PRACA ORYGINALNA

Characteristic of voltage-dependent changes generating the bursting activity of the intergeniculate leaflet neurons – – patch clamp *in vitro* study

Charakterystyka napięciowo-zależnych zmian zaangażowanych w generowanie aktywności erupcyjnej neuronów listka ciała kolankowatego bocznego – badania *in vitro* patch clamp

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ABSTRACT

INTRODUCTION

The intergeniculate leaflet of the thalamus (IGL) is one of the two major neuronal structures of the mammalian biological clock. IGL neurons in rats, investigated *in vivo*, show a characteristic isoperiodic infra-slow oscillatory pattern (ISO) of activity. The mean period of this activity is about 120 s. The function of oscillatory activity, commonly observed in other biological clock structures, could be connected with the secretion of neuropeptides. Molecular study of the bases of bursting activity evoked *in vitro*, can help to better understand the mechanism of these oscillations. It was shown that the key element in this phenomenon is the expression of HCN family nonselective cationic channels and voltage-dependent T-type calcium channels. The generated h-current (the base of voltage sag) and t-current (causing low threshold spikes; LTS) are the topic of many studies revealing the mechanism of rhythmic neuronal activity.

MATERIALS AND METHODS

Based on our results of a *patch clamp* study, we have proposed the division of IGL cells into groups (using cluster analysis) based on different amplitudes of voltage components evoked by the h-current and t-current. The location of the investigated neurons chosen for further analysis was confirmed by immunohistochemical staining and confocal microscopy.

RESULTS AND CONCLUSIONS

The IGL neurons were classified into four groups showing different amplitudes of voltage sag and LTS. This classification obtained during experiments conducted at *in vitro* conditions, may provide information about the oscillatory nature of the neuron observed in the *in vivo* study.

KEY WORDS

intergeniculate leaflet, bursting activity, ISO, h-current, t-current, patch clamp

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STRESZCZENIE

WSTĘP

Listek ciała kolankowatego bocznego wzgórza (*intergeniculate leaflet* – IGL) jest jedną z dwóch głównych neuronalnych struktur zegara biologicznego ssaków. U szczura, w badaniach *in vivo*, neurony IGL wykazują charakterystyczny izoperiodyczny wzór infrawolnych oscylacji (*infra-slow oscillation* – ISO) generowania potencjałów czynnościowych, o okresie około 120 s. Przypuszcza się, że taka aktywność neuronalna, będąca cechą wspólną struktur zegara biologicznego, może ułatwiać sekrecję neuropeptydów. Badania molekularnych podstaw aktywności erupcyjnej, która może być wywołana w warunkach *in vitro,* przybliżają nas do poznania mechanizmu powstawania tych oscylacji. Wykazano, że kluczowym elementem, w generowaniu tego rodzaju oscylacji, jest obecność nieselektywnych kanałów kationowych z rodziny HCN oraz napięciowozależnych kanałów wapniowych typu-T. Przepływający przez nie prąd-h (powodujący *sag* napięciowy) oraz prąd-t (manifestujący się występowaniem niskoprogowych potencjałów wapniowych (*low threshold spike* – LTS) to obiekty wielu badań, dotyczących rytmicznej aktywności komórek nerwowych.

MATERIAŁY I METODY

Opierajac się na wynikach własnych badań, metodą elektrofizjologicznej rejestracji aktywności pojedynczych neuronów – *patch clamp* – autorzy zaproponowali podział neuronów IGL (na podstawie analizy klastrów), w zależności od amplitudy zmian napięcia, wywołanych prądem-h i prądem-t. Lokalizacja analizowanych neuronów została potwierdzona dzięki barwieniom immunohistochemicznym i mikroskopii konfokalnej.

WYNIKI I WNIOSKI

Otrzymano cztery grupy neuronów IGL, różniących się amplitudą *sagu* napięciowego oraz LTS. Przeprowadzony podczas badań *in vitro* podział może dostarczyć informacji na temat zdolności komórek do generowania aktywności oscylacyjnej w warunkach *in vivo*.

SŁOWA KLUCZOWE

listek ciała kolankowatego bocznego, aktywność erupcyjna, ISO, prąd-h, prąd-t, *patch clamp*

INTRODUCTION

The intergeniculate leaflet of the thalamus (IGL) is an important neuronal structure of the mammalian biological clock, intercalated between the dorsal and the ventral part of the lateral geniculate nucleus [1]. It is known that this small structure is a homologue of the pregeniculate nucleus of primates [2]. The IGL is one of the two most important components of the biological clock, staying in reciprocal relation to the suprachiasmatic nuclei (SCN) [3] – the master generator of the biological clock. The main function of IGL is to integrate photic information derived from the retina with nonphotic information from nonspecific brain systems [for review see: 4]. Histologically and functionally, the IGL is composed of two distinct neuronal populations of γ-aminobutaric acid (GABA)ergic cells, characterised by the coexpression of different neuropeptides such as neuropeptide Y (NPY) and enkephaline (ENK) [1]. The NPY positive cells reach the SCN via the geniculohypothalamic tract [3,5], whereas the neurons producing ENK connect bilateral IGLs forming the geniculogeniculate pathway [6]. A very interesting feature of IGL cells, which was shown *in vivo*, is their light-dependent isoperiodic infra-slow oscillatory activity (ISO) [7,8,9]. It has been recently published by our group that ISO is exclusively generated by ENK cells [10]. The function of ISO is not exactly known, although high frequency periods in the firing pattern during bursting activity are essential for neuropeptide secretion. Moreover, oscillatory activity is widely observed among many biological clock structures as the olivary pretectum nuclei (OPN) or the SCN [11,12]. The *in vitro* extracellular electrophysiological studies, performed in our laboratory, indicated that 10% of IGL neurons exhibit bursting activity with the high frequency periods lasting for several to a dozen seconds [13].

The molecular bases of the bursting neuronal activity in single neurons and neuronal networks assume the cooperation of two currents flowing through the neuronal cellular membrane [for review see: 14]. The first one is h-current (I_h) generated by hyperpolarisation-activated cyclic nucleotides-gated (HCN) channels. The dense expression of HCN3 was previously described in IGL [15]. The second component of bursting activity is called t-current (I_t), as it is generated by

voltage-dependent T-type calcium channels. I_t is the cause of low threshold spikes (LTS) robustly expressed in many thalamic nuclei [16].
The results of our patch clamp *in vitro* study showed a variety of I_h and I_t generation by IGL cells exhibited by different amplitudes of voltage sag (caused by the current via HCN channels) and LTS. Therefore, we can hypothesise about the oscillatory or non-oscillatory character of the neuron activity observed in the *in vivo* study.

MATERIALS AND METHODS

Animals and tissue preparation

The experiments were approved by the Local Ethics Committee (Jagiellonian University, Krakow) and 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). All possible effort was made to minimise the animals' suffering and number. The experiments were conducted with the use of male Wistar rats (14–21 days old). The animals were housed and bred in a colony at the Institute of Zoology Animal Facility in standard 12/12 light/dark conditions (lights-on at 8 a.m.) with food *ad libitum*. Before sacrifice (always between 1 and 2 ZT), the animals were anesthetized with izofluran (2 ml/kg body weight, Baxter) and subsequently decapitated. The entire brain was quickly removed from the skull and immersed in ice-cold artificial cerebrospinal fluid (ACSF), composed of (in mM): NaCl 118, $NaHCO_3$ 25, KCl 3, NaH_2PO_4 1.2, $CaCl_2$ 2, $MgCl_2$ 2, glucose 10 (pH 7.4; osmolarity ~ 295 mOsmol/l). Acute coronal slices (250 μm thick) were cut on a Leica vibrotome (VT1000S). Slices containing the IGL were placed in a pre-incubation chamber for 30 min at 32°C and then for another 60 min at room temperature (21°C). Before each recording, the slice with the investigated structure was transferred into the recording chamber and perfused continuously (2.5 ml/min) with oxygenated (95% O_2, 5% CO_2) ACSF enhanced with 0.5 μM tetrodotoxin (TTX; Tocris, Bristol, UK).

Electrophysiological patch clamp recordings

For whole-cell patch clamp recordings, borosilicate glass pipettes (Sutter Instruments, Novato, USA) with the resistance of 5–9 MΩ were used. Each pipette was filled with an intrapipette solution composed of (in mM): potassium gluconate 125, KCl 20, HEPES 10, $MgCl_2$ 2, Na_2ATP 4, Na_3GTP 0.4, EGTA 1 and biocytin (0.1%; Tocris, Bristol, UK) (pH = 7.4; osmolarity ~ 300 mOsmol/l). To locate the IGL neurons, cells in the slice were visualized with a 60x objective on a Zeiss Examiner microscope fitted with video-enhanced infrared differential interference contrast (Göttingen, Germany). All the recordings were performed in the current clamp mode (current = 0). During each registration, every 30 s, the hyperpolarising rectangular current pulses (-0.08 nA; duration: 1 s) were adjusted to measure the amplitude of the voltage sag during the pulse, and LTS respectively. The amplitudes taken for analysis were collected and averaged from 7 subsequent voltage responses after establishing the stable membrane potential for 60 s. Neurons with a membrane potential more positive than -50 mV (after the junction potential was adjusted) were excluded from further analysis. The signal was low-passed filtered at 5 kHz and digitized at 15 kHz. The data was recorded using Signal and Spike2 (CED) software.

Immunohistochemical staining

At the end of each electrophysiological experiment, slices with the tested cells were fixed in a 4% paraformaldehyde solution (PFA) in phosphate-buffered saline (PBS, pH = 7.4) at 4°C overnight. Then, the slices were rinsed in PBS (2 x 10 min) and subsequently placed in a 10% solution of normal donkey serum, dissolved in a 0.6% solution of Triton X-100 in PBS for 24 h. In the next step, the slices were incubated in PBS containing primary antibodies against neuropeptide Y (rabbit – anti NPY, 1 : 8000, Sigma-Aldrich) for 72 h. ExtrAvidyn conjugated Cy3 (1 : 250, Sigma-Aldrich) was added to visualize cells filled with biocytin after recording. Consequently, the slices were rinsed in PBS (2 x 10 min) and then incubated with secondary antibodies conjugated with Alexa Fluor 647 (1 : 300, JacksonImmuno Research). After 24 h the slices were rinsed again (5 x 10 min) in PBS and mounted on gelatine-coated glass slides with Vectashield (Fluoroshield™ with DAPI, Sigma-Aldrich). After successful staining, the slices were examined using a confocal laser microscope (Axiovert 200; Zeiss, Jena, Germany). All the neurons which were not stained or were located beside IGL borders, were excluded from further analysis.

Data analysis

All the electrophysiological data were analysed with the use of Statistica 10.0 (StatSoft, Inc. USA). The neurons were grouped in clusters using *Cluster analysis: Tree clustering* mode with a *Euclidean distances* scale. The parameters applied for the analysis were calculated as follows: *voltage sag parameter* (amplitude of voltage sag/minimal voltage value within the sag) and *LTS parameter* (amplitude of LTS/mean resting membrane potential). All the calculations were

made to minimise the voltage-dependency of the investigated phenomena and therefore to normalise the parameters taken for *cluster analysis*. Data were expressed as a mean value ± SEM.

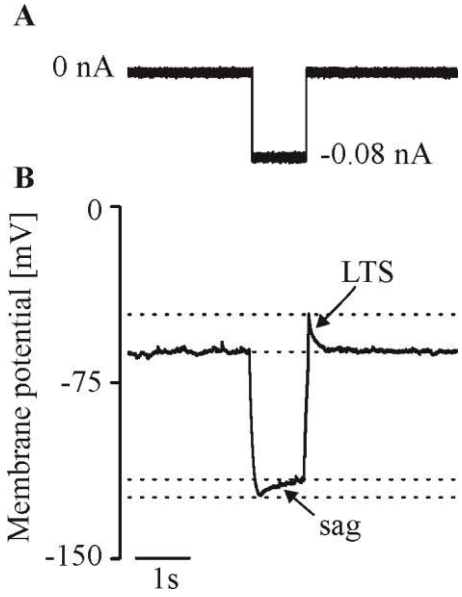

Fig. 1. A – Rectangular current pulse used during each experiment: duration – 1 s, value – -0.08 nA. B – Changes in membrane potential in response to hyperpolarising current pulse (see A). Please note characteristic voltage components: voltage sag and low threshold spike (LTS). Dashed lines assign amplitude of measured phenomena (in mV).
Ryc. 1. A – Prostokątne pulsy prądowe stosowane podczas każdego doświadczenia: czas trwania – 1 s, wartość – -0.08 nA. B – Zmiany potencjału błonowego w odpowiedzi na hiperpolaryzujący puls prądowy (patrz: A). Proszę zwrócić uwagę na charakterystyczne komponenty napięciowe: sag napięciowy oraz niskoprogowe potencjały wapniowe (LTS). Przerywane linie wyznaczają zakres amplitudy mierzonych zjawisk (w mV).

RESULTS

Twenty one IGL neurons were recorded and further analysed. All the patch clamp recordings were made in the presence of TTX (0.5 μM). Therefore, the recorded changes in the amplitude of two phenomena: voltage sag and LTS, were the voltage components derived from single, isolated neurons.

The mean resting membrane potential of the 21 recorded IGL neurons amounted to -55.55 mV ± 1.5 mV. During each registration, the 0.08 nA current pulse was adjusted which hyperpolarised the membrane to -114.19 mV ± 2.81 mV. The amplitude of voltage sag, measured during the current pulse, was on average 4.31 mV ± 0.72 mV and the mean LTS amplitude (following the hyperpolarisation) was 8.59 mV ± ± 1.26 mV (Fig. 1). Considering the values above, the mean *voltage sag parameter* (-0.037 ± 0.006) and *LTS parameter* (-0.151 ± 0.021) were calculated (see *Materials and methods*)

During our experiments, we speculated the existence of several IGL neuronal groups having different amplitudes of voltage sag and LTS. To test this hypothesis *Cluster analysis* was used. Taking into account the *voltage sag parameter* and *LTS parameter* of each recorded IGL neuron, the whole population of 21 cells was divided into 3 clusters. Because of the heterogeneity of the second cluster, it was further partitioned into two groups: *Cluster 2a* and *Cluster 2b* (Fig. 2). The exemplary voltage responses within each cluster were shown in Figure 3, whereas the mean amplitudes of voltage sag and LTS within each cluster were summarised in the graph (Fig. 4).

The first cluster (*Cluster 1*) consisted of 4 out of 21 IGL cells (approximately 19%). The mean values of the investigated phenomena were as follows: *voltage sag parameter* = -0.081 ± 0.005; voltage sag amplitude = 10.04 mV ± 0.92 mV; *LTS parameter* = = -0.129 ± 0.024; LTS amplitude = 7.1 mV ± ± 1.23 mV. The average resting membrane potential characterising this group was -55.48 mV ± 1.37 mV.

Cluster 2 consisted of 13 cells and was further divided. *Cluster 2a* constituted about 19% of the total investigated IGL population (3 out of 21 neurons). It was characterised by: *voltage sag parameter* = = -0.047 ± 0.003; voltage sag amplitude = 5.6 mV ± ± 1.54 mV; *LTS parameter* = -0.249 ± 0.019; LTS amplitude = 13.49 mV ± 1.54 mV; with a mean resting membrane potential = -54.05 mV ± 3.7 mV. *Cluster 2b* was the biggest recorded IGL neuronal group (10 out of 21 neurons; approximately 48%). The average values in this cluster were as follows: *voltage sag parameter* = -0.016 ± 0.002; voltage sag amplitude = = 1.77 mV ± 0.27 mV; *LTS parameter* = -0.172 ± ± 0.032; LTS amplitude = 10.23 mV ± 2 mV. Moreover, the mean membrane potential amounted to -57.62 mV ± 4.87 mV.

The last group (*Cluster 3*) was of the same size as *Cluster 1* (4 out of 21 IGL cells; about 19%), but did not share the same characteristics: *voltage sag parameter* = -0.038 ± 0.002; voltage sag amplitude = = 3.97 mV ± 0.26 mV; *LTS parameter* = -0.044 ± ± 0.015; LTS amplitude = 2.3 mV ± 0.86 mV; mean resting membrane potential = -51.58 mV ± 2.78 mV.

All the results were collected in Table I.

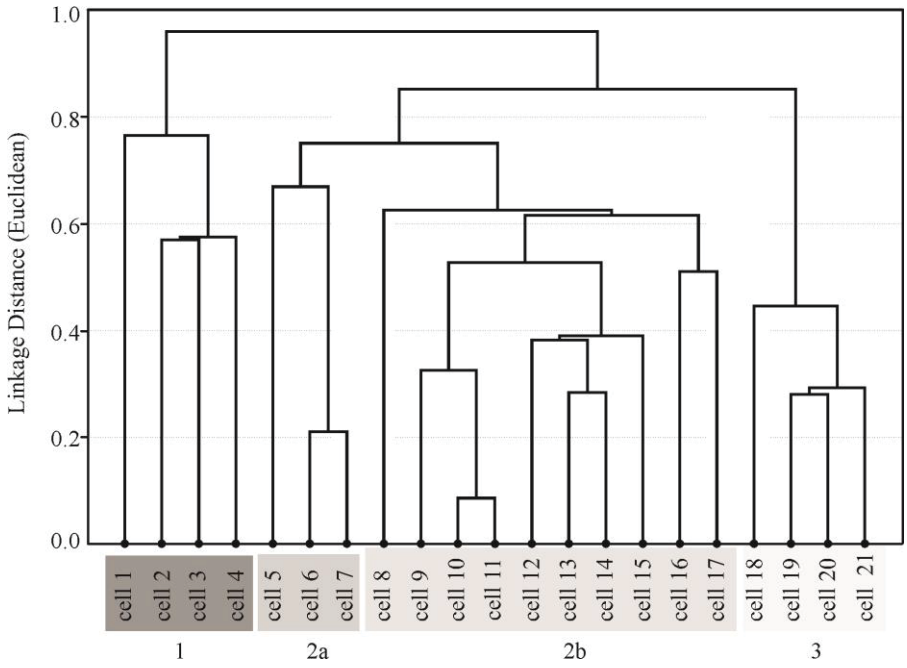

Fig. 2. Results of cluster analysis grouped by *voltage sag parameter* and *LTS parameter* (for detailed information see Materials and methods). Analysis showed clusters (4 groups after Cluster 2 division into Cluster 2a and 2b) of IGL neurons with different voltage sag and LTS amplitude.
Ryc. 2. Wyniki analizy klastrów grupujące komórki w oparciu o wartość *parametru sagu napięciowego* i *parametru niskoprogowych potencjałów wapniowych* (szczegółowe informacje w sekcji Materiały i metody). Analiza wykazała istnienie skupisk (4 grupy po podziale Klastra 2 na Klaster 2a i 2b) neuronów IGL z różnymi amplitudami sagu napięciowego i LTS.

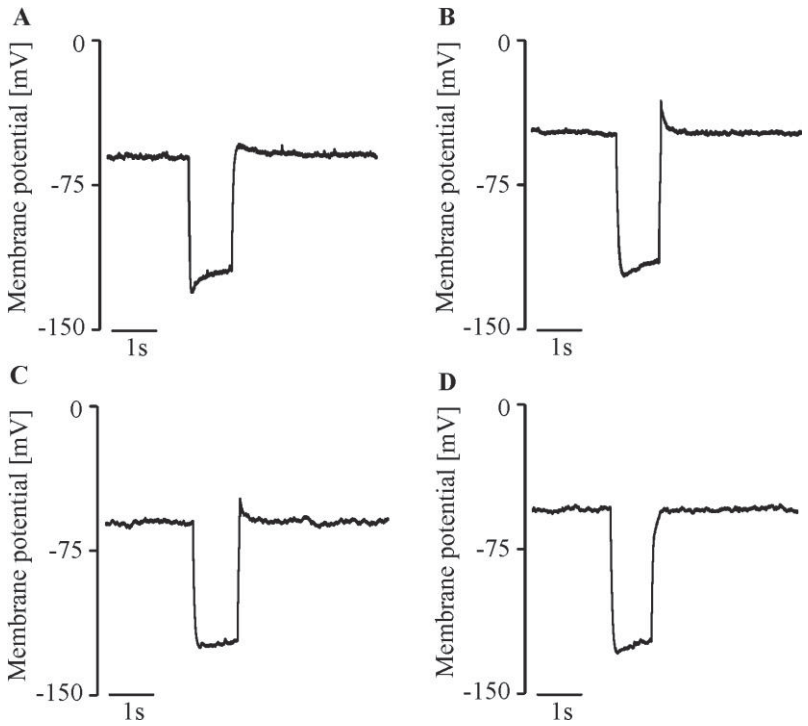

Fig. 3. Examples of voltage changes in response to rectangular current pulse (duration – 1 s, value – -0.08 nA) of IGL neurons from: A. Cluster 1, B. Cluster 2a, C. Cluster 2b, D. Cluster 3.
Ryc. 3. Przykłady zmian napięciowych w odpowiedzi na prostokątne pulsy prądowe (czas trwania – 1 s, wartość – -0.08 nA) neuronów IGL z: A. Klastra 1, B. Klastra 2a, C. Klastra 2b, D. Klastra 3.

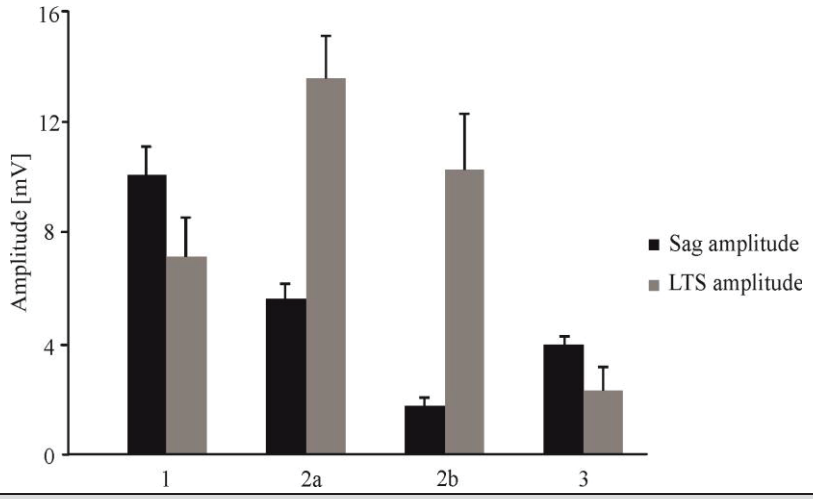

Fig. 4. Graph showing amplitudes [mV] of voltage sag and LTS in IGL neurons clusters (1, 2a, 2b and 3) ± SEM.
Ryc. 4. Graf ilustrujący amplitudy [mV] sagu napięciowego i LTS w klastrach neuronów IGL (1, 2a, 2b i 3) ± SEM.

Table I. Characteristics of IGL neurons in different clusters. For details see Results
Tabela I. Charakterystyka neuronów IGL w poszczególnych klastrach. Dokładne informacje w sekcji Wyniki

Cluster	1	2a	2b	3
Voltage sag parameter	-0.081	-0.047	-0.016	-0.038
Voltage sag amplitude [mV]	10.04	5.6	1.77	3.97
LTS parameter	-0,129	-0,249	-0,172	-0,044
LTS amplitude [mV]	7.1	13.49	10.23	2.3

DISCUSSION

In the present study, we have presented the possible division of IGL neurons into four groups with different amplitude of voltage components responsible for the bursting activity. The first group (*Cluster 1*) can be characterised by the highest voltage sag amplitude and therefore by generation of the biggest I_h. Moreover, the neurons within this group exhibited a high amplitude of LTS – the component resulted from I_t crossing the neuronal membrane. These two characteristics are the base for the assumption of the possible oscillatory nature *in vivo* of the *Cluster 1* neurons. The neurons from *Cluster 2* (*2a* and *2b*) share the same proportion of investigated voltage changes: high LTS amplitude with low voltage sag amplitude, however, the general amplitude of the voltage components in *Cluster 2a* is higher than in *Cluster 2b*. Considering this group of cells (whole *Cluster 2*), it is hard to suggest their firing pattern *in vivo*, because robust I_t is the common feature in thalamic neurons [16], not always involved in generating the oscillatory pattern. It is important to highlight here that only the cooperation of I_h and I_t can create the oscillatory activity pattern [for review see: 14]. The characteristic feature of the IGL cells forming *Cluster 3* was the low amplitude of voltage sag as well as LTS. Therefore, it can be hypothesised that these neurons *in vivo* did not generate the characteristic oscillatory pattern because the I_h and I_t are too small.

These results confirm the previous findings indicating the role of I_h in the physiological functioning of IGL cells. It was shown that in IGL, the HCN3 channels are exclusively expressed [15]. It is interesting that whilst the HCN3 (like HCN1) channels posses a different activation mechanism than other types – they are gated by phosphatidylinositol diphosphate (PIP2) and not by cyclic adenosine monophosphate (cAMP) [15]. Gating by PIP2 elevates the voltage activation threshold of HCN3 channels from -95 mV to -75 mV. The depolarising current flowing through the open HCN3 brings the membrane potential to the voltage activation threshold of T-type calcium channels, which initiates the burst [14]. There is no data indicating the role of I_t in IGL oscillatory or bursting activity, although it is widely investigated in other brain structures exhibiting rhythmic activity [for review see: 14,17].

The previous studies performed by our group [7,8,9] showed the characteristic infra-slow isoperiodic (120 s) oscillatory pattern (ISO) of activity generated by IGL cells *in vivo*. Besides the endogenous properties of IGL neurons enabling the bursting and therefore oscillatory activity [13,15], ISO is not observed

in vitro. That is why it is known that ISO is dependent on other neurons disconnected from the structure during the slice preparation. The possible candidates essential for the oscillatory activity of IGL neurons are the GABAergic neurons because the blockade of $GABA_A$ receptors disrupts ISO [18], moreover GABA is the most abundant neurotransmitter in IGL and the entire biological clock system [19]. Between the periods of high frequency firing pattern in ISO, there are periods in which the neuron is silent. It was previously suggested that this hyperpolarised membrane state is caused by enkephaline from ENK IGL neurons [10]. What is interesting, this neuropeptide derives rather from axonal collaterals of ipsilateral IGL neurons, because it was shown that the electrical lesion of IGL does not influence the rhythmic activity of the contralaterally located counterpart [20]. There is a strong correlation between the ISO in IGL and OPN but only on the ipsilateral side [12]. It is also known that these two structures of the subcortical visual system are densely innervated by intrinsically photosensitive retinal ganglion cells from the ipsilateral eye [22]. Therefore, the ISO in IGL and OPN shows light dependency [20,21]. The neurotransmitters released on the axon terminals consist of glutamate as well as pituitary adenylate cyclase-activating polypeptide (PACAP) [22] conveying photic information to the IGL [for review see: 9].

The modulation of I_t and I_h by nonphotic cues derived from nonspecific brain systems has not yet been described in IGL neurons, although both currents are the aim of different neurotransmitters in various brain nuclei [14,23]. It was shown in our laboratory at *in vivo* conditions that ISO is affected by serotonergic input from dorsal raphe nuclei [24]. Therefore, it can be hypothesised that serotonin has a direct influence on I_h or I_t, as shown in other neurons in different brain structures [25,26,27], or uses a different mechanism which affects ISO activity.

CONCLUSIONS

In conclusion, our research was aimed at the classification of various types of IGL neurons with different voltage sag and LTS amplitude. Therefore, we can speculate the magnitude of I_h and I_t respectively. The widely described effect of these two neuronal currents in bursting activity sheds new light on understanding the characteristic oscillatory pattern of IGL neurons observed *in vivo*. Our newly proposed division can help to determine the neuronal nature while investigating single IGL neurons using the patch clamp technique. This method provides a new tool, useful during pharmacological studies at *in vitro* conditions. Further research is needed to establish the role of possible modulation of I_h and I_t by various neurotransmitters affecting IGL and therefore changing the bursting activity and ISO pattern.

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