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Diogo Fernandes da Silva

Old-onset caloric restriction effects in neuropeptide Y, somatostatin and cholinergic varicosities in the hippocampal dentate hilus of aged rats

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Old-onset caloric restriction effects in neuropeptide Y, somatostatin and cholinergic varicosities in the hippocampal dentate hilus of aged rats

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Old-onset caloric restriction effects on neuropeptide Y- and somatostatin-containing neurons and on cholinergic varicosities in the rat hippocampal formation

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Abstract Caloric restriction is able to delay age-related neurodegenerative diseases and cognitive impairment. In this study, we analyzed the effects of old-onset caloric restriction that started at 18 months of age, in the number of neuropeptide Y (NPY)- and somatostatin (SS)-containing neurons of the hippocampal formation. Knowing that these neuropeptidergic systems seem to be dependent of the cholinergic system, we also analyzed the number of cholinergic varicosities. Animals with 6 months of age (adult controls) and with 18 months of age were used. The animals aged 18 months were randomly assigned to controls or to caloric-restricted groups. Adult and old control rats were maintained in the ad libitum regimen during 6 months. Caloric-restricted rats were fed, during 6 months, with 60 % of the amount of food consumed by controls. We found that aging induced a reduction of the total number of NPY- and SS-positive neurons in the hippocampal formation accompanied by a decrease of the cholinergic varicosities. Conversely, the 24-month-old-onset caloric-restricted animals maintained the number of those peptidergic neurons and the density of the cholinergic varicosities similar to the 12-month control rats. These results suggest that the aging-associated reduction of these neuropeptide-expressing neurons is not due to neuronal loss and may be dependent of the cholinergic system. More importantly, caloric restriction has beneficial effects in the NPY- and SS-expressing neurons and in the cholinergic system, even when applied in old age.

Keywords Caloric restriction · Hippocampus · Neuropeptide Y · Somatostatin · Acetylcholine

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Introduction

Aging is a natural process that is characterized by accumulation of several biological alterations that lead to a progressive decline of physical and cognitive functions (Fontana et al. 2010). Until know, caloric restriction, i.e., reducing caloric consumption without causing malnutrition, is the only known nongenetic intervention capable of increasing the mean and maximal lifespan and delay age-related diseases and cognitive decline (Weindruch 1996; Roth et al. 2001; Anton and Leeuwenburgh 2013; Cava and Fontana 2013). This radical nutritional intervention also induces several beneficial effects in the health of many species, including mammals and nonhuman primates (Fontana et al. 2010; Roth and Polotsky 2012). These effects include amelioration or reduction of obesity,

diabetes mellitus, nephropathy, cancer, and cardiovascular diseases (Andrade et al. 2002; Colman et al. 2009; Fontana et al. 2010; Mattison et al. 2012). Furthermore, in the brain, caloric restriction has been shown to protect neurons from several metabolic and ischemic insults (Bruce-Keller et al. 1999; Duan and Mattson 1999; Andrade et al. 2002, 2006), increase the resistance to epileptic seizures (Bough et al. 1999; Azarbar et al. 2010), and decrease or delay age-associated cognitive decline and some neurodegenerative diseases (Gillette-Guyonnet and Vellas 2008; Del Arco et al. 2011).

Several mechanisms appear to be involved in the abovementioned beneficial effects of caloric restriction in the central nervous system (CNS). One mechanism can be related to the reduction of the oxidative damage due to the decrease of the mitochondria glucose availability and consequently to diminished oxidative free radical release that alter several biomolecules (Sohal et al. 1994; Merry 2004; Marchal et al. 2013; Picca et al. 2013). Another possible mechanism is that the mild stress induced by caloric restriction induces the increase of the expression of neuroprotective bioactive compounds such as heat-shock proteins and several neurotrophic factors, such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) (Duan et al. 2001; Lee et al. 2002) that can protect neurons and increase their survival or prevent aging-associated neuronal degeneration (Lee et al. 2002; Cuello 2012; Perovic et al. 2013).

The gamma-aminobutyric acid (GABA)-ergic interneurons are one of the neuronal populations more vulnerable to the normal aging process (Vela et al. 2003). Indeed, it has been shown that aging is associated with a reduction of the GABAergic subpopulations that express neuropeptide Y (NPY) and somatostatin (SS) in several brain areas such as the hypothalamus, striatum, and cortical regions (Kowalski et al. 1992; Cha et al. 1996; Zhang et al. 1998; Cadacio et al. 2003; Cardoso et al. 2006; Stanley et al. 2012; Pereira et al. 2013; Spiegel et al. 2013). One of those cortical regions is the hippocampal formation (HF), a limbic area involved in several cognitive functions, including spatial learning and memory (Morris 1984; Eichenbaum 1999; Aggleton and Brown 2006; Cardoso et al. 2011). In fact, the interneurons of the HF expressing NPY and SS represent one of the neuronal populations most affected by aging (Cadacio et al. 2003; Vela et al. 2003; Patrylo et al. 2007).

Taking into account that aging provokes reduction of the NPY and SS expression in the HF of the Rat, we sought to analyze if caloric restriction has the capacity to prevent the loss of the neuronal expression of these neuropeptides in the dentate hilus, CA3, and CA1 subfields. Furthermore, knowing that in the cortex, the NPY- and SS-ergic systems seem to be dependent of cholinergic system (Jolkkonen et al. 1997; Zhang et al. 1998; Cardoso et al. 2006; Potier et al. 2006), we also studied the effects of caloric restriction upon the cholinergic fibers by analyzing the vesicular acetylcholine transporter (VAChT) immunoreactive (IR) varicosities density. Finally, because humans have difficulties engaging in caloric restriction regimen over the long-term (Scheen 2008; Anton and Leeuwenburgh 2013), we decided to start the nutritional deprivation at 18-month-old rats (old-onset) and to verify if the chronic treatment starting at this age is enough to induce the expected beneficial effects. In other words, we aimed to verify if caloric restriction started at this advanced age presents at least some of the numerous benefits observed in younger rats (Andrade et al. 2002; Rich et al. 2010; Roth and Polotsky 2012; Cardoso et al. 2013). We centered this study of old-onset caloric restriction in the HF because it is one of the brain regions most affected by nutritional deficits (Cintra et al. 1990; Hipólito-Reis et al. 2013) and aging (Cadacio et al. 2003; Stanley et al. 2012), added to its importance in cognitive functions (Morris 1984; Eichenbaum 1999; Aggleton and Brown 2006; Cardoso et al. 2011).

Material and methods

Animals and diets

Male Wistar rats obtained from the colony of the Institute of Molecular and Cell Biology (Porto, Portugal) were maintained under standard laboratory conditions (20–22 °C and a 12-h light/dark cycle) with free access to food and water. In the present study, 6 animals aged 6 months and 12 animals aged 18 months were used. At the beginning of the experimental study, the animals were housed individually to allow daily quantification of liquid and food consumption. Rats were weighed weekly and bedding was changed at the same time minimizing stress due to handling. The animals with 6 months of age (adult controls) have maintained the ad libitum consumption of standard laboratory chow (Mucedola, Italy) containing proteins (17 %) supplemented with lysine (0.7 %), methionine (0.3 %) and cysteine (0.5 %), carbohydrates (57 %), fat (4 %), and salts (7 %) throughout the entire experimental period (6 months). The animals with

18 months of age were randomly assigned to old control and old caloric-restricted groups. Old control rats maintained the ad libitum consumption of standard laboratory chow, referred above, throughout the entire experimental period (6 months). Caloric-restricted rats were fed, during 6 months, with 60 % of the amount of food consumed by old control animals (Andrade et al. 2002; Cardoso et al. 2013). These caloric-restricted rats were fed once a day at 0900 hours, and food was available until depletion. All diets were supplemented with diet vitamin fortification mixture (MP Biomedicals, USA), to avoid differences due to the micronutrient intake. All rats had free access to water throughout the experimental period. At the end of the experimental study, we had a group of 12-month adult controls, a group of 24-month-old controls, and a group of 24-month-old caloric-restricted animals. All animals were euthanized at the same time.

The handling and care of the animals followed the Principles of Laboratory Animal Care (NIH Publication No. 86–23, revised 1985) and the European Communities Council Guidelines in Animal Research (86/609/UE). All efforts were made to minimize the number of animals used and their suffering.

Tissue preparation

General procedures

Animals were deeply anesthetized with sodium pentobarbital (80 mg/kg body weight, intraperitoneal) and transcardially perfused with 150 ml of 0.1 M phosphate buffer followed by a fixative solution containing 4 % paraformaldehyde in phosphate buffer at pH 7.6. The brains were removed from the skulls, coded for blind processing and analysis, and separated by a midsagittal cut into right and left halves. The frontal and occipital poles were removed, and the remaining blocks of tissue containing the HF were separated and processed for glycolmethacrylate embedding or immunocytochemistry. Because prior studies have shown that the HF of rodents display right/left asymmetries (Slomianka and West 1987), the blocks were alternately sampled from the right and left hemispheres, in order that whatever the procedure performed, the HF from both sides were included.

Glycolmethacrylate embedding

After perfusion, the blocks containing the HF destined to glycolmethacrylate embedding were postfixed during 60 days in a fixative solution containing 1 % paraformaldehyde and 1 % glutaraldehyde in 0.12 M phosphate buffer at pH 7.4. After that, the blocks were dehydrated through a graded series of ethanol solutions and embedded in glycolmethacrylate, as described in detail elsewhere (West et al. 1991). These blocks were then serially sectioned in the coronal plane at a nominal thickness of 40 μm using a Jung Multicut microtome. Every tenth section was collected using a systematic random sampling procedure (Gundersen and Jensen 1987), mounted serially, and stained with a modified Giemsa solution (West et al. 1991).

Immunocytochemistry for NPY, SS, and VAChT

After perfusion, the blocks destined to immunocytochemistry containing the HF were stored for 1 h in the fixative solution used in the perfusion and maintained overnight in the 10 % sucrose solution at 4 °C. Blocks were then mounted on a vibratome, serially sectioned in the coronal plane at 40 μm and collected in phosphate-buffered saline (PBS). From each brain, three sets of vibratome sections containing the HF were selected, using a systematic random sampling procedure, to be used for immunostaining of NPY, SS, and VAChT (Cardoso et al. 2006, 2010). Sections were washed twice in PBS, treated with 3 % H_2O_2 for 10 min to inactivate endogenous peroxidase and incubated overnight at 4 °C with the primary polyclonal antibody against either NPY or SS (Bachem; 1:10,000 dilution in PBS) or VAChT (Chemicon; 1:15,000 dilution in PBS). Thereafter, the sections were washed twice and incubated with the respective biotinylated secondary antibody (Vector Laboratories, Burlingame, CA, USA; 1:400 dilution in PBS). Sections were then treated with avidin-biotin peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories; 1:800 dilution in PBS). In the two last steps, the incubation was carried out for at least 1 h at room temperature. Following treatment with the peroxidase complex, sections were incubated for 10 min in 0.05 % diaminobenzidine (Sigma) to which 0.01 % H_2O_2 was added. Sections were rinsed with PBS for at least 15 min between each step. To increase the tissue penetration, 0.5 % Triton X-100 was added to PBS that was used in all immunoreactions and washes. Specificity of the immune reactions was controlled by omitting the incubation step with primary antisera. All immunochemical reactions and washings described above were carried out in 12-well tissue culture plates,

four sections in each well, to assure that staining of the sections from all groups analyzed was performed in parallel and under identical conditions. Following termination of the staining procedures, sections were mounted on gelatin-coated slides and air-dried. They were then dehydrated in a series of ethanol solutions (50, 70, 90, and 100 %) and coverslipped using Histomount (National Diagnostics, Atlanta, GA, USA).

Morphometric analysis

Estimation of total number of neurons in dentate hilus

The total number of neurons was estimated on glycolmethacrylate-embedded sections by applying the optical fractionator method (West et al. 1991). The boundaries of the granular layer of the dentate gyrus and hilus were consistently defined at all levels along the septotemporal axis of the HF on the basis of cell morphology and cytoarchitectonic criteria (Fig. 1) (Amaral and Witter 1995). Estimations were carried out using the C.A.S.T.-Grid System (Olympus, Denmark) in an average of 14 sections per animal. Beginning at a random starting position, visual fields were systematically sampled along the *x*- and *y*-axes, using a raster pattern procedure. Neurons were counted in every frame using the optical disector at a final magnification of ×2000. The coefficient of error (CE) of the individual estimates was calculated according to Gundersen et al. (1999) and ranged between 0.07 and 0.09.

Estimation of the total number of NPY-IR and SS-IR cells in the dentate hilus, CA3, and CA1 subfields

Neurons immunoreactive to NPY and SS were identified as darkly stained perikarya and on the basis of their location and morphology (Figs. 1, 2, and 3). The total number of these neurons was estimated using the optical fractionator method (West et al. 1991). The boundaries of the dentate hilus, CA3, and CA1 *stratum pyramidale* subfields were consistently defined at all levels along the septotemporal axis of the HF on the basis of cytoarchitectonic criteria (Amaral and Witter 1995) and using the Rat brain atlas of Paxinos and Watson (1998). Neuron counting was carried out using the Olympus C.A.S.T.-Grid System (Denmark), and a mean of 12 systematically sampled sections was used per animal. Beginning at a random starting position, visual fields were systematically sampled along the *x*- and *y*-axes, using a raster pattern procedure. The neuronal nuclei were selected as a convenient counting unit. They were counted in every frame using the optical disector at a final magnification, at the level of the monitor, of ×2000. The CE of the individual estimates was calculated according to Gundersen and collaborators (1999) and ranged between 0.08 and 0.10.

Estimation of the areal density of VAChT-positive varicosities in the dentate hilus, CA3, and CA1 subfields

The cholinergic varicosities stained with VAChT were counted using a computer-assisted image analyzer (Leica QWin) fitted with a Leica DMR microscope and Leica DC 300F video camera. For each animal, ten VAChT-stained sections, adjacent to those used for counting NPY-IR and SS-IR cell profiles, were analyzed. Measurements were performed at a final magnification of ×1000. The varicosities were defined as darkly stained axonal dilations (Figs. 1, 2, and 3) with size greater than 0.25 μm^2 (Wong et al. 1999; Cardoso et al. 2006). A sample frame (3.86×10^3 μm^2) was laid over each field of view, and the number of varicosities falling within it was counted. Within each section, four different placements of the frame, each time at a randomly selected position, were used to obtain a mean count for the dentate hilus, CA3, and CA1 *stratum pyramidale* subfields. The results were expressed as areal densities (number/mm^2).

Statistical analysis

Before conducting statistical comparisons, data were tested for normality using the Kolmogorov–Smirnov one-sample test. Because all data samples passed the normality tests, they were analyzed using one-way ANOVA test. Post hoc analyses were performed whenever appropriate, using the Newman–Keuls test. Differences were considered significant at the $p<0.05$ level. Results are expressed as means±SD.

Results

Animals and diets

Daily food intake, measured at 0900 hours every day, was in average 31.6±1.25 g in 12-month adult control

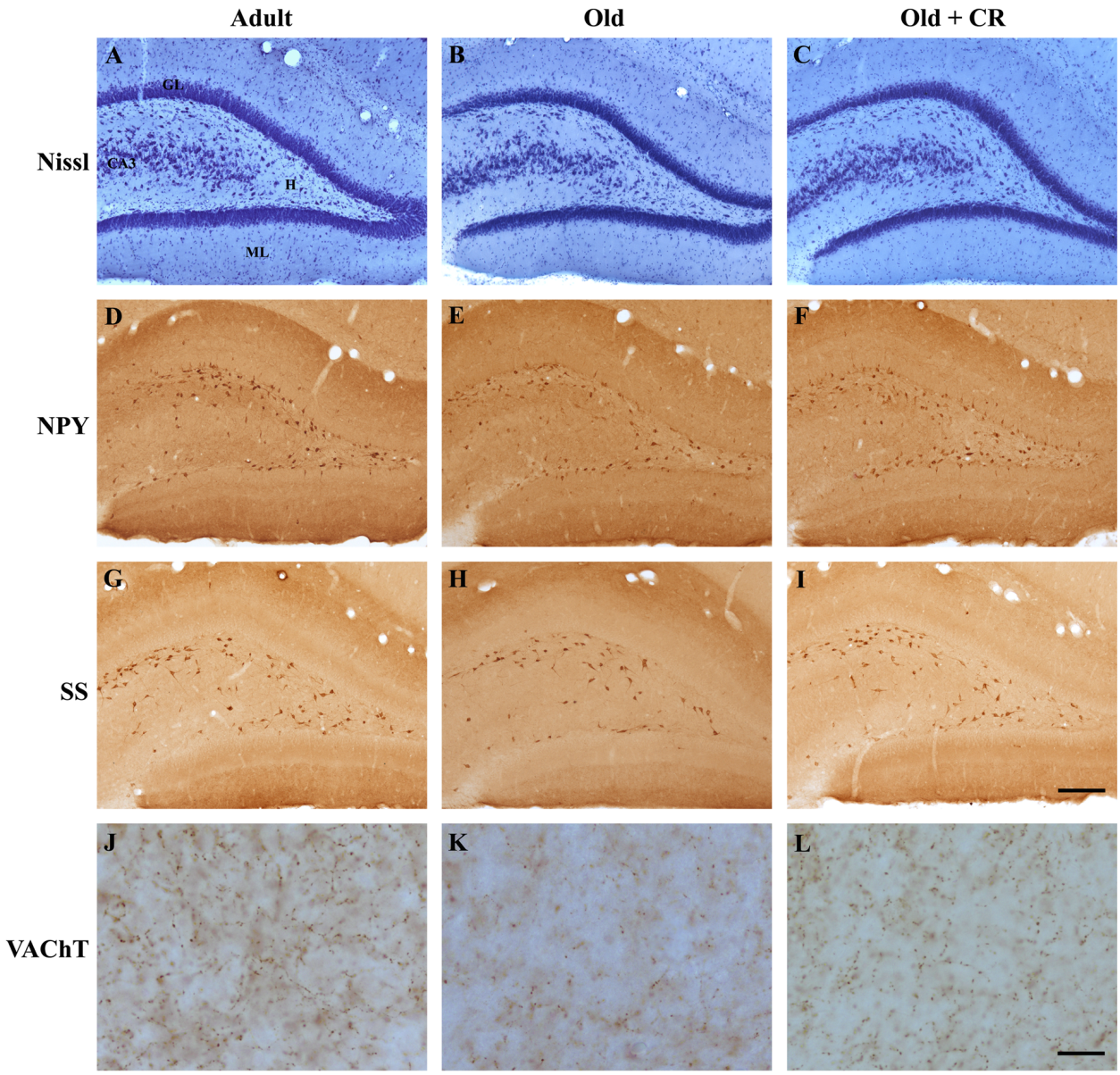

Fig. 1 Representative photomicrographs of level-matched coronal sections of the dentate gyrus from 12-month adult control (adult; **a**, **d**, **g**, **j**), 24-month-old control (old; **b**, **e**, **h**, **k**), and 24-month-old caloric-restricted (old+CR; **c**, **f**, **i**, **l**) rats. Sections shown in **a**, **b**, **c** were Nissl-stained, whereas **d**, **e**, **f** were immunostained for NPY, those shown in **g**, **h**, **i** were immunostained for SS and those shown in **j**, **k**, **l** were immunostained for VAChT. Note that there are no differences in the density of hilar cells in the Nissl-stained sections of the three groups. Note also that the density of NPY-IR and SS-IR cells and the density of VAChT-IR varicosities in the dentate hilus is decreased in the 24-month-old control rat when compared with the 12-month adult control rat, and conversely increased in 24-month-old caloric-restricted rat when compared to the 24-month-old control rat. There are no differences in the density of the NPY-IR and SS-IR cells and in the density of VAChT-IR varicosities between the 24-month-old caloric-restricted and the 12-month adult control rat. *ML* dentate gyrus molecular layer, *GL* granule cell layer, *H* dentate hilus, *CA3* pyramidal cell layer of CA3 hippocampal field. *Scale bar*=200 μm in A-I and 20 μm in **j**–**l**

rats, 29.4±4.10 g in 24-month-old control animals, and 19.2±0.90 g in the 24-month-old caloric-restricted group. By the end of the experiment, the mean body weight of 24-month-old control rats (560±23.25 g) was similar to 12-month adult control rats (535±25.30 g). On average, the body weight of 24-month old caloric-

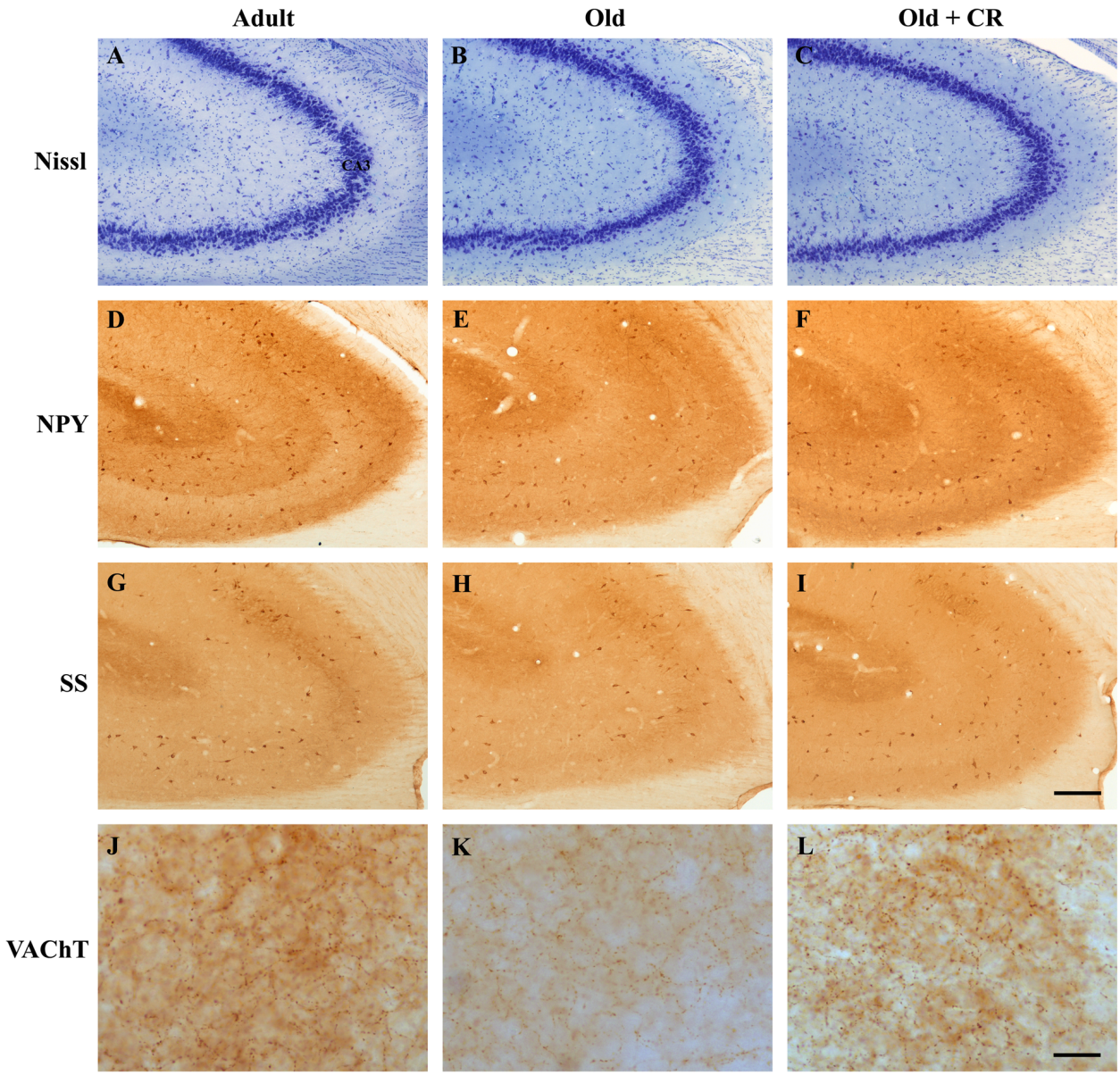

Fig. 2 Representative photomicrographs of level-matched coronal sections of the CA3 subfield from 12-month adult control (adult; **a**, **d**, **g**, **j**), 24-month-old control (old; **b**, **e**, **h**, **k**), and 24-month-old caloric-restricted (old+CR; **c**, **f**, **i**, **l**) rats. Sections shown in **a**, **b**, **c** were Nissl-stained, whereas **d**, **e**, **f** were immunostained for NPY, those shown in **g**, **h**, **i** were immunostained for SS and those shown in **j**, **k**, **l** were immunostained for VAChT. Note that there are no differences in the density of CA3 pyramidal cells in the Nissl-stained sections of the three groups. Note also that the density of NPY-IR and SS-IR cells and the density of VAChT-IR varicosities in the CA3 subfield is decreased in the 24-month-old control rat when compared with the 12-month adult control rat and conversely increased in 24-month-old caloric-restricted rat when compared to the 24-month-old control rat. There are no differences in the density of the NPY-IR and SS-IR cells and in the density of VAChT-IR varicosities between the 24-month-old caloric-restricted and the 12-month adult control rat. CA3, pyramidal cell layer of CA3 hippocampal subfield. *Scale bar*=200 μm in **a**–**i** and 20 μm in **j**–**l**

restricted animals was 40 % lower than that of 24-month-old control rats ($p<0.001$). No significant difference was detected between the mean brain weights of 12-month adult controls (1.54±0.03 g), 24-month-old control (1.53±0.05 g), and 24-month-old caloric-restricted (1.54±0.04 g) animals.

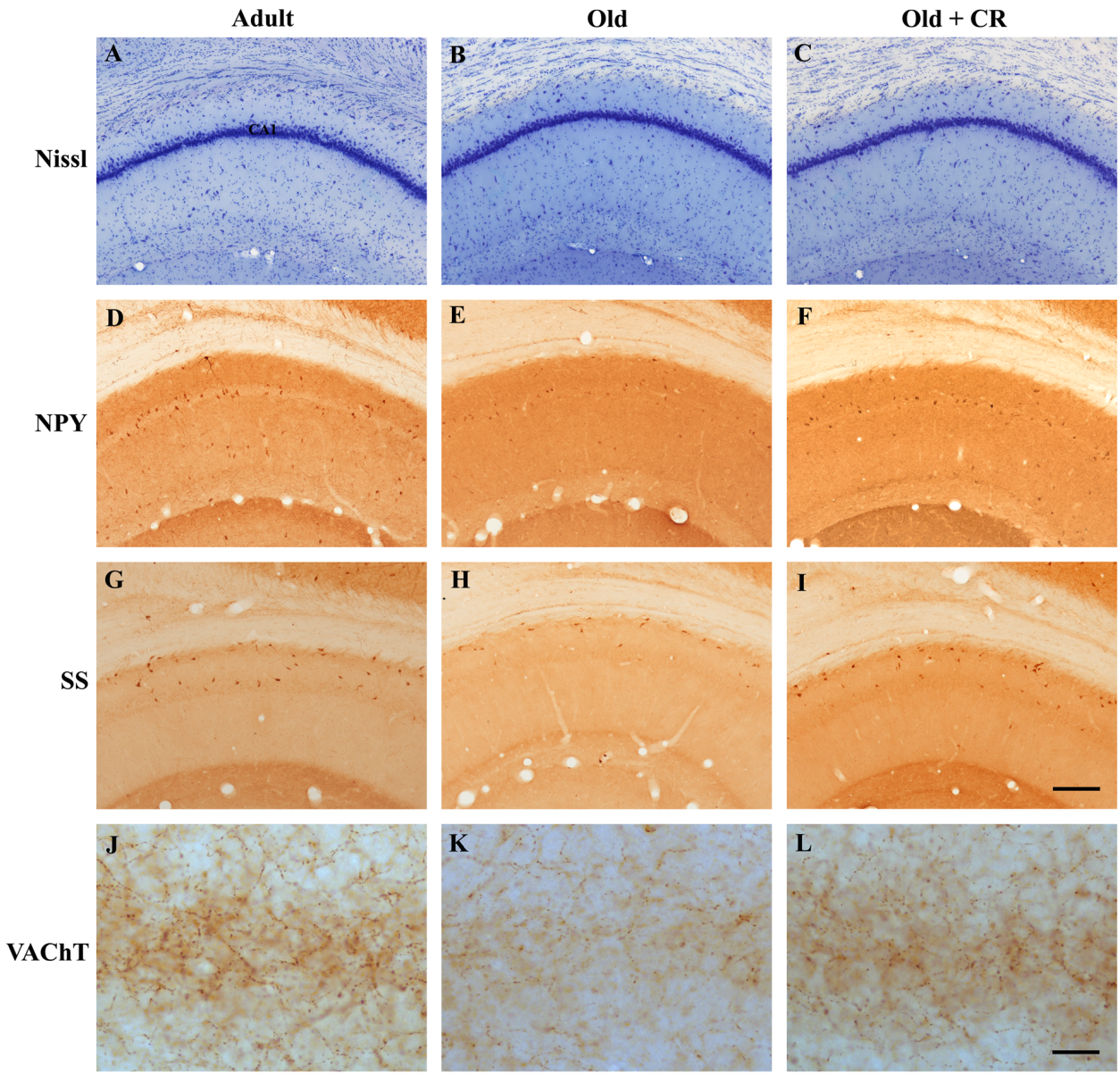

Fig. 3 Representative photomicrographs of level-matched coronal sections of the CA1 subfield from 12-month adult control (adult; **a**, **d**, **g**, **j**), 24-month-old control (old; **b**, **e**, **h**, **k**), and 24-month-old caloric-restricted (old+CR; **c**, **f**, **i**, **l**) rats. Sections shown in **a**, **b**, **c** were Nissl-stained, whereas **d**, **e**, **f** were immunostained for NPY, those shown in **g**, **h**, **i** were immunostained for SS and those shown in **j**, **k**, **l** were immunostained for VAChT. Note that there are no differences in the density of CA1 pyramidal cells in the Nissl-stained sections of the three groups. Note also that the density of NPY-IR and SS-IR cells and the density of VAChT-IR varicosities in the CA1 subfield is decreased in the 24-month-old control rat when compared with the 12-month adult control rat and conversely increased in 24-month-old caloric-restricted rat when compared to the 24-month-old control rat. There are no differences in the density of the NPY-IR and SS-IR cells and in the density of VAChT-IR varicosities between the 24-month-old caloric-restricted and the 12-month adult control rat. CA1, pyramidal cell layer of CA1 hippocampal subfield. *Scale bar*=200 μm in **a**–**i** and 20 μm in **j**–**l**

Total number of neurons

The analysis of the total number of neurons estimated in the hilus of the dentate gyrus from 12-month adult control, 24-month-old control, and 24-month-old caloric-restricted groups revealed that there was no significant effect of the treatment ($F_{2,15}$=0.76, p=0.49).

Total number of NPY-IR neurons

The estimates of the total number of NPY-IR neurons in the dentate hilus, CA3, and CA1 *stratum pyramidale* subfields of the HF are shown in Fig. 4. Analysis of the data showed that there was a significant effect of the treatment in the total number of NPY-IR neurons in the dentate hilus ($F_{2,15}$=8.15, p<0.01), CA3 ($F_{2,15}$=12.74, p<0.01), and CA1 ($F_{2,15}$=52.79, p<0.001). Post hoc comparisons showed that there was a significant reduction of the total number of NPY-IR neurons in the dentate hilus (18 %, p<0.05), CA3 (30 %, p<0.05), and CA1 (53 %, p<0.001) subfields of 24-month-old control rats when compared to 12-month adult control rats. Conversely, it was found a significant increase of the total number of NPY-IR neurons in the hilus (29 %, p<0.01), CA3 (53 %, p<0.01), and CA1 (94 %, p<0.001) subfields of 24-month-old caloric-restricted rats when compared to the 24-month-old control rats. Furthermore, the total number of NPY-IR neurons in the hilus, CA3, and CA1 subfields of 24-month old caloric-restricted rats was similar to the 12-month adult control rats.

Total number of SS-IR neurons

The estimates of the total number of SS-IR neurons in the dentate hilus, CA3, and CA1 *stratum pyramidale* subfields of the HF are shown in Fig. 5. Analysis of the data showed that there was a significant effect of the treatment in the total number of SS-IR neurons in the dentate hilus ($F_{2,15}$=14.02, p<0.01), CA3 ($F_{2,15}$=57.63, p<0.001), and CA1 ($F_{2,15}$=47.40, p<0.001). Post hoc comparisons showed that there was a significant reduction of the total number of SS-IR neurons in the dentate hilus (42 %, p<0.01), CA3 (38 %, p<0.001), and CA1 (56 %, p<0.001) subfields of 24-month-old control rats when compared to 12-month adult control rats. Conversely, it was found a significant increase of the total number of SS-IR neurons in the hilus (45 %,

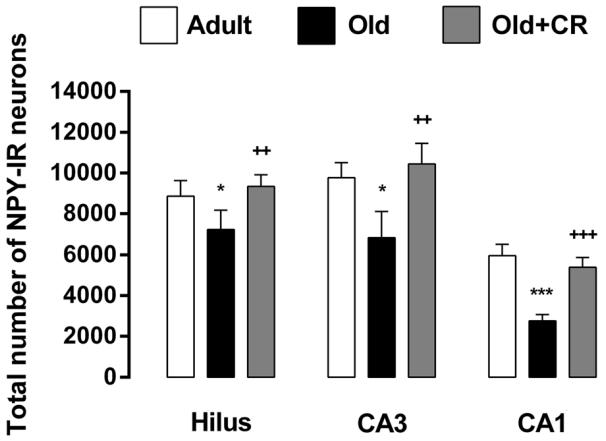

Fig. 4 Graphic representation of the total number of NPY-IR cells in the dentate hilus and CA3 and CA1 subfields of 12-month adult control (adult), 24-month-old control (old), and 24-month-old caloric-restricted (old+CR) rats. Note that there is a significant reduction of the total number of NPY-IR neurons in the dentate hilus (18 %, p<0.05), CA3 (30 %, p<0.05), and CA1 (53 %, p<0.001) subfields of 24-month-old control rats when compared to 12-month adult control rats. Conversely, it was found a significant increase of the total number of NPY-IR neurons in the hilus (29 %, p<0.01), CA3 (53 %, p<0.01), and CA1 (94 %, p<0.001) subfields of 24-month-old caloric-restricted rats when compared to the 24-month-old control rats. There are no significant differences in the total number of NPY-IR neurons in the hilus, CA3, and CA1 subfields between the 24-month-old caloric-restricted rats and the 12-month adult control rats. Data are presented as mean±SD. *p<0.05 and ***p<0.001 versus 12-month adult control group; ^{++}p<0.01 and ^{+++}p<0.001 versus 24-month-old control group

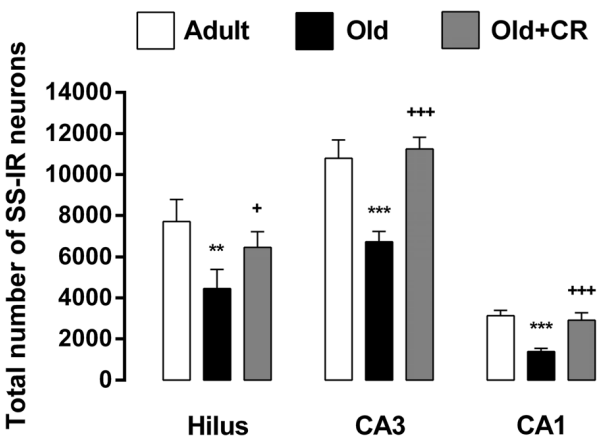

Fig. 5 Graphic representation of the total number of SS-IR cells in the dentate hilus and CA3 and CA1 subfields of 12-month adult control (adult), 24-month-old control (old), and 24-month-old caloric-restricted (old+CR) rats. Note that there is a significant reduction of the total number of SS-IR neurons in the dentate hilus (42 %, p<0.01), CA3 (38 %, p<0.001), and CA1 (56 %, p<0.001) subfields of 24-month-old control rats when compared to 12-month adult control rats. Conversely, it was found a significant increase of the total number of SS-IR neurons in the hilus (45 %, p<0.05), CA3 (67 %, p<0.001), and CA1 (110 %, p<0.001) subfields of 24-month-old caloric-restricted rats when compared to the 24-month-old control rats. There are no significant differences in the total number of SS-IR neurons in the hilus, CA3, and CA1 subfields between the 24-month-old caloric-restricted rats and the 12-month adult control rats. Data are presented as mean±SD. **p<0.01 and ***p<0.001 versus 12-month adult control group; ^{+}p<0.05 and ^{+++}p<0.001 versus 24-month-old control group

$p<0.05$), CA3 (67 %, $p<0.001$), and CA1 (110 %, $p<0.001$) subfields of 24-month-old caloric-restricted rats when compared to the 24-month-old control rats. Furthermore, the total number of SS-IR neurons in the hilus, CA3, and CA1 subfields of 24-month old caloric-restricted rats was similar to the 12-month adult control rats.

Areal density of cholinergic varicosities

The results of the areal density of VAChT-IR varicosities in the dentate hilus, CA3, and CA1 *stratum pyramidale* subfields of HF are shown in Fig. 6. Analysis of the data revealed that there was a significant effect of the treatment in the areal density of VAChT-IR varicosities in the dentate hilus ($F_{2,15}=7.88$, $p<0.01$), CA3 ($F_{2,15}=8.64$, $p<0.01$), and CA1 ($F_{2,15}=20.81$, $p<0.001$). Post hoc comparisons demonstrated that there was a significant reduction of the density of VAChT-IR varicosities in the dentate hilus (30 %, $p<0.05$), CA3 (23 %, $p<0.01$), and CA1 (30 %, $p<0.001$) subfields of 24-month-old control rats when compared to 12-month adult control rats. Conversely, it was found a significant increase of the density of VAChT-IR varicosities in the dentate hilus (29 %, $p<0.05$), CA3 (21 %, $p<0.05$), and CA1 (38 %, $p<0.05$) subfields of 24-month old caloric-restricted rats when compared to the 24-month old control rats. There were no statistically significant differences in the density of VAChT-IR varicosities in the dentate hilus, CA3, and CA1 between 24-month-old caloric-restricted rats and 12-month adult control rats.

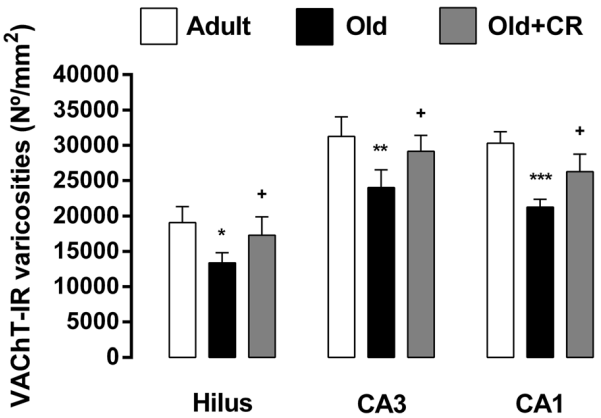

Fig. 6 Graphic representation of the areal density of VAChT-IR varicosities in the dentate hilus and CA3 and CA1 subfields of 12-month adult control (adult), 24-month-old control (old), and 24-month-old caloric-restricted (old+CR) rats. Note that there was a significant reduction of the density of VAChT-IR varicosities in the dentate hilus (30 %, $p<0.05$), CA3 (23 %, $p<0.01$), and CA1 (30 %, $p<0.001$) subfields of 24-month-old control rats when compared to 12-month adult control rats. Conversely, it was found a significant increase of the density of VAChT-IR varicosities in the dentate hilus (29 %, $p<0.05$), CA3 (21 %, $p<0.05$), and CA1 (38 %, $p<0.05$) subfields of 24-month-old caloric-restricted rats when compared to the 24-month-old control rats. There are no statistically significant differences in the density of VAChT-IR varicosities in the hilus, CA3, and CA1 subfields between 24-month-old caloric-restricted rats and 12-month adult control rats. Data are presented as mean±SD. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ versus 12-month adult control group; $^{+}p<0.05$ versus 24-month old control group

Discussion

The main finding of the present study is that prolonged old-onset caloric restriction started at 18 months of age, maintained the total number of NPY-IR and SS-IR neurons in the dentate hilus, CA3, and CA1 subfields of the HF, and prevented the natural decrease of the expression of these neuropeptides, generally reported during aging. Furthermore, it was found that the old-onset caloric restriction was also capable to maintain the hippocampal cholinergic varicosities to values similar to the younger adult controls aged 12 months. Finally, and as expected (Andrade et al. 2002; Cardoso et al. 2013), the old-onset caloric restriction treatment did not induce neuronal loss in the aged animals.

In fact, regarding the total number of neurons, the present study corroborates previous works (Rapp and Gallagher 1996; Sousa et al. 1998) demonstrating that aging did not induce neuronal loss in the hilus of the dentate gyrus. Interestingly, we have found that old-onset caloric restriction treatment during 6 months did not induce neuronal death in the dentate hilus. It was already known that moderate caloric restriction in adult rats did not lead to gross morphological alterations in the brain, even in chronic treatments (Andrade et al. 2002; Cardoso et al. 2013), although subtle neuronal changes, such as dendritic alterations, were described (Andrade et al. 2002). However, there are few studies that analyzed if the caloric restriction would be capable to promote morphological and functional changes when started in advanced age (Kim and Choi 2000; Adams et al. 2008; Del Arco et al. 2011; Singh et al. 2012), a period where the brain is particularly vulnerable to environmental aggressions (Shetty et al. 2011). As follows, at a gross morphological level, the present results

revealed that caloric restriction did not induce neuronal loss in the dentate hilus, suggesting that this type of nutritional deprivation has no serious morphological consequences, even when applied in old rats and during several months. Taking into account that one of the great problems of dietary and caloric restriction treatments in humans is the difficulty to maintain this very restrict diet during long periods (Scheen 2008; Anton and Leeuwenburgh 2013), the present results suggest that caloric restriction can be successfully applied safely at older ages, maybe avoiding the necessity of long periods of treatment during the adult period of life, where the compliance to this diet is poor or it is difficult to maintain (Anton and Leeuwenburgh 2013).

Applying unbiased stereological methods, the present findings fully support previous studies (Cadacio et al. 2003; Hattiangady et al. 2005) demonstrating that aging leads to significant reduction of the total number of NPY-IR neurons in the dentate hilus and also in the CA3 and CA1 subfields, in line with numerous other studies that showed reduction of NPY-IR neurons in several brain regions in old animals (Cha et al. 1996; Huh et al. 1997; Zhang et al. 1998; Cadacio et al. 2003; Cardoso et al. 2006). Furthermore, we have also found, in the same hippocampal regions, that aging induced significant reduction of the total number of SS-IR neurons. This finding was as well described by others that found reduction not only in the hippocampal SS-IR neuronal number (Spiegel et al. 2013) and density (Potier et al. 2006; Gavilán et al. 2007; Stanley et al. 2012) but also in its mRNA levels (Vela et al. 2003; Gavilán et al. 2007). The reduction of NPY-IR and SS-IR neurons during aging could be due to several reasons including cell death, decrease of activity, or even alteration of the protein conformation (Cadacio et al. 2003; Vela et al. 2003; Gavilán et al. 2007). However, several factors, such as treatment with drugs and neurotrophins, are capable to induce partial or total recover of those neuropeptide levels to adult control values in aged rats (Cardoso et al. 2006; Pereira et al. 2013; Spiegel et al. 2013). Therefore, it is likely that this reduction does not reflect irreversible loss of neurons. Supporting this hypothesis is also the present data showing that old-onset caloric-restriction treatment recovers the total number of NPY-IR and SS-IR neurons to levels similar to the younger adult control values. Furthermore, given that NPY- and SS-IR neurons are part of the hippocampal interneuronal population, the present results also corroborate previous studies where it was showed that age-related decrease of the number of glutamate decarboxylase-67 immunopositive hippocampal interneurons reflects a reduction of the protein expression rather than cell death (Stanley and Shetty 2004; Spiegel et al. 2013).

Although it seems that aging does not provoke neuronal death of NPY-IR and SS-IR neurons in the hippocampus, it is clear that it induces significant reduction of their levels detected by immunocytochemistry. These age-related reductions can have functional consequences in the old brain because these neuropeptides are widely expressed in CNS and have important roles in the regulation of emotions, cognitive functions, and feeding behavior (Wettstein et al. 1995; Thorsell et al. 2006; Martel et al. 2012). Interestingly, this old-onset caloric restriction model was capable to maintain the total number of NPY-IR and SS-IR neurons in the dentate hilus and hippocampus proper, showing that moderate caloric restriction was able to prevent the reduction of their levels, generally associated with the aging process. This is very important because caloric restriction can be used in the therapeutics of diseases associated with the decrease of neuropeptide levels, such as epilepsy (Azarbar et al. 2010; Hartman and Stafstrom 2013). It is known that aging brain is more susceptible to epileptic seizures (Hauser 1992; Hattiangady et al. 2011) that was associated to the reduced levels of NPY and SS, neuropeptides known to have anticonvulsive properties (Baraban 2004; Stanley et al. 2012). Indeed, it is known that caloric restriction reduces the epileptic seizures (Bough et al. 1999; Azarbar et al. 2010). These capabilities could be linked, among other factors, to the increase of NPY and SS levels (Cardoso et al. 2010; Drexel et al. 2012).

Bearing in mind that NPY- and SS-ergic systems are dependent of the cholinergic innervation in the cerebral cortex (Milner et al. 1997; Zhang et al. 1998; Cardoso et al. 2006; Potier et al. 2006), we also sought to analyze the effects of aging and caloric restriction upon the cholinergic system of the HF. We have found that aging induced a significant decrease of the density of cholinergic varicosities in the dentate hilus and also in the CA3 and CA1 subfields of the HF, in accordance with other works that described aging deleterious effects upon the cholinergic system in several brain regions (Zhang et al. 1998; Lukoyanov et al. 1999; Cardoso et al. 2006; Potier et al. 2006; Ypsilanti et al. 2008). Interestingly, we have also found that old-onset caloric-restricted rats presented a higher density of cholinergic varicosities in

the dentate hilus and in the CA3 and CA1 hippocampal subfields when compared to age-matched 24-month-old controls. At the best of our knowledge, there are few studies that analyzed the effects of caloric restriction in the cholinergic system. Indeed, Del Arco and collaborators (2011) have shown that basal dialysate concentration of acetylcholine in the prefrontal cortex was significantly decreased by aging and conversely increased by caloric restriction, both in adult and aged animals. Moreover, others had shown that caloric restriction had neuroprotective effects in the choline acetyltransferase activity in fronto-parietal cortex of animals subjected to ibotenic lesion of nucleus basalis magnocellularis (Contestabile et al. 2004). Furthermore, Kim and Choi (2000) had also found that caloric restriction increased the acetylcholine levels in the hippocampus of adult mice. Increasing evidence suggests that acethylcholine exerts trophic effects upon target areas of the basal forebrain cholinergic projections, including the cerebral cortex (Wettstein et al. 1995; Zhu and Waite 1998; Cardoso et al. 2006). It has been described that NPY-IR and SS-IR neurons are innervated by cholinergic terminals (Lamour and Epelbaum 1988; Wettstein et al. 1995; Jolkkonen et al. 1997; Potier et al. 2006) and that the selective lesion of basal forebrain cholinergic nucleus decreases the number of cortical NPY- and SS-IR neurons (Jolkkonen et al. 1997; Milner et al. 1997; Zhang et al. 1998). Furthermore, we have previously reported that infusion of the neurotrophin NGF, known to have neurotrophic effect upon cholinergic neurons (Cuello et al. 1992; Niewiadomska et al. 2002), resulted in the recovery of VAChT cholinergic varicosities in the somatosensory cortex of old rats that was paralleled by a complete recovery of expression of NPY in the same region (Cardoso et al. 2006). Those results suggest that loss of NPY-IR neurons in the somatosensory cortex of aged rats could be the consequence of the lack of trophic support otherwise provided by cholinergic neurons of basal forebrain nucleus. Similarly, the present data corroborate this hypothesis revealing that the maintenance of the total number of NPY-IR as well as SS-IR neurons in the hilus of aged caloric-restricted animals was accompanied by a parallel preservation of the density of the cholinergic varicosities.

One caveat that should be mentioned is that it is not known which process underlies the recovery of the cholinergic innervation after caloric restriction. The previous view that aging is associated with significant cholinergic cell loss has been challenged by recent studies. The current view suggests that aging is associated with a gradual loss of the cholinergic function caused by dendritic, axonal, and synaptic degeneration and decrease in the trophic support, among other factors, without any or significant cell loss (Ypsilanti et al. 2008; Niewiadomska et al. 2002, 2011; Schliebs and Arendt 2011). Indeed, it was demonstrated, applying stereological methods, that cholinergic cell number and size in the medial septal nucleus/diagonal band of Broca are not significantly different between adult and old rats (Ypsilanti et al. 2008). Given this, it is plausible to suggest that the reduction of the cholinergic innervation during aging observed in this study and several others (Zhang et al. 1998; Lukoyanov et al. 1999; Cardoso et al. 2006; Potier et al. 2006; Ypsilanti et al. 2008) is not related to neuronal loss. However, to the best of our knowledge, it is not yet clear if the reduction of the hippocampal cholinergic innervation during aging is derived from axonal and synaptic degeneration or if there is a cholinergic downregulation that precludes the detection of the cholinergic innervation. Taking this in account, the recovery of the cholinergic varicosities after caloric restriction, observed in the present study, could be explained by cholinergic upregulation to detectable levels or sprouting of local intact cholinergic axons. Future studies are necessary to clarify this issue.

In conclusion, we have found that aging induced a significant reduction of the total number of NPY-IR and SS-IR neurons in the dentate hilus, CA3, and CA1 subfields that was accompanied by a decrease of the cholinergic varicosities. The old-onset caloric restriction had the capacity to reverse these events characteristic of aging. In other words, the number of NPY-IR and SS-IR neurons in the 24-month-old caloric-restricted rats was similar to those found in the 12-month adult control rats. More importantly, the cholinergic innervation of the 24-month-old caloric-restricted animals did not decrease with aging as verified in the 24-month-old control rats fed ad libitum. Therefore, the aging-associated reduction of these neuropeptide-expressing neurons is likely not due to neuronal loss and appear to be dependent of the cholinergic system. Therefore, the moderate caloric restriction even when started in old age has beneficial effects upon NPY-IR and SS-ergic systems, which have important role in the regulation of cognition and feeding behavior. Finally, we can safely state that even a late-onset caloric restriction regimen is a viable dietary treatment displaying beneficial effects in the aged CNS. This

knowledge is specially needed in order to fulfill the promise of the use of the caloric restriction in human to counteract neurodegenerative diseases associated with aging and a healthier lifespan.

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Conflict of interest All authors state that there are no actual or potential conflicts of interest.

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Anexos

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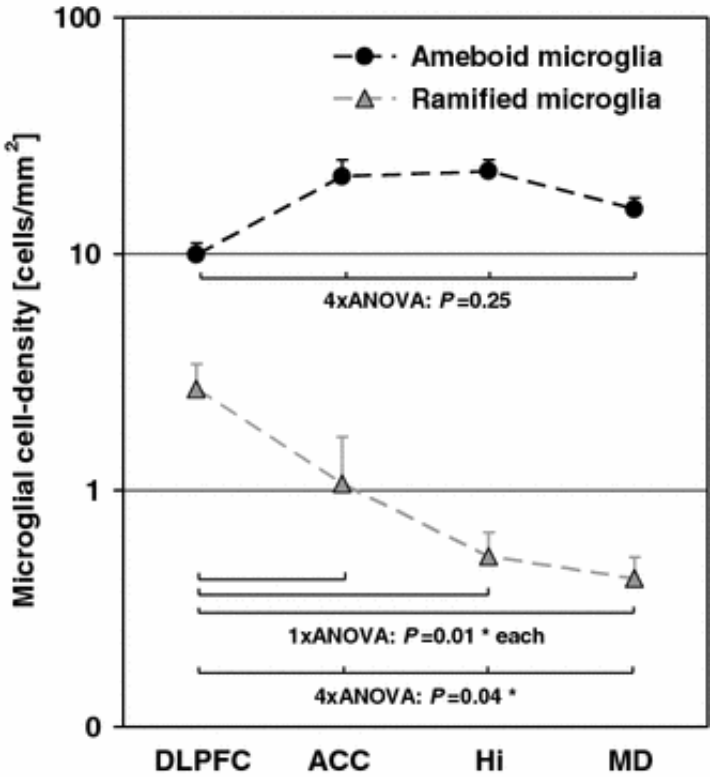

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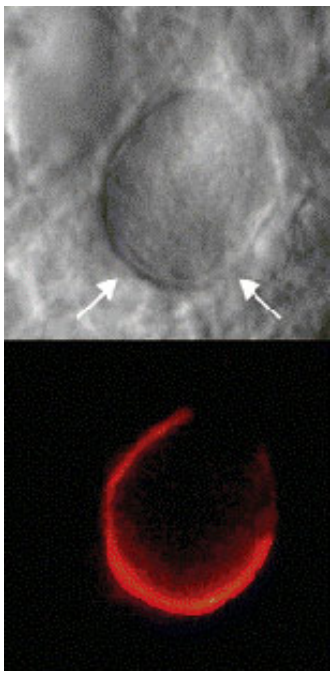

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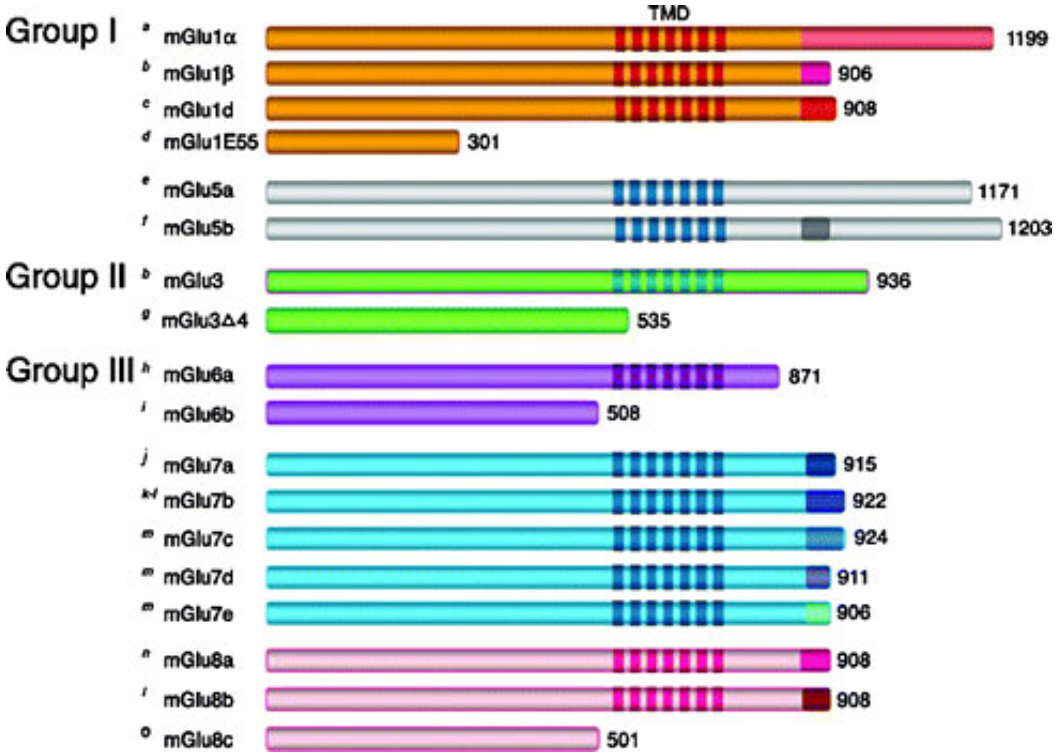

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