

K. PALUS, L. CHROBOK, M.H. LEWANDOWSKI

## DEPOLARIZATION OF THE INTERGENICULATE LEAFLET NEURONS BY SEROTONIN - *IN VITRO* STUDY

Jagiellonian University, Institute of Zoology, Department of Neurophysiology and Chronobiology, Cracow, Poland

The intergeniculate leaflet of the thalamus (IGL) is a part of the mammalian biological clock which integrates photic and non-photoc information and conveys it to the master biological clock - suprachiasmatic nuclei (SCN). One of the non-photoc cues is delivered by the serotonergic projection from dorsal raphe nucleus. *In vitro* electrophysiological recordings were performed from single IGL neurons using whole-cell patch clamp technique. We investigated the influence of serotonin (serotonin creatinine sulfate complex, 5HT) on 'spontaneous' neuronal activity in this structure. In most of recorded cells, 5-HT caused significant increases in firing rate. In majority of cases the effect was presynaptic. However, in some cases we observed postsynaptic depolarization. To our knowledge, depolarizing influence of 5HT on the single neurons in the IGL has been shown here for the first time.

**Key words:** *biological clock, intergeniculate leaflet, biological clock, non-photoc information, patch clamp, 5-hydroxytryptamine, raphe nucleus, hyperpolarization*

### INTRODUCTION

The intergeniculate leaflet of the thalamus (IGL), is a structurally and functionally important component of the rodent biological clock. In the laboratory rats this small structure is located between the dorsal lateral geniculate (DLG) and ventral lateral geniculate (VLG) parts of the lateral geniculate body (4.4–4.8 mm anterior to bregma, 3.9–4.1 mm lateral to midline and 5.0–5.2 mm ventral to dura) (1). A vast body of physiological and behavioral studies indicate the involvement of the IGL in the regulation and modulation of biological rhythms. The IGL integrate photic information with non-photoc cues and conveys this information to the master biological clock - suprachiasmatic nuclei (SCN) (2–4). The neurons in the IGL are usually identified as members of two major groups: neuropeptide Y (NPY) positive and enkephalinergic (ENK) cells. Both neuropeptides are colocalized with  $\gamma$ -aminobutyric acid (GABA). In rat the NPY-positive neurons are the major component of geniculohypothalamic tract (GHT) projecting to the SCN (3, 5), whereas ENK cells constitute the main source of geniculogeniculate pathway connecting contralaterally located IGLs. This population of neurons express characteristic isoperiodic oscillatory pattern of the activity, observed in rat during *in vivo* recordings (6–8). One of the sources of non-photoc information arriving to the biological clock is serotonergic projection from the raphe nuclei, which constitutes the primary source of serotonin (5-hydroxytryptamine, 5HT) in the brain (9). The SCN receives numerically strongest input from medial raphe nucleus (MRN), whereas the IGL is mostly innervated by the neurons in the dorsal part of the raphe nucleus (DRN). The DRN is involved in the control of sleep-wake cycle and may be responsible for relaying non-photoc information

associated with arousal induced by *i.e.* the locomotor activity and altered waking (10–12).

*In vivo* experiments performed in our laboratory demonstrated that electrical stimulation of the DRN decreases firing rate of isoperiodic oscillatory neurons in the rat IGL, while lesion of this structure disinhibits their activity (13, 14). Increased 5HT secretion into IGL was also observed after electrical stimulation of the DR in another murid rodent - the hamster (15). Also, the inhibitory influence of 5HT on spontaneous and light-evoked activity of the hamster IGL neurons has been reported previously (16). These studies suggest that serotonin may significantly contribute to regulation of the IGL activity. The aim of the present study was to determine the effect of 5HT action on the single IGL neurons. It appears that 5HT has the diverse effect on the IGL neurons - either presynaptic or postsynaptic effects were observed.

### MATERIALS AND METHODS

#### *Preparation*

All experiments were performed in accordance with the European Community Council Directive of 24 November 1986 (86/0609/EEC) and Polish Animal Welfare Act of 23 May 2012 (82/2012). They were approved by Local (Cracow) Ethical Commission. Animals were held in 12h/12h light/dark condition (light on 8.00 am; light off 8.00 pm) with water and food *ad libitum* in Jagiellonian University Animal Facility. Every effort was made to minimize the number and suffering of animals used. Experiments were performed on 20 young male Wistar rats which were kept in 12h/12h light/dark regime for 14 to 21 days.

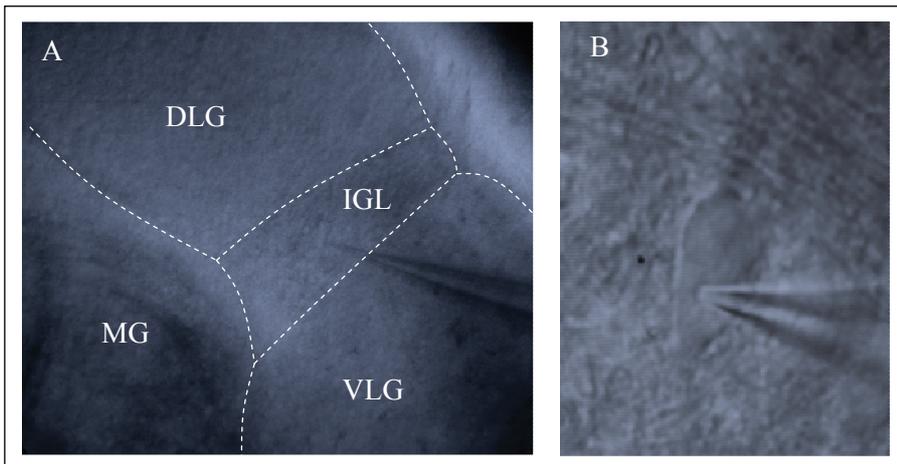


Fig. 1. (A) Intergeniculate leaflet of the thalamus (IGL) localization on the brain slice (10 $\times$  magnification). DLG, dorsal lateral geniculate nucleus; VLG, ventral lateral geniculate nucleus; MG, medial geniculate nucleus. (B) IGL neuron during the patch clamp recording (60 $\times$  magnification).

Animals were anesthetized with izofluran (2 ml/kg body weight, Baxter) and decapitated (between 9.00 and 10.00 am). Brain was rapidly removed from the skull and immersed in ice-cold artificial cerebrospinal fluid (ACSF), composed of (in mM): NaCl 118, NaHCO<sub>3</sub> 25, KCl 3, NaH<sub>2</sub>PO<sub>4</sub> 1.2, CaCl<sub>2</sub> 2, MgCl<sub>2</sub> 2, glucose 10 (pH 7.4; osmolarity  $\sim$ 295 mOsmol/l). A block of tissue containing thalamus was sectioned at 250  $\mu$ m thick coronal slices on Leica vibrotome (VT1000S). Slices containing the IGL were placed in pre-incubation chamber for 30 min at 32 $^{\circ}$ C and then another 60 min in room temperature (21 $^{\circ}$ C). Subsequently, the slice with investigated structure was transferred to the recording chamber and perfused continuously with oxygenated ACSF (95% O<sub>2</sub>, 5% CO<sub>2</sub>), kept at 32 $^{\circ}$ C.

#### Electrophysiological recordings and data analysis

Whole-cell patch-clamp recordings were performed on the IGL neurons (with no preference of the localization of the neurons) between 4 and 10 Zeitgeber time (ZT) and no ZT-dependent changes in the responses were observed. The recording electrode were placed in the IGL region under visual, microscopic control (Fig. 1). For the recordings borosilicate glass pipettes (Sutter Instruments, Novato, USA) were used. The pipettes (resistance 5–7 M $\Omega$ ) were filled with internal pipette solution containing in mM: potassium gluconate 125, KCl 20, HEPES 10, MgCl<sub>2</sub> 2, Na<sub>2</sub>ATP 4, Na<sub>3</sub>GTP 0.4, EGTA 1 (pH=7.4 adjusted with 5 M KOH; osmolarity  $\sim$ 300 mOsmol/l). The IGL neurons were visualized with 60 $\times$  objective on Zeiss Examiner microscope fitted with infrared differential interference contrast (Goettingen, Germany). Whole-cell recordings in current clamp mode (CC) were performed using a SC 05LX (NPI, Tamm, Germany) amplifier and Spike 2 software (CED). Neurons were held at 0 current in CC to record their spontaneous activity. During each recording, current rectangular pulses (1 s, 80 pA) were injected to monitor membrane resistance. Neurons with the membrane potential more positive than  $-35$  mV were excluded from analysis ( $-50$  mV when adjusted for the junction potential).

The signal was low-passed filtered at 5 kHz and digitized at 15 kHz. Data was recorded using the following software: Signal and Spike2 (CED).

#### Reagents

Stock solutions of serotonin creatinine sulfate (5HT, Sigma-Aldrich, St Louis, MO, USA) and tetrodotoxin (TTX, Tocris, Bristol, UK) were prepared in distilled water (100 $\times$  concentrated) and kept in  $-20^{\circ}$ C. Working solutions (5HT, 5  $\mu$ M and TTX, 0.5  $\mu$ M) were prepared in ACSF on the day of experiment. All drugs

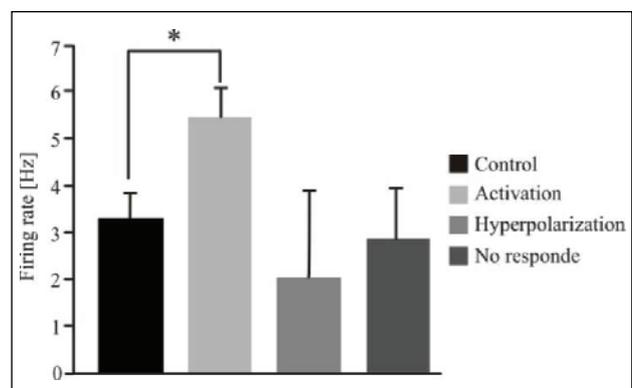


Fig. 2. Changes in mean firing rate before and after serotonin creatinine sulfate (5HT) application on the intergeniculate leaflet of the thalamus (IGL) neurons. Different responses were observed after drug application: activation (n=12, Student's paired t-test, \*  $p < 0.05$ ), inhibition (n=2) and lack of response (n=4).

were delivered by bath perfusion and  $\sim$ 200 s was needed for the substance to reach the recording chamber.

#### Statistical analysis

Analysis was performed in MATLAB (MathWorks, Inc., USA) and Statistica 10.0 (StatSoft, Inc. USA). Changes in firing rate or membrane potential were considered to be significant if they were different from baseline by more than three S.D. All data was expressed as mean value  $\pm$  S.E.M. Student's t-test was used and  $p < 0.05$  was considered as significant.

## RESULTS

In total 28 neurons were investigated in current clamp mode. Spontaneous activity of 18 IGL neurons was recorded in normal ACSF. Majority of tested cells (n=17) expressed regular pattern of activity with mean firing rate of  $3.86 \text{ Hz} \pm 0.1 \text{ Hz}$ , only one neuron was silent. On average the firing rate changed from  $3.31 \text{ Hz} \pm 0.07 \text{ Hz}$  to  $4.52 \text{ Hz} \pm 0.13 \text{ Hz}$  (n=18) after 5HT application, when all tested cells were included. However, not all of the recorded neurons were sensitive to the applied substance. The activity of 4 out of 18 cells did not change after drug administration (before  $2.87 \text{ Hz} \pm 0.17 \text{ Hz}$  and after  $2.87 \text{ Hz} \pm 0.12 \text{ Hz}$ ). Among neurons sensitive to 5HT, the firing rate of 12 increased (from  $3.39 \text{ Hz} \pm 0.08 \text{ Hz}$  to  $5.48 \text{ Hz} \pm 0.21 \text{ Hz}$ ,

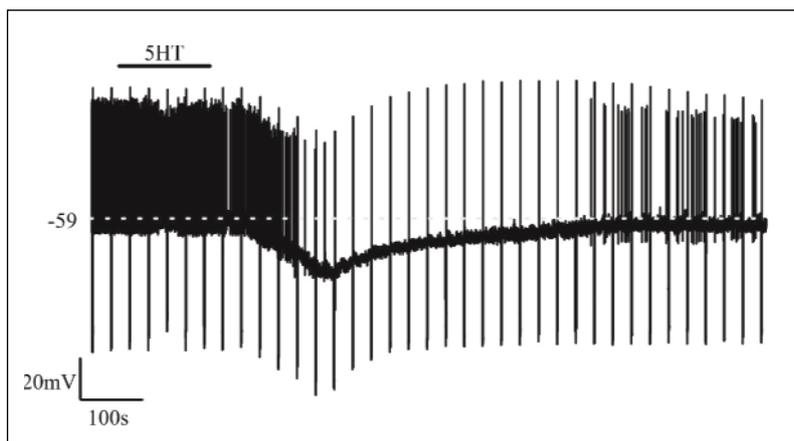


Fig. 3. Representative hyperpolarizing effect of serotonin creatinine sulfate (5HT) (5  $\mu$ M, solid horizontal bar) on intergeniculate leaflet of the thalamus (IGL) neuron. 5HT application caused significant hyperpolarization of the recorded neuron. Downwards deflections represent the responses of IGL cell to a hyperpolarizing current injection (80 pA).

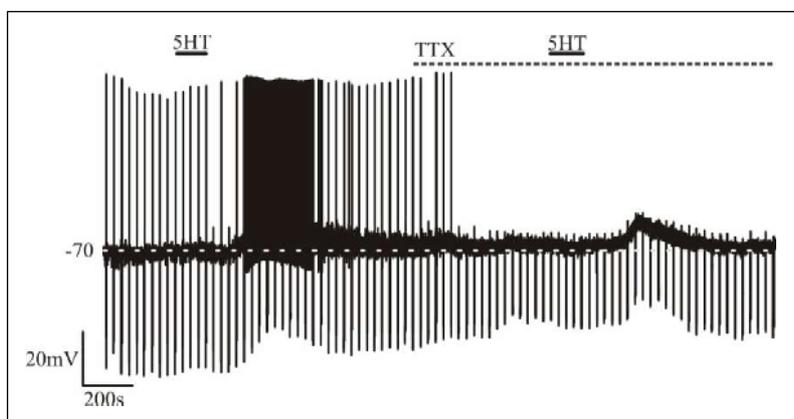


Fig. 4. Representative depolarizing effect of serotonin creatinine sulfate (5HT) (5  $\mu$ M, solid horizontal bar) on intergeniculate leaflet of the thalamus (IGL) neuron. First 5HT application in normal artificial cerebrospinal fluid (ACSF) caused increase in the spontaneous firing rate of recorded neuron. Second 5HT (solid, horizontal bar) application was performed in ACSF containing 0.5  $\mu$ M tetrodotoxin (TTX) (dashed, horizontal bar) and significant depolarization was observed after 5HT application. Downwards deflections represent the responses of IGL cell to a hyperpolarizing current injection (80 pA).

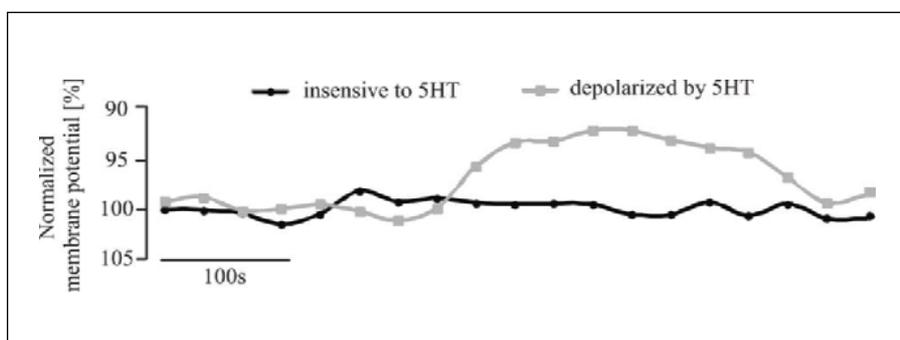


Fig. 5. Mean changes in membrane potential (normalized) before and after serotonin creatinine sulfate (5HT) application (5  $\mu$ M, solid horizontal bar) in artificial cerebrospinal fluid (ACSF) containing tetrodotoxin (TTX). Each data point represents the % of the mean baseline value. Black line - mean membrane potential of insensitive neurons to 5HT (n=9), grey line - mean membrane potential of neurons depolarized by 5HT (n=7).

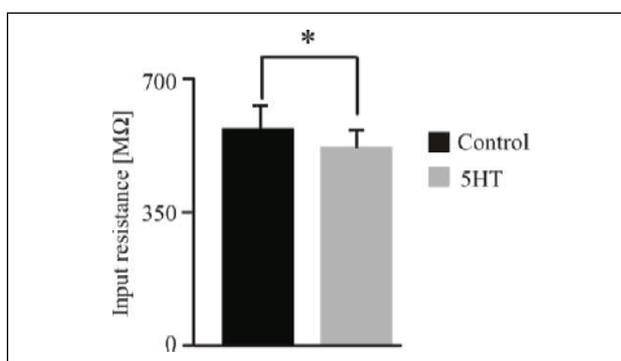


Fig. 6. Decrease in input resistance after application of serotonin creatinine sulfate (5HT) in the presence of tetrodotoxin (TTX), n=8, Student's paired t-test, \*  $p < 0.05$ .

$T=2.612$ ,  $p=0.02$ , paired Student's t-test), and 2 decreased (from 3.39 Hz  $\pm$  0.32 Hz to 2.03 Hz  $\pm$  0.14 Hz) in response to 5HT, respectively (Figs. 2-4). It should be mentioned that large discrepancy between hyperpolarizing effects was observed.

Results of previous *in vivo* experiments (13-16) suggest that 5HT may influence activity of IGL neurons by direct postsynaptic action. Therefore, in order to evaluate if the observed effect of 5HT is pre- or postsynaptic, in 7 neurons (out of 18) after recording the response to 5HT in normal ACSF, TTX (0.5  $\mu$ M) was added to recording solution. In these conditions the membrane potential of 4 neurons did not change after repeated application of 5HT ( $-55.46$  mV  $\pm$  0.23 mV versus  $-54.80$  mV  $\pm$  0.26 mV). Three cells were depolarized by 5HT in the presence of TTX (from  $-59.03$  mV  $\pm$  0.30 mV to  $-55.89$  mV  $\pm$  0.69 mV, Fig. 4). The results indicate that serotonin mainly acts by influencing synaptic activity but direct action on postsynaptic receptors cannot be ruled out.

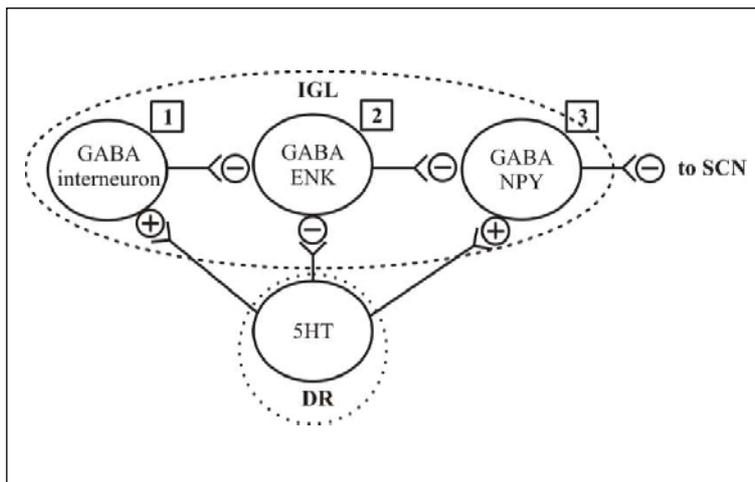


Fig. 7. Hypothetic mechanism of serotonin creatinine sulfate (5HT) action in the intergeniculate leaflet (IGL) neuronal network. [1] Postsynaptic depolarizing effect of 5HT on "GABAergic interneurons", which exert inhibitory effect on enkephalin (ENK) + neurons. In addition, 5HT may directly hyperpolarize ENK + cells. [2] Either postsynaptic or presynaptic inhibition (*via* GABAergic interneuron) can be observed while recordings from "GABA ENK" cells after 5HT application. [3] Direct postsynaptic effect of 5HT on "GABA neuropeptide Y (NPY)" neurons, (imitating direct 5HT effect from dorsal raphe - DR *in vivo*) or indirect depolarization, as a result of diminished inhibitory influence from ENK + neurons. The consequence of these interactions is increase in neuropeptide Y (NPY) release to the suprachiasmatic nuclei (SCN).

Table 1. All 5HT effects described by our study. For detailed information - see RESULTS.

Administration	5HT in normal ACSF	5HT in TTX after 5HT in normal ACSF (taken from previous group)	5HT in TTX only
Excitation	67% (+2.09 Hz, n=12)	43% (+3.14 mV, n=3)	40% (+3.02 mV, n=4)
Inhibition	11% (-1.36 Hz, n = 2)	0% (n=0)	10% (-4.87 mV, n=1)
No effect	22% (+0.001Hz, n = 4)	57% (+0.66 mV, n = 4)	50% (-0.34 mV, n=5)
Total	n=18 (+1.21 Hz)	n=7 (+1.60 mV)	n=10 (+0.73 mV)

Serotonin creatinine sulfate complex is known to be difficult to rinse out from the brain slices after application (17). Therefore, we have studied the effect of 5HT on activity of 10 IGL neurons in the presence of TTX in ACSF from the beginning of recording. By using this protocol, we administered 5HT once only per each tested neuron, and thereby eliminated desensitization, being also possible, observed in previous studies (18) and during our recordings (n=3, data not shown). On average, membrane potential changed from  $-58.97 \text{ mV} \pm 0.15 \text{ mV}$  to  $-58.24 \text{ mV} \pm 0.37 \text{ mV}$  (n=10) after 5HT application. However, in this group of neurons, four were depolarized ( $-61.56 \text{ mV} \pm 0.19 \text{ mV}$  to  $-58.54 \text{ mV} \pm 0.37 \text{ mV}$ ) and one was hyperpolarized (from  $-61.85 \text{ mV}$  to  $-66.71 \text{ mV}$ ) by 5HT. The remaining five neurons were insensitive to applied drug ( $-54.46 \text{ mV} \pm 0.23 \text{ mV}$  versus  $-54.80 \text{ mV} \pm 0.26 \text{ mV}$ ). The data representing all depolarized and non-sensitive to 5HT cells investigated in ACSF containing TTX were collected together in the Fig. 5. We also observed decrease in input resistance during the responses either depolarization and hyperpolarization (this data was pulled together) after 5HT application (from  $570 \text{ M}\Omega \pm 68 \text{ M}\Omega$  to  $530 \text{ M}\Omega \pm 61 \text{ M}\Omega$ ,  $T=2.86$ ,  $p=0.02$ , paired Student's t-test, Fig. 6). All the results are summarized in Table 1.

## DISCUSSION

In the present study, we have demonstrated diverse effects of 5HT on the activity of single IGL neurons. We observed both presynaptic and postsynaptic effects accompanied by the decrease in input resistance. Previous studies performed in our laboratory showed inhibitory effect of serotonergic projection from the DR on the IGL activity (13, 14). Moreover, in the majority of cells application of 5HT or 8-OH-DPAT into the IGL caused a significant decrease of firing rate (16). Interestingly, in the present study only three neurons were hyperpolarized after 5HT

treatment. The discrepancies between previous and present results could be related to different techniques used in both studies. Thus unlike *in vivo* recordings performed in the earlier studies, present experiments were focused on the activity of single IGL neurons in the isolated brain slices, where the connections between the IGL and other structures were limited. Another possible explanation may arise from the fact that authors of previous articles focused mainly (16) or exclusively (13, 14) on examination of only certain group of the IGL neurons, sensitive to the light (13, 14, 16) and/or exhibiting characteristic oscillatory pattern of activity (13, 14).

Serotonin creatinine sulfate complex, a nonspecific 5HT receptors agonist, used in our studies may exert its effect through interaction with various receptor subtypes (19). It has been shown that  $5HT_{1A}$ ,  $5HT_7$  and  $5HT_{5A}$  may be responsible for serotonin action in the hamster IGL (16, 20, 21). Activation of these receptors may contribute to different electrophysiological responses. For example,  $5HT_{1A}$  evokes hyperpolarization and/or inhibitory effect on spontaneous activity of cells in several brain areas, including the IGL (16, 22). The  $5HT_7$  mediates depolarizing-excitatory responses in anterodorsal thalamus (23), modulate glutamatergic synaptic transmission in the rat frontal cortex (24) and GABAergic transmission in rat hippocampal CA1 area (25). The modulation of cell excitability by  $5HT_{5A}$  is the least explored. However, recently (26) it was demonstrated that  $5HT_{5A}$  elicits inwardly rectifying  $K^+$  current in subpopulation of layer V of pyramidal neurons. The same current inhibited by cAMP was observed in mPFC pyramidal neurons (27). Consistent with our findings others (28) demonstrated multiple action of 5HT in the SCN neurons. They have shown not only postsynaptic and presynaptic inhibition but also postsynaptic depolarization after serotonin application (28). This diversity of responses were explained by different 5HT receptor types attendance in this brain structure. Ying and Rusak (16) suggested that in addition to  $5HT_{1A}$ , other receptor type was probably involved in response to

5HT in the IGL. It should be also noticed, that our data are consistent with hypothesis proposing a network of different 5HT-responding cell types in the IGL (29). Clearly, further research is needed to elucidate the possible involvement of different 5HT receptors expression depending on different cell type in the neuronal network of the IGL.

The IGL is the second after the SCN most important component of the biological clock. Its main function is the integration of photic and non-photoc information, and further rely it to the SCN through NPY-containing pathway (30). The NPY injection or synaptic release of this neuropeptide to the SCN at the end of the subjective night, causes the active phase advance (31). Similar effect (29) could be induced by increased locomotor activity (wheel-running).

That is why, the activation of the DR by the locomotor activity, plays an important role in arousal-associated information input to the biological clock (32). It has been shown that phase-shifting effect of the locomotor activity requires projection from the DR to the IGL (33). It could be hypothesized here, that serotonin originating from the DR, directly or indirectly activates NPY-positive neurons in the IGL.

The second population of IGL neurons, connecting contralateral IGLs, are ENK-positive. It was shown (34), that in both: NPY-positive and ENK-positive neurons, the main inhibiting brain neurotransmitter - GABA is co-expressed. What is more, it has been studied that enkephalinergic neurons of the IGL in rats are light sensitive (35) and generate light-dependent oscillations (6-8, 36). Therefore, modulatory effect of the ENK neurons on the NPY neurons was proposed (14). The previous hypothesis that 5HT can inhibit the ENK neurons and disinhibits the NPY neurons is able to explain the observed network mechanism of the secretion of NPY to the SCN (29) (Fig. 7).

The data presented in the present paper can improve our understanding of the role of the reciprocal connections and the role of 5HT in modulation the IGL neuronal network. We have shown that presynaptic depolarizing effect which could be exerted by abolishing inhibitory inputs to the recorded neurons. The increase in NPY secretion to the SCN after 5HT treatment may be related to the observed postsynaptic depolarization of presumed NPY-positive neurons. However, the direct connection between DR the NPY-positive cells is not proven or for that matter disproven by our data. The further electrophysiological studies combined with immunohistochemical stainings are necessary to evaluate our statements.

In conclusion, the present study shows the effects (including depolarizing effect, shown for the first time) of the serotonin-creatinine complex on single IGL neurons. Moreover, our results provide a basis for the new level of understanding of neuronal network in the investigated structure. We believe that our findings are likely to provide the cornerstone for untangling arousal effect of 5HT on mammalian biological clock.

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Author's address: Prof. Marian H. Lewandowski Jagiellonian University, Institute of Zoology, Department of Neurophysiology and Chronobiology, 9 Gronostajowa Street, 30-387 Cracow, Poland.  
E-mail: marian.lewandowski@uj.edu.pl