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Original article

# The influence of bilberry fruit on memory and the expression of parvalbumin in the rat hippocampus

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## Abstract

Therefore, the aim of the present study was to evaluate the possible effect of bilberry fruit (*Vaccinium myrtillus* L.) supplement in a daily diet on the cognitive behaviour of the rats and the expression of parvalbumin (PV) in populations of hippocampal neurons. It has been postulated that the antioxidants present in bilberry fruit may act as neuroprotective factors playing also a significant role as memory enhancements. Forty Wistar rats with a similar average body weight ( $460 \pm 0.4$  g) were divided into four groups (n=10 per group). The control group received standard feed (210 g/week), whereas animals of experimental groups received standard feed supplemented with bilberry (*per os*) at consumed doses of 2 g (group I), 5 g (group II), and 10 g/kg b.w./day (group III). After three months of feeding with bilberry, the modified elevated plus-maze test (*mEPM*) was performed. After 32 weeks of feeding, brains were collected and PV-immunoreactive (ir) neurons were immunohistochemically visualized. In the modified elevated plus-maze test, transfer latency examined 2 h and 24 h after the acquisition session was significantly shorter ( $p < 0.05$ ) in the group II in comparison with the control group. In CA1 and CA2/CA3 hippocampal fields as well as dentate gyrus of all experimental groups, a significant ( $p < 0.05$ ) decrease in number of PV-ir neurons were found. In relation to the control group, the mean subpopulation of PV-ir neurons found in groups II and III were significantly reduced. The subpopulations of PV-ir neurons found in DG of all experimental groups were significantly reduced in comparison to the control. In conclusion the in the present paper we demonstrated a relationship between the diet rich in a bilberry fruit and process of memory as well as numbers of calcium-binding protein-expressing hippocampal neurons. Our results may be source of basic knowledge for further research aiming at neuroprotective role of the bilberry fruit.

**Key words:** bilberry, calcium-binding proteins, hippocampus, memory, rat

## Introduction

Bilberry (*Vaccinium myrtillus* L.) is well-known as a source of antioxidants (Baum et al. 2014), including polyphenolic compounds (Veberic et al. 2015). Oral administration of an extract prepared from bilberry fruit has been shown to reduce low-grade inflammation (Kolehmainen et al. 2012) to decrease lipoprotein levels (Madihi et al. 2013), and to protect against atherogenesis (Mauray et al. 2010). Ramirez et al. (2005) showed that a diet containing *Vaccinium* berries enhanced memory, especially the working and short-term memory in adult rats.

The limbic system is the structure of the brain that plays an important role in the learning mechanisms and the memory processes. It consists of a number of interconnected neuronal structures including the main centre – the hippocampus. It is associated with the processes of the memorizing the new information in the declarative memory (descriptive) and transferring this to the long-term memory (El-Falougy and Benuska 2006). In rats, hippocampus is the place of location for “cognitive maps” that allow animals to orientate themselves in space during their migrations. Pyramidal neurons within the hippocampus are known as “the place cells” and become active when the animals enter a particular place in their environment (Moser et al. 2008). Calcium-binding proteins (CaBPs) also act in the proper functioning of the limbic system via the reduction of calcium ion ( $\text{Ca}^{2+}$ ) levels in the neuroplasm, because their excess can lead to neurotoxicity and cell death. Any irregularities in the functioning of CaBPs may lead to diseases of the central nervous system (CNS) (Schwaller et al. 2002). Parvalbumin (PV) is one of the most characteristic CaBPs in CNS (Müller et al. 2007). PV is a buffering protein and its main task is to reduce the level of  $\text{Ca}^{2+}$  in the cytoplasm.

The aim of the current study was to find out whether bilberry fruit supplemented to a daily diet influences the expression of PV in the hippocampus, and thus to determine whether the components present in the bilberry fruit can modify and improve the memory processes.

## Materials and Methods

### Plant material

Fresh bilberry fruit (10 kg) harvested in forests in Lublin province was purchased at the local market in Lublin (51°14'29.3604" N, 22°29'47.9544" E). The identity of fruit samples was authenticated by Prof Kazimierz Główniak (Department of Pharmacognosy with the Medicinal Plant Laboratory, Medical Universi-

ty of Lublin, Poland). Bilberries were gradually mashed up using a Thermomix TM31 (Vorwerk, Germany) for 3 min (maximal speed at ambient temperature) followed by portioning and freezing ( $-20^{\circ}\text{C}$ ) until use.

### Animals

Forty male Wistar rats were obtained from the Centre for Experimental Medicine of the Medical University of Białystok, Poland. This study was performed in compliance with institutional guidelines and approved by the 2<sup>nd</sup> Local Ethics Committee at the University of Life Sciences in Lublin, Poland (License No. 105/2010 and 34/2012). Every effort was made to minimize animal suffering. Animals were caged individually with free access to standard rodent feed (LSM, Agropol S.J., Poland) and water *ad libitum*, and they were maintained on a 12-h-light/12-h-dark cycle, at  $23 \pm 1^{\circ}\text{C}$ .

### Experimental diet

Twenty-eight-week-old animals (weight 400 – 530 g) were divided into four groups ( $n=10$ ) with a similar average body weight ( $460 \pm 0.4$  g). The control group received standard rodent feed (210 g/week), whereas experimental groups of animals received standard feed supplemented with bilberry (*per os*) at consumed doses of 2 g (group I), 5 g (group II), and 10 g/kg b.w./day (group III). All rats received their respective diets until euthanization at the age of 60 weeks. Body weight and the weight of unconsumed feed were recorded every week.

### Modified elevated plus-maze test (mEPM)

Cognitive behaviour was evaluated using the mEPM learning task. The procedure was performed after 3 months of the experiment. The plus-maze was made of wood and consisted of two open arms ( $50 \times 10$  cm) and two enclosed arms ( $50 \times 10 \times 25$  cm). The arms were connected by a central platform ( $10 \times 10$  cm). The maze was painted black and elevated to 50 cm above the floor. The experiment was conducted in a quiet, dark room, and the central platform of the maze was illuminated by red light. In the acquisition session, each rat was gently placed at the distal end of an open arm of the maze and transfer latency (TL1) was recorded as described by Kruk et al. (2012). The retention sessions were performed 2 h (TL2) and 24 h (TL3) after the acquisition session (TL1).

### Immunohistochemistry and antibodies

The brains from 40 male rats were stored in 10% buffered formalin (pH=7) for 12 h at  $4^{\circ}\text{C}$ , dehydrated in ethyl alcohol, followed by embedding in paraffin

Table 1. Body weight, brain weight and feed intake in rats during supplementation with bilberry. Values are given as mean ± SD from 10 rats in each group: control (no fruit); I (2.0 g bilberry fruit/kg b.w./day); II (5.0 g bilberry fruit/kg b.w./day); III (10.0 g bilberry fruit/kg b.w./day). Values sharing various letters between groups (vertically, a–b) differ significantly at p<0.05.

Group	Initial body weight [g]	Initial feed intake [g/week]	Body weight after 12 weeks [g]	Feed intake after 12 weeks [g/week]	Body weight after 32 weeks [g]	Feed intake after 32 weeks [g/week]	Brain weight after 32 weeks [g]
Control	459.9 ± 62.7 <sup>a,A</sup>	138.4 ± 16.2 <sup>b,A</sup>	472.2 ± 56.1 <sup>a,A</sup>	146.4 ± 14.6 <sup>a,A</sup>	485.6 ± 58.2 <sup>a,A</sup>	129.6 ± 14.7 <sup>a,A</sup>	2.025 ± 0.059 <sup>a</sup>
I	460.4 ± 37.1 <sup>a,A</sup>	121.9 ± 9.7 <sup>a,A</sup>	483.9 ± 40.7 <sup>a,A</sup>	148.4 ± 9.1 <sup>a,C</sup>	502.0 ± 39.7 <sup>a,A</sup>	132.6 ± 8.9 <sup>a,B</sup>	2.193 ± 0.104 <sup>ab</sup>
II	461.8 ± 27.2 <sup>a,A</sup>	123.5 ± 7.4 <sup>a,A</sup>	491.3 ± 34.5 <sup>a,AB</sup>	153.6 ± 10.1 <sup>a,B</sup>	517.6 ± 43.4 <sup>a,B</sup>	134.4 ± 12.3 <sup>a,A</sup>	2.222 ± 0.077 <sup>b</sup>
III	460.8 ± 26.5 <sup>a,A</sup>	123.0 ± 13.6 <sup>a,A</sup>	480.9 ± 30.3 <sup>a,AB</sup>	158.7 ± 15.7 <sup>a,C</sup>	509.9 ± 28.9 <sup>a,B</sup>	139.4 ± 11.4 <sup>a,B</sup>	2.212 ± 0.198 <sup>b</sup>

Values sharing various letters within a group during the experiment (horizontally, A–C) differ significantly at p<0.05.

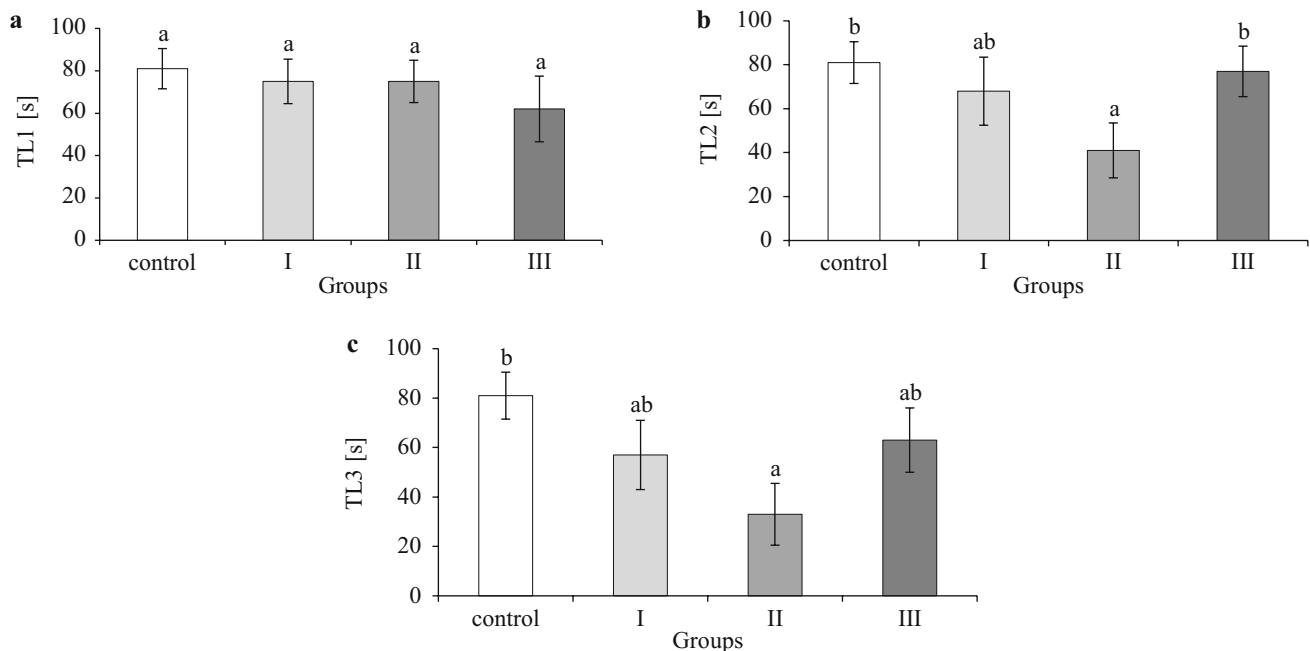


Fig. 1. Effect of the feeding of rats with bilberry on the transfer latency during the acquisition session – TL1 (a) and after 2 h – TL2 (b) or 24 h – TL3 (c). Groups: control (no fruit); I (2 g/kg b.w./day); II (5 g/kg b.w./day); III (10.0 g/kg b.w./day). Values are given as mean ± SD (n=10). Values sharing different letters (a–c) differ significantly at p<0.05

blocks according to a previously described method (Szalák et al. 2015). Briefly, the paraffin blocks were cut into 5 µm-thick sections which were placed on silanized glass slides (SuperFrostPlus, Germany). The sections were incubated for 24 h at 4°C with primary monoclonal antibodies raised against PV (1:2000; SWANT, PV25, Switzerland). Next day, the slides were washed in a washing buffer (2 × 15 min) and covered with anti-mouse/rabbit Ig (ImPRESS<sup>TM</sup>; MP-7500 Vector, USA) for 1 h. For the visualization of primary antisera, 3,3'-diaminobenzidine (DAB, Vector, USA) chromogen was used. A working solution of DAB was applied onto the slides and the process was monitored under a light microscope. Finally, the slides were rinsed in distilled water. Counterstaining (for 20 min) with Mayer's hematoxylin was performed. After washing in distilled water, the slides were dehydrated in an ethyl alcohol series, cleared in xylene,

mounted in Canadian balm and coverslipped. The slides were viewed under a light microscope (Axiolab, Zeiss, Germany) connected to a digital camera. From each animal, approx. 25–30 sections immunostained for PV were analyzed. No less than one hundred of PV-ir neurons in CA1, CA2/CA3 and DG and were viewed and counted. The specificity of the antibodies used was verified using a negative control in which primary antibodies were replaced with the same concentrations of appropriate non-immune IgG.

### Statistical analysis

Results were expressed as mean ± standard deviation (SD). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test (p<0.05).

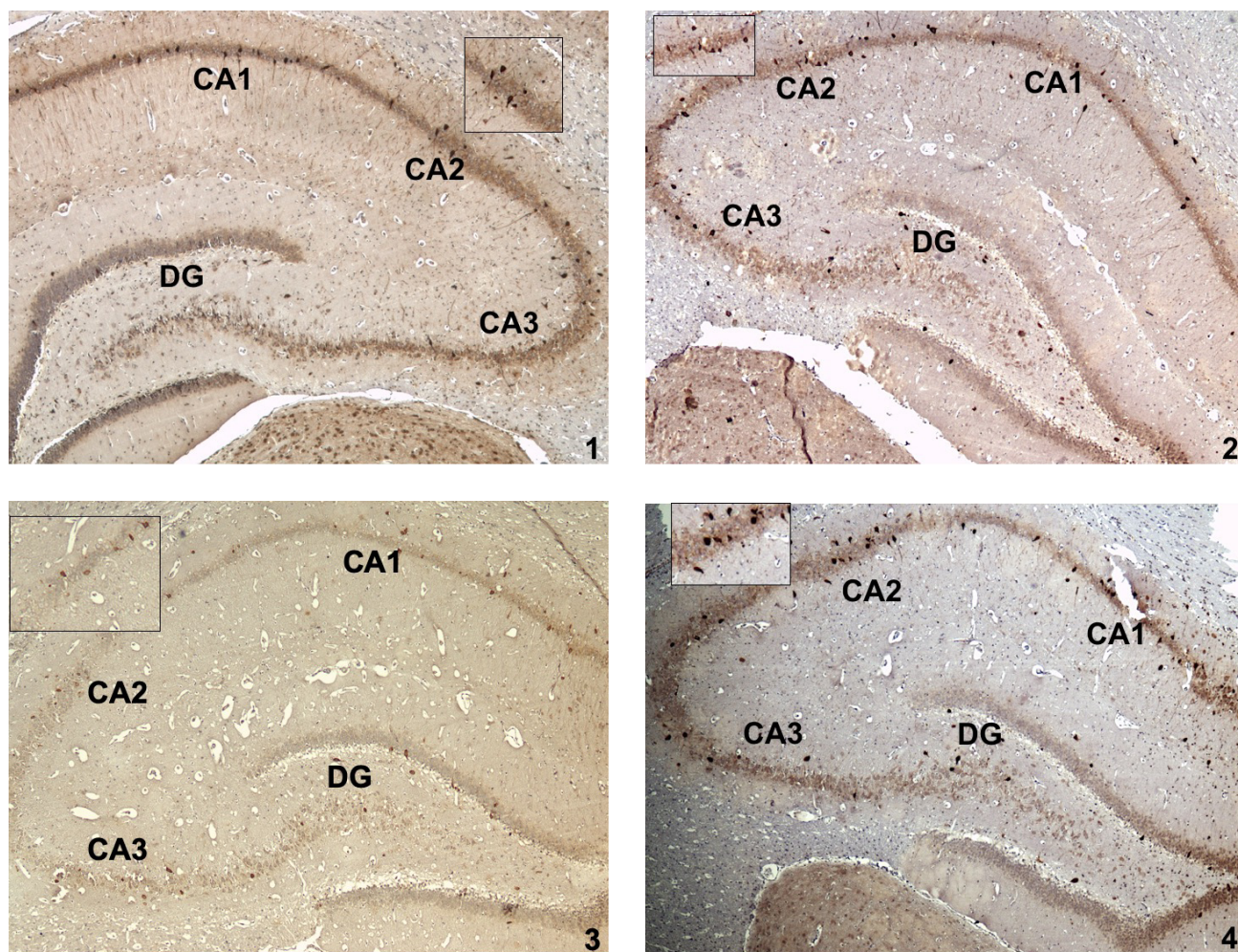


Fig. 2. Immunoreactivity to PV in moderate numbers of neurons of the rat fields CA1, CA2/CA3 and DG of the hippocampus are seen in (1–4): 1. Control group; 2. Experimental group I (2.0 g bilberry fruit/kg b.w./day); 3. Experimental group II (5.0 g bilberry fruit/kg b.w./day); 4. Experimental group III (10.0 g bilberry fruit/kg b.w./day). Some PV-ir neurons were exposed. Magnification  $\times 4$  in (1–4)

## Results

### Body weight, brain weight and feed intake

The changes in body weight, brain weight and feed intake in the adult rats during the supplementation with bilberry for 32 weeks are presented in Table 1. In the first week of the study, feed intake in the control group was significantly higher ( $p < 0.05$ ) than in experimental groups fed bilberry. However, there were no differences in standard feed intake between the groups or in the body weight between all experimental groups during the subsequent weeks of the study. Brain weight was significantly higher ( $p < 0.05$ ) in experimental groups fed bilberry in comparison with the control group. On the other hand, rats in the groups II and III had a significantly ( $p < 0.05$ ) increased body weight during the study. The consumption of standard feed enriched with bilberry fruit was notably ( $p < 0.05$ ) increased over the period.

### Memory evaluation

After three months of the experiment, the cognitive behaviour of all rats was determined (Fig. 1). There were no significant ( $p < 0.05$ ) differences in the average transfer latency in the acquisition session between all experimental groups (Fig. 1a). The average transfer latency ranged from  $62.0 \pm 15.5$  s (in the group III) to  $81.0 \pm 9.5$  s (in the control group). However, TL2 (Fig. 1b) and TL3 (Fig. 1c) were significantly ( $p < 0.05$ ) shorter in the case of the group II in comparison with the control group. The average time TL2 and TL3 were  $41.0 \pm 12.5$  s (2 h) and  $33.0 \pm 12.5$  s (24 h), respectively.

### Immunohistochemistry

In both the control group and the experimental groups, immunoreactivity to PV was found in relatively numerous neurons of all studied fields of the rat hippocampus (CA1 and CA2/CA3), as well as in neurons of the dentate gyrus (DG). Additionally, in the control group, immunoreactivity to PV was observed in numer-

Table 2. The average numbers of PV-ir neurons in CA1, CA2/CA3 hippocampal fields as well as DG of animals from control and experimental groups: I (2.0 g bilberry fruit/kg b.w./day); II (5.0 g bilberry fruit/kg b.w./day); III (10.0 g bilberry fruit/kg b.w./day). Statistically significant differences are marked A (vs. control), B (vs. group I), or C (vs. group II) ( $p < 0.05$ )

	CA1 (neurons PV-ir)	CA2/CA3 (neurons PV-ir)	DG (neurons PV-ir)
Control group	246.8 ± 6.7	191.1 ± 4.4	52.1 ± 2.9
Group I	103.9 ± 3.3 <sup>A</sup>	81.3 ± 2.1 <sup>A</sup>	31.5 ± 2.1 <sup>A</sup>
Group II	70.1 ± 1.8 <sup>A,B</sup>	58.2 ± 3.6 <sup>A,B</sup>	41.4 ± 3.7 <sup>A,B</sup>
Group III	75.6 ± 1.4 <sup>A,B</sup>	72.0 ± 2.6 <sup>A,C</sup>	32.1 ± 2.5 <sup>A</sup>

ous nerve fibers emerging from parvalbumin-immunoreactive (PV-ir) neurons of CA1 and CA2/CA3 hippocampal fields, while in DG, only in the case of a few PV-ir neuronal perikarya was PV-ir nerve processing visible (Fig. 2). The average numbers of PV-ir neurons found in fields of the hippocampus as well as in DG of control and experimental groups are summarized in Table 2. When compared to the control group in all hippocampal fields as well as DG of all experimental groups, significantly decreased numbers of PV-ir neurons were found ( $p < 0.05$ ). In relation to the control group, the average subpopulation of PV-ir neurons found in experimental groups I, II, and III was reduced to approx. 57%, 70%, and 65.5%, respectively. In comparison to the controls, the subpopulations of PV-ir neurons found in DG of experimental groups I, II, and III were significantly reduced to approx. 40%, 21%, and 38%, respectively ( $p < 0.05$ ).

## Discussion

In the present study, the average brain weight of rats fed bilberry was significantly ( $p < 0.05$ ) higher in comparison with the control group. 32 weeks into the experiment, rats were 60–64 weeks old which can be compared to humans aged 40–45 years (Sengupta 2013). Aksenov et al. (2013) also showed that the supplementation of the diet of 64–72-week-old mice with a complex dietary supplement (vitamins, plant extracts, and minerals) resulted in a 7–11% increase in the weight of brains ( $p < 0.02$ ). In contrast, brain weight decreases during normal aging (Ferretti et al. 2010).

To evaluate the effect of feeding rats with bilberry fruit on short- and long-term memory, the modified elevated plus-maze (mEPM) test was used. This is based upon the aversive behavior of rodents to open and high spaces (Kruk et al. 2012). As a result of training (repeated exposure of animals to open arms), transfer latency is expected to be shorter. Given this result, it was assumed that a diet enriched with 5 g of bilberries/kg b.w./day (group II) for 3 months can positively affect cognitive functions. Our results

confirmed the findings presented in previous studies on the bilberry and other *Vaccinium* berries. Ramirez et al. (2005) showed that a diet enriched with berries enhanced memory, especially the working and short-term memory in adult rats. Similar results were presented by Krikorian et al. (2010), who investigated the administration of blueberry juice to older adults (aged 70–80 years) for 12 weeks. Due to the results of the improved paired associated learning and word list recall tests, memory improvement and the reduction of depressive symptoms could be proven in this study. It is also worth mentioning other studies. First, Vepsäläinen et al. (2013) showed that the long-term supplementation of a diet with bilberry extract caused a decrease in amyloid  $\beta$  ( $A\beta$ )  $A\beta$ 1-40 and  $A\beta$ 1-42 levels in brains of aged APdE9 mice and an alleviated deficit of the spatial working memory in these animals. Secondly, Yamakawa et al. (2016) suggested that a diet containing 1% of anthocyanoside extract from bilberry might protect mice suffering from Alzheimer's disease (AD) from cognitive degeneration. Moreover, this protection was accompanied by an increase in the levels of insoluble deposits of  $A\beta$ 1-42. Finally, Rahman et al. (2008) reported on the prevention of oxidative stress in mice brains induced by psychological stress (whiskers cut model) as a result of orally administered anthocyanins isolated from bilberry. Therefore, bilberry anthocyanins could be useful for the treatment of neurodegenerative diseases associated with oxidative stress and it is assumed that bioactive compounds from bilberry fruit could prevent memory deficiency.

$Ca^{2+}$  ions are one of the most important factors influencing the proper functioning of brain neurons, thus regulating the fundamental cellular mechanisms underlying vital mental processes such as learning and/or memory (Schwaller et al. 2002). Amongst many neuronal synthesized CaBPs, PV seems to be of a particular importance because of its ability to reduce the concentration of free  $Ca^{2+}$ . Our preliminary study clearly indicated that after feeding with bilberry fruit, a significant reduction of PV-expressing hippocampal neurons was observed in all experimental groups. The group II was characterized by the lowest percent-

age of PV-positive hippocampal neurons. It seems that changes in the expression of CaBPs may be present during certain neurodegenerative diseases such as AD, but these findings are sometimes contradictory. For example, a significant reduction of PV-ir neurons has been observed in the neocortex of patients with AD (Satoh et al. 1991), but in other studies this effect has not been noted (Ferrer et al. 1991). As mentioned above, the expression of PV-positive neurons was significantly reduced in the brains of the experimental animals, which may lead to the belief that antioxidants present in bilberry fruit probably directly downregulate the cellular levels of Ca<sup>2+</sup> and potentially protect neurons from cellular damage. Antioxidants have been previously shown to exhibit neuroprotective activity, mainly by enhancing blood flow and angiogenesis in the brain (Spencer 2010) and by downregulation of pro-apoptotic proteins of the Bcl-2 family (Vauzour et al. 2007). Several studies have indicated that antioxidants are able to evoke hippocampal neurogenesis and the process of neuronal differentiation may be continued throughout the whole adult life (Gould et al. 1999). Given the above results it is likely that dietary supplementation with bilberry fruit may have a beneficial neuroprotective effect, resulting in enhanced memory processes.

## Conclusions

In summary, the findings of our study demonstrated that the administration of bilberry (5 g/kg b.w./day) to adult Wistar rats for 32 weeks resulted in the improvement of short- and long-term memory ( $p < 0.05$ ) in comparison to the control group. In our opinion, the inclusion of bilberry fruit in the diet could contribute to memory enhancement. The long-term administration of bilberry seems to cause an increase in the weight of the brain. Since the number of PV-expressing hippocampal neurons was significantly reduced after the supplementation of the diet with the bilberry fruit, we suggest that antioxidants present in bilberry fruit may influence the metabolism of neuronal Ca<sup>2+</sup> ions. Taking into account the results of our study, it is possible that high dose of bilberry fruit supplemented to the daily diet may potentially exhibit memory enhancing effects, however further *in vivo* and *in vitro* investigations clarifying these mechanisms are needed.

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