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Original article

Immunoreactivity of acetylcholinesterase and M1 muscarinic receptors in the hippocampus and striatum of rats treated with Rebaudioside A

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Abstract

Rebaudioside A (RebA) is a steviol glycoside used for production of sweeteners. It was shown that the glycosides affect memory and learning processes. The aim of the study was to investigate neurons immunoreactive for acetylcholinesterase (AChE) and M1 muscarinic receptors (mAChRs-M1) of the hippocampal CA1 and CA3 fields and striatal caudateputamen (CP) and globus pallidus (GP) in rats receiving RebA. RebA was administrated to adult rats for 45 days in dilutions of 1 mg and 2 mg RebA/ml water. Indirect peroxidase-antiperoxidase immunohistochemical reaction was conducted on frontal sections containing the hippocampus and striatum with use of antibodies against AChE and mAChRs-M1. Immunoreactive for the studied proteins neurons were morphologically and morphometrically assessed in hippocampal CA1 and CA3 fields and in CP and GP. Microscopic observations did not reveal significant changes in morphology of immunoreactive neurons, which suggests no neurotoxic effect of the studied glycoside on these cells. Morphometric analyses revealed an increase in the density of AChE and mAChRs-M1 immunoreactive neurons. A decrease in reaction intensity of AChE-positive neurons was also demonstrated in the hippocampal CA1 field and in GP. In contrast, an increase in reaction intensity of mAChRs-M1-positive neurons was found in CA1, CA3 fields and in CP and GP. The results of our preliminary studies indicate that RebA administrated to rats has an impact on cholinergic neurons in the studied area. The results suggest a possible increase in the activity of the cholinergic system, responsible for memory and learning processes, after administration of RebA.

Key words: rebaudioside A, stevia, acetylcholinesterase, hippocampus, striatum, rat

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Introduction

Rebaudioside A (RebA) was isolated from Stevia rebaudiana Bertoni plant. This substance is used in sweeteners production (Tao and Cho 2020). Steviol glycosides have been shown to have therapeutic properties including antihypertensive, anti-diabetic, anti-oxidant, anti-inflammatory and anti-cancer effects (Dyduch-Siemińska et al. 2020). Few studies indicate a positive effect of oral administration of steviol glycoside extracts on memory in rats and the reduction in acetylcholinesterase (AChE) levels in brain homogenates of these animals (Juyal et al. 2010a,b, Sharma et al. 2010, Jahanshahi et al. 2013, Chayushyan et al. 2017). It is thought that RebA, through its antidiabetic, memory-improving, antioxidant and anti-inflammatory effects may be used as dietary supplement in diseases with an increased risk of dementia (Abdel-Aal et al. 2021, Veurink et al. 2021).

Acetylcholine (ACh) is the main cholinergic neurotransmitter in the brain that enhances various types of memory engram encoding and modulates long-term potentiation (LTP) (Bazzari and Parri 2019). The concentration of ACh in synapses is regulated by acetylcholinesterase (AChE) which rapidly hydrolyzes and inactivates this neurotransmitter. In neurons AChE is present in the perikaryon, in the cellular membrane, in axons, in dendrites and in synapses (Janeczek et al. 2018). Acetylcholine stimulates 2 types of receptors: muscarinic (mAChRs) and nicotinic. Among the mAChRs there are M1-M5 types of receptors. These receptors belong to the metabotropic receptors which function is related to the G protein and the phosphatidylinositol system (Radu et al. 2017).

The hippocampus is composed of CA1-CA4 fields divided into layers: the stratum oriens (SO), stratum pyramidale (SP), stratum radiatum (SR) and stratum lacunosum-moleculare (SLM). This area is primarily responsible for declarative memory (El Falougy et al. 2008). The striatum belongs to the basal ganglia and consists of the caudate, putamen and globus pallidus. In rats, the caudate nucleus and the putamen are not separated from each other (caudateputamen – CP). The striatum is responsible for procedural memory (Ni et al. 2018).

In the hippocampus and the striatum the majority of acetylcholine receptors are postsynaptic muscarinic M1 receptors (mAChRs-M1). In the hippocampus, they are present in the pyramidal neurons of all fields, in the granular cells of the dentate gyrus and in interneurons. In the striatum, mAChRs-M1 are present in 78% of neurons, especially in the postsynaptic spiny dendrites (Betterton et al. 2017, Mann et al. 2018).

A crucial aspect of this study was the assessment of AChE and mAChRs-M1 immunoreactivity in hippocampal and striatal neurons after oral administration of RebA. So far, the influence of this glycoside on the immunoreactivity of AChE and mAChRs-M1 in nervous cells of the brain has not been studied.

Materials and Methods

Fifteen male, 55-day-old, Wistar rats were used for the study. The animals were kept individually in separate cages (20°C, 30% humidity) with constant access to fodder and water. The approval of the 2nd Local Ethics Committee for Experiments on Animals in Lublin no. 22/2013 was obtained for the research.

Ten animals were randomly divided into 2 groups that received 25 ml of RebA in separate drinkers in dilutions of 1 mg and 2 mg RebA/ml water (group I, 65 mg/kg b.w. and group II, 119 mg/kg b.w. respectively) for 45 days. The remaining five rats received only fresh water (control group, C). The day after the last administration of RebA, the animals were euthanized (xylasine, 10% ketamine *i.m.*). Their brains were fixed in 10% formalin, embedded into paraffin blocks and cut in the frontal 4µm-thick sections.

The indirect peroxidase-antiperoxidase immunohistochemical reaction was performed on the brain sections containing the hippocampus and striatum from every animal. Antibodies and reagents used in staining were from Sigma-Aldrich (St. Louis, Missouri, USA) and were diluted in 0.5M Tris buffered saline (pH 7.6). Antigens were retrieved by heating in 0.01M citrate buffer (pH=6.0). Sections were incubated with 3% H_2O_2 for 30 min and goat serum at room temperature for 20 min. The primary antibodies, mouse monoclonal anti-acetylcholinesterase antibody (A6970, 1:1000) and rabbit polyclonal anti-muscarinic M1 antibody (M9808, 1:1000), were used for the slides for 16h in 4°C. Next, peroxidase-conjugated secondary IgG antibodies (A9917, 1:150, A9169 1:400, 1h, room temperature) were used. Diaminobenzidine (DAB, 32750 Fluka) was used as a chromogen. The slices were counterstained with the Mayer's hematoxylin (MHS80) and mounted in DPX (44581 Fluka). A specificity control for the reactions was performed. The micrographs were taken using a BX51 light microscope (Olympus, Tokyo, Japan).

AChE-IR and mAChRs-M1-IR neurons were morphologically and morphometrically assessed in the SP of the hippocampal CA1 and CA3 fields and in the striatal CP and GP. The number of the nerve cells was semi-quantitatively assessed using a scale: (- / +) few, (+) moderately numerous, (++) numerous, (+++) very numerous.

Morphometric analyzes were performed using the Cell^D program. The density of neurons was presented as the ratio of the density of immunopositive neurons

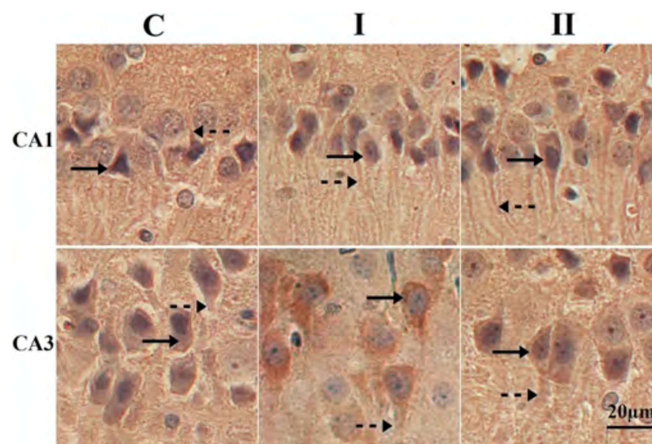

Fig. 1. Acetylcholinesterase immunoreactive (AChE-IR) neurons in rat hippocampal CA1 and CA3 area of control rats (C), and animals which received 1 mg RebA/ml of water (I) and 2 mg RebA/ml of water (II). Moderately numerous (+) AChE-IR neurons in the C group of CA1 area and numerous (++) neurons in the other groups and in CA3 area (full arrow). The presence of the reaction product in initial sections of nerve fibres (dotted arrow). Immunoperoxidase labeling for AChE. x40.

to all nerve cells, counted in 50 fields of a grid with an area of $8x10^{-3}$ mm^2. The AChE-IR and mAChRs-M1-IR neurons of the striatum were single and unevenly distributed, hence they were not suitable material for the quantification of neuron density. The immunostaining intensity of the studied proteins in neurons was measured in a $1µm^2$ square of the cytoplasm. The results were converted so that the higher values represent a darker color (Nguyen et. al. 2013). The surface area of the neurons was measured by manual outlining of their bodies. The nuclear-cytoplasmic index (N/C) was calculated as the ratio of the nuclear surface area to the cytoplasm area of the cellular bodies. For each analysis, 100 measurements were made in each test area and group but no more than 5 immunopositive cells in one photomicrograph and were presented as means with standard deviation.

Results were statistically assessed with the one-way analysis of variance (ANOVA) test and the Tukey HSD *post hoc* test. The non-parametric Kruskal-Wallis test was used when the data did not meet the conditions of normal distribution and homogeneity of variance. The significance levels of all tests were set at α=0.05. Statistical analyzes were conducted in the R 3.3.1 program (GNU General Public License).

Results

AChE-IR and mAChRs-M1-IR neurons were observed in the SP of studied hippocampal fields, in the CP and GP of the striatum in all groups of animals. The reaction product was characterized by diffuse immunostaining in the neuronal bodies and in some cells it was pinpointed at the cellular membrane and in the processes.

Morphological analyzes

In the SP of the hippocampal CA1 field, in the control animals, moderately numerous (+) AChE-IR neurons were present. In animals receiving RebA, AChE-IR cells were numerous (++). In all studied groups, these cells were pyramidal with an oval or round nucleus located centrally in the neuroplasm. In CA3 field, there were numerous (++), pyramidal and oval immunostained neurons in all studied groups. A brown reaction product was visible in the initial sections of the dendrites, especially in animals from group II (Fig. 1).

In the CP and GP of the striatum, there were single (+/-) variform and large neurons similarly immunostained for AChE in all groups of studied animals. In the central part of the perikaryons there were oval or round nuclei. Moreover, moderately numerous (+) and small AChE-IR neurons with large round nuclei were observed in animals from groups I and II. In GP the neurons of the group II were characterized by weaker immunostaining compared to the other groups of rats. In the examined areas, pinpointed immunostaining was also seen in the nerve fibers grouped into bundles (Fig. 2).

In SP of the hippocampal CA1 field, in the control group, there were moderately numerous (+) mAChRs-M1-IR neurons which were pyramidal with a large oval nucleus in the central part of the perikaryons. Similar cells were found in groups I and II but they were more numerous (++) and were characterized by more intense immunostaining compared to those in animals from group C. In CA3, immunopositive, pyramidal and oval neurons with oval or round nuclei were numerous (++) in all groups and more intensely immunostained in groups I and II than in C (Fig. 3).

In striatal CP, in control rats, there were single (+/-), large, spindle-shaped mAChRs-M1-IR neurons with oval nuclei in the widest part of the cell. In animals

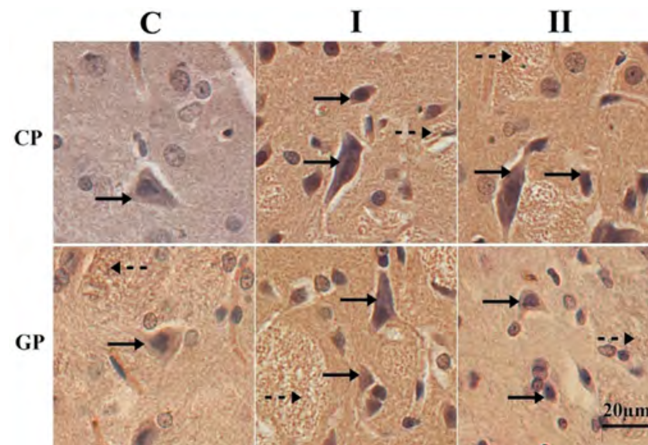

Fig. 2. The AChE-IR neurons in rat striatal CP and GP of the control rats (C), and animals which received 1 mg RebA/ml of water (I) and 2 mg RebA/ml of water (II). Single (+/-), large AChE-IR neurons in every studied group and moderately numerous (+) small neurons in groups I and II (full arrow). Immunostaining product in nerve fibres (dotted arrow). Immunoperoxidase labeling for AChE. x40.

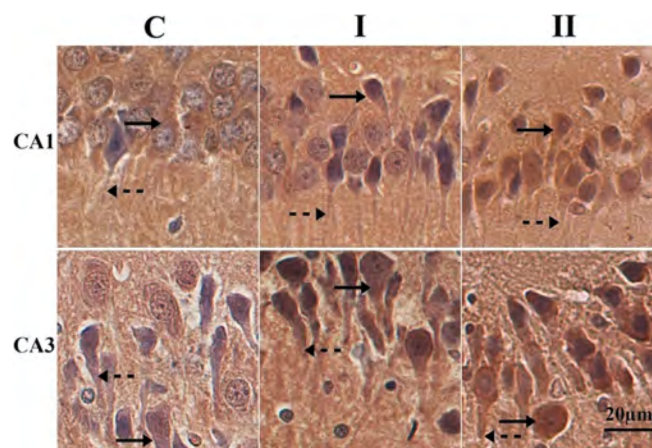

Fig. 3. M1 muscarinic receptor immunoreactive (mAChRs-M1-IR) neurons in rat hippocampal CA1 and CA3 area of the control rats (C), and animals which received 1 mg RebA/ml of water (I) and 2 mg RebA/ml of water (II). Moderately numerous (+) mAChRs-M1-IR neurons in CA1 area in the C group and numerous (++) more intensely stained neurons in the other groups. Numerous mAChRs-M1-IR neurons in all the groups in CA3 with more intensely stained cells in group I and II (full arrows). The presence of the reaction product in initial sections of nerve fibres (dotted arrow). Immunoperoxidase labeling for mAChRs-M1. x40.

from groups I and II numerous (++) mAChRs-M1-IR oval cells with similarly shaped nuclei were present. These neurons were much smaller and more intensely immunostained than in C animals. In the studied groups of rats, the pinpointed reaction product was visible in the bundles of nerve fibers. Similar results were found in striatal GP (Fig. 4).

Morphometric analyzes

Density of AChE-IR neurons

In the SP of the CA1 field of the hippocampus statistically significant increase in the density of AChE-IR neurons was found in animals consuming the RebA at a dose of 1 mg/ml of drinking water compared to group C. In the SP of the CA3 field no statistically significant differences were found in the AChE-IR neuron density between all studied groups (Table 1).

Immunostaining intensity of AChE-IR neurons

In the SP of the hippocampal CA1 field there was a decrease in the mean immunostaining intensity of AChE-IR neurons in animals consuming the sweetener, slightly more evident in group I. In the SP of the CA3 field of the hippocampus the immunostaining intensity of the AChE-IR neurons was similar to that observed in group C. The AChE-IR neurons in the striatal CP were similarly immunostained regardless of the sweetener dose. In striatal GP the neurons of group II were weaker immunostained for AChE in comparison to the control rats (Table 1).

Density of mAChRs-M1-IR neurons

In the SP of the hippocampal CA1 field a statistically significant increase in the density of immunoreactive neurons occurred in rats from both groups consum-

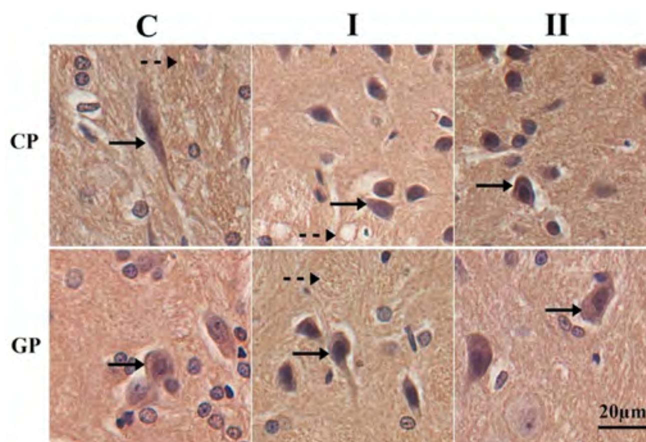

Fig. 4. The mAChRs-M1-IR neurons in rat striatal CP and GP of the control rats (C), and animals which received 1 mg RebA/ml of water (I) and 2 mg RebA/ml of water (II). Single (+/-), large mAChRs-M1-IR neurons in C group and numerous (++), more intensely stained neurons in groups I and II in CP and GP (full arrows). The presence of the reaction product in initial sections of nerve fibres (dotted arrows). Immunoperoxidase labeling for mAChRs-M1. x40.

Table 1. The density, immuno-staining intensity, square area and nuclear-cytoplasmic (N/C) index of AChE-IR neurons in hippocampal CA1, CA3 areas and striatal CP, GP in control rats (C), and animals which received 1 mg RebA/ml of water (I) and 2 mg RebA/ml of water (II). Data presented as mean with standard deviation. Different letters show statistically significant differences for p<0,05 in ANOVA test (A) or Kruskal-Wallis test (K).

	AChE											
	CA1			CA3			CP			GP		
	C	I	II	C	I	II	C	I	II	C	I	II
Density	0.11± 0.03^{a}	0.26 ±0.11$^{b(A)}$	0.16 ±0.09^{ab}	0.46 ±0.12	0.52 ±0.06	0.45 ±0.07	-	-	-	-	-	-
Immuno-staining intensity	55.29 ±12.9^{a}	38.17 ±12.18$^{b(K)}$	42.99 ±8.42$^{c(K)}$	54.96 ±13.09	57.03 ±19.28	50.49 ±11.15	53.48 ±12.52	47.05 ±15.57	48.57 ±14.49	52.49 ±15.58^{a}	48.69 ±12.99^{ab}	41.95 ±12.28$^{b(A)}$
Area (µm^2)	132.84 ±54.3^{a}	85.03 ±23.83$^{b(K)}$	96.78 ±28.31^{ab}	169.38 ±59.92	180.75 ±44.34	148.43 ±50.14	135.16 ±10.92^{a}	82.74 ±34.69$^{b(K)}$	136.06 ±64.81^{ab}	149.07 ±17.72^{a}	110.58 ±38.3$^{b(K)}$	104.41 ±35.12$^{b(K)}$
N/C index	0.47 ±0.1	0.5 ±0.12	0.48 ±0.1	0.45 ±0.11	0.47 ±0.11	0.43 ±0.09	0.38 ±0.05^{a}	0.56 ±0.14$^{b(K)}$	0.37 ±0.13^{ab}	0.42 ±0.04^{a}	0.45 ±0.11^{ab}	0.4 ±0.1$^{b(K)}$

ing the sweetener. In the SP of the CA3 field an increase in the mean density of mAChRs-M1-IR neurons was found in both groups of animals receiving the sweetener (Table 2).

Immunostaining intensity of mAChRs-M1-IR neurons

In the SP of the hippocampal CA1 field the mean immunostaining intensity for mAChRs-M1 was higher in the rats receiving RebA at both dilutions, especially in group II, than in the control group. In the SP of the CA3 field of the hippocampus a statistically significant increase in the intensity of immunostaining was observed in both groups of animals receiving RebA compared to nerve cells in the control rats. In the CP of the striatum an increase in the studied parameter was found in animals receiving the sweetener, irrespective of the dose. The increase in the intensity of immunostaining of mAChRs-M1-IR neurons in the striatal GP was observed in particular in group II of animals (Table 2).

Analyzes of other parameters

The surface area of AChE-IR cells was smaller in the CA1 and CP area in group I animals and in GP in both groups of rats receiving RebA. The N/C index was different in CP in group I and in GP in group II (Table 1).

mAChRs-M1-IR cells with much smaller surface areas were found in CP in both animal groups receiving RebA and in GP in rats from group I. The N/C index was higher in group I of animals in CA3, and in groups I and II in CP (Table 2).

Discussion

Immunohistochemical studies demonstrated the presence of AChE-IR and mAChRs-M1-IR neurons in the SP layer of the hippocampal CA1 and CA3 fields, and in CP and GP of the striatum in rats receiving 25 ml of RebA in dilutions of 1 mg/ml of water and 2 mg/ml

Table 2. The density, immuno-staining intensity, square area and nuclear-cytoplasmic (N/C) index of mAChRs-M1-IR neurons in hippocampal CA1, CA3 areas and striatal CP, GP in control rats (C), and animals which received 1 mg RebA/ml of water (I) and 2 mg RebA/ml of water (II). Data presented as mean with standard deviation. Different letters show statistically significant differences for p<0,05 in ANOVA test (A) or Kruskal-Wallis test (K).

	mAChRs-M1											
	CA1			CA3			CP			GP		
	C	I	II	C	I	II	C	I	II	C	I	II
Density	0.13 ±0.05^{a}	0.36 ±0.09$^{b(A)}$	0.35 ±0.06$^{b(A)}$	0.44 ±0.12^{a}	0.62 ±0.09$^{b(A)}$	0.57 ±0.11$^{b(A)}$	-	-	-	-	-	-
Immuno-staining intensity	39.64 ±9.79^{a}	51.94 ±12.27$^{b(A)}$	58.63 ±14.06$^{c(A)}$	18.79 ±7.46^{a}	56.5 ±13.94$^{b(K)}$	54.19 ±16.15$^{b(K)}$	33.7 ±8.8^{a}	51.4 ±14.1$^{b(A)}$	52.5 ±17.66$^{b(K)}$	40.6 ±12.91^{a}	59.74 ±11.08$^{b(A)}$	65.01 ±11.24$^{c(A)}$
Area (μm^2)	146.75 ±24.49	139.52 ±19.81	132.41 ±31.3	138.43 ±2.98	137.47 ±23.2	134.61 ±22.75	173.38 ±38.45^{a}	70,91 ±22.24$^{b(K)}$	63.41 ±12.52$^{b(K)}$	158.25 ±29.71^{a}	103.42 ±29.81$^{b(A)}$	130.84 ±34.16^{ab}
N/C index	0.58 ±0.08	0.53 ±0.12	0.53 ±0.1	0.42 ±0.08^{a}	0.49 ±0.08$^{b(A)}$	0.44 ±0.09^{ab}	0.41 ±0.09^{a}	0.5 ±0.1$^{b(A)}$	0.53 ±0.09^{b}	0.42 ±0.08	0.42 ±0.08	0.41 ±0.08

of water daily for 45 days. Morphological analyzes showed no significant changes in the structure of the immunopositive neurons after administration of RebA. Some studies performed by other authors have indicated possible negative, toxic and mutagenic effects of consuming steviol sweeteners (Nunes et al. 2007, Pasqualli et al. 2020). Although, most analyzes demonstrated no negative impact of steviol sweeteners on humans and animals (Momtazi-Borojeni et al. 2017, Casas-Grajales et al. 2019, Dyduch-Siemińska et al. 2020).

The presence of a cytoplasmic reaction for mAChRs-M1 can by explained by the engagement of G protein-coupled metabotropic receptors in signaling in the perikaryon and in the cellular nucleus. The intracellular localization of mAChRs-M1 may be the result of constitutive internalisation by a clathrin dependent mechanism or of internalisation stimulated by agonists of these receptors (Radu et al. 2017).

We found a higher number of AChE-IR neurons in the hippocampal CA1 field which was confirmed by morphometric analyses and an increase in the density of mAChRs-M1-IR neurons in the studied areas. A characteristic result in the CP and GP of the striatum in animals consuming the sweetener was an increase in the number of small, reactive neurons. The increase in the density of AChE-IR and mAChRs-M1-IR neurons in the hippocampus and striatum may indicate an increase in the activity of cholinergic transmission in these areas in rats receiving RebA for 45 days. This may induce synaptic plasticity and increase the excitability of these areas by influencing the presynaptic release of glutamate and gamma-aminobutyric acid (GABA) (Picciotto et al. 2012). The studied receptors modulate long-term potentiation and are highly expressed in areas of the brain where multiple cholinergic synapses are responsible for information acquisition and storage (Scarpa et al. 2020). Most reports indicate cholinergic cell loss in dementia-related disorders (Hampel et al. 2018, Ihara et al. 2018).

The results of own microscopic analysis revealed weaker immunostaining of AChE-IR neurons in CA1 and GP areas in the RebA-treated rats. It is difficult to explain the more pronounced decrease in the immunostaining intensity after using a lower dose of RebA in the CA1 field of the hippocampus. Research indicates that even twice the dose of steviol glycosides may not show the expected effect (Hazali et al. 2014). Moreover, the performance of these sweeteners may be dependent on various factors, e.g. the presence of extracellular Ca^{2+} ions (Abudula et al. 2004). In contrast, more intensely stained mAChRs-M1-IR neurons were present in the CA1 and CA3 areas of the hippocampus, and striatal CP and GP in rats from groups 1 and 2 than in the control animals.

The weaker immunostaining intensity of AChE-IR neurons may indirectly indicate an increase in the concentration of acetylcholine in the synapses, which allows the neurotransmitter to bind more mAChRs-M1 and to affect active sites for a longer time. Stimulation of mAChRs-M1 leads to an improvement in cognition, which was demonstrated in patients with Alzheimer's dementia (Sharma et al. 2010, Betterton et al. 2017, Arpita et al. 2019, Yi et al. 2020). Thus, the increase in the immunostaining of mAChRs-M1-IR neurons found in the present study may suggest the influence of RebA on the activity of the studied receptors, which determine neurotransmission in memory and learning processes (Krawczyk et al. 2021). In addition, stevioside, a glycoside from the stevia plant, reverses the negative effects of scopolamine which increases AChE activity in rats. A single, high dose of stevioside administered together with scopolamine for 30 minutes, prior to the test performed in the Morris water maze,

reversed the effect of the dementia-inducing alkaloid significantly, by decreasing the AChE activity, improving memory in comparison to rats that received only the mAChRs antagonist. The steviol sweetener counteracts the scopolamine-induced oxidative stress in the brain. Furthermore, immunohistochemical studies have shown that scopolamine significantly reduces the density of pyramidal, mAChRs-M1-IR neurons in the rat hippocampus (Sharma et al. 2010, Jahanshahi et al. 2013). Few publications describe the positive effects of steviol sweeteners on memory and learning. An alcoholic extract of Stevia rebaudiana administered orally for 16 days significantly improved learning abilities and memory in young and older rats reducing the AChE activity. The authors explain that the effect obtained can be attributed to the antioxidant and anti-inflammatory properties of stevia (Juyal et al. 2010a,b). This is also indicated by more recent studies (Chayushyan et al. 2017).

Morphometric analysis of other parameters of AChE-IR and mAChRs-M1-IR cells, i.e. N/C index and surface area did not reveal large differences that could be considered significant and influencing functional changes in the brains of animals receiving RebA. The most characteristic changes were found in the striatum where small cells reactive for both AChE and mAChRs-M1 were observed in rats receiving RebA. Hence, morphometric analyzes showed a decrease in AChE-IR cells surface areas in the striatal CP in group I, and in the striatal GP in groups I and II. Similar results were obtained by examining mAChRs-M1-IR neurons in striatal CP groups I and II and striatal GP group I.

Based on physiological and biochemical studies a positive effect of steviol glycosides on memory and learning was established (Juyal et al. 2010a,b, Sharma et al. 2010). Our own analyzes indicate that RebA influences the immunoreactivity of AChE and mAChRs-M1 in hippocampal and striatal neurons at a lower and higher dose. On this basis, it can be concluded that the studied steviol glycoside, through its effect on the cholinergic system, may play a positive role in the functioning of memory and learning processes. This sweetener has no significant effect on the morphology of nerve cells. Further long-term studies of neurons in multiple regions of the brain using steviol glycosides at acceptable daily doses are required.

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