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ELECTROPHYSIOLOGY AND PHARMACOLOGY OF THE OPTIC INPUT TO THE RAT INTERGENICULATE LEAFLET *IN VITRO*

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The mammalian intergeniculate leaflet (IGL) of the thalamus is a neuronal element of the circadian timing system, which receives direct photic input from the retina. The purpose of this study was to analyze responses of rat IGL neurons *in vitro* to optic tract stimulation and to identify neurotransmitters released from the terminals of retinal ganglion cells in this structure. Following optic tract stimulation, most of the responding IGL cells were excited and only a minority of them were inhibited. Neurons showing the excitatory response were tested in the presence of AP-5, a selective antagonist of NMDA receptors. In most cases the responses were only partially inhibited by the presence of AP-5. Complete disappearance of excitatory responses was achieved by adding CNQX, an AMPA/kainate receptor-selective antagonist, to the standard incubation fluid. Inhibitory responses were blocked or considerably attenuated in the presence of bicuculline, a $GABA_A$ receptor antagonist, in the ACSF. This study demonstrated that glutamate is the main neurotransmitter mediating optic tract input to the IGL, acting mainly via non-NMDA ionotropic receptors. It was also shown that NMDA and $GABA_A$ receptors are involved in passing photic input to the IGL, albeit to a much lesser extent.

Key words: *photic input; glutamate; circadian rhythms*

INTRODUCTION

Light is the most important and powerful environmental cue for synchronization of mammalian circadian rhythms. The suprachiasmatic nucleus (SCN) is a structure recognized as the circadian pacemaker. The SCN receives photic information via two principal pathways: directly from the retina through the retinohypothalamic tract (RHT); and indirectly from the retinorecipient intergeniculate leaflet (IGL) (1).

The IGL is known primarily as an important component of the mammalian biological clock. The main, accepted function of the intergeniculate leaflet, as part of the circadian timing system, is to integrate photic and non-photic information in order to modify SCN activity (2).

Due to its anatomical connections, the IGL, a homologue of the pregeniculate nucleus in the primates (3), is also thought to be associated with the visuomotor (4, 5) and sleep/arousal systems (5). Morphological evidences show that intergeniculate leaflet participates in signaling pathway from the retina to the hypothalamic neuroendocrine cells (6). The IGL can fulfill its accepted and putative functions primarily thanks to monosynaptic input from the retina (7). Its common origin with the retinohypothalamic tract is one of the characteristic features of this pathway. Some retinal ganglion cells (RGCs) projecting to the SCN bifurcate to make synaptic contact with neurons in the intergeniculate leaflet (8).

Notwithstanding the significance of photic input for circadian as well as visuomotor and sleep/arousal

circuits, in which the IGL is strongly suggested to play a part, the chemical nature of the retinal input to the intergeniculate leaflet remains unknown. On the other hand, numerous studies employing a wide range of techniques have identified the neurotransmitters responsible for conveying photic information to the SCN (for review see 9). The main transmitter released from RGC endings in the SCN is glutamate, co-stored in terminals with pituitary adenylate cyclase-activating polypeptide (PACAP) (10). Based on the common origin of the RHT and the retino-intergeniculate pathway, glutamate is thought to be a good candidate for the neurotransmitter released from the axons of retinal ganglion cells in the IGL. This assumption is additionally reinforced by several findings. The first one is study described by Shinohara *et al*. (11) showing that elevation of IGL derived NPY in the SCN after light pulses is blocked by NMDA receptor antagonist. The second source of support comes from our previous studies showing IGL cells to be highly sensitive to glutamate application (12). What is more, PACAP immunoreactivity was found within the IGL (13), and PACAP is known, as mentioned, to be co-stored with glutamate in the RHT endings; this suggested that the same may occur in the retino-intergeniculate tract. However, the common origin of the RHT and the retino-intergeniculate pathway or the presence of glutamatergic receptors in IGL does not definitely implicate glutamate as a retino-intergeniculate transmitter, particularly since some data might lead to the opposite conclusion. Edelstein and Amir (14) have shown that light-induced Fos protein expression in the IGL is not dependent on an ionotropic glutamate receptor mechanism. Since treatment with NMDA and AMPA/kainate glutamatergic receptor antagonists did not attenuate Fos expression in the IGL, they concluded that "glutamate may not be involved in light transmission to the IGL". Moreover, Fujiyama *et al*. (15) have revealed weak immunoreactivity for VGluT1 and VGluT2 transporters in the IGL. The same paper showed that the immunoreactive profiles of VGluT1 and VGluT2 were only slightly reduced after bilateral enucleation. This may indicate an extraretinal origin of glutamatergic innervation of the IGL. The aims of our present study were to characterize the electrophysiology of optic tract (OT) input to the IGL and the role of glutamate as putative neurotransmitter released from the retinal ganglion cell terminals to the IGL.

MATERIALS AND METHODS

All procedures were conducted in accordance with the European Community Council Directive of 24 November 1986 (86/609/EEC) and under Polish law. Male Wistar rats (100-120 g; 4 weeks old) used in this study were obtained from the local animal facility, where environmental conditions were kept constant (22-23°C, 12 h photoperiod, lights on at 8:00 a.m.), with food and water available *ad libitum*.

The animals were decapitated under pentobarbital anesthesia (Morbital 1.5 ml/kg, Biowet Pulawy, Poland). After decapitation, the brain was quickly removed and submerged in oxygenated ice-cold artificial cerebrospinal fluid (ACSF; composition (mM): NaCl 132, KCl 2, $MgSO_4$ 1.25, KH_2PO_4 1.25, $NaHCO_3$ 26, $CaCl_2$ 1.87, glucose 10). Coronal brain slices (400 µm thick) containing the thalamus with the lateral geniculate complex were cut on a microtome (Leica VT1000S, Berlin) and transferred to the interface recording chamber. Slices were perfused with warmed (34°C) carbogenated ACSF for 1.5 h prior to recording in the same conditions.

Single-cell neuronal activity was recorded extracellularly with glass microelectrodes made from borosilicate capillaries, using a horizontal puller (Sutter Instruments, Novato, USA) and filled with 2M NaCl solution (resistance; 3-5 MΩ). Single units were discriminated using Spike2 software. Cells from different regions of the IGL were recorded under visual microscopic control. Signals were amplified (10,000x) and bandpass-filtered between 500 Hz and 2000 Hz using an X-3Cell amplifier (FHC Inc., Bowdoin, USA) and sampled (20 kHz) with 1401plus (CED, Cambridge, England) equipment and software (Spike2) for storage and analysis.

Well described and commonly used protocol of electrical stimulation of the axonal endings in the optic tract *in vitro* was applied (16-18). Stimulation of retinal ganglion cell afferents to the IGL was accomplished with a bipolar concentric electrode. The stimulating electrode was placed into the optic tract approximately 300 µm below the boundary of the IGL, to deliver single electrical pulses from a WMT stimulator (200-300µs/200-500µA).

Before starting the stimulation protocol, baseline neuronal activity was recorded for 600 s. "Silent" neurons not showing spontaneous discharge activity were identified by their sensitivity (expressed as burst discharge) to mechanical stimulation with the recording pipette.

After achieving stable responses to 50 consecutive stimulation sweeps in normal ACSF, for each neuron the procedure was repeated in the presence of one of the following drugs: the NMDA receptor antagonist AP-5 (D(-)-2-amino-5-phosphonopentanoic acid, 50 µM), the AMPA/kainate receptor antagonist CNQX (6-Cyano-7-nitroquinoxaline-2,3-dione disodium salt, 10 µM), or the $GABA_A$ receptor antagonist bicuculline (30 µM). All drugs were administered by

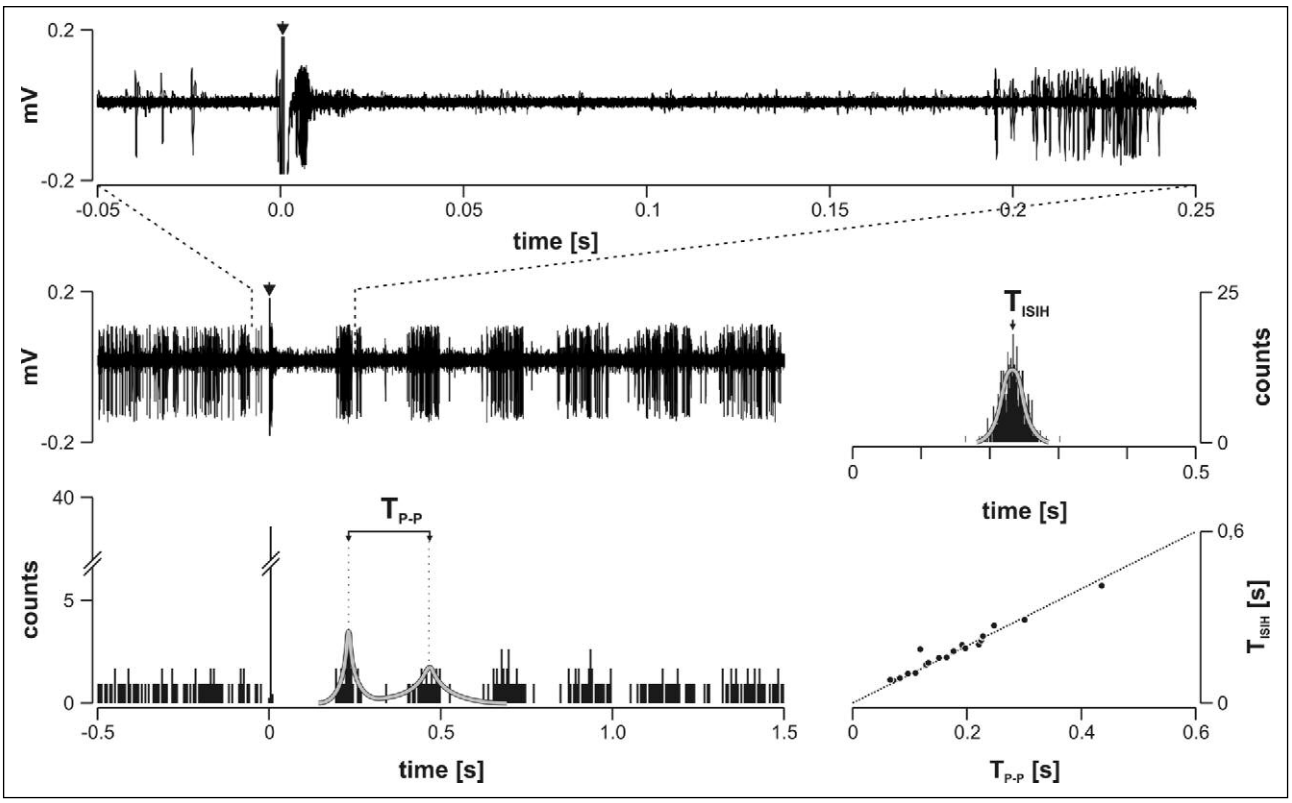

Fig. 1. Overlaid sweeps of raw signal (top left; 50 sweeps) and peristimulus time histogram (bottom left; PSTH - 50 sweeps), showing the response of the IGL neuron to electrical stimulation of the optic nerve (stimulus at time 0 s). The initial excitatory response is followed by decaying oscillatory firing. The sum of two Gaussian functions (grey, solid line) has been fitted to the first two oscillatory increases of activity. $T_{P\text{-}P}$ - period of oscillation.
Top right - interspike interval histogram (ISIH) with Gaussian function fitted to it (grey, solid line).
Bottom right - graph showing the linear relationship between the mean interspike interval of spontaneous activity (T_{ISIH}) and the period of decaying oscillatory firing ($T_{P\text{-}P}$). The linear function ($y = 1.01*x$; $R^2 = 0{,}94$; dotted line) has been fitted to the data points. Row trace in the middle has the same time scale as the PSTH below. Bin size on all graphs = 1 ms.

bath application. All chemicals were purchased from Sigma.

Optic tract stimulation effects on IGL neuronal activity were observed on peristimulus time histograms (PSTH) generated on-line. Each PSTH was generated for 50 consecutive stimulations and consisted of 2000 1 ms bins; 500 bins (0.5 s) before the stimulus was taken as the baseline. The mean number of counts per bin was calculated for the baseline, and the response of the neuron was determined in relation to this value. Bins occurring after stimulation and having their amplitude (A_{bin}) above the baseline were considered excitation if the amplitude (CA_{excit}) and duration (C_D) criteria were fulfilled; the CA_{excit} criterion is A_{bin} > baseline + 1SD, and the C_D criterion is that the number of consecutive bins fulfilling CA_{excit} is higher than the number of consecutive baseline bins fulfilling CA_{excit}. Bins occurring after stimulation and having their amplitude (A_{bin}) below the baseline were considered inhibition if the amplitude (CA_{inhib}) and duration (C_D) criteria were fulfilled; the CA_{inhib} criterion is that A_{bin} < baseline - 1SD or $A_{bin} = 0$, and the C_D criterion is that the number consecutive bins fulfilling CA_{inhib} is higher than the number of consecutive baseline bins fulfilling CA_{inhib}. For cells having an oscillatory component in the response, the period of oscillation was determined based on the centroid coefficients of the curve, being the sum of two Gaussian functions, fitted to the PSTH. For these cells the interspike interval of spontaneous activity was determined based on the centroid coefficient of the Gaussian function fitted to the interspike interval histogram (ISIH). The latency, duration and amplitude (no. of counts per bin) were calculated for each response. All descriptive statistics are given as means ± S.E.M. Changes of neuronal firing in the response were normalized to the baseline and are given as percentages ± S.E.M. All statistical comparisons were made with Student's t test for independent samples, and differences at $p < 0.05$ were considered significant. All statistical tests employed STATISTICA software (StatSoft, Inc., USA). Histogram analysis (PSTHs and ISIHs,

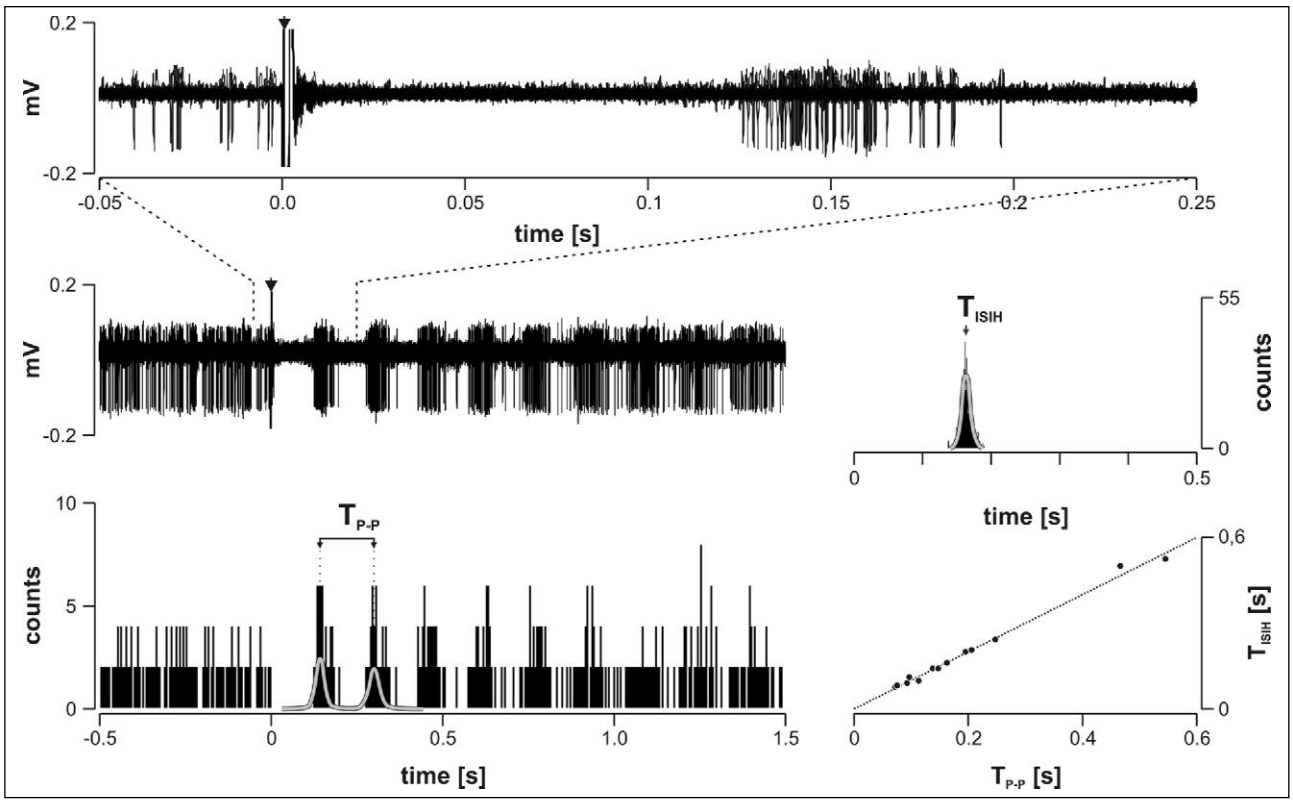

Fig. 2. Overlaid sweeps of raw signal (top left; 50 sweeps) and peristimulus time histogram (bottom left; PSTH - 50 sweeps), showing the response of the IGL neuron to electrical stimulation of the optic nerve (stimulus at time 0 s). The initial inhibitory response is followed by decaying oscillatory firing. The sum of two Gaussian functions (grey, solid line) has been fitted to the first two oscillatory increases of activity. $T_{P\text{-}P}$ - period of oscillation.
Top right - interspike interval histogram (ISIH) with Gaussian function fitted to it (grey, solid line).
Bottom right - graph showing the linear relationship between the mean interspike interval of spontaneous activity (T_{ISIH}) and the period of decaying oscillatory firing ($T_{P\text{-}P}$). The linear function ($y = 1.04*x$; $R^2 = 0{,}99$; dotted line) has been fitted to the data points. Row trace in the middle has the same time scale as the PSTH below. Bin size on all graphs = 1 ms.

response detection and analysis, curve fitting) was done with a custom-made script in MATLAB (MathWorks, Inc., USA).

RESULTS

A total 106 intergeniculate leaflet neurons were tested for responsiveness to optic tract stimulation. Of these, 58% (62/106) showed clear responses to stimulation and the remaining 44 cells were unresponsive. Only the IGL cells that responded to optic tract stimulation were submitted to further analysis and are described below.

The majority of the responsive cells (35%; 22/62) showed initial excitatory responses to optic tract stimulation followed by decaying oscillatory firing (*Fig. 1*). The period of decaying oscillation was equal to the interspike interval in each case. The correlation between these two parameters was linear for all the cells responding in this way (*Fig. 1*).

Another group of IGL cells responsive to optic tract stimulation (21%; 13/62) showed initial inhibitory responses followed by decaying oscillatory firing (*Fig. 2*). The period of decaying oscillation was equal to the interspike interval in each case. The correlation between these two parameters was linear for all the cells responding in this way (*Fig. 2*).

A different group of IGL neurons (21%; 13/62) responsive to optic tract stimulation showed monophasic excitatory responses (*Fig. 3*, top). A smaller share of neurons (18%; 11/62) exhibited a biphasic response to optic tract stimulation, with initial excitation followed by transient inhibition (*Fig. 3*, middle). The least frequent response of IGL neurons to optic tract stimulation was monophasic inhibition (*Fig. 3*, bottom).

Parameters of different response types (latency, duration, no. of counts per response, and normalized percent change in firing) were determined, averaged and compared with each other. The latency of the excitatory components did not differ significantly between the different response types (*Fig. 4A*). Nor did the average duration of the responses or their components (in the case of polyphasic ones) differ, except for the duration of excitatory responses. The

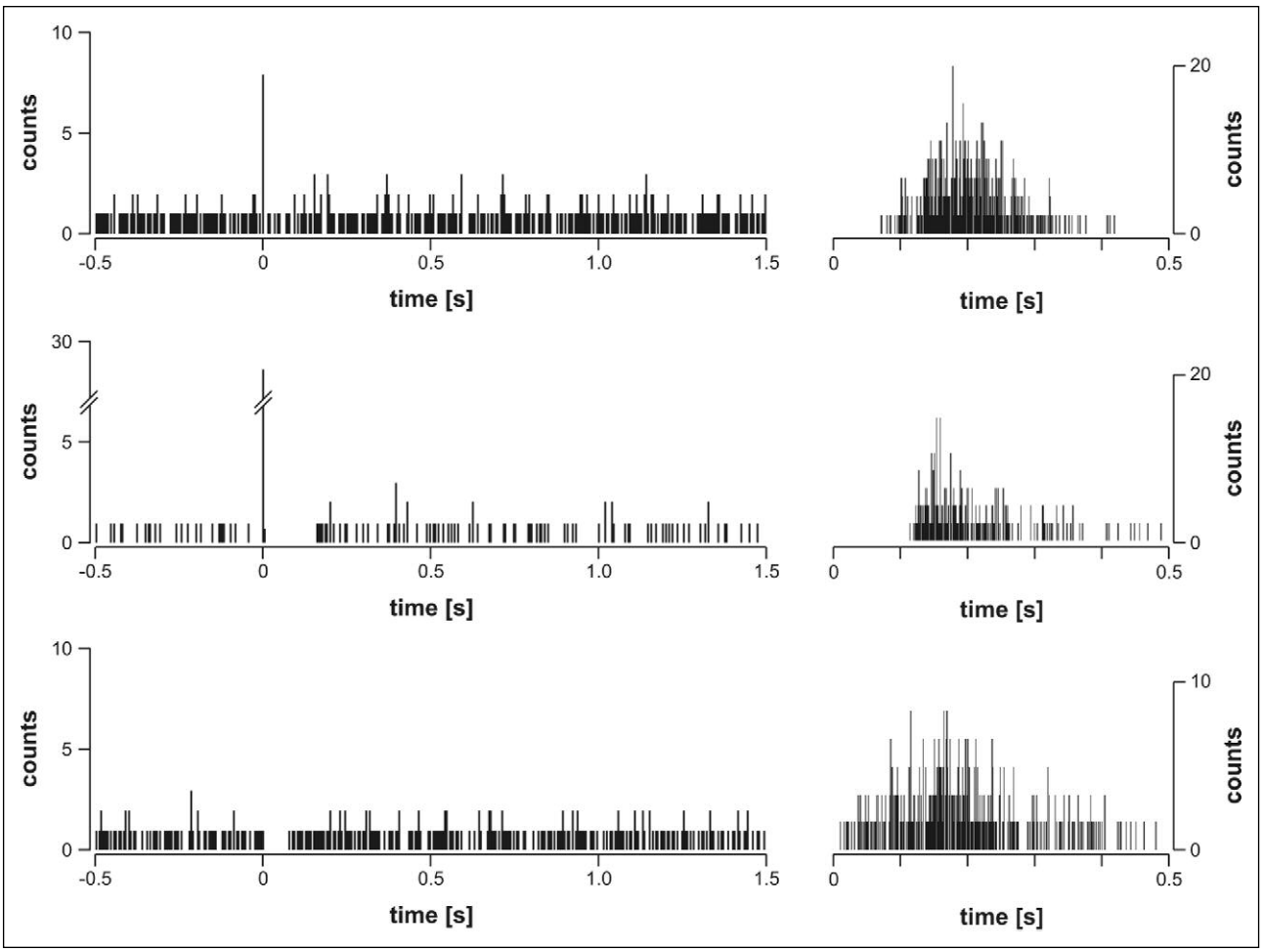

Fig. 3. Peristimulus time histograms (top/middle/bottom left; PSTH - 50 sweeps), showing the different responses of IGL neurons to electrical stimulation of the optic nerve (stimulus at time 0 s).
Top left - example of monophasic excitatory response.
Middle left - example of biphasic response, with initial excitation followed by transient inhibition.
Bottom left - example of monophasic inhibitory response. Interspike interval histograms (ISIH) for these sample neurons are shown at right. Bin size on all graphs = 1 ms.

duration of monophasic excitation was significantly longer than the duration of excitation followed by decaying oscillatory firing (*Fig. 4B*). No statistically significant differences were detected in the number of spikes per response or in the normalized percent change in firing in the different components of each response type observed after optic tract stimulation (*Fig. 4C*).

The effect of the NMDA receptor antagonist (AP-5) on the observed response to optic tract stimulation was tested in 18 intergeniculate leaflet neurons. *Table 1* summarizes the changes in response induced by AP-5. *Fig. 5A* gives an example of the most frequent effect of AP-5: attenuation of IGL neuron response to optic tract stimulation.

The effect of the AMPA/kainate receptor antagonist (CNQX) on the observed response to optic tract stimulation was tested in 12 intergeniculate leaflet neurons. *Table 1* summarizes the changes in the response induced by CNQX. *Figs 5B* and *5C* gives an example of the most frequent effect of CNQX on monophasic excitation and the biphasic response: the disappearance of IGL neuron response to optic tract stimulation.

The effect of the $GABA_A$ receptor antagonist (bicuculline) on the observed response to optic tract stimulation was tested in 8 intergeniculate leaflet neurons. *Table 1* summarizes the changes in the response induced by bicuculline. *Fig. 5D* gives an example of the most frequent effect of bicuculline: the disappearance of IGL neuron inhibitory response to optic tract stimulation.

DISCUSSION

Monosynaptic retinal input to the intergeniculate leaflet of the lateral geniculate body was described

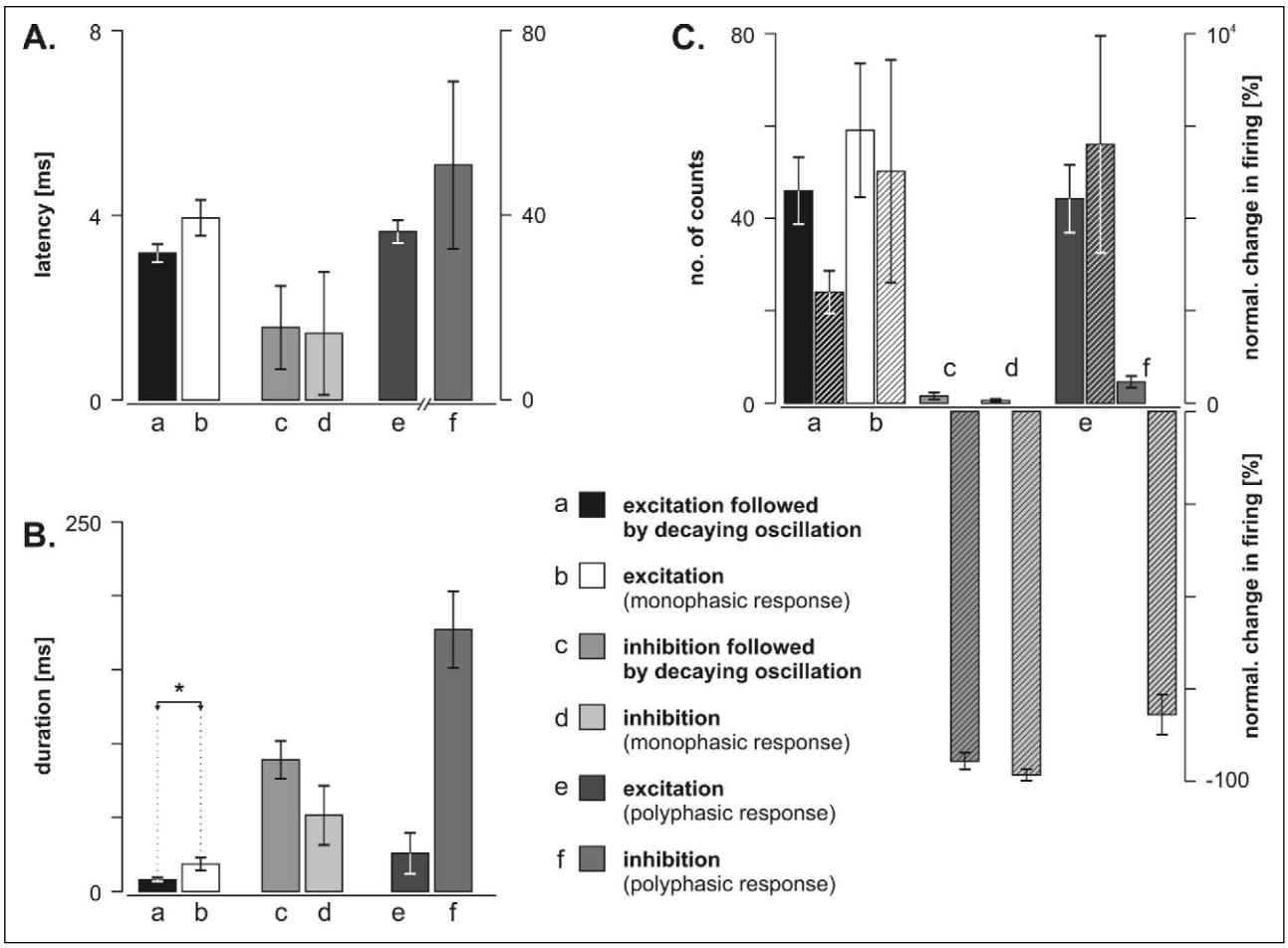

Fig. 4. Bar graphs of the descriptive statistics for the different response types.
A - latencies (± S.E.M.) of the excitatory components of the different response types. No statistically significant differences were found ($p >> 0.05$).
B - duration (± S.E.M.) of the excitatory components of the different response types. The duration of monophasic excitation was significantly longer than the duration of excitation followed by decaying oscillatory firing ($p < 0.05$; asterisked).
C - number of spikes per response and normalized percent change (± S.E.M.) in firing in the different components of each response type. No statistically significant differences were found ($p >> 0.05$). Bars are described in the legend.

over three decades ago by Hickey and Spear (7). Since then, numerous experiments has established the role of the IGL as an element passing photic information to the subcortical visual system, the hypothalamic neuroendocrine cells and the suprachiasmatic nuclei - the main circadian oscillator (5, 6, 19-21). Nevertheless, the neurotransmitters released from the optic tract to the IGL remained unidentified.

The results described in this study characterize the responses of intergeniculate leaflet neurons to electrical stimulation of the OT, and implicate glutamate as a major neurotransmitter released from retinal ganglion cell terminals in the IGL.

The different responses of IGL cells to electrical stimulation of the OT observed in this study correspond to the responses of suprachiasmatic nucleus cells to analogous stimulation protocols described by Jiao and Rusak (22), who assigned terms to the different responses of SCN cells to optic nerve stimulation: activation, suppression, and mixed response. A more detailed classification suits the present results; it additionally subdivides the mixed responses into activation followed by rhythmic oscillation, activation followed by inhibition, and inhibition followed by rhythmic oscillation. These categories resemble those put forward by Shibata *et al*. (23), with the exception of inhibition followed by oscillation, a type of response they did not mention in that paper. It clearly occurs within the SCN, as can be inferred from the figures in Jiao and Rusak (22) (*Fig. 1C* in the original paper). For the responses having an oscillatory component in the SCN and IGL, the short oscillation intervals correlate with a high frequency of spontaneously generated spikes, and the long oscillation intervals correlate with low firing rates. Clearly, the value of the peak-to-peak intervals of

Table 1. Summary of the effects of AP-5, CNQX and bicuculline on the different types of response to optic nerve stimulation. Excitatory responses (monophasic, and excitation followed by oscillatory discharge) are grouped together. Inhibitory responses (monophasic, and inhibition followed by oscillatory discharge) are grouped together. The numbers indicate how many times the particular change was observed. Changes: ↑ - increase, ↓ - decrease, disapp. - disappearance, no change.

Antagonist	Effect	Response type		
		excitation	**inhibition**	**biphasic**
AP - 5	↑	1	2	1
	↓	5	0	1
	disapp.	2	0	0
	no change	4	1	1
CNQX	↑	0	0	0
	↓	2	0	1
	disapp.	4	1	1
	no change	1	2	0
Bicuculline	↑	0	0	1
	↓	0	3	0
	disapp.	0	3	0
	no change	1	0	0

oscillations induced by stimulation of retinal ganglion cell axons depends on the intervals between spontaneously generated action potentials.

The ability to respond to stimulation with regularly spaced action potentials arises most likely from cell membrane properties; apparently these properties do not differ between some populations of SCN and IGL neurons. The oscillatory activity we induced by OT stimulation was characteristic of cells with a regular pattern of spontaneous firing rates. The same was found for SCN neurons (23-25). Probably it is the slow outward conductance occurring after the action potential that is responsible for the spontaneous regular firing of IGL and SCN neurons. In fact it has been shown that the action potentials of regularly firing IGL neurons are characterized by a long-lasting post-hyperpolarization phase (26). Ca^{2+}-independent depolarization-activated K currents are believed to contribute to this phenomenon, since those currents are known to participate in the generation of regular spiking (27). The recorded variation of peak-to-peak interval values between neurons probably arises from differences in the density, distribution and kinetics of ion channels. Differences in cell membrane properties also most likely underlie the differences in the duration of the responses classified as excitatory and as excitatory followed by oscillations. This variation of the responses of IGL cells to OT stimulation reflects the heterogeneity of neuronal morphology and electrophysiology in this nucleus, shown in other studies (4, 26, 28-35).

The general classification of IGL neuronal responses to OT stimulation clearly shows the predominance of excitatory over inhibitory responses (74% and 26%, respectively). The proportion was even more skewed in observations in vivo. The majority of hamster IGL neurons recorded by Ying *et al*. (36) were activated by light, and only 6% of light-sensitive cells were inhibited. Our previous results also show that the shift from dark to light periods provokes high neuronal activity followed by oscillation of IGL neurons *in vivo* (31). Ying *et al*. (36) showed the presence of light-sensitive IGL neurons that were not active spontaneously, which resemble some cells described in this paper. A characteristic feature of those neurons recorded *in vitro* and *in vivo* is the occurrence of substantial, sharp increases in their firing rates after stimulation.

Experiments with selective receptor antagonists allowed us to determine the type of glutamatergic receptors responsible for conveying photic information to the IGL. AP 5 could not completely abolish but rather only attenuated the responses to OT stimulation in the majority of the recorded neurons. This suggested the involvement of other, non-NMDA glutamate receptors, confirmed by the observation that CNQX, a competitive AMPA/kainate glutamate receptor antagonist, was able to block the responses evoked by OT stimulation completely. This means that the recorded responses were mediated mostly by non-NMDA ionotropic glutamate receptors. The results for rat SCN and IGL are not consistent at this point:

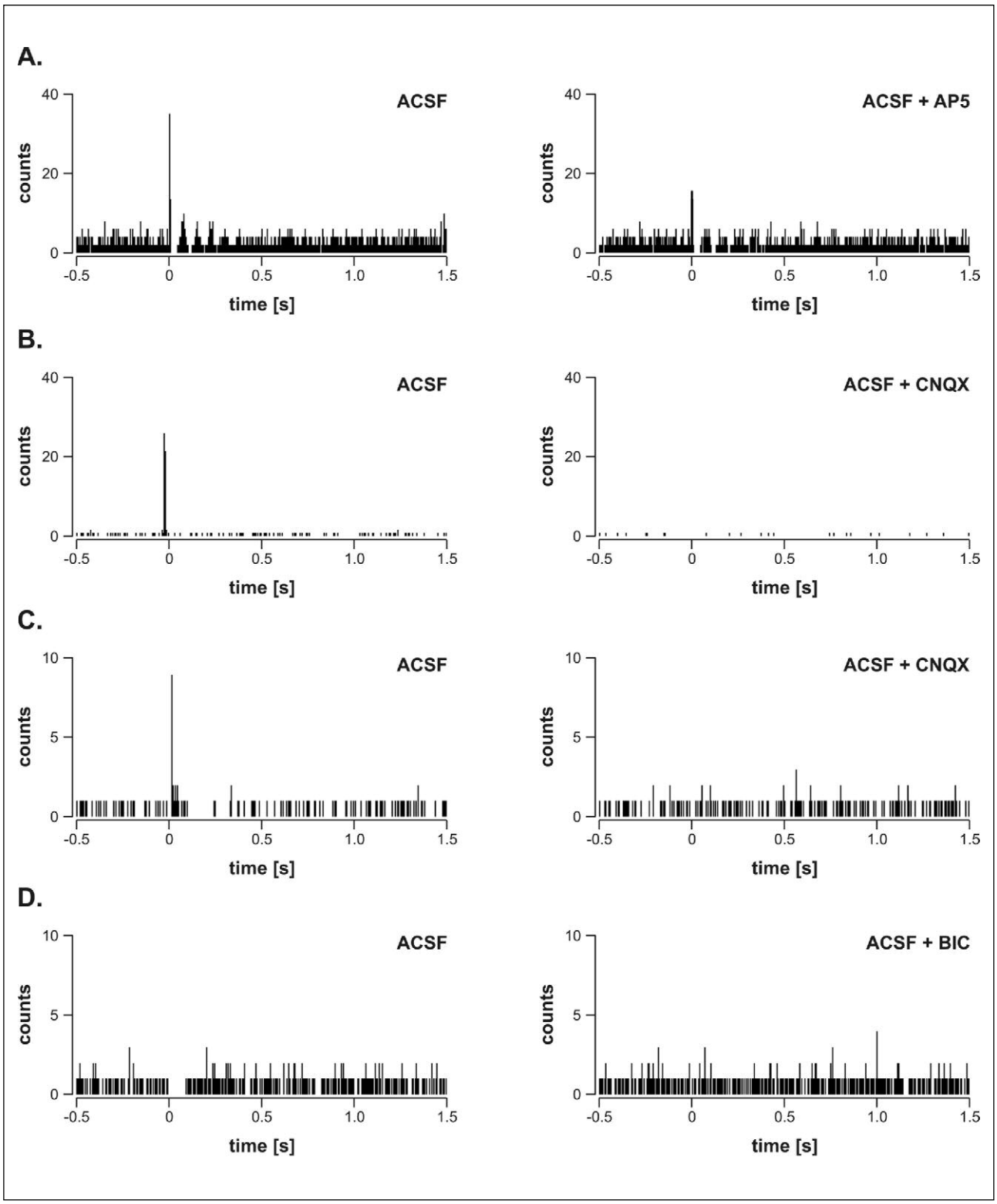

Fig. 5. Peristimulus time histograms (PSTH - 50 sweeps), showing changes in the response of IGL neurons to electrical stimulation after application of different drugs.

A - example of the most frequent effect of AP-5 application. The excitatory response (at left) is attenuated by AP-5 present in the ACSF (at right).

B - example of the most frequent effect of CNQX on monophasic excitation. The excitatory response (at left) is completely blocked by CNQX present in the ACSF (at right).

C - example of the most frequent effect of CNQX on the biphasic response. The response (at left) is completely blocked by CNQX present in the ACSF (at right).

D - example of the most frequent effect of bicuculline (BIC) on the inhibitory response. Inhibition (at left) is completely blocked by BIC present in the ACSF (at right). Bin size on all graphs = 1 ms.

for the SCN, NMDA receptor blockage was found to be enough to inhibit ON stimulation or glutamate-evoked responses (22, 37). In the majority of recorded cases, blockage of NMDA receptors did not completely prevent IGL neurons from responding to OT stimulation. The AP-5 concentration (50 μM) used in this study was potent enough to eliminate NMDA-evoked IGL neuron responses (12). Thus the observed partial reduction of response was rather not the effect of incomplete NMDA blocking.

Although we have shown the dominant role of the AMPA/kainate glutamate receptor, these results are not enough to preclude the involvement of other receptor types in transmission of photic information to the IGL. Further experiments with glutamate and other neurotransmitter receptor antagonists need to be done.

Blockage of the $GABA_A$ receptors with bicuculline influenced all inhibitory effects of OT stimulation. Complete disappearance or substantial attenuation of inhibitory responses in the presence of bicuculline point to the role of $GABA_A$ receptors in photic information transmission and processing within the IGL. The suggestion that GABA is directly released from stimulated ganglion cell endings is supported by the observation that GABA is located in RGC axons (38). Moreover, inhibitory responses of SCN cells to ON stimulation described by Jiao and Rusak (22) have shown their lack of sensitivity to AP-5, an antagonist potent enough to inhibit the great majority of excitatory responses. Those results point to the role of GABA as a neurotransmitter of the optic nerve, as is suggested by the authors. An alternative hypothesis explaining the observed inhibitory effect of OT stimulation is based on the fact that GABA is the most abundant neurotransmitter within the IGL (32, 39). Immunohistochemical data show that GABA is co-stored with other neurotransmitters in nearly all IGL neurons (32). Suppression of the neuronal activity evoked by OT stimulation might be a transsynaptic effect of secondary release of GABA from interneurons within the IGL. Blockage of $GABA_A$ receptors would result in abolition of stimulation-evoked inhibition in the case of direct release of GABA from RGC terminals or excitation of local interneurons. Thus, the observed attenuation of the inhibitory response in the presence of bicuculline does not constitute evidence for either of the two hypotheses.

The present study provides the first direct demonstration that glutamate is neurotransmitter released to the IGL from retinal axonal endings. It also points aut GABA as agent involved in the processing of photic information within this structure.

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