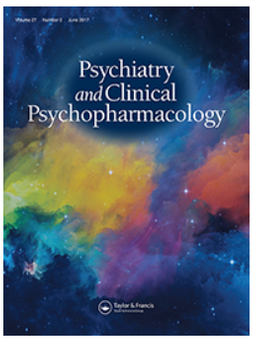

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Effects of agmatine on cognitive functions during vascular dementia in biological aging through eNOS and BDNF expression

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ABSTRACT

Objective: Biological aging has been recognized to cause impairment of memory and the development of vascular dementia. Based on our previous work, agmatine has been shown to have a beneficial effect and might have therapeutic potential on cognitive functions, including learning and memory. The aim of the present study was to examine the possible effect of agmatine on biological aging-induced vascular endothelial dysfunction and associated dementia in rats.

Methods: We used three different age groups (4-month-olds, 18-month-olds and 24-month-olds; $n = 12$ in each group) of control and agmatine-treated rats. Control animals received physiological saline for 8 weeks. Agmatine sulfate (40 mg/kg, twice daily) was given to the agmatine groups orally for 8 weeks. Herein, we investigated the effects of agmatine on systolic blood pressure (SBP), nitric oxide (NO)-mediated endothelium-dependent and -independent vasorelaxant responses in thoracic aorta, cognitive performance (passive avoidance test; PAT, and Morris water maze test; MWMT), endothelial nitric oxide synthase (eNOS) expression and both hippocampal and amygdaloid brain-derived neurotrophic factor (BDNF) expression in aged rats.

Results: We found cognitive decline, endothelial dysfunction and reduced eNOS and BDNF expression in aged rats. All these changes may result from aging-induced vascular dementia. We also found that chronic treatment with agmatine may improve amygdala-dependent emotional and spatial learning and memorial performance, and endothelial function, and may regulate eNOS and BDNF protein expression in aged rats.

Conclusion: Results of the current study point out that chronic agmatine treatment may prevent endothelial dysfunction associated with vascular dementia through eNOS and BDNF expression in aged rats.

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Aging; agmatine; passive avoidance; Morris water maze; vascular dementia

Introduction

Cognitive decline in biological aging is well known; however, the exact cause of it is unclear. Several lines of evidence suggest that vascular causes of dementia are more common in older patients [1,2]. Vascular dementia, in which cerebrovascular pathologies are correlated with cognitive decline, is the most widely recognized type of dementia as well as Alzheimer's disease [3]. Aging of the cerebrovascular circulation and the effects of vascular changes on the brain are responsible for biological aging of the brain [4,5]. In addition, there is growing evidence that damage to the vascular system is related to an increased risk of cognitive decline in aging. Hemodynamic flow has been shown to be disturbed by cardiovascular risk factors, such as hypertension, and thus to result in cerebral hypoperfusion [6]. It is well known that chronic brain hypoperfusion can expedite the onset of cognitive symptoms [7,8]. Previous clinical and preclinical researches demonstrate that aging is connected with systemic inflammation, vascular endothelial dysfunction [9], increased production of reactive oxygen species and decreased bioavailability of nitric oxide [10]. Taken together, we can introduce that drugs that improve endothelial dysfunction will have the ability to ameliorate vascular cognitive deficits in aged individuals. This hypothesis was recently proposed by our study in diabetic vascular dementia [11].

Agmatine, a putative neurotransmitter, was discovered in mammalian brains in 1994 [12], which interacts with several receptor subtypes, such as N-methyl-D-aspartate (NMDA) receptors, and inhibits neuronal and inducible nitric oxide synthase (NOS) (nNOS, iNOS) [13]; however, it stimulates endothelial nitric oxide synthase (eNOS) in the rat brain after cerebral ischemia [14]. Agmatine has a variety of pharmacological

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effects in the central nervous system (CNS) such as anticonvulsant [15], neuroprotective [16–18], anti-stress, anxiolytic and antidepressant activity [19,20] and prevents tolerance and attenuates withdrawal signs in morphine dependence [21,22], providing analgesia and reducing thermal and mechanic hyperalgesia in a neuropathic pain model [23,24]. Recently, endogenous agmatine has been documented to have a crucial role as a neurotransmitter in the cognitive functions [25–27]. Aging-induced changes in the agmatine level were also found in memory-related structures of brain [28] and age-related cognitive decline and agmatine might be benefical to streptozotocin- [29] and scopolamine-induced dementia in rats [30] as well as aging [31,32]. Latest findings suggest that agmatine has a crucial role in the processes of cognition under normal and/or pathological situations. Therefore, herein our study, we examined the possible effect of agmatine on emotional and spatial memory, blood pressure and vascular response in young, middle-aged and aged male rats.

Materials and methods

Experimental animals

In the present study, male Wistar Albino rats aged 4 months old (220–300 g), 18 months old (300–350 g) and 24 months old (400–500 g) have been used. The rats were obtained from Kocaeli University Experimental Medical Research and Application Center (DETAB, Kocaeli, Turkey). Every behavioral trial was directed from 9:00 am to 12:00 pm under standard conditions (22 ± 2°C room temperature; 12-h light/dark cycle with lights on at 7:00 am). The animals were fed with tap water and food pellets ad libitum. Ethical approval was granted by the Kocaeli University Animal Research Ethics Committee (Project number: HADYEK-5/2-2010).

The rats were randomly divided into groups (n = 12 in each group) such as young (4 months old), middle-aged (18 months old), aged (24 months old) control groups and matching-aged agmatine groups. Control animals received physiological saline for 8 weeks, whereas agmatine sulfate (40 mg/kg, twice daily) was given to the agmatine groups (4 months, 18 months and 24 months) orally by gavage for 8 weeks.

Locomotor activity test

Locomotor movements were evaluated with an animal activity monitoring system (May AMS 9701, Commat Ltd., Ankara, Turkey) including a plexiglass box (40 cm × 40 cm × 30 cm), a computer and open-field activity software. Total locomotor activity was represented as the sum of the vertical, ambulatory and stereotypic activities of the animals for 5 min.

Passive avoidance test

Passive avoidance test (PAT; model 7551, Ugo Basile, Italy) was utilized for the assessment of emotional memory based on contextual fear conditioning, as previously described elsewhere [30]. Briefly, rats learn to avoid a specific place associated with an aversive event. The diminishment of latency to avoid was used as learning. A guillotine door separated two-compartment-containing (light and dark chamber) apparatus was used. The rats were placed in the light chamber after 20 s, the guillotine door separating was opened and the initial latency to enter the dark chamber was recorded. When the rats entered the dark chamber, they were given a foot shock of 0.5 mA for 3 s through the grid floor of the dark compartment. At that point, the rats came back to their home cage. Twenty-four hours later, the retention latency time was measured similarly as in the acquisition trial; however, foot shock was not conveyed. The cut-off time was limited to 300 s.

Morris water maze test

A water tank (150 cm in diameter) was used to measure spatial memory as previously described elsewhere [30]. The platform was put in the center of the southwest quadrant and submerged 1.5 cm below the surface of water, and small black plastic balls were put on the water surface. The platform was not changed during the first four days, and latency to discover the platform was determined. Every trial was ended when the rat had climbed onto the escape platform or at the end of 60 s. Each rat was permitted to remain on the platform for 20 s. The rats that could not discover the platform within 60 s were put on the platform and were permitted to stay there for 20 s. A probe trial was utilized to evaluate the rat's spatial retention of the location of the hidden platform on day 5. During this trial, the platform was removed from the maze and the rat was permitted to search the pool for 60 s in order to spend time in the quadrant that previously contained the hidden platform, the so-called target quadrant.

Systolic blood pressure measurement

An automatic blood pressure and heart rate recorder system (May BPHR 9610; Commat Ldt, Ankara, Turkey) was used to assess indirect systolic blood pressure (SBP) and heart rate (HR). The average value of BP and HR in each rat was obtained from three consecutive inflation–deflation cycles of the cuff.

Organ bath studies

The rats were sacrificed by decapitation and the thoracic aorta from the aortic arch to the diaphragm was extracted. Vessels were put in Krebs solution, dissected

cautiously and rings were prepared. The rings were placed in 20 ml organ baths containing Krebs solution that were maintained at 37°C by utilizing a thermoregulated water circuit and aerated continuously with 95% O_2 and 5% CO_2. The pH of the fluid was 7.4. The rings were equilibrated for 60 min and during this period, the bath solution was changed each 15 min. Resting tension was set at 1 g by repeated adjustment and stayed unaltered throughout the investigation. Each ring was connected to a force-displacement transducer (May FDT 10A; Commat Ltd., Ankara, Turkey) for the estimation of isometric force, which was displaced continuously and recorded online on a computer by utilizing a four-channel transducer data acquisition system (MP 30B-CE; Biopac Systems, Santa Barbara, CA, USA), using software (BSL Pro 3.7; Biopac Systems) that could analyze the data.

Agonist-induced contractions

Aortic rings were exposed to 80 mM KCl for 5 min. Tissues were then washed, and phenylephrine (10^{-9}–10^{-4} M)-induced contractile responses were obtained cumulatively.

Agonist-induced relaxations

Each aortic ring was pre-contracted with a submaximal concentration of phenylephrine (10^{-6} M). These concentrations resulted in 85–87% of the maximal response to phenylephrine. After the phenylephrine-induced contraction had reached a plateau, the concentration–response relationships for carbachol (10^{-8}-10^{-5} M), sodium nitroprusside (SNP; 10^{-8}–10^{-4} M) or papaverine (10^{-5}–10^{-4} M) were obtained by the addition of one of these agents to the bath in a cumulative fashion. The agonist concentration in the bath was incremented approximately threefold at each step after the response to the previous dose had reached a plateau. Between successive concentration response curves, the tissues were rinsed with fresh buffer and they were allowed to recover for 30 min. During this period, tension returned to basal levels.

Immunohistochemical analyses for eNOS and brain-derived neurotrophic factor

Rats were fixed with 10% neutral buffered formalin. After routine histological tissue procedure, the tissues were cut into 3 μm thickness and embedded in paraffin. Routine immunohistological procedure was performed and sections were incubated with polyclonal primary antibody against eNOS (sc654, Santa Cruz, CA, USA) and with secondary biotinylated antibody, streptavidin peroxidase and diaminobenzidine (DAB) solution.

The primary rabbit polyclonal anti-brain-derived neurotrophic factor (BDNF) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was applied overnight at a 1:100 dilution at room temperature. Negative control samples were prepared by replacing the primary antibody with the antibody diluent solution (Ab-diluent reagent solution, Invitrogen, Carlsbad, CA, USA) at the same concentration. After several washes, the slides were incubated with a biotinylated secondary antibody (Histostain-Plus Kit, Broad Spectrum, Invitrogen, Carlsbad, CA, USA) for 20 min at room temperature, and DAB (DAB Substrate Kit, Invitrogen, Carlsbad, CA, USA) was applied for visualization. The sections were briefly counterstained with Mayer's hematoxylin (Invitrogen, Carlsbad, CA, USA) and mounted with ClearMount (Invitrogen, Carlsbad, CA, USA) on glass slides.

Both aorta and brain slides were examined under a light microscope (Olympus BX 50, Tokyo, Japan). The photomicrographs were taken with a Leica DM 100 system (Leica DFC 290HD, Wetzlar, Hessen, Germany). All samples were treated following the same protocol. One researcher who was blind to the current study graded the staining intensity based on a semi-quantitative scale ranging from no expression (0) to very weak (1+), moderate (2+), strong (3+) and very strong (4+) expression. The percentage of positive cells was defined as 0, < 5%; 1, 6–15%; 2, 16–50%; 3, 51–80%; and 4, > 80% positive cells.

Measurement of blood glucose

Blood glucose level was detected with a commercial glucose meter and glucose-sensitive dipsticks (Accutrend-Alpha glucometer, Boehringer, Mannheim, Germany). In brief, blood was withdrawn from the tail vein. A drop of blood put on the glucometer strip was included in the glucometer for the determination of blood glucose level.

Drugs and treatments

Agmatine was dissolved in saline and given orally by gavage in a volume of 0.2 ml per 100 g body weight of the rat. Agmatine (40 mg/kg) was administered twice daily for 8 weeks. Behavioral testing commenced 24 h after the last drug treatment. The control group was received physiological saline (orally by gavage, 0.2 ml/100 g). Agmatine sulfate, phenylephrine hydrochloride, carbachol chloride, SNP and papaverine hydrochloride were used (obtained from Sigma Aldrich). Drugs were prepared fresh every day and kept in cold until injected.

Statistical analysis

Data are the mean ± standard error of the mean (SEM). Acquisition (1–4 day) latency scores in the Morris water maze test (MWMT) were measured by two-way analyses of variance (ANOVA). The following were measured by one-way ANOVA: scores of the time spent in the escape platform quadrant in the

Table 1. Body weights (g), blood glucose (mg/dl) and systolic BP (mm/Hg), locomotor activity, passive avoidance acquisition test phases and MWMT of the time spent in the target quadrant on day 5 of rats in 4- (4 M), 18-(18 M), 24-month-old (24 M) and agmatine-treated age-matching groups.

	4 M control ($n = 6$)	4 M+ Agmatine ($n = 6$)	18 M ($n = 6$)	18M + Agmatine ($n = 6$)	24 M ($n = 6$)	24 M + Agmatine ($n = 6$)
Body weight (g)	266.83 ± 6.42	275.33 ± 4.89	311.16 ± 4.1*	312.33 ± 4.16*	441 ± 6.31*	431.83 ± 6.36*
SBP (mm/Hg)	103.9 ± 3.034	102.7 ± 3.20	109.9 ± 1.18	104.4 ± 2.94	114 ± 1.46	109.5 ± 2.21
Blood glucose (mg/dl)	88.67 ± 3.72	89.17 ± 3.33	88.16 ± 2.47	88.83 ± 3.56	84.5 ± 3.26	80.17 ± 5.56
Total locomotor activity	1135 ± 48.41	1065 ± 57.04	1031 ± 40.65	1054 ± 54.44	938.8 ± 57.86	971.3 ± 71.71
First-day latency (s)	80.95 ± 21.57	69.68 ± 14.92	80.99 ± 25.10	99.98 ± 33.12	79.92 ± 21.70	78.67 ± 25.59
Time spent in escape platform's quadrant (s)	28.67 ± 2.36	29.42 ± 1.80	19.33 ± 0.61*	22.17 ± 1.80	15.75 ± 1.40*	23.33 ± 1.94#

Note: Values are arithmetic means ± SEM, n = number of animals used.
*$P < .05$, statistically different from 4-month-old control rats and #$P < .05$, statistically different from 24-month-old rats.

MWMT; first day and retention latencies in PAT scores; total locomotor activity scores and foot shock sensitivity scores. Further statistical analyses for individual groups were carried out using the Bonferroni test. In isolated organ bath experiments, contractile force is expressed as milligrams of developed tension. The relaxant responses are expressed as the percentage of pre-contraction to phenylephrine. Concentration–response curves were fitted by nonlinear regression with the simplex algorithm, and maximum responses (E_m) and pD_2 (–log EC_{50}) calculated using the software of the transducer data acquisition system. Briefly, the cumulative concentration–response curve data were fitted as described previously to a four-parameter logistic equation: $E = E_m/1 + (EC_{50}/[D]n)$, where E denotes the observed effect in grams of tension, $[D]$ denotes the concentration of agonist, E_m denotes the calculated maximal effect, EC_{50} denotes the $[D]$ at 0.5 E_m and n is the slope factor parameter. The significances were conducted with one-way ANOVA followed by a post hoc Tukey–Kramer test. The immunoreactivity scores were compared by the Kruskal–Wallis test following Dunn's multiple comparison test; $P < .05$ was considered significant.

Results

Body weight was significantly different between groups ($F_{(5,71)} = 199$, $P < .0001$), whereas agmatine treatment has no effect (Table 1).

Behavioral assessment

Locomotor activity test

Agmatine administration of 40 mg/kg, twice daily for 8 weeks had no effect on locomotor activity in young and aged groups compared to controls ($F_{(5,71)} = 1.572$, $P = .183$) (Table 1). There was no statistical difference in locomotor activity among the different age groups and agmatine-treated matching groups (Table 1).

Passive avoidance test

Retention times were evaluated in order to examine possible changes both by aging and by agmatine treatment by using PAT. There were no significant differences between the groups ($F_{(5,71)} = 0.1667$, $P = .9739$) on day 1 (training session) (Table 1).

On the second day, however, there was a significant reduction in retention time in 18-month-old and 24-month-old groups compared to controls. There was no significant change between 4-month-old rat responses with or without agmatine treatment, whereas agmatine treatment significantly reversed the reduction in retention time in both 18-month- and 24-month-old groups ($F_{(5,71)} = 7.377$, $P < .0001$) (Figure 1).

Morris water maze test

All rats exhibited lower latency time during the first 4 days of the test to get to the platform ($F_{(3,264)} = 16.90$, $P < .0001$). There was a further statistical difference between groups by aging and by agmatine treatment

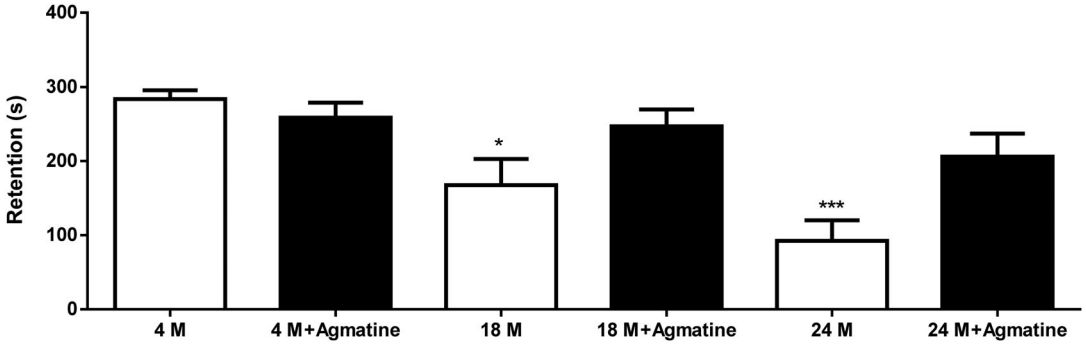

Figure 1. Passive avoidance retention test phases of young (4-month-old), middle-aged (18-month-old), aged (24-month-old) and agmatine (40 mg/kg)-treated matching groups ($n = 12$ rats in each group). Data were presented as mean ± SEM and *$P < .05$ and ***$P < .001$ compared to the young control group (4-month-old).

($F_{(5,264)}$ = 24.82, P < .0001). There was no significant difference between days related to age and treatment ($F_{(15\ 264)}$ = 0.52, P = .9312) (Figure 2).

There was a significant difference between 18-month and 24-month groups on the 5th day of the experiment in terms of time spent in the escape platform quadrant during the probe trial of the MWMT. Also, agmatine treatment reversed the reduction in the time spent in escape platform's quadrant back to control values (P < .001) ($F_{(5,71)}$ = 9.293, P < .001) (Table 1).

Blood glucose level estimation

Blood glucose levels were detected in all groups. There was no significant difference in blood glucose levels among the groups (Table 1).

Effect of aging and systemic administration of agmatine on the expression of BDNF protein

There was a significant decrease in BDNF protein expression in the hippocampal CA1 and CA3 regions in the aged rats (P < .05, Figure 3), whereas treatment with 40 mg/kg agmatine significantly increased the levels of BDNF protein in the hippocampal CA1 and CA3 regions in 24-month-old rats (P < .05, Figure 3). In 24-month-old rats receiving 40 mg/kg agmatine, BDNF protein expressions were similar to those in the control group. Similarly, in the amygdala, BDNF protein levels were significantly lower in the aging rats compared to the control rats (P < .05, Figure 3). In the 40 mg/kg agmatine group, levels of BDNF protein were significantly increased compared with those in the aging group (P < .05, Figure 3 and Table 2).

Effects of aging and systemic administration of agmatine on SBP and vascular reactivity

Any significant change was not detected in the SBP of rats (4-month-old control = 103.9 ± 3.034 mmHg; 4-month-old control + agmatine = 102.7 ± 3.20; 18-month-old = 109.9 ± 1.18 mmHg; 18-month-old + agmatine = 104.4 ± 2.94 mmHg; 24-month-old = 114 ± 1.46 mmHg; 24-month-old + agmatine = 109.5 ± 2.21 mmHg; n = 6 for each group; P > .05) (Table 1).

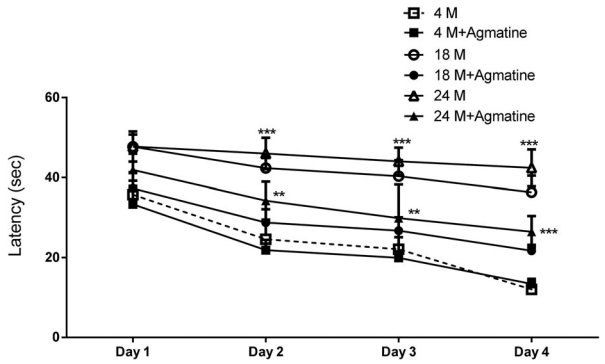

Figure 2. Escape latency (s) in MWMT results of 1–4 days by rats in young (4-month-old), middle-aged (18-month-old), aged (24-month-old) and agmatine (40 mg/kg)-treated matching groups (n = 12 rats in each group). Data were presented as mean ± SEM and **P < .01 and ***P < .001 compared to the young control group (4-month-old).

Contractile responses induced with 80 mM KCl did not differ in all groups. No significant difference was observed between the maximum responses of rings of the 4-, 18- and 24-month-old groups, or agmatine-treated rats (P > .05) (Table 3).

Carbachol and SNP induced relaxations in thoracic aortic rings pre-contracted with a submaximal concentration of phenylephrine (10^{-6} M). There was no significant difference in the pre-contractile tone among the groups (Table 3). In rings pre-contracted with phenylephrine at submaximal concentrations, carbachol (10^{-8}–10^{-5} M) evoked concentration-dependent relaxations. The carbachol-induced endothelium-mediated relaxation was decreased significantly in both the 18-month-old and 24-month-old groups compared with that in the 4-month-old control, agmatine-treated 4-month-old control and agmatine-treated 18- and 24-month-old groups (P > .05, Figure 4). The cumulative concentration–response curve for carbachol was shifted to the right with significantly lower values of E_m and pD_2 in the 18- and 24-month-old groups than in 4-month controls (P < .05, Table 3 and Figure 4). However, the impairment in the two agmatine treatment groups was returned to that seen in 4-month-old controls. No significant difference was found in the values of pD_2 and E_m between 4-month-old control and agmatine-treated rats (P > .05, Figure 4 and Table 3).

In pre-contracted rings, SNP, an NO donor (10^{-8}–10^{-4} M), induced concentration-dependent relaxation (Figure 5), but no significant differences were found in the values of E_m and pD_2 among the groups (Table 3). In addition, the papaverine-induced vasorelaxations were similar for all groups (Table 3).

Effects of aging and systemic administration of agmatine on the expression of eNOS protein

In the 4-month-old rats, eNOS immunopositivity was established in the cytoplasm of the endothelial cells of the aorta (Figure 6(a)). eNOS immunopositivity decreased in the 24-month-old group compared with that in 4-month-old controls (P < .01, Figure 6(b)). In the agmatine-treated aging rats, eNOS immunopositivity was similar to that of the 4-month-old control group (Figure 6(c) and Table 2).

Discussion

This study revealed the behavioral function, endothelium-dependent vasorelaxant responses and the

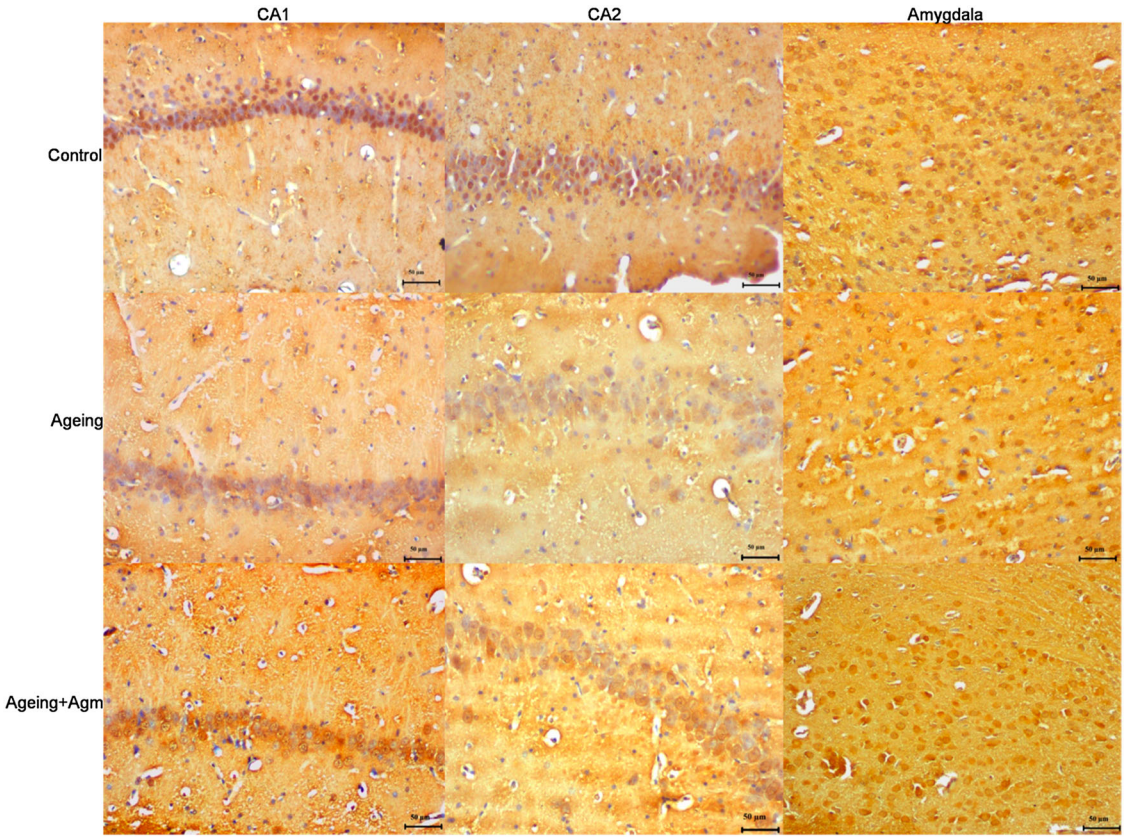

Figure 3. Representative image illustrating BDNF expression (arrows) in hippocampal formation. Control (a), 24-month (b) and 24-month + agmatine (c) groups in the CA1 region, and control (d), 24-month (e) and 24-month + agmatine (f) groups in the CA3 region. control (g), 24 month (h) and 24-month + agmatine (i) groups in the amygdala. BDNF expression was decreased in 24 month in the CA1 and CA3 regions of the hippocampus, and amygdala, whereas in 24-month + agmatine rats BDNF expression was similar to those in the control group in the all regions. Scale bars: 50 µm.

age-induced changes in BDNF and eNOS expressions. Chronic agmatine administration increased the emotional (amygdala-dependent) and spatial learning-memorial (hippocampus-dependent) performance of the aged rats, and maintained the endothelium-dependent vasorelaxant responses. Furthermore, the long-term agmatine treatment significantly prevented the age-related reduction in BDNF immunoreactivity in the hippocampus CA1 and CA3 zones and amygdala, and eNOS immunoreactivity in the thoracic aorta, which may to some extent contribute to correct the behavioral functions in the aged rats.

Aging is an unavoidable process and it is well known that it is one of the causes of cognitive dysfunctions. Learning and memory deficits in aging are mostly related to hippocampal CA1, CA3 and dentate gyrus, (DG) entorhinal, perirhinal, parahippocampal and prefrontal cortex dysfunctions. Besides, it has been shown in several studies that NO may play a major role in the aging process. It regulates cerebellar blood flow

Table 2. Semi-quantitative distribution of BDNF-immunoreactive neurons of the CA1 and CA3 regions of the hippocampus and amygdala and eNOSimmunoreactivity of aorta in rats.

Hippocampal CA1	Animal 1	Animal 2	Animal 3	Animal 4	Animal 5	Animal 6
4 M	2+	1+	2+	3+	2+	3+
24 M	1+	1+	2+	1+	2+	0
24 M + agmatine	2+	1+	1+	3+	2+	2+
Hippocampal CA3						
4 M	2+	2+	3+	2+	2+	2+
24 M	1+	2+	1+	0	0	2+
24 M + agmatine	1+	3+	2+	2+	2+	2+
Amygdala						
4 M	3+	2+	3+	2+	2+	3+
24 M	1+	1+	1+	2+	2+	1+
24 M + agmatine	1+	3+	2+	2+	3+	2+
eNOS						
4 M	3+	3+	2+	2+	2+	3+
24 M	0	1+	1+	2+	1+	1+
24 M + agmatine	2+	3+	2+	2+	3+	1+

Note: The staining intensity was classified as no expression (0), very (1+), moderate (2+), strong (3+) or very strong (4+) expression. The immunopositivity was decreased in 24-month-old rats group (24M) compared to the 4-month-old control group (4M) ($P < .01$, Kruskal–Wallis test). In the agmatine-treated group, both BDNF and eNOS immunoreactivity were similar to that of the control group.

Table 3. E_m (% of 10^{-6} M phenylephrine) values for carbachol, sodium nitroprusside and papaverine, E_m values (g) for 80 mM KCl and pD_2 values (–log EC_{50}) for carbachol, and sodium nitroprusside in rings of thoracic aorta obtained from 4- (4 M), 18-(18 M), 24-month-old (24 M) and agmatine-treated age-matching group rats.

	4 M control ($n = 6$)	4 M + Agmatine ($n = 6$)	18 M ($n = 6$)	18 M + Agmatine ($n = 6$)	24 M ($n = 6$)	24 M + Agmatine ($n = 6$)
KCl E_m (g)	0.70 ± 0.10	0.71 ± 0.09	0.88 ± 0.12	0.79 ± 0.07	0.77 ± 0.10	0.77 ± 0.11
Papaverine E_m (%)	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
Carbachol E_m (%)	61.8 ± 3.99	56.7 ± 5.52	37.5 ± 3.83*	48.5 ± 7.1	29.2 ± 3.7*	46.8 ± 3.4
Carbachol pD_2	6.82 ± 0.54	6.89 ± 0.32	6.72 ± 0.36	6.82 ± 0.42	5.74 ± 0.39*	6.80 ± 0.52
SNP E_m (%)	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
SNP pD_2	6.38 ± 0.24	6.30 ± 0.22	6.46 ± 0.36	6.46 ± 0.32	5.92 ± 0.52	6.39 ± 0.44

Note: Values are arithmetic means ± SEM. n = the number of thoracic aortic rings used.
*Statistical significance was $P < .05$.

and several other functions at physiological levels. Though it functions as a neuronal messenger and regulates the cerebral blood flow at a physiological level, NO is a strong oxidizing agent that is excessively excreted through iNOS, and may cause inflammation, nitro-oxidative stress, apoptosis and mitochondrial dysfunction [33,34]. It has been demonstrated that an increase in iNOS expression and nNOS activity or protein expression might result in a number of cognitive disorders in aged rats [35–37]. Nevertheless, some other authors reported that the number of NOS-containing neurons reduced in the aged animals, accompanied with a reduction in the NOS activity as well as nNOS and eNOS protein expressions, and no iNOS activity was observed in the memory-related structures [38–43].

It was recently reported that the agmatine levels in the memory-associated cerebral structures demonstrated some changes specific to the age-related zone [39,40,44]. The recent empiric studies evoke that exogenously administered agmatine recovers the learning and memory functions, and has a therapeutic potential [45,46]. Both the behavioral and neurochemical useful effects of agmatine in the aged rats were first reported by Rushaidhi et al. [31]. Agmatine (40 mg/kg once a day, i.p) corrected the spatial working memory and object recognition memory in the aged male rat after 10–16 injections, while the spatial reference memory and exploratory behavior demonstrated no change after a single injection of agmatine [31].

In this study, it was detected that the chronic agmatine treatment corrected the emotional and spatial memory broken down in the age-related PAT and water maze test, and these results are in compliance with the findings obtained by the previous researchers. For instance, systemically or centrally administered agmatine increases various learning and memory performances in naive animals, and ensures protection against the learning and memorial deficits caused by the administered amyloid beta or scopolamine [41,47–50]. In addition to the behavioral studies, it was reported that chronically administered agmatine significantly repressed the age-related increase in total NOS activity in dentate gyrus and prefrontal cortex [31,32].

In this study, chronically administered agmatine did not affect the locomotor activity of the aged rats. The animals were trained to find the hidden platform to determine spatial learning and memory. The retention time to find the platform was adversely affected in the

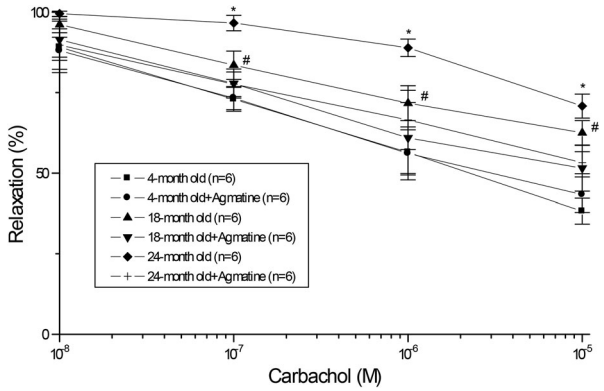

Figure 4. Carbachol concentration–response curves in isolated thoracic aortic rings pre-contracted with phenylephrine (10^{-6} M). The concentration–response curve for carbachol was shifted to the right with significantly lower values of E_m and pD_2 in the 24-month-old group than in 4-month-old controls (*$P < .05$). Impairment of relaxation in the agmatine treatment group was returned to that seen in the controls. Each point is expressed as a percentage of the contraction induced by phenylephrine and is represented as the mean ± SEM. The values in parentheses indicate the number of preparations used; *$P < .05$, different from the response of tissue rings from 4-month-old young control rats.

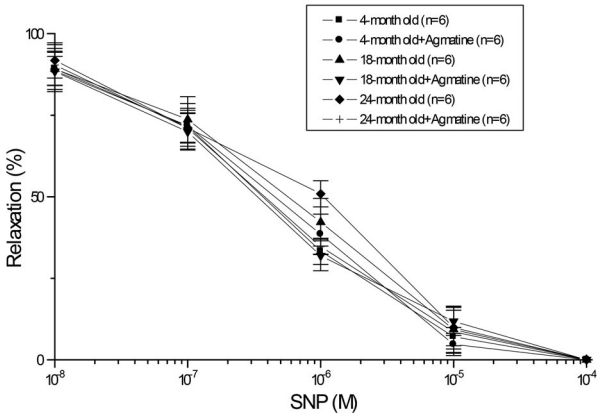

Figure 5. Sodium nitroprusside concentration–response curves in isolated thoracic aortic rings pre-contracted with phenylephrine (10^{-6} M). The relaxation response to SNP was similar among vascular tissues from all groups. Each point is expressed as a percentage of the contraction induced by phenylephrine and is expressed as the mean ± SEM. The values in parentheses indicate the number of preparations used.

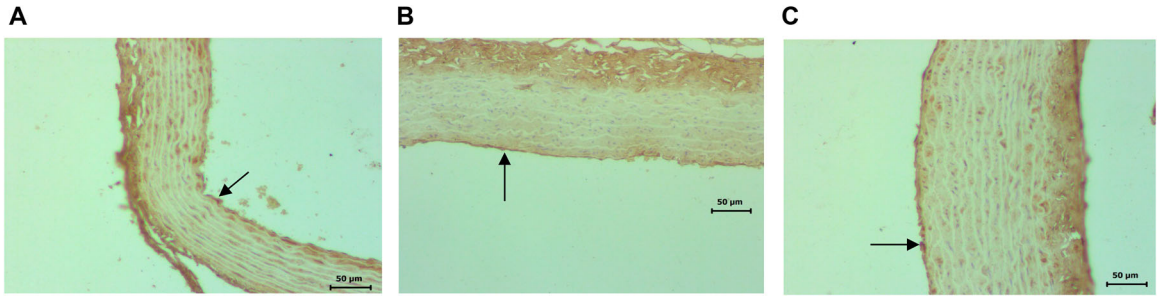

Figure 6. Representative light microscopy of control, 24-month-old and agmatine-treated 24-month-old groups in the endothelium of the aorta. Decreased eNOS (arrows) (b) immunoreactivity in the aorta in 24-month-old rats compared to 4-month-old controls (a) and increased eNOS (c) immunoreactivity in endothelial tissue in the agmatine-treated 24-month-old group compared to 24-month-old groups (b). Scale bars: 20 µm.

course of the memoric direction finding, and the exploratory time was reduced and the spatial memory functions were distorted in the platform area, depending on the age compared to the young controls even in the probe test. During the locomotor activity test, no significant difference was found among the groups. On the last day of the water tank trial, a probe test was carried out to evaluate the performance of the animals in learning the place of the platform. The animals in the two different aged age groups performed the explorative behavior in the quadrant of the secret platform in a shorter time, compared to the young controls, while the agmatine-treated aged animals demonstrated a similar performance to that of the young groups. In the PAT, the first-day performance was identical in all the groups, while the performances evaluated 24 hours later (on the second day) were found to be lowered depending on age. The agmatine-treated aged animals had a latency identical to the young controls on the second day. Considering all the behavioral data, the findings obtained in the present study are consistent with the previously published findings. In consequence, 40 mg/kg chronic agmatine treatment selectively corrects behavioral performance in the aged rats [31,32].

Agmatine has been reported to lower blood glucose levels in animal models of diabetes [51]. In our study, blood glucose levels were measured at the end of the treatment and no change in blood glucose levels have been found in any group. Hence, the effect of agmatine on cognitive dysfunction is independent from the glucose levels in aged rats.

NO and cGMP mediate physiological effects not only in the central nervous system, but also in the cardiovascular system. Reducing the NO-dependent signal transduction during biological brain aging may be an important factor in cognitive deficits. In addition to this decrease in the central nervous system, an age-related decrease in eNOS activity was reported, pointing out that the changes in vascular reactivity would likely result in age-related vascular dysfunction [52]. NO has a basic role in the regulation of vascular function [53]. It is reported that aging is closely related to vascular aging, and the vascular structural and functional changes might be the most important cause of vascular aging [54]. In this study, the endothelium-mediated relaxing responses obtained by carbachol in the isolated aorta strips were significantly reduced depending on age, while no significant change was determined among the groups in terms of the NO-donor SNP relaxing responses. The agmatine-treated aged animals were determined to have such endothelium-dependent relaxing responses as are comparable to those of the young control group. Likewise, the chronic agmatine treatment restored the reduced eNOS immunoreactivity detected in the aged animals to that of the young control groups. However, no age-related change was detected in the KCI and papaverine responses. In addition to such data, it was demonstrated that eNOS immunoreactivity in the thoracic aorta was reduced in the aged animals compared to the young animals, but a long-term agmatine application prevented this reduction. Moreover, in a stress model, down-regulated gene expressions of all stress-induced NLRP3 inflammasome components, such as NLRP3, IL-1β and IL-18, have been shown by exogenously administered agmatine in the prefrontal cortex and hippocampus. Agmatine decreased pro-inflammatory cytokine levels in these brain regions and in serum. In addition, stress decreased IL-4 and IL-10 levels and agmatine treatment restored these anti-inflammatory cytokines to normal in the prefrontal cortex. Therefore, it was claimed that the mechanisms underlying the neuroprotective role of agmatine would inhibit the TNF-α and IL-1β secretion, and clean the reactive oxygen types, or block the intrinsic apoptotic pathway, so that the NOS activity or protein expression might be affected [44,53,55–57].

Furthermore, it was reported that aging causes an increase in the iNOS activity, and a distortion in the eNOS/iNOS balance [58]. It is well known that agmatine plays an important role in regulating the NO production [59–61]. The treatment with agmatine by inhibiting iNOS has a neuroprotective effect, and may correct distorted balance. In addition to the age-related increase in iNOS activity, the levels of free oxygen radicals were found to be increased [62]. Agmatine is known to have a strong antioxidant effect, and it's protective effect on

the age-related endothelial dysfunction might be explained also with the anti-oxidizing effect of agmatine [56].

BDNF, a member of the neurotrophine family of growth factors, has a crucial role in cognitive functions [63] and is shown to play a critical role in the aging and neurodegenerative process. The mechanism by which aging impairs memory involves decreased BDNF levels. Several lines of findings support this concept. Accordingly, our findings support that the significant decrease in BDNF immunoreactivity in the hippocampal CA1 and CA3 zones as well as amygdala of the aged rats in comparison with the young control group provides direct evidence for the role of BDNF in memory deficit. Furthermore, agmatine treatment caused a significant increase in the age-related BDNF reduction in the hippocampus and amygdala, thus showing a neuroprotective effect.

Taken together, the findings of this study show that long-term agmatine administration increases the BDNF levels in both the hippocampus and amygdala, and also peripherally the NO synthesis and/or bioavailability, and corrects the age-related endothelial dysfunction, and hence may help in recovering vascular aging and vascular dementia.

Disclosure statement

No potential conflict of interest was reported by the authors.

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