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The Effects of Muscarinic Cholinergic Receptor Antagonist on Slow Bursting Neuronal Activity in the Rat Intergeniculate Leaflet*

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The intergeniculate leaflet (IGL) of the thalamus is an important neuronal element of the mammalian circadian time-keeping system. It receives direct input from retinal ganglion cells. In addition, there are, among other projections, afferents to the IGL from the brainstem cholinergic nuclei. The aim of the present study was to determine the influence of intravenous (i.v.) application of atropine – a muscarinic acetylcholine receptor antagonist, on the ultra-slow isoperiodic oscillations of the IGL neurons. Spontaneous neuronal activity was extracellularly recorded from the rat IGL. Different concentrations of atropine were administrated after recording baseline activity. In all experiments, the period of oscillation became longer after injection of atropine. In some of cases we also noted a temporary disturbance of the oscillatory pattern of neuronal activity. These data suggest that the oscillatory firing of IGL cells can be modulated by cholinergic influence.

Key words: Atropine, intergeniculate leaflet, intravenous, *in vivo*, mAChRs, ultradian oscillations.

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In mammals, the circadian time-keeping system (CTS) is established by two major neuronal structures: the suprachiasmatic nuclei (SCN) of the anterior hypothalamus and the intergeniculate leaflet (IGL), a distinctive subdivision of the lateral geniculate nucleus (LGN) of the thalamus (MOGA & MOORE 1997; MOORE *et al*. 2002). Light is a principal zeitgeber that synchronizes circadian rhythms to the environmental light-dark (LD) cycle. Both mentioned structures receive direct input from retinal ganglion cells. Additionally, the IGL receives non-photic information and is responsible for the integration of this information with photic effects transmitted by the optic tract (WEBER & REA 1997). The main neuronal pathways conveying non-photic information arrive from the origin of non-specific projection localized in the brain stem. Several anatomical, pharmacological and electrophysiological studies confirm the existence of such afferents to the intergeniculate leaflet from locus coeruleus (LC), dorsal raphe nuclei (DRN) (BLASIAK *et al*. 2006; MOORE & CARD 1994) or brainstem cholinergic nuclei – mesopontine laterodorsal tegmental (LDTg) and pedunculpontine tegmental nuclei (PPTg) (BRAUER *et al*. 1984; HOROWITZ *et al*. 2004; LEVEY *et al*. 1987; PEKALA *et al*. 2007).

Considerable data support a role of cholinergic agents as regulators of the circadian system. Electrophysiological studies have shown that SCN neurons are responsive to the iontophoretical application of acetylcholine (ACh), both *in vivo* and *in vitro* (KOW & PFAFF 1984). Also carbachol – an agonist of the cholinergic receptors, has been shown to shift the phase of circadian rhythms in rodents when administered intraventricularly or directly into the suprachiasmatic nucleus (BINA & RUSAK 1996; LIU & GILLETTE 1996). Moreover, nicotine administration induces c-Fos-like immunoreactivity in the SCN in a time- and dose-dependent manner (FERGUSON *et al*. 1999).

Despite the well-studied role of acetylcholine in the circadian system, little is known about the functional significance of the connection between

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brainstem reticular formation and the IGL. Experiments with potentials evoked in the intergeniculate leaflet (by stimulating the contralateral SCN) and inhibition after LDTg stimulation (LEWANDOWSKI & BLASIAK 1999) suggest that the activity of the IGL can be influenced by the cholinergic projection from LDTg. Moreover, recent results obtained by our group indicate the modulatory effect of carbachol on the glutamate-induced activity of the IGL neurons (PEKALA *et al*. 2007).

In previous studies, we have demonstrated the existence of isoperiodic ultradian oscillations in the neuronal firing of the rat IGL (LEWANDOWSKI *et al*. 1999) which are suggested as a basic activity of this structure as well as the whole neuronal mechanism of CTS (LEWANDOWSKI & BLASIAK 2004). However, the cholinergic influence on this rhythmic bursting is unknown.

Therefore, the aim of the present study was to determine whether and how the intravenous injection of atropine – the antagonist of muscarinic cholinergic receptors, affects the rhythmic oscillation in the activity of intergeniculate leaflet neurons.

Material and Methods

The experiments were successfully performed on 10 male Wistar rats weighing 300-400 g, kept on a 12:12 light-dark cycle (light on at 08:00 h), bred in our laboratory. Water and food were available *ad libitum*. All the animal handling and surgical procedures were carried out in accordance with the European Community Council Directive of 24 November 1986 (86/609/EEC) and Polish national law. The animals were anesthetized with intraperitoneal injection of Urethane (1.5 g/kg, dissolved in 2 ml of saline, supplemented later if necessary; SIGMA). The femoral vein was then catheterized for intravenous drug administration and the animals were mounted in a stereotaxic apparatus (ASI). During the experiment, the animal's body temperature was monitored and maintained thermostatically at 37±0.5°C. A sagittal incision was made and the upper surface of the skull was exposed and cleaned. After exposing the skull surface, the incisor bar was adjusted until the levels of lambda and bregma were equal. Craniotomies were performed above the level of the IGL and visual areas of the cortex to permit microelectrode penetration into the IGL and implantation of an EEG recording electrode. EKG and EEG signals were monitored to verify the physiological state of the animals.

The extracellular recording of neuronal activity within the IGL (stereotaxic coordinates: AP: -4.5 mm, LM: 3.9 mm, DV: 4.5-5.0 mm from the bregma) was performed using glass microelectrodes (impedance 5 Mohm) filled with 2 M NaCl and 2% Chicago Sky Blue. An EEG signal from the visual cortex was epidurally recorded with a silver ball electrode. The recording was carried out under constant illumination (300 lux at the animal's eye level). The signal from the electrodes was amplified (10 000× for MUA; 5000× for EEG), filtered (0.3-3 kHz for MUA; 1-200 Hz for EEG) using an Axon Instruments CyberAmp 380, and sampled by Micro 1401 mk II (CED) equipment and software (Spike2) for storage and further analysis.

In each experiment, after achieving baseline activity, Ringer solution (vehicle for atropine) was administrated intravenously. After another 20 minutes of recording, atropine (ATR, Sigma) in a dose of 4 or 8 mg/kg (4% or 8% concentration respectively), diluted in the Ringer solution, was infused intravenously. Only one infusion of the control solution and one of the drug was performed on the same animal.

At the end of the experiment, a current of -5 μA was passed through the recording electrode for 3.5 min in order to mark the exact position of the tip of the electrode.

During the experiment, rate-meter histograms of IGL neuronal activity (1-s bins) were generated. To determine the period of rhythmic bursting observed in the IGL, Fast Fourier Transforms were applied to 1200-s intervals in the rate-meter histograms before vehicle infusion, and directly before and immediately after drug application.

All values were tested for a normal distribution using Shapiro-Wilk's W test (normality was assumed for W significance of $P>0.05$). The changes induced by drug application were analyzed by a one-way repeated-measure ANOVA. Statistical significance was judged at $P<0.05$.

All the descriptive statistical data are given as means ±S.E.M. Statistical calculations including Fast Fourier Transforms, autocorrelation function analysis and generation of the histograms of firing frequency distribution were made using Statistica software (StatSoft).

Results

After recording the baseline of slow bursting neuronal activity in the IGL (at least 20 min), Ringer solution was intravenously injected into the femoral vein. As the administration of the vehicle alone caused no changes in the pattern of neuronal activity ($P=0.995$, $F=0.00004$) (Figs 1, 3), after another 20 min of recording, the muscarinic cholinergic receptor antagonist – atropine, was ap-

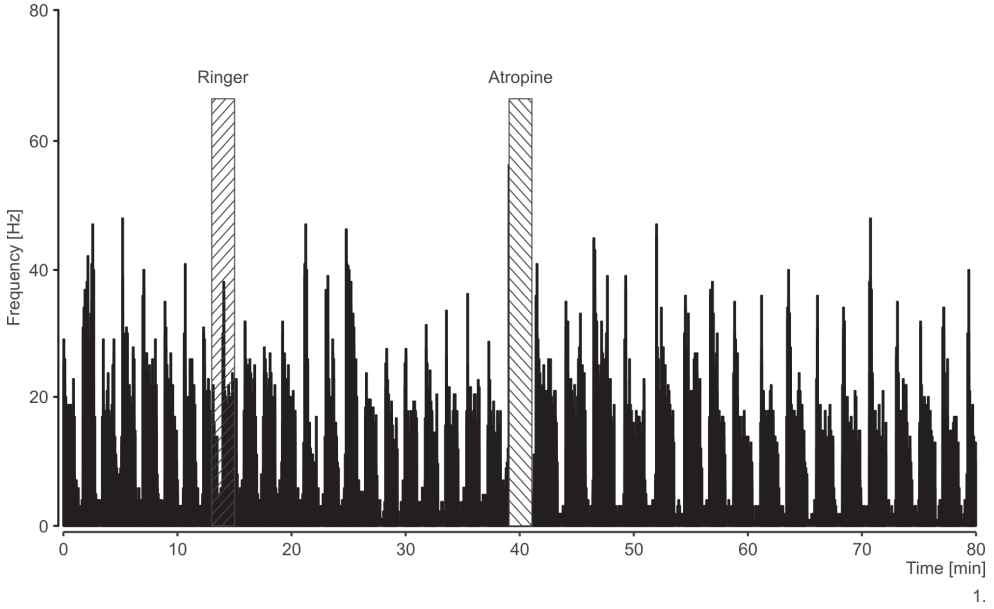

Fig. 1. Firing rate histogram showing rhythmic, slow bursting activity of IGL neurons. Vertical bars indicate the time of i.v. injection of vehicle and atropine. Bin size =1 s.

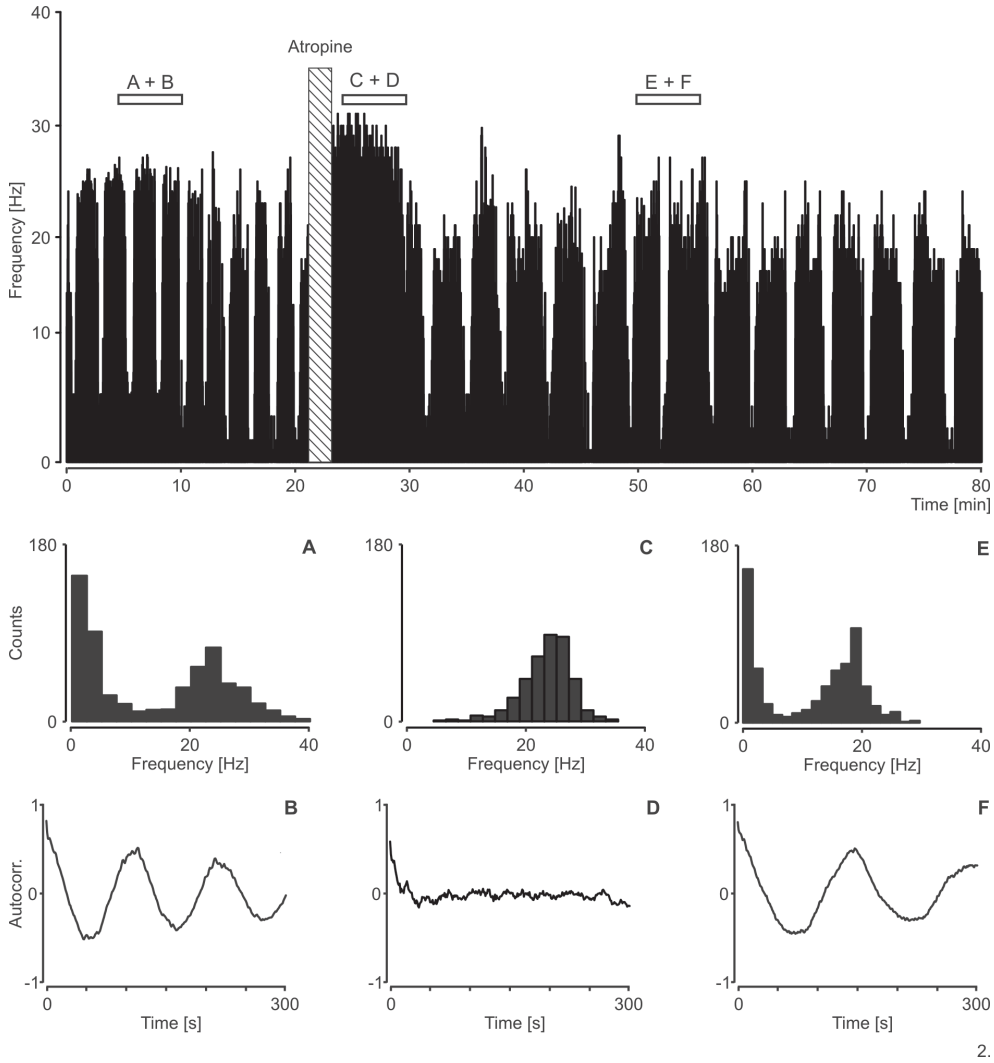

Fig. 2. (Top) Firing rate histogram of the IGL neuronal activity before and after drug application (vertical bar). Bin size = 1 s. Horizontal, hollow bars indicate intervals for which the distribution of firing frequencies and the autocorrelation functions were estimated. (Bottom) A, C, E – histograms showing the distribution of the firing frequencies of IGL neurons before (A) and after (C, E) drug application. Bin size = 2Hz. B, D, F – curves of the autocorrelation function of IGL neuronal activity before (B) and after (D, F) injection of atropine.

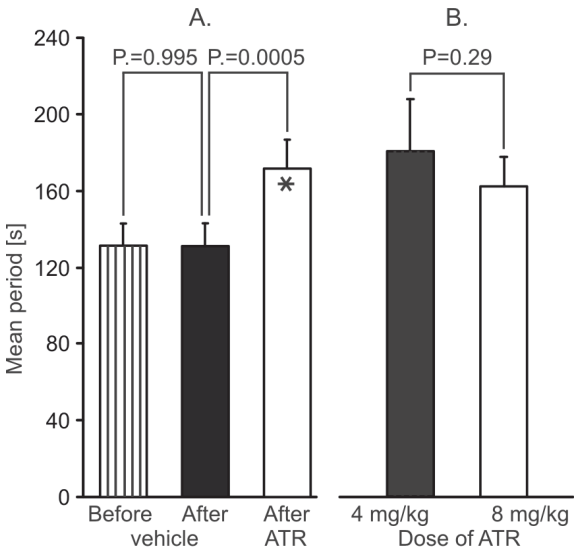

Fig. 3. Bar graph showing the mean periods of neuronal oscillation within the IGL. A – vertically dashed bar indicates the mean period before vehicle injection (baseline); solid bar – the mean period after vehicle injection (control); hollow bar – the mean period after drug injection. B – solid bar indicates the mean period after atropine injection in a dose of 4 mg/kg; hollow bar – the mean period after an injection in a dose of 8 mg/kg. Asterisk shows statistically significant difference. Each bar shows the mean period ± S.E.M.

plied. In all experiments, after atropine injection, the period of observed oscillation became longer (P=0.0005, F=31.27) (Figs 1, 2, 3). In 5 cases, immediately after drug injection, a temporary disappearance (no more than 600 s) of rhythmic slow bursting activity was observed (Fig. 2). During this period, neuronal firing was characterized by a flat curve of the autocorrelation function and by an unimodal distribution of frequencies in the firing rate histogram (Fig. 2C, D). After this transient disturbance a recovery of the slow bursting activity was observed, but the period of neuronal oscillation, as in the remaining cases, was significantly longer than before drug application (Figs 1, 2E, F, 3A). After drug application, recording was carried out in a few cases even up to 5 hours and no recovery to the previous period's length was observed.

The period's broadening did not differ statistically between the two applied doses (P=0.29, F=1.26). However, after injection of ATR in a dose of 8 mg/kg, the increase of the mean period of the observed oscillation was smaller (Fig. 3B).

The injection of atropine had no statistically significant effect on the mean firing rate (P=0.45, F=0.63).

Discussion

In the present study, we have clearly demonstrated that intravenous injection of a general muscarinic receptor antagonist – atropine – alters the neuronal firing of the oscillating cells within the rat IGL. In all investigated cases, the blockade of muscarinic receptors caused an enlargement of recorded ultradian oscillations. In addition, in half of the cases, we observed temporary disturbances of the rhythmic pattern of neuronal activity. Interestingly similar effects on the neuronal oscillations were previously observed after blockade of $GABA_A$ receptors by bicuculline (BLASIAK & LEWANDOWSKI 2004).

A significant limitation of the present study is that general administration of atropine does not determine the precise site of the action of the drug. Therefore, in our discussion we can only suggest the probable locus of atropine effect.

The work by MOORE and SPEH (1993) has demonstrated that virtually all IGL neurons are GABA-ergic and that GABA is colocalized with enkephalin in the population of neurons projecting to the contralateral IGL, and with neuropeptide Y in the population of neurons projecting to the SCN. They showed also that most of the SCN cells are GABA-producing and proposed to consider GABA as the principal neurotransmitter of the circadian system. On the basis of this anatomical study we can consider the action of atropine on GABAergic neurons.

Central mAChRs are most frequently found at presynaptic sites and their activation leads to the modulation of neurotransmitter release. They have been known to regulate GABAergic transmission with diverse effects: acetylcholine, as well as muscarinic receptor agonists, can either enhance or suppress GABA release. There are some data indicating that activation of mAChRs stimulates the release of GABA in rat substantia nigra, globus pallidus or neostriatum (HARSING Jr & ZIGMOND 1998; KAYADJANIAN *et al*. 1994; KAYADJANIAN *et al*. 1997). However, in rat cortex, corpus striatum or midbrain dopaminergic neurons (GRILLNER *et al*. 2000; HASHIMOTO *et al*. 1994; MARCHI *et al*. 1990) it is able to inhibit GABAergic synaptic transmission and GABA release. It is interesting to note that these opposing effects were antagonized by atropine. The nature of the response was dependent, first of all, on the receptor subtypes involved, but there were also essential discrepancies between different brain regions and effective doses of muscarinic drugs required to obtain modulatory effects.

There is much data demonstrating the distribution of mAChRs and showing the action of the cholinergic agents in many regions of CNS. PLUMMER *et al*. (1999) have identified specific muscarinic receptors for acetylcholine in the rat LGN, although they do not distinguish IGL from

the lateral geniculate complex. They have revealed especially dense immunoreactivity in the entire LGN for a m2 receptor subtype known as an autoreceptor or heteroreceptor, regulating the release of neurotransmitters. Additionally, they have demonstrated that neurons immunopositive for the m2 receptor have a somatodendritic morphology of internurons. Because IGL cells involved in the observed oscillations form an interconnected network of GABAergic interneurons (similar to that formed by GABAergic cells in the SCN) (BLASIAK & LEWANDOWSKI 2004), it can be presumed that the m2 receptor may be the predominant muscarinic receptor subtype associated with these inhibitory neurons in the IGL, as in the LGN and the entire thalamus. Furthermore, there are numerous studies describing the suppressed effect of cholinergic modulation on the inhibitory action of interneurons (MCCORMICK & PAPE 1988; MCCORMICK 1992). Therefore, we suggest that the application of atropine, by acting on the m2 receptor subtype, could have decreased the release of GABA and consequently suppressed the action of this neurotransmitter on the IGL bursting cells, thus inducing the longer action of neuronal firing. This kind of atropine action might be therefore consistent with the similar effect of bicuculline on IGL oscillations, suggesting a close relationship between the cholinergic and the GABAergic system within this nucleus.

Acetylcholine acting on muscarinic receptors also modulates other types of neurotransmission, such as dopaminergic or glutaminergic (FORSTER & BLAHA 2003). Activation of the mAChRs located on glutaminergic axon terminals in rat hippocampus can inhibit the release of glutamate (Glu) and this effect is blocked by atropine (MARCHI *et al*. 1989). There is anatomical evidence showing the localization of GluRs mRNAs in the rat IGL (SATO *et al*. 1993). Electrophysiological data from our laboratory (BLASIAK *et al*. 2007; PEKALA *et al*. 2007) has shown responsiveness of the IGL cells to Glu, therefore confirming the existence of Glu innervation of the IGL. For this reason, another possible locus for atropine effects is a blockade of mAChRs located on glutamatergic nerve terminals and excitation of Glu release.

Because of the systemic injection of the drug, we could not exclude affecting the muscarinic receptors of neurons in other brain regions. It is possible that the observed effect is due to the blockade of mAChRs in the structures that simultaneously receive cholinergic innervations and whose neurons project to the IGL. What is even more probable, the observed amplification of the oscillations in extracellular activity of the IGL cells could be a consequence of altered neuronal activity of afferent structures and direct action of atropine on the IGL cells.

In half of the cases, immediately after injection of atropine, we noticed transient disturbances in the rhythmic neuronal activity. This effect was neither dependent on the applied dose of the drug nor on the circadian time of injection. Interestingly, this disappearance of rhythmic slow bursting in IGL activity appears after intracerebroventricular infusion of bicuculline or picrotoxin and also after a shift from dark to light (BLASIAK & LEWANDOWSKI 2004; LEWANDOWSKI *et al*. 2000). An injection of atropine can enhance ACh release by blockading ACh autoreceptors in mesencephalic reticular formation (MRF) and on the axon terminals (BAGHDOYAN *et al*. 1998; MOOR *et al*. 1995; ROTH *et al*. 1996). Therefore, it could increase the action of ACh on nicotinic receptors, as their activation requires higher concentrations of ACh than do muscarinic receptors (LEWANDOWSKI *et al*. 1993). Activation of MRF (due to an atropine injection or a light pulse) has been known to trigger a state of arousal accompanied by desynchronization of EEG. Perhaps this temporary tonic firing mode of IGL neurons is the time required to stabilize the modulatory action of ACh released from MRF cells. The fact that it appears only in 50% of our experiments could be dependent on the physiological state of an animal. However, this assumption should be tested in successive experiments.

The present results suggest that the oscillatory firing of IGL cells can be modulated by cholinergic influences. However, to fully answer the question concerning localization of the site of action of the cholinergic drug and to define which particular receptor subtypes are involved in the observed effect, experiments with local drug application of agents selective for particular ACh receptor subtypes are required.

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