNv (Fig. 1). This electrode was used to provide conditioning stimulation trains. Conditioning stimulation trains (3-8 pulses long, 50-1000 μ A intensity, 200-300 μ s duration at 25 Hz) were triggered at a frequency of 0.008-0.017 Hz and were delivered 0.2 – 1.0 sec prior to optic tract stimulation. Signals were obtained with an Axopatch-200B amplifier (Axon Instruments, Foster City, CA) in the voltage-clamp mode. Data was acquired with Clampex Ver.7.0 (Axon Instruments).

Data Analysis

To quantify evoked non-NMDA glutamatergic synaptic transmission, 2-3 consecutive responses evoked by optic tract stimulation were obtained before and then during exposure to an exogenous cholinergic agonist, either in normal ACSF or in the presence of atropine. These responses were averaged and plotted with Origin 7.0. A similar approach was taken for conditioning stimulation experiments, except that the conditioning stimulus train took the place of the exogenous cholinergic agonist. The percentage blockade of evoked non-NMDA glutamatergic synaptic transmission produced by exposure to agonists or conditioning stimulation under various conditions was calculated using the following formula: $(1 - (A/B)) \times 100\%$ (where A = amplitude in the presence of ACSF containing agonist or following conditioning stimulation; B = amplitude in normal ACSF without agonist or conditioning stimulation). The Repeated Measures One-Way ANOVA with a Tukey post-test (InStat 3.0, GraphPad Software) was used to test for statistical differences between 3 or more groups. A two-tailed t-test was used to compare the two data sets under conditioning stimulation, either in normal ACSF or in the presence of various antagonists.

The Mini Analysis Program ver. 6.0.7(Synaptosoft) was used to analyze non-NMDA glutamatergic miniature PSCs. The main parameters for miniature PSC detection were: threshold 7 pA; period to search a local maximum 10 msec; time before a peak for baseline 18 msec; period to search a decay time 10 msec. Analyses of 60 sec of continuous data were done under control conditions and in the presence of 10 μ M muscarine. Tetrodotoxin (0.3 μ M), bicuculline (10 μ M) and AP5 (50 μ M) were included in all bath solutions. The Kolmogorov-Smirnov test was used to determine significant differences between cumulative distributions of miniature PSC amplitudes and intervals.

For all statistical analyses, $p < 0.05$ was chosen as the level of significance. Data are expressed as mean $\pm$ S.E.M.

Agonist and antagonist applications

When examining muscarinic effects, ACh and muscarine were applied by bath perfusion for 2 min at 10-15 min intervals with or without atropine. A separate, gravity-fed U-tube system (Alkondon and Albuquerque, 1993) was used to deliver glutamate to LGNv neurons when examining possible muscarinic modulation of non-NMDA postsynaptic responses to exogenously applied glutamate (e.g., Fig. 7). The tip of the U-tube had a diameter of 150 μm , and the lower rim of the U-tube tip was placed 2-3 mm from the LGNv neuron being recorded from. The in-flow for all solutions delivered by U-tube into the recording chamber was gravity-fed, whereas the U-tube out-flow from the chamber was regulated by vacuum to provide a steady out-flow suction pressure. This out-flow suction system prevented any leakage of agonist solutions into the bath between agonist applications.

Materials

Drugs used were obtained from the following sources: acetylcholine chloride, atropine sulfate, bicuculline methiodide, (3-Aminopropyl)(diethoxymethyl)phosphinic acid hydrate (CGP), L-glutamic acid, ($\pm$)- α -Methyl-(4-carboxyphenyl)glycine (MCPG), muscarine chloride and naloxone methiodide were purchased from Sigma (St. Louis, MO); 4-diphenylacetoxy-N-(2-chloroethyl)-piperidine hydrochloride (4-DAMP), dihydro- β -erythroidine (DH β E) and AP5 were from RBI/Sigma (Natick, MA); tetrodotoxin (TTX) was from Calbiochem (San Diego, CA). Triptamine was a generous gift from Professor Carlo Melchiorre (Departimento di Scienze Farmaceutiche, Universita di Bologna, Italy). N-[2-[2-[(Dipropylamino)methyl]-1-piperidinyl]ethyl]-5,6-dihydro-6-oxo-11H-pyrido[2,3-b][1,4]benzodiazepine-11-carboxamide (AFDX 384) was from Tocris (Ellisville, MO).

RESULTS

Biphasic effects of ACh on optic tract-evoked non-NMDA glutamatergic PSCs

Whole-cell recordings were made from LGNv lamina interna neurons in embryonic chick brain slices (Fig. 1). Electrical stimulation of the optic tract produced monosynaptic glutamatergic PSCs under the recording conditions used (Fig. 2Aa). Throughout this study, all bath solutions contained 10 μM bicuculline to block GABA_A receptor-mediated responses and 50 μM AP5 to block NMDA receptors. Previous work demonstrated that >95% of the optic tract-evoked glutamatergic response under these conditions was mediated by non-NMDA receptors (Guo et al., 2005). Bath application of ACh (100 μM for 2 min) had a biphasic effect on optic tract-evoked PSCs. During the 2 min exposure to ACh, the amplitude of the evoked PSCs was significantly increased ($66.0 \pm 13.9\%$ increase, $n=13$, $p<0.001$; Fig. 2Ab). After 2 min washout of ACh, PSC amplitude was significantly decreased by $40.4 \pm 5.0\%$ compared with control ($p<0.05$, $n=13$; Fig. 2Ac). Recovery from the decreased amplitude required another 1-5 min of washout (Fig. 2Ad). In the presence of 1 μM atropine, the initial increase in PSC amplitude produced by ACh was identical to that seen without atropine, indicating that the initial enhancement was due entirely to activation of nicotinic receptors (Fig. 2Bb). However, in the presence of atropine the depression of PSC amplitude during washout of ACh was no longer observed, and instead PSC amplitude returned to the control level by 2 min of washout ($p>0.05$, $n=6$, Fig. 2Bc), suggesting that muscarinic receptors played a role in mediating the depression.

Muscarine depresses retinogeniculate transmission

To confirm that activation of muscarinic receptors can decrease retinogeniculate transmission we examined the effects of muscarine, a pure muscarinic agonist. Muscarine (10 μM for 2 min) significantly decreased the optic tract-evoked glutamatergic PSCs by $44.7 \pm 6.0\%$ compared to control ($p<0.001$, $n=11$; Fig. 3A). Muscarine produced a significant decrease in retinogeniculate transmission after 20 sec (Fig. 3C). The effect of muscarine was completely

prevented by 1 μ M atropine ($p > 0.05$, $n = 5$, Fig. 3B). These results demonstrate that the onset of an observed decrease in evoked PSC amplitude due to muscarinic receptor activation is much more rapid in the absence of a simultaneous nicotinic potentiation of the evoked PSCs such as that produced by ACh.

Effects of conditioning stimulus trains on glutamatergic PSCs

The LGNv is innervated by a dense network of cholinergic fibers (Sorenson et al., 1989; Fig. 1). Endogenous release of ACh from these fibers can activate nicotinic receptors and increase evoked glutamatergic transmission in the LGNv (Guo et al., 2005). We therefore sought to determine whether endogenous release of ACh in LGNv could act on muscarinic receptors to modulate retinogeniculate transmission. For these experiments a second stimulating electrode, termed the conditioning electrode, was placed in the lateral edge of the LGNv while recording from LGNv lamina interna neurons in the central portion of the nucleus (see Fig. 1). Trains of conditioning stimuli were then given prior to optic tract stimulation. At a low intensity of conditioning stimulation (200 μ A) there was an enhancement of non-NMDA glutamatergic PSCs evoked by subsequent optic tract stimulation (Fig. 4A). However, as the intensity of conditioning pulses was increased, the enhancement of optic tract-evoked responses decreased (Fig. 4B) and at high conditioning stimulation intensities there was a marked decrease in retinogeniculate transmission (Fig. 4C). To exclude nicotinic actions, we examined the effects of conditioning stimulation in the presence of the nicotinic antagonist dihydro- β -erythroidine (DH β E). With 30 μ M DH β E present, conditioning stimulation never enhanced, but only decreased, the non-NMDA glutamatergic response to optic tract stimulation (decreased by $56.9 \pm 7.7\%$, $n = 5$, $p < 0.01$; Fig. 5A). The magnitude of this decrease was significantly reduced when 1 μ M atropine was present ($32.9 \pm 4.4\%$; $p < 0.05$ compared to conditioning stimulation in absence of atropine, $n = 5$, Fig. 5B,C). Recovery from the decrease in PSC amplitude caused by conditioning stimulation was seen after 20 sec (Fig. 5A,B).

Conditioning stimulation may result in the release of neurotransmitters other than ACh, and some of these transmitters might modulate retinogeniculate transmission. We therefore examined the actions of conditioning stimulation in the presence of an antagonist cocktail containing the following antagonists: AP5 (50 μ M), bicuculline, (10 μ M), CGP (100 μ M), MCPG (250 μ M), naloxone (1 μ M), and DH β E (30 μ M). Conditioning stimulation continued to decrease optic tract-evoked glutamatergic PSCs in the presence of the antagonist cocktail, although the magnitude of the effect was reduced by the cocktail (decreased by $33.2 \pm 8.6\%$, $n = 4$, $p < 0.05$; Fig. 6A). Muscarinic receptors mediated the remaining decrease, since it was prevented by the addition of 1 μ M atropine to the antagonist cocktail ($9.9 \pm 4.3\%$ decrease; $p < 0.001$ compared to antagonist cocktail without atropine, $p > 0.05$ compared to control, $n = 4$, Fig. 6B,C).

Localization of muscarinic receptors mediating the effects

To determine whether postsynaptic muscarinic receptors played any role in this depression, we examined the effect of ACh and muscarine on the response of LGNv neurons to exogenously applied glutamate in the presence of tetrodotoxin (TTX; 0.3 μ M). U-tube application of glutamate (200 μ M) for 2 s produced inward currents in LGNv neurons (Fig. 7A). A 5 s application of the same concentration of glutamate produced a larger response (last trace, bottom row), indicating that the response to 2 s of glutamate was submaximal. Bath application of ACh (100 μ M, second trace, top row) or muscarine (10 μ M, second trace, bottom row) did not alter the magnitude of the inward current produced by exogenously applied glutamate ($p > 0.05$, $n = 4$ for both). The results suggest that activation of presynaptically located muscarinic receptors was responsible for the observed decreases in optic tract-evoked glutamatergic transmission in the LGNv.

We also determined the effects of 10 μ M muscarine on miniature PSCs in LGNv neurons in the presence of 0.3 μ M TTX. Cumulative distribution plots of the data are shown for an LGNv neuron in Figure 7C. While the miniature PSC amplitudes observed in the presence of 10 μ M muscarine were not significantly different from control amplitudes, the interval distribution of miniature PSCs was significantly different in the presence of muscarine in all 4 neurons tested (Kolmogorov-Smirnov test, $p < 0.05$). The mean decrease in frequency produced by muscarine was 62% (control, 0.98 ± 0.22 Hz, muscarine 0.37 ± 0.09 Hz, $n = 4$, $p < 0.05$ by paired t-test). This provides additional support for the conclusion that the muscarinic receptors are located on presynaptic glutamatergic terminals and when activated cause a reduction in glutamate release.

Pharmacology of muscarinic receptors modulating retinogeniculate transmission

In this series of experiments, the ability of several subtype-preferring muscarinic antagonists to block the inhibitory effects of muscarine was examined (Table 1). The M2-preferring antagonists triptamine and AFDX 384 blocked all of muscarine's effects at 1 μ M. In contrast, the M3-preferring antagonist 4-DAMP did not produce a significant block at 1 μ M ($p > 0.05$, $n = 6$) and required 10 μ M for a complete block (Table 1).

DISCUSSION

The results demonstrate that ACh released from cholinergic fibers in the chick LGNv modulates neurotransmission through this retinorecipient nucleus in a complex manner. The amplitudes of the monosynaptic non-NMDA glutamatergic PSCs evoked in LGNv projection neurons by optic tract stimulation were altered by synaptically released ACh as well as by exogenously applied ACh. A cholinergic depression of retinogeniculate transmission was mediated by activation of muscarinic receptors, whereas activation of nicotinic receptors led to enhanced transmission. Essentially all of the LGNv neurons tested exhibited this biphasic response to exogenous ACh and to conditioning stimulation when appropriate intensities of both optic tract and conditioning stimulation were used.

Afferent cholinergic fibers are present in a dense network throughout the chick LGNv (Sorenson et al., 1989), and our results indicate that sufficient ACh can be released from these fibers by brief electrical stimulation to activate both classes of cholinergic receptors. Our results are consistent with these muscarinic receptors being located presynaptically on the terminals of retinal ganglion cells, since there was an absence of postsynaptic effects of ACh on LGNv neurons or on their responses to exogenous glutamate, and muscarine significantly decreased the frequencies but not the amplitudes of non-NMDA glutamatergic miniature PSCs.

Activation of muscarinic receptors by either muscarine or ACh decreased PSC amplitude in LGNv neurons. While it is difficult to fully characterize the muscarinic receptor subtypes mediating this response due to a lack of pure subtype-selective antagonists, two M2-preferring antagonists, triptamine and AFDX 384 (Maggio et al., 1994; Gilsbach and Hein, 2008), blocked all of muscarine's effects at 1 μ M. Our functional data imply that the muscarinic receptors are located on retinal ganglion cell terminals, and this is consistent with a report that found M2 receptors expressed in the "vast majority" of chick retinal ganglion cells (Fischer et al., 1998). These authors also identified M4 receptors on retinal ganglion cells, but not M3 receptors. The M1 subtype is not found in the chick (Yin et al., 2004).

When ACh was applied exogenously in the present study, the optic tract-evoked PSC amplitude was markedly increased throughout the 2 min exposure to agonist, and the amount of increase was the same whether or not atropine was present. Thus, this cholinergic enhancement of retinogeniculate transmission was due entirely to activation of nicotinic receptors. In a previous study we showed that the nicotinic receptors mediating enhanced retinogeniculate transmission were located presynaptically on retinal ganglion cell terminals, and that α -bungarotoxin-

sensitive receptors mediated responses to exogenous ACh while α -bungarotoxin-insensitive receptors were activated by synaptically released ACh (Guo et al., 2005). The persistence of the nicotinic response throughout the 2 min ACh exposure is consistent with previous reports that α -bungarotoxin-sensitive receptors are resistant to desensitization at lower agonist concentrations, taking into consideration that the U-tube application method results in a 3-4 fold dilution of the agonist before it reaches the receptor sites (Guo et al., 1998; Woollorton et al., 2003).

Modulation of retinogeniculate transmission by ACh is thus a complex process with both excitatory and inhibitory components. In the retinal ganglion cell terminals the activation of nicotinic receptors, which are cationic ligand-gated ion channels, would result in increased glutamate release and enhanced PSCs as a result of depolarization of the terminal membrane and increased intraterminal Ca^{2+} . In contrast, activation of presynaptic muscarinic receptors, which are G-protein coupled metabotropic receptors, decreases glutamate release to decrease PSC amplitudes. One possible mechanism whereby muscarinic receptors could produce this effect is hyperpolarization of the presynaptic membrane by activation of K^+ channels. While the specific mechanism mediating the observed effects is not known, chick M2 receptors can hyperpolarize neuronal membranes due to activation of K^+ channels (Gadbut et al., 1996).

A second set of experiments examined whether endogenous ACh released from the cholinergic fibers in the LGNv would be able to modulate optic-tract evoked PSCs. In these conditioning stimulation experiments, the excitatory nicotinic effect predominated at low stimulation intensities resulting in increased PSC amplitudes. At higher stimulation intensities, the effect was instead a marked reduction in evoked glutamatergic PSC amplitude. A portion of this reduction was blocked by atropine, demonstrating that the inhibitory muscarinic activation was effective under these conditions. In contrast to the bath applied ACh experiments, not all of the inhibitory response was blocked by atropine. This points out a significant difference between optic tract stimulation, which selectively activates only glutamatergic optic tract fibers, and the conditioning stimulation, which in addition to activating cholinergic fibers also activates other fibers containing different neurotransmitters. In an attempt to further isolate the muscarinic receptor-mediated inhibitory response during conditioning stimulation, we added antagonists for the inhibitory receptors GABA_B , metabotropic glutamate and opioid to our bath solution. Under these conditions, the magnitude of the inhibitory response was substantially reduced, indicating that neurotransmitters other than ACh did reduce the optic-tract evoked response. Under these conditions, atropine was able to block the remaining inhibitory response, confirming its muscarinic character.

A possible concern in these conditioning experiments is that at the higher stimulation intensities used there is a depletion of presynaptic vesicles that contributes to the observed reduction in amplitude of the evoked PSCs. However, if vesicle depletion is occurring it does not represent a major factor contributing to the decreased PSC amplitude, since there was little or no reduction in evoked PSC amplitude when the combination of the antagonist cocktail plus atropine was present in the bath prior to applying the conditioning stimulation (see Fig. 6B).

We have demonstrated that exogenous ACh and muscarine as well as the release of endogenous neurotransmitters by conditioning stimulation can have a marked effect on the amplitude of evoked PSCs in LGNv neurons. One could therefore anticipate that adding an antagonist to the bath solution might directly result in changes in evoked PSC amplitude due to a blockade of endogenous release of transmitters. However, this was not the case, as we did not see changes in PSC amplitudes following addition of atropine, $\text{DH}\beta\text{E}$ or the antagonist cocktail to the bath solution. This may be a consequence of the unavoidable severing of afferent and efferent connections that occurs in a brain slice preparation, so that tonic synaptic activity is greatly reduced. Previous work has shown that $\text{DH}\beta\text{E}$ does not effect basal glutamatergic spontaneous

PSCs in LGNv neurons, but does block the increase in these spontaneous currents produced by nicotinic agonists (Guo et al., 1998).

The avian LGNv shares many characteristics with the mammalian LGNv, such as complex afferent and efferent connections to other nuclei within both the thalamofugal and tectofugal visual pathways and a role in visuomotor control functions (Crossland and Uchwat, 1979; Gunturkun and Karten, 1991; Shimizu and Karten, 1993; Harrington, 1997; Moore et al., 2000). In Fig. 3 of Guo et al. (2005) and in Fig. 1 of the present paper, we have presented images of lamina interna neurons. They are columnar, typically with between 1-3 dendrites descending into the LGN neuropil layer and 1-3 shorter dendrites ascending to the area dorsal to the lamina interna layer. The ventral dendrites form a complex branching pattern that is localized within a limited area of the neuropil layer. It is within the neuropil layer that the retinal ganglion cell afferents are likely to innervate the lamina interna neurons. Lamina interna neurons are projection neurons since their axons originate from the soma or a major dendrite and exit the LGNv dorsally. The axon can often be seen running medial to the nucleus rotundus in the apparent direction of the dorsal portion of the LGN (LGNd), where the LGNv has been shown to project (Tombol et al., 2004). The specific connections of the lamina interna neurons are not well understood. Axon collaterals are seen branching off the main axon in the area between the nucleus rotundus and the lamina interna layer. Sometimes these collaterals descend into the lamina interna layer or even into the neuropil layer. Although the lamina interna neurons we have visualized to date have similar morphological characteristics, further studies to determine connectivity and neurotransmitters and receptors expressed by the neurons would need to be done to more confidently conclude that they all belonged to one class. In mammalian species, the LGNv also sends a projection to the LGNd (Woolf and Butcher, 1986; Nakamura et al., 2005). The functional properties of neurons within the mammalian LGNv are poorly understood compared with the much better characterized neurons in the LGNd, which innervate the primary visual cortex. However, several groups have recently used patch-clamp recording within brain slices to examine functional activity within the rat LGNv. Govindaiah and Cox (2009) identified presynaptic and postsynaptic inhibitory effects on rat LGNv neurons following activation of metabotropic glutamate receptors. Born and Schmidt (2008) described a number of GABAergic pathways to and from the rat LGNv where transmission was mediated by GABA_A and possibly GABA_C receptors. The LGNv in mouse and rat contains abundant nicotinic and muscarinic receptors, and eye enucleation experiments indicate that many of the nicotinic receptors are localized on terminals of the retinal ganglion cells innervating the LGNv (Swanson et al., 1987; Tonnaer et al., 1988; Plummer et al., 1999). We have recently demonstrated that the mouse LGNv contains a cholinergic fiber network and that activation of nicotinic receptors markedly enhances optic-tract evoked glutamatergic PSCs in mouse LGNv neurons (Chiappinelli et al., 2007). A difference we found in the mouse is that LGNv neurons respond to nicotinic agonists with a direct depolarizing response, indicating that they express nicotinic receptors on their soma/dendrites, whereas we do not detect such postsynaptic responses in avian LGNv neurons (McMahon et al., 1994; Tredway et al., 1999; Chiappinelli et al., 2007).

Cholinergic afferents and receptors are prominent within the LGNv. The present study demonstrates that endogenously released ACh modulates neurotransmission through the chick LGNv by both excitatory responses mediated by nicotinic receptors and inhibitory effects mediated by muscarinic receptors.

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Abbreviations

ACh	acetylcholine
ACSF	artificial cerebrospinal fluid
AFDX 384	N-[2-[2-[(Dipropylamino)methyl]-1-piperidiny]ethyl]-5,6-dihydro-6-oxo-11H-pyrido[2,3-b][1,4]benzodiazepine-11-carboxamide
AP5	D(-)-2-amino-5-phosphonopentanoic acid
CGP	(3-Aminopropyl)(diethoxymethyl)phosphinic acid hydrate
4-DAMP	4-diphenylacetoxyl-N-(2-chloroethyl)-piperidine hydrochloride
DH β E	dihydro- β -erythroidine
LGNd	dorsal lateral geniculate nucleus
LGNv	ventral lateral geniculate nucleus
MCPG	($\pm$)- α -Methyl-(4-carboxyphenyl)glycine
PSC	postsynaptic currents
TTX	tetrodotoxin

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Fig. 1.

Positioning of recording pipette and stimulation electrodes on brain slice containing the LGNv. Stimulating electrodes were positioned with micromanipulators onto the optic tract (*Optic Tract Stimulation*) and the lateral portion of the LGNv (*Conditioning Stimulation*). A patch pipette (*Recording Pipette*) was used to record electrical activity in LGNv neurons in the lamina interna. This section has been immunohistochemically stained for choline acetyltransferase (Sorenson et al., 1989) to illustrate the dense network of cholinergic fibers present in the LGNv. **INSET:** Streptavidin-Alexa 568 projection image of biocytin-filled LGNv neuron prepared after the completion of a recording session. Abbreviations: li, lamina interna; ne, neuropil, Rt, nucleus rotundus.

Fig. 2.

Biphasic effects of ACh on optic tract-evoked non-NMDA glutamatergic PSCs in LGNv neurons. **A:** **a)** Control (CTR), electrical stimulation of the optic tract evokes an inward non-NMDA glutamatergic PSC in a representative LGNv principal neuron. **b)** The evoked current is increased in amplitude during bath application of ACh (100 μ M for 2 min). **c)** After washout of ACh for 2 min the evoked PSC is significantly decreased from the control level, then recovers to the original amplitude after 7 min (**d**). **B:** **a)** Control response in the same LGNv neuron is unchanged when 1 μ M atropine is added to the bath solution. **b)** In the presence of 100 μ M ACh, the evoked response is increased to the same extent as before. **c)** During washout of ACh the evoked response does not decrease below control amplitude, and returns to the control level by 2 min. Bar graphs below traces in **A** and **B** show amplitudes (mean $\pm$ S.E.M.) for each condition, with $n=13$ in **A** and $n=6$ in **B**. *, significantly different from control using ANOVA as described in Experimental Procedures. **C:** Time-Line showing the effect of 2 min exposure to ACh in a representative LGNv neuron. Optic tract stimulation occurs once every 20 sec throughout the experiment, and the amplitude of each evoked response is plotted in the absence (black squares) or presence (red circles) of 1 μ M atropine. Neurons were held at -70 mV. In this and all later figures, all bath solutions contained bicuculline (10 μ M) and AP5 (50 μ M).

Fig. 3.

Muscarine decreases evoked retinogeniculate transmission in LGNv neurons. **A:** The optic tract-evoked non-NMDA glutamatergic PSC in an LGNv neuron (**a**) was markedly decreased in the presence of 10 μ M muscarine (**b**). After washout of muscarine, the evoked glutamatergic PSC recovered to control level (**c**). **B:** In the presence of atropine (1 μ M), the muscarine-mediated decrease in evoked PSC was blocked (**b**). The neuron was held at -70 mV. Muscarine (10 μ M) was bath applied for 2 min. Bar graphs below traces in **A** and **B** show amplitudes (mean $\pm$ S.E.M.) for each condition, with $n=11$ in **A** and 5 in **B**. *, significantly different from control using ANOVA as described in Experimental Procedures. **C:** Time-Line showing the effect of 2 min exposure to muscarine in a representative LGNv neuron. Optic tract stimulation

occurs once every 20 sec throughout the experiment, and the amplitude of each evoked response is plotted in the absence (black squares) or presence (red circles) of 1 μ M atropine. All bath solutions contained bicuculline (10 μ M) and AP5 (50 μ M).

Fig. 4.

Effects of varying conditioning stimulation intensity on retinogeniculate transmission. Non-NMDA glutamatergic responses in an LGNv neuron evoked by optic tract stimulation (**at arrows**) are shown in the absence (**Left**) and presence (**Right**) of a preceding conditioning stimulation train. The conditioning train (12 pulses, 200 μ sec pulse duration, 25 Hz) is delivered 900 msec prior to optic tract stimulation. **A:** At low conditioning stimulus intensity (200 μ A), the optic tract-evoked response is potentiated. **B:** At 300 μ A, the response is similar in both cases. **C:** At 400 μ A conditioning stimulation, a marked decrease in the evoked response to optic tract stimulation is observed. The neuron was held at -70 mV. All bath solutions contained bicuculline (10 μ M) and AP5 (50 μ M).

Fig. 5.

The decrease in optic tract-evoked non-NMDA glutamatergic PSCs induced by conditioning stimulation was partially reversed by atropine. **A:** Three consecutive responses to optic tract stimulation (20 sec apart) were recorded in an LGNv neuron in the presence of DHβE (30 μM) to block nicotinic receptors. Left: Initial retinogeniculate PSC. Center: A conditioning stimulation train of 400 μA (12 pulses, 200 μsec pulse duration, 25 Hz) was delivered 900 msec prior to optic tract stimulation, and resulted in a decrease in the evoked response. Right: Recovery of response 20 sec after previous evoked response. **B:** Three consecutive responses to optic tract stimulation (20 sec apart) were recorded in the same LGNv neuron after addition of atropine (1 μM) to the previous recording solution. Left: Initial retinogeniculate PSC. Center:

Conditioning stimulation train identical to that in **A** resulted in a smaller decrease in the evoked response. Right: Recovery of response 20 sec after previous evoked response. The neuron was held at -70 mV. **C**: Comparison of amplitudes (mean $\pm$ S.E.M.) of evoked PSCs as a percentage of control for conditioning stimulation in the absence and presence of $1\text{ }\mu\text{M}$ atropine ($n=5$ neurons). *, significantly different from conditioning stimulation in the absence of atropine. All bath solutions contained bicuculline ($10\text{ }\mu\text{M}$) and AP5 ($50\text{ }\mu\text{M}$).

Fig. 6.

Muscarinic decrease of evoked responses persists in antagonist cocktail. **A:** Three consecutive responses to optic tract stimulation (20 sec apart) were recorded in an LGNv neuron in the presence of the antagonist cocktail (50 μ M AP5, 10 μ M bicuculline, 100 μ M CGP, 250 μ M MCPG, 1 μ M naloxone, and 30 μ M DH β E). Left: Initial retinogeniculate PSC. Center: A conditioning stimulation train of 800 μ A (200 μ sec pulse duration, 25 Hz, 5 pulses) was delivered 900 msec prior to optic tract stimulation, and resulted in a decrease in the evoked response. Right: Recovery of response 20 sec after previous evoked response. **B:** Three consecutive responses to optic tract stimulation (20 sec apart) were recorded in the same LGNv neuron after addition of atropine (1 μ M) to the previous recording solution. Left: Initial

retinogeniculate PSC. Center: Conditioning stimulation train identical to that in **A** resulted in a smaller decrease in the evoked response. Right: Recovery of response 20 sec after previous evoked response. The neuron was held at -70 mV. **C**: Comparison of amplitudes (mean $\pm$ S.E.M.) of evoked PSCs as a percentage of control for conditioning stimulation in cocktail in the absence and presence of $1 \mu\text{M}$ atropine ($n=4$ neurons). *, significantly different from conditioning stimulation in the absence of atropine. All bath solutions contained bicuculline ($10 \mu\text{M}$) and AP5 ($50 \mu\text{M}$).

Fig. 7.

A: Muscarinic agonists have no effect on postsynaptic currents evoked by exogenously applied glutamate. Fast application of 200 μ M glutamate (2 s; **small red bars above records**) in ACSF containing 0.3 μ M TTX produces an inward current in an LGNv neuron that is unchanged in amplitude when either ACh (100 μ M, **top row, second trace**) or muscarine (10 μ M, **bottom row, second trace**) were present in the bath. Fast application of glutamate (200 μ M) for 5 s (**large red bar above record**) produced a larger inward current (**final response**). **B:** Miniature non-NMDA glutamatergic PSCs recorded in 0.3 μ M TTX under control conditions (Ctr) and in the presence of 10 μ M muscarine (mus) applied for 2 min. **C:** Cumulative distribution plots of miniature PSCs measured in an LGNv neuron. Plots of data were constructed from 60 sec of continuous data (91 events in control, 32 events in muscarine). The Kolmogorov-Smirnov test was used to determine significant differences between cumulative distributions. The amplitude distributions (**right**) were not significantly different ($p > 0.05$) in muscarine than in control (mean amplitude in muscarine, 13.2 pA; in control, 13.6 pA). However, the interval between miniature PSCs (**left**) was significantly increased in the presence of 10 μ M muscarine (Kolmogorov-Smirnov test, $p < 0.05$). Mean interval between events was 1909 msec in muscarine; 636 msec in control. All neurons were held at -70 mV. All bath solutions contained TTX (0.3 μ M), bicuculline (10 μ M) and AP5 (50 μ M).

TABLE 1

Effects of selective muscarinic antagonists on the inhibition by muscarine of evoked glutamatergic currents in the LGNv

Muscarine (10 μ M) in:	% of blockade (Mean $\pm$ S.D.)	N (cells)
Normal ACSF	46.0 $\pm$ 19.2	10
Atropine (1 μ M)	2.9 $\pm$ 7.3 *	5
Tripitramine (1 μ M)	5.8 $\pm$ 10.2 *	6
AFDX 384 (1 μ M)	4.5 $\pm$ 16.4 *	6
4-DAMP (10 μ M)	-1.6 $\pm$ 8.1 *	5

Muscarine (10 μ M) was applied by bath perfusion for 2 min. Percentage blockade of evoked glutamatergic response was calculated by the following formula: $(1 - (A/B)) \times 100\%$ (where A = amplitude in ACSF containing 10 μ M muscarine and indicated antagonist; B = amplitude in normal ACSF).

* significantly different from Normal ACSF at $p < 0.05$.