doi: 10.4149/gpb_2024002

Putative identification of proopiomelanocortin and neuropeptide-Y neurons of the arcuate nucleus by their response to leptin: *in vivo* electrophysiology study in male and female rats

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Abstract. The arcuate nucleus (ARN) of the hypothalamus is involved in multiple biological functions, such as feeding, sexual activity, and the regulation of the cardiovascular system. It was reported that leptin increased *c-Fos* expression in the proopiomelanocortin (POMC)- and decreased it in the neuropeptide-Y (NPY)-positive neurons of the ARN, suggesting that it stimulates the former, and inhibits the later. This study aimed at the direct electrophysiological examination of the effect of leptin on ARN neurons and to investigate potential sex-dimorphic changes. Wistar rats were anesthetized with urethane and the electrodes were inserted into the ARN. After a spontaneous active neuron was recorded for at least one minute, leptin was administered intravenously, and the firing activity of the same neuron was recorded for two additional minutes. It was found that approximately half of the ARN neurons had an excitatory, and another half an inhibitory response to the leptin administration. The excitability of the neurons with excitatory response to leptin was not different between the sexes. The average firing rate of the neurons with inhibitory response to leptin in females was, however, significantly lower comparing to the males. The obtained results demonstrate that the ARN neurons with stimulatory response to leptin are POMC and those with inhibitory response are NPY neurons. NPY Y1 receptor be might responsible, at least in part, for the sex differences in the excitability of the neurons putatively identified as NPY neurons.

Key words: Arcuate nucleus (ARN) — Lateral hypothalamus — Leptin — Proopiomelanocortin (POMC) — Neuropeptide-Y (NPY) receptor 1 (Y1)

Abbreviations: ARN, arcuate nucleus; GABA, γ-aminobutyric acid; ISI, interspike interval; NPY, neuropeptide-Y; POMC, proopiomelanocortine.

Introduction

The arcuate nucleus (ARN) is located in the ventromedial hypothalamus, in proximity of the third cerebral ventricle (Sapru 2013). The ARN neurons are diverse from the neurochemical, neuroanatomical, and functional points of view. Different ARN neurons release different amino acids, monoamines, and neuropeptides, such as γ-aminobutyric acid (GABA), dopamine, prolactin, dynorphin, neuropeptide-Y (NPY), as well as various proopiomelanocortins (POMC), such as ß-endorphin. ARN neurons projects to the other nuclei of the hypothalamus, such as periventricular nucleus, as well as to the other areas of the central nervous system (CNS), as distant as the spinal cord (Bouret et al. 2004). ARN is involved in the multiple biological functions, such as feeding (Garza et al. 2023; Ullah et al. 2023), sexual activity (Cortes et al. 2022; Zhou et al. 2022), and the central regulation of the cardiovascular system (Pan et al. 2023; Sun et al. 2023).

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The neurons located in the lateral section of the ARN are believed to be involved in the central regulation of appetite and metabolism. Two lines of evidence support this suggestion. Firstly, these neurons are sensitive to the peripheral metabolic markers, such as leptin, a hormone produced by adipose cells (Elias et al. 1999). Secondly, they project to the lateral hypothalamus and regulate the activity of the melanin-concentrating hormone-secreting neurons located therein. Melanin-concentrating hormone, in turn, is known as one of the key appetite regulators (Bouret et al. 2004; Bodnar 2013).

The lateral section of the ARN is rich in POMC- and NPY-expressing neurons. POMC, as well as NPY neurons project to the lateral hypothalamus and to express leptin receptor. The effect of the leptin on the POMC and NPY neurons is, however, different. It was reported that leptin putatively stimulates POMC and suppresses NPY neurons of the ARN (Elias et al. 1999). Elias and colleagues estimated that leptin has a stimulatory effect on the POMC and inhibitory effects on the NPY cells using the assessment of *c-Fos* proto-oncogene expression in these neurons. A later study (Srour et al. 2023) confirmed that leptin depolarized a subpopulation of the ARN cells, which are putatively POMC neurons, in the isolated brain slices. However, to the authors best knowledge, the effect of leptin on the action potentials generation in ARN neurons, as well as the sex-related differences in the excitability of the POMC and NPY neurons, were not yet investigated in *in vivo* conditions. Considering the role of these circuits in the metabolic, eating, and mood disorders (Qu et al. 2020), and taking into account the sex-related differences in frequency of these disorders (Merikangas and Almasy 2020), it is of interest to assess the excitability of the ARN neurons in both sexes.

The aim of the current study was to identify the spontaneously active neurons in the lateral section of the ARN of male and female Wistar rats and the examine the effect of the acute administration of the recombinant rat leptin on these neurons.

Materials and Methods

Animals

Adult male (n = 12) and female (n = 11) Wistar rats (250–300 g) were ordered from the Animal Breeding Facility of the Institute of Experimental Pharmacology and Toxicology, Centre of Experimental Medicine, Slovak Academy of Sciences (Dobra Voda, Slovakia). Animals were housed under standard laboratory conditions (temperature: 22 ± 2°C, humidity: 55 ± 10%) with a 12 h light/12 h dark cycle (lights on at 7 a.m.). Pelleted food and tap water were available ad libitum. All experimental procedures were approved by the Animal Health and Animal Welfare Division of the State Veterinary and Food Administration of the Slovak Republic (Permit number Ro 3592/15-221) and conformed to the Directive 2010/63/EU of the European Parliament and of the Council on the Protection of Animals Used for Scientific Purposes. In this type of investigation, the use of animals could not be avoided (Homberg et al. 2021).

Chemicals

Recombinant rat leptin was purchased from the National Hormone & Peptide Program, Harbor-UCLA Medical Center (Torrance, CA, USA) and dissolved in saline (0.9% sodium chloride: NaCl). Other chemicals were purchased from Merck Life Science s.r.o, Bratislava, Slovakia.

In vivo electrophysiological experiments

In vivo electrophysiological experiments were performed as previously described (Paliokha et al. 2023). Animals were anesthetized by urethane (1.25 g/kg, *intraperitoneally*) and mounted in the stereotaxic frame (David Kopf Instruments, Tujunga, CA). The body temperature was maintained between 36 and 37°C with a heating pad (Gaymor Instruments, Orchard Park, NY, USA). The scalp was opened, and a 3 mm hole was drilled in the skull for insertion of electrodes. Glass-pipettes were pulled with a DMZ-Universal Puller (Zeitz-Instruments GmbH, Martinsried, Germany) to a fine tip approximately 1 μm in diameter and filled with 2 M NaCl solution. Electrode impedance ranged from 4 to 6 MΩ. The pipettes were inserted into the lateral part of the ARN (3.8–4.0 mm posterior to bregma, 0.6–0.7 mm lateral to the midline, and 9.4–9.8 mm ventral to brain surface) (Paxinos and Watson 2014). The action potentials generated by the spontaneously active neurons were recorded using the AD Instruments Extracellular Recording System (Dunedin, New Zealand). One neuron *per* animal was recorded. The neuronal firing rate and burst activity characteristics were calculated using the burstiDAtor software (www.github.com/nno/burstidator). The onset of a burst was signified by the occurrence of two spikes with an interspike interval (ISI) <14 ms and its termination of a burst was defined as an ISI >14 ms, in accordance with a previous study describing periventricular nucleus-projecting ARN nucleus in *in vivo* conditions (Dickson et al. 1994).

Leptin administration

Leptin (1 mg/kg) was administered *via* a catheter placed in a femoral vein, after a spontaneously active neuron was identified and its basal firing activity was recorded for at least one minute. The dose of leptin was chosen according to the previous behavioral study (Elias et al. 1999).

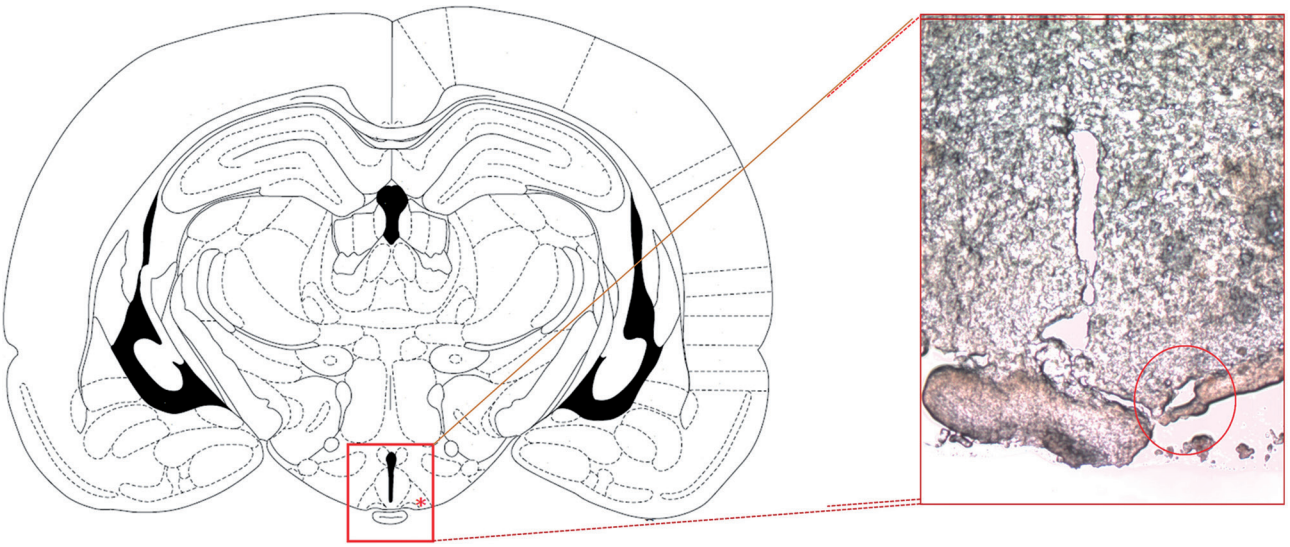

Figure. 1. Histological verification of the electrode tip location in the ARN. Tip location shown by the circle (◯) or star (*).

Histological verification of the electrode tip location

After completion of electrophysiological recordings, the animals were euthanized by an overdose of urethane. In selected animals, the electrode tip location was labeled by electrolytic lesion using a direct current of 0.5 mA for 15 s, as previously described (Csatlosova et al. 2021; Dremencov et al. 2022). The brains were removed and fixed in 10% paraformaldehyde for 24 h, and afterward in 30% sucrose for 7 days. Frozen sections (thickness: 50 μm) were cut and examined under a light microscope to verify the placement of the electrode tip in the lateral ARN (Fig. 1).

Statistical analysis

The effect of leptin on the excitability of hippocampal glutamate and brainstem monoamine neurons, the spikes frequency after the administration of leptin was expressed as percentage of the basal firing activity of the same neurons. The effects of leptin in male and female rats were examined using the two-way analysis of variance for repeated measures (RM ANOVA; first factor of comparison: time-before or after leptin administration; second factor of comparison: sex-male or female), followed by Bonferroni post-hoc test. The sex differences in the basal firing rate and burst activity of the neurons were examined using the two-tailed Student's *t*-test. The normal distribution of the data was verified using the Shapiro-Wilk test.

Results

Spontaneously active neurons were detected in the rat ARN under urethane anesthesia

We were able to detect between one to three spontaneously active neurons *per* one electrode descent through the ARN.

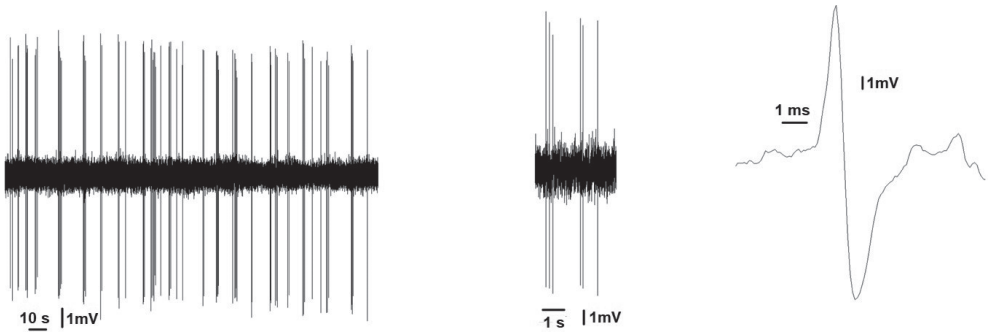

Figure 2. Firing pattern and the action potential waveform of the neurons of rat ARN.

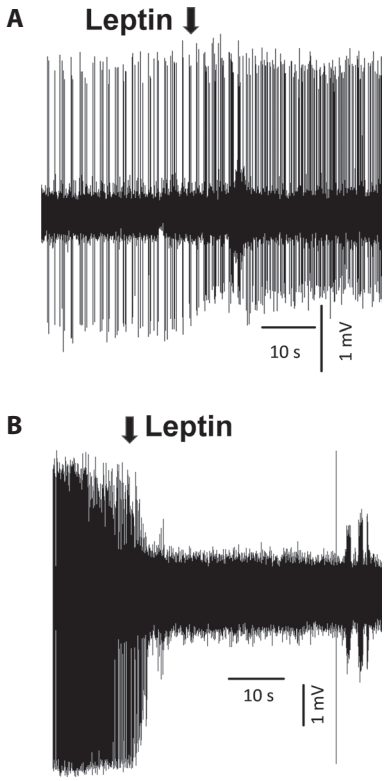

Figure 3. Effect of leptin on the excitability of the representative pro-opiomelanocortin (POMC; **A**) and neuropeptide-Y (NPY; **B**) neurons of the rat ARN.

The neurons' excitability pattern and the waveforms of their action potentials are shown in Figure 2.

Leptin excited one and inhibited another sub-portion of the ARN neurons

Acute administration of leptin increased the firing activity of 7 out of 12 ARN neurons in males and 6 out of 11 ARN neurons in females (Figs. 3A and 4A). In 5 out of 12 ARN neurons in males and 5 out of 11 ARN neurons in females, the firing activity decreased after leptin administration (Figs. 3B and 4B). The two-way repeated measures ANOVA effects of time were significant in both cases ($F_{12,157} = 2.88$, $p = 0.02$ and $F_{12,118} = 2.66$, $p = 0.004$, respectively). The effects of sex and time × sex interactions in both cases were not statistically significant.

Sex-related differences in the basal firing of the ARN neurons

The mean basal rate (one-minute-long recording before leptin administration) of the neurons excited by leptin (presumably POMC neurons) was not different between the sexes. The mean basal firing rate of the neurons inhibited by leptin (presumably NPY neurons) in female rats was significantly lower, comparing to the males ($p = 0.01$, two-tailed Student's *t*-test, Fig. 5).

Burst firing of the ARN neurons

The burst firing characteristics of the ARN neurons characterized by stimulatory and inhibitory response to leptin in male and female rats are summarized in the Table 1. Neurons stimulated by leptin in males had higher frequency of the burst firing, comparing to females ($p = 0.01$, two-tailed Student's *t*-test). These results should be, however, taken cautiously, since the number of the neurons exhibiting burst firing and stimulated by leptin detected at the present study was low.

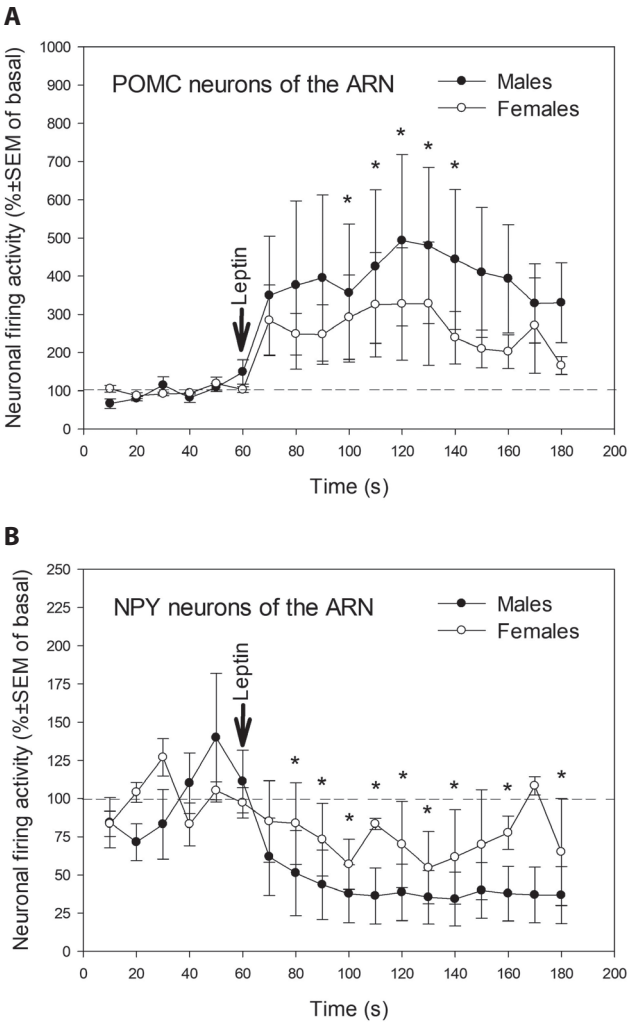

Figure 4. Effect of leptin on the excitability of pro-opiomelanocortin (POMC; **A**) and neuropeptide-Y (NPY; **B**) neurons of the ARN in male and female rats. Error bars show the standard error of the mean (SEM). * $p \leq 0.05$ in comparison with baseline (one-minute-long recording *prior* to leptin injection), Bonferroni *post-hoc* test.

Discussion

In the present study, the action potentials generated by the spontaneously active neurons in the lateral section of the rat ARN was performed and the effect of leptin om the excitability of these neurons examined. Approximately half of the neurons in both sexes showed excitatory response to the acute intravenous administration of leptin. Based on the previous study of Elias and colleagues (Elias et al. 1999), reporting the activation of the *c-Fos* proto-oncogene expression in the POMC-positive hypothalamus-projecting neurons of the lateral ARN, these neurons are presumably POMC-expressing. Another half of the neurons showed inhibitory response to the leptin. Based on the abovementioned Elias at al.'s study, these neurons are presumably NPY-positive. The stimulatory effect of leptin on the POMC neurons, as well as its inhibitory effect on the NPY neurons, tended to be more potent in males comparing to females, even though this difference was not statistically significant. Higher sensitivity of the POMC neurons to leptin in males comparing to females has been also reported in a previous study using electrophysiology in the isolated slices from murine brain (Srour et al. 2023).

The results of our study suggest that the POMC and NPY neurons are equally distributed across the lateral ARN. Our finding, based on the assessment of *in vivo* electrophysiological response to the acute leptin administration, is consistent with the findings of the previous study (Elias et al. 1999), based on *ex vivo* assessment of *c-Fos* expression following *in vivo* leptin administration.

POMC neurons are characterized by the slightly higher spontaneous firing rate, compared to the NPY cells. Both populations of the neurons exhibit action potentials (spikes) of high amplitude and long duration. Significant portion of the ARN neurons, exhibit burst mode of the firing, as it was previously reported (Dickson et al. 1994). According to the results of our study, POMC and NPY neurons cannot be distinguished by their firing mode and the burst firing.

We found that the basal firing rate of NPY neurons in male rats was higher compared to the females. Similarly, to the finding of the previous study (Srour et al. 2023), POMC neurons had similar firing rates across the sexes. However, POMC cells in males might be characterized by a higher frequency of the burst firing, compared to females. Considering the role of the NPY in the eating disorders (Kawai et al. 2017), the sex difference in the basal activity of NPY neurons may explain, at least in part, the sex differences in their frequency (Merikangas and Almasy 2020).

Interestingly, previous studies reported no sex differences in the brain NPY mRNA (Hill et al. 2014) or protein (Henn et al. 2021; Carboni et al. 2022; Linhares et al. 2022) levels. With regards to the specific receptors mediating NPY transmission, the density of the cortical NPY receptors Y1 was higher in males, while their affinity was greater in females (Michel et al. 1995). The sensitivity of the peripheral vascular

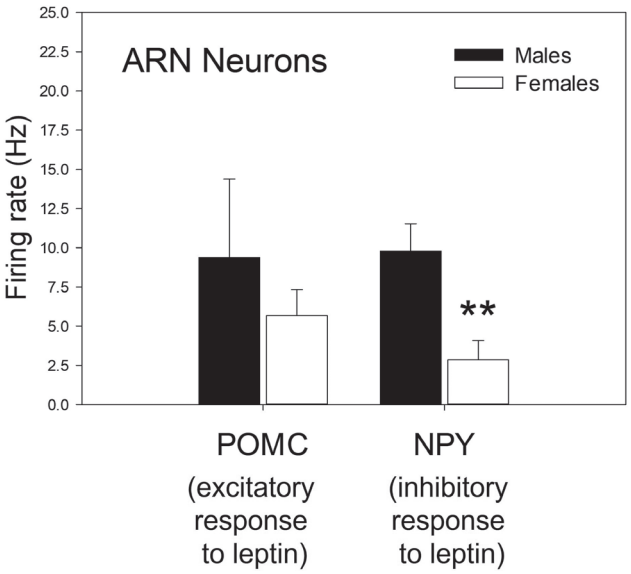

Figure 5. Basal firing rate of pro-opiomelanocortin (POMC) and neuropeptide-Y (NPY) neurons of the (ARN) in male and female rats. Error bars show the standard error of the mean (SEM). ** $p \leq 0.01$ in comparison with males, two-tailed Student's *t*-test.

Table 1. Characteristics of the burst firing of pro-opiomelanocortin (POMC) and neuropeptide-Y (NPY) neurons of the ARN in male and female rats

Characteristic	Male rats		Female rats	
	POMC	NPY	POMC	NPY
% of neurons with burst firing	43	80	80	50
Number of bursts/s	2.73 ± 0.47	0.49 ± 0.28	0.60 ± 0.32**	0.06 ± 0.01
% of spikes occurring in bursts	53 ± 8	29 ± 17	27 ± 19	2 ± 0
Mean number of spikes/burst	3.16 ± 0.36	5.19 ± 0.27	2.28 ± 0.20	2.00 ± 0.00

** $p < 0.01$ in comparison with males, two-tailed Student's *t*-test.

Y1 receptors in females was greater than in males as well (Glenn et al. 1997; Liu et al. 2016).

Y1 receptors are densely expressed in the ARN (Mikkelsen and Larsen 1992; Fuxe et al. 1997). These receptors were reported to be coupled to the $\alpha_{I/O}$ G-protein (Michel et al. 1995; Raimondi et al. 2002). Multiple $\alpha_{I/O}$ G-protein-coupled receptors of the CNS, such 5-$HT_{1A/1B}$, α_2-adrenergic, and D_2 receptors, are involved in the negative autoregulation of the neurons secreting corresponding neurotransmitter, e.g., serotonin (5-HT), noradrenalin, and dopamine (Pavlovicova et al. 2015). It is this possible that Y1 receptors are involved in the autoregulation of excitability of NPY neurons, and their higher sensitivity in females is putatively responsible for the reduced excitability of NPY neurons in comparison to the males. Since Y1 receptors are expressed on the POMC cells (Fuxe et al. 1997), decreased burst firing of the ARN POMC neurons in females might be explained by increased Y1-mediated signaling as well. Further studies are required to test this hypothesis.

The two primary limitations of this study are (1) the lack of the exact neurochemical examination of the neurons being recorded and (2) testing of the effect of one dose of leptin only. In the future studies, the POMC expression of the neurons stimulated by leptin, and NPY expression in the neurons inhibited by leptin, should be confirmed using the "juxtacellular" (Tang et al. 2014) and/or optogenetic (Grzelka et al. 2023) methods should be performed. In addition, the effect of the several doses of leptin on the excitability of ARN neurons should be tested, in order to examine the possible dose-dependent nature of leptin's effect and its putative sex-related differences.

Summarizing, the present study, to the authors' best knowledge, was first to examine the excitability of the lateral ARN neurons in *in vivo* condition and to distinguish out of them POMC and NPY cells, based on their response to leptin. Even though this study is preliminary and has certain limitation, it might motivate further *in vivo* neurophysiological research of the ARN-lateral hypothalamus neuronal circuits and POMC/NPY neurotransmission, which are fundamental in the eating behaviour and metabolic disorders.

Acknowledgments. This work was supported by the Slovak Research and Development Agency (grant APVV-20-0202) and Scientific Grant Agency of Ministry of Education of Slovak Republic and Slovak Academy of Sciences (grant VEGA-2/0057/22). Authors thank Nick Oosterhof and Chris Oosterhof for their valuable assistance with the data analysis, as well as for the critical proof-reading of this paper.

Conflict of interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Received: October 16, 2023
Final version accepted: January 30, 2024
First published online: February 22, 2024