

The effects of black garlic ethanol extract on the spatial memory and estimated total number of pyramidal cells of the hippocampus of monosodium glutamate-exposed adolescent male Wistar rats

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Abstract Monosodium glutamate (MSG) is believed to exert deleterious effects on various organs, including the hippocampus, likely via the oxidative stress pathway. Garlic (*Alium sativum* L.), which is considered to possess potent antioxidant activity, has been used as traditional remedy for various ailments since ancient times. We have investigated the effects of black garlic, a fermented form of garlic, on spatial memory and estimated the total number of pyramidal cells of the hippocampus in adolescent male Wistar rats treated with MSG. Twenty-five rats were divided into five groups: C– group, which received normal saline; C+ group, which was exposed to 2 mg/g body weight (bw) of MSG; three treatment groups (T2.5, T5, T10), which were treated with black garlic extract (2.5, 5, 10 mg/200 g bw, respectively) and MSG. The spatial memory test was carried out using the Morris water maze (MWM) procedure, and the total number of pyramidal cells of the hippocampus was estimated using the physical disector design. The groups treated with black garlic extract were found to have a shorter path length than the C– and C+ groups in the escape acquisition phase of the MWM test. The estimated total number of pyramidal cells in the CA1 region of the hippocampus was higher in all treated

groups than that of the C+ group. Based on these results, we conclude that combined administration of black garlic and MSG may alter the spatial memory functioning and total number of pyramidal neurons of the CA1 region of the hippocampus of rats.

Keywords Monosodium glutamate · Black garlic · Spatial memory · Pyramidal cells · Hippocampus

Introduction

Monosodium glutamate (MSG) has been used widely all over the world as a flavour enhancer due its well-known umami taste (Sand 2005; Freeman 2006). However, MSG is also suspected to be capable of exerting various negative effects on various organs, including the brain (Farombi and Onyema 2006; Collison et al. 2009). In fact, the brain is thought to be one of the most vulnerable organs to the effects of MSG due to its high metabolism, high content of polyunsaturated fatty acids, low anti-oxidant capacity and the hard-to-replicate property of its neuronal cells (Blaylock 1997; Singh et al. 2003; Noor and Mourad 2010), with the hippocampus, characterized by a high density of glutamate receptors (Blaylock 1997), being one of the most susceptible areas (Noor and Mourad 2010).

The hippocampus is the site of selection of important information and consolidation of spatial memory. Environmental sensory inputs received by the cortical sensory and association areas are transmitted in sequential order via the entorhinal cortex and hippocampal formation [dentate gyrus (DG), a series of cornu ammonis regions (CA3, CA2, CA1), subiculum] before finally transferred back to the cortical association areas (Andersen et al. 2007). *N*-methyl-D-aspartate receptors (NMDARs) are one

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type of glutamate receptor present in CA regions. NMDARs play an important role in spatial memory acquisition in the CA1 regions and, in contrast, mediate spatial memory retrieval in the CA3 regions. The naturally constant alteration of sensory inputs activates parts of stored memories, upon which CA3 NMDARs stimulate the firing of the recurrent network in order to assemble the complete pattern of the memories (termed “pattern completion”) (Nakazawa et al. 2004). The DG–CA3 network compares novel environmental inputs to recently acquired representations (e.g. within a fraction of minutes), while CA1 cells compare the inputs to longer term acquired representations (e.g. within the past hours or days) (Lee et al. 2005). CA1 cells are essential for associating information received gradually when there are considerable temporal intervals between components of stimuli. Of note, it is possible that CA1 and CA3 cells have an overlapping role in memory which involves temporal ordering associated with spatial location (Langston et al. 2010). Given the roles of the CA3 and CA1 regions in spatial memory formation, it follows that any disruption to the pyramidal cells of the CA2–CA3 and CA1 regions may cause spatial learning and memory deficits.

Garlic (*Alium sativum* L.) has been used as traditional remedy for various ailments across continents since ancient times (Sato et al. 2006; Park et al. 2009). Meticulous scientific investigations have revealed that the efficacy of garlic depends on its various properties, including its action against oxidative stress and modulation on anti-oxidant enzyme activities (Afzal et al. 2000; Borek 2001; Ide and Lau 2001; Numagami and Ohnishi 2001; Atif et al. 2009; Morihara et al. 2011; Colin-Gonzalez et al. 2012) which suggest its potential role as a neuroprotector (Mathew and Biju 2008). Garlic extract has also been shown to prevent neuronal death and reduce glutamate level in the ischaemic model of rats (Saleem et al. 2006). The fermentation of garlic under specific conditions produces black garlic with a relatively higher anti-oxidant activity (Sato et al. 2006; Wang et al. 2010; Kim et al. 2013) owing to its higher polyphenol level, scavenging activity and effectiveness to prevent DNA damage (Kim et al. 2013) compared to fresh garlic extract.

We have previously reported the effects of black garlic on both the estimated total number of Purkinje cells in the cerebellum and motor coordination of rats treated with MSG (Aminuddin et al. 2014). It remains largely undetermined whether MSG causes spatial memory and hippocampal pyramidal cells deficits and whether black garlic extract could reverse these deficits. The aim of this study was to investigate these effects in a rat model.

Materials and methods

Animals and treatments

Twenty-five male Wistar rats aged 3–4 weeks were used in this experiment. They were obtained from the animal house of Universitas Gadjah Mada (Gadjah Mada University), Yogyakarta, Indonesia. The experimental procedure was approved by the Ethical Committee of the Faculty of Medicine, Universitas Gadjah Mada (approval number KE/FK/302/EC). The rats were randomly divided into five groups: (1) C– (negative control) group, treated with isotonic saline solution orally and intraperitoneally; (2) C+ (positive control) group, given isotonic saline solution orally and injected with MSG intraperitoneally [2 mg/g body weight (bw)]; (3–5) treatment groups T2.5, T5 and T10, fed with garlic extract solution orally (2.5, 5 and 10 mg/200 g bw, respectively) 30 min prior to an intraperitoneal injection of MSG (2 mg/g bw). All treatments were carried out for 10 consecutive days. The rats were fed pellets and water ad libitum throughout the experiment and were maintained under a 12/12-h light/dark cycle.

A solution of 99 % MSG in isotonic saline solution was made fresh daily. The garlic extract used in the study was black garlic prepared through spontaneous fermentation, based on the protocol described by Sato et al. (2006). Briefly, fresh garlic was weighed and fermented without any additives for 40 days at 60 °C and 85–95 % relative humidity (Sato et al. 2006; Wang et al. 2010). Once prepared, the black garlic was pulverized and soaked in 70 % ethanol solution for 3 days. The ethanol solution was subsequently evaporated from this mixture using a rotary evaporator followed by evaporation in a water bath. The filtrate of this process was weighed and was ready for use. It has been suggested that humans can reap the beneficial effects of fresh garlic with a daily intake of 4 g/day (Amagase et al. 2001; Tattelman 2005). The equivalent dosage for rats can be calculated by multiplying this dosage by 0.018 (Pamidiboina et al. 2010; Mursito et al. 2011), which is equal to 72 mg/day of fresh garlic or 5 mg/day of garlic extract. The equivalence of the weights of fresh garlic and black garlic extract was derived by determining the weight of fresh garlic (prior to fermentation) and that of black garlic extract (subsequent to fermentation). The garlic extract was also given to the treatment groups of rats as half (2.5 mg/day) and twice (10 mg/day) this standard dosage (5 mg/day) in order to determine the optimum dose. Total phenol content of the extract was determined using a spectrophotometer (Shimadzu Corp., Kyoto, Japan) run at a wavelength of 755 nm and found to be 5.12 % of the total phenol gallic acid equivalent.

Morris water maze test

Morris water maze (MWM) tests were performed 1 day after the last treatment. The protocol described by Partadiredja and Bedi (2011) was used in this spatial memory test, with slight modifications. The apparatus consisted of a circular tank with a diameter of 1.5 m and a height of 0.4 m. The tank was divided into four equally imaginary quadrants, which were designated as A, B, C and D. A circular platform made of a white tin container (diameter 13 cm, height 16.5 cm) was placed in the middle of one randomly selected quadrant. The position of the platform was kept constant for each rat throughout the experiment. Eight evenly spaced starting points were marked around the inner wall of the tank. Several different colour pictures were also attached at the circumference wall of the tank. The pool was filled with water up to a height of 18–19 cm (i.e. 1.5–2.5 cm above the platform). The water temperature was around 25 °C and made opaque by adding coconut milk to hide the platform. A video camera was placed above the centre of the pool and relayed the image of the tank and the movement of the animals to an adjacent computer.

On the day prior to the trial days, the rats were moved to the test room in order to habituate to their surroundings. On the days of trials, the platform was placed in the middle of a randomly selected quadrant for each rat. A number between one and eight was chosen randomly in each trial, and this number indicated the starting point where a given rat was placed to start swimming. The trial started by placing the rat in the pool at this starting point, with its head facing towards the inner wall of the tank. The rat would be forced to swim in order to escape from the water and was expected to accidentally locate the platform and climb onto it. The time needed by the rat from the start of swimming until climbing onto the platform was recorded as “escape latency”. The rat was allowed to be on the platform for 1 min before being removed, dried and returned to its cage for 1 min before the next trial. Each trial was limited to a maximum of 3 min. Those rats which failed to locate the platform within 3 min were removed from the pool, dried and returned to their cages for 1 min before the next trial. In this case, 3 min was recorded as the escape latency. Each rat performed eight trials per day on 3 consecutive days (“escape acquisition phase”). On days 10 and 17, the rats were assigned single trials per day (“memory persistence test phase”) to examine whether they were able to retain the spatial memory on the location of the submerged platform (Partadiredja and Bedi 2011) following the initial escape acquisition phase. All trials were recorded with a video camera. The length of the swimming track of the rat was measured using a curvimeter (Comcurve 10; Koizumi Sokki Mfg, Nagaoka-shi, Niigata,

Japan). The data collected on escape latency and path length of the swimming track were used to examine the spatial memory function of the rats.

On days 18 and 19, the proximal cues task/sensorimotor test was carried out in order to examine the possible sensory, motor or motivational deficits. In this version of the MWM test, the room and apparatus used were exactly the same as those used in the spatial memory test (“the distal cues version”), with the exception that the platform was made visible (i.e. the surface of the platform was above the water surface) and a yellow flag was attached to the platform (Kandel et al. 2000). This non-spatial test is designed to exclude any possible interference of sensory or motor defects and does not require hippocampus function. Therefore, provided that there is no significant difference between groups in the escape latency and path lengths during this visible platform version of the MWM test, any difference between groups that later arise from the escape latency and path lengths in the non-visible platform test is liable to the difference in memory ability. The test consisted of four trials per day, with each trial lasting for a maximum of 30 s. The platform was placed in the quadrant which was different from that in the spatial memory test, but it was kept in a constant position in this quadrant during this test (Sarnyai et al. 2000; Craig et al. 2008). The performances of the rats were recorded using a video camera and a laptop computer. The time needed to locate and climb onto the platform and the swimming tracks were also measured.

Tissue preparation

Two days after the last sensorimotor test, the rats were sacrificed by trans-cardial perfusion with 4 % formaldehyde in phosphate buffer solution (PBS), under ketamine HCl anesthesia (ketamine HCl 40 mg/kg bw; PT Guardian Pharmatama, Jakarta, Indonesia). The cerebrums were then removed from the skulls of the rats and weighed. The right hippocampus was dissected out of the cerebrum, dehydrated in graded series of alcohol concentrations, cleared in toluene solution and finally embedded in paraffin blocks. The blocks were sectioned at a thickness of 3 µm using a Leica RM 2235 microtome (Leica Biosystems, Wetzlar, Germany). Examples of micrographs of CA1 and CA2–CA3 regions of all groups are presented in Fig. 1. One pair of sections per 200 sections was taken for stereological analysis. The number of sections taken for analysis by light microscopy was determined randomly by lottery. The slides were then processed and stained with toluidine blue.

Stereological analyses

The volume of the CA1 region and of the CA2–CA3 region of the hippocampus was estimated using the Cavalieri

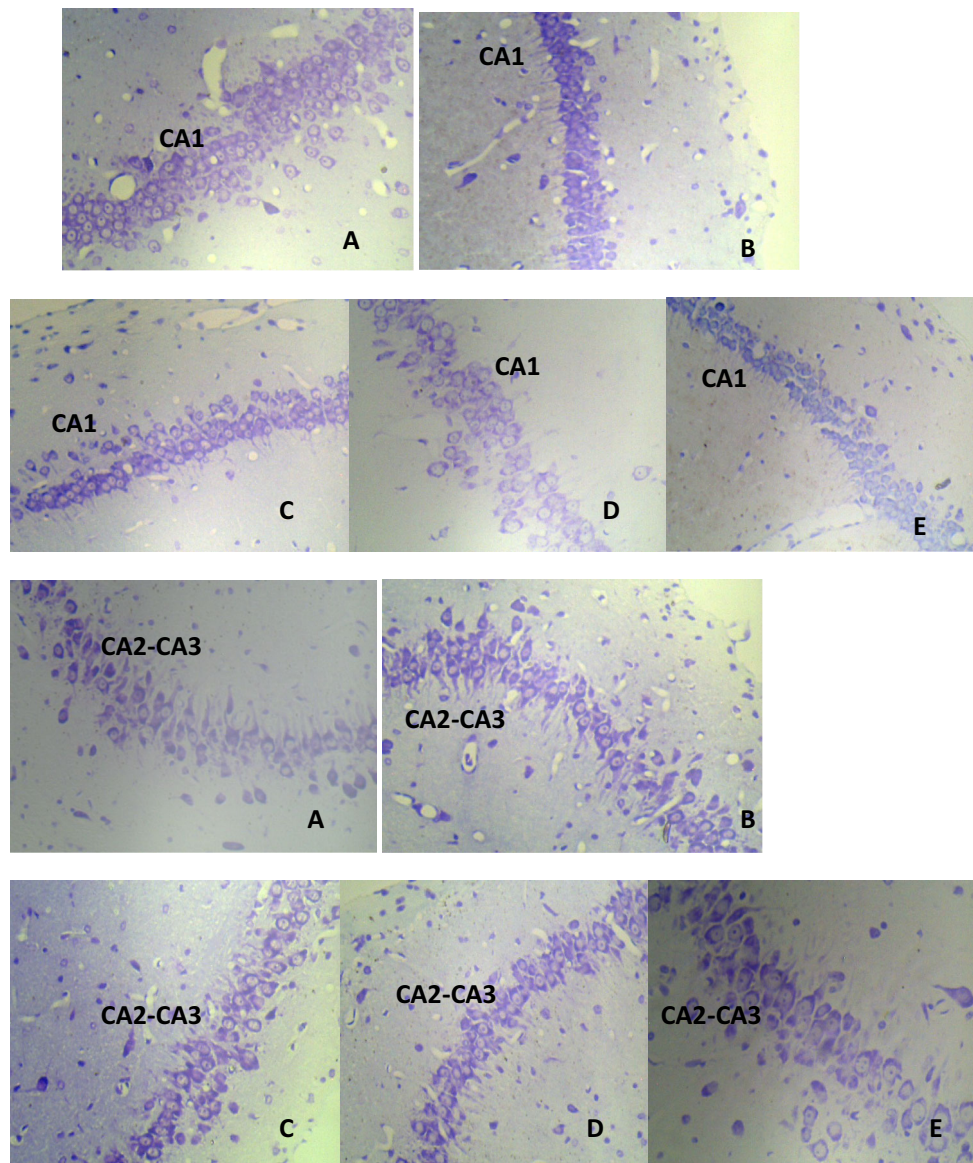


Fig. 1 Micrographs representing the pyramidal cells layer of the CA1 and CA2–CA3 regions of the hippocampus of rats of the C– (a), C+ (b), T2.5 (c), T5 (d) and T10 (e) groups. C– negative control group, NaCl 0.9 % intraperitoneally (ip) + NaCl 0.9 % orally (po); C+ positive control group, monosodium glutamate (MSG) 2 mg/g

body weight (bw) (ip) + NaCl 0.9 % (po); T2.5 treatment group 2.5, MSG 2 mg/g bw (ip) + *Allium sativum* 2.5 mg/200 g bw (po); T5 treatment group 5, MSG 2 mg/g bw (ip) + *A. sativum* 5 mg/200 g bw (po); T10 treatment group 10, MSG 2 mg/g bw (ip) + *A. sativum* 10 mg/200 g bw (po)

principle (Gundersen and Jensen 1987; Michel and Cruz-Orive 1988; Miki et al. 2000, 2004; Partadiredja and Bedi 2010). One section from each pair of sections was analyzed using a digital camera (Optilab, Miconos, Indonesia) connected to a light microscope (XSZ, PR China) and a laptop computer. Pictures of the sections were taken and combined using Adobe Photoshop CS4 (Adobe System Inc., San Jose, CA) to enable the entire hippocampal area to be shown in a section. The ImageJ software program (NIH Image; National Institutes of Health, Bethesda, MD) was used to make a virtual grid composed of test points spaced 10 mm apart. This grid was superimposed to the picture of

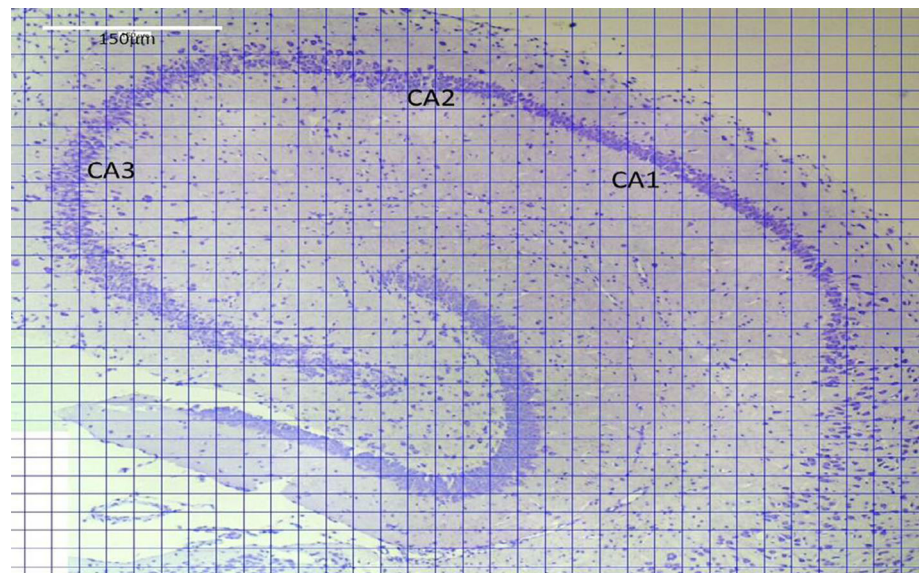
hippocampus (Fig. 2). The number of points of the grid falling on the CA1 and CA2–CA3 regions of the hippocampus was then counted.

The total number of points (P) for each rat was obtained from the sum of all points falling on all sections in a given rat. The volume of the hippocampus (V) was calculated using the formula (Bedi 1991; Howard and Reed 2005; Partadiredja and Bedi 2010):

$$[V = P \cdot u \cdot t \cdot N/n]$$

where P is the total number of points, u is the area represented by each point of the grid, t is the thickness of the

Fig. 2 A micrograph showing a section of cornu ammonis (regions CA1–CA3) of the hippocampus superimposed with a grid having a regular array of test points



serial section, N is the total number of serial sections through hippocampus and n is the number of serial sections sampled used for counting.

The numerical density of pyramidal cells of the hippocampus was estimated using the physical disector probe (Sterio 1984; Partadiredja and Bedi 2010). Briefly, a pair of consecutive sections was assigned as a *test section* and a *look up section*, respectively. Systematic random sampling was used to determine the section taken. A picture was taken of these sections using a digital camera connected to the light microscope and computer. The cell count (Q -) was obtained from the number of cell nuclei that appeared in the test section, but not in the look-up section (see the example in Fig. 3). The procedure was done using a counting frame as described by Sterio (1984) and Partadiredja and Bedi (2010). In order to increase the efficiency of counting the cells, the sections which were first assigned as test sections were then assigned as look up sections, and vice versa.

The numerical density (N_v) of pyramidal cells were calculated using the formula (Sterio 1984; Howard and Reed 2005; Partadiredja and Bedi 2010):

$$[N_v = \Sigma Q - /a \cdot h]$$

where Q - is total number of nuclear profiles counted, a is total area of test section and h is the thickness of sections.

The hippocampus may shrink due to tissue processing which would cause bias in the calculation of hippocampal volume and pyramidal cell numerical density. To avoid potential bias, we calculated this shrinkage using the method described by Partadiredja et al. (2003) with a slight modification. Briefly, a grid made up of test points regularly spaced 1 cm apart was superimposed on the pictures of hippocampal tissues taken before and after the tissues

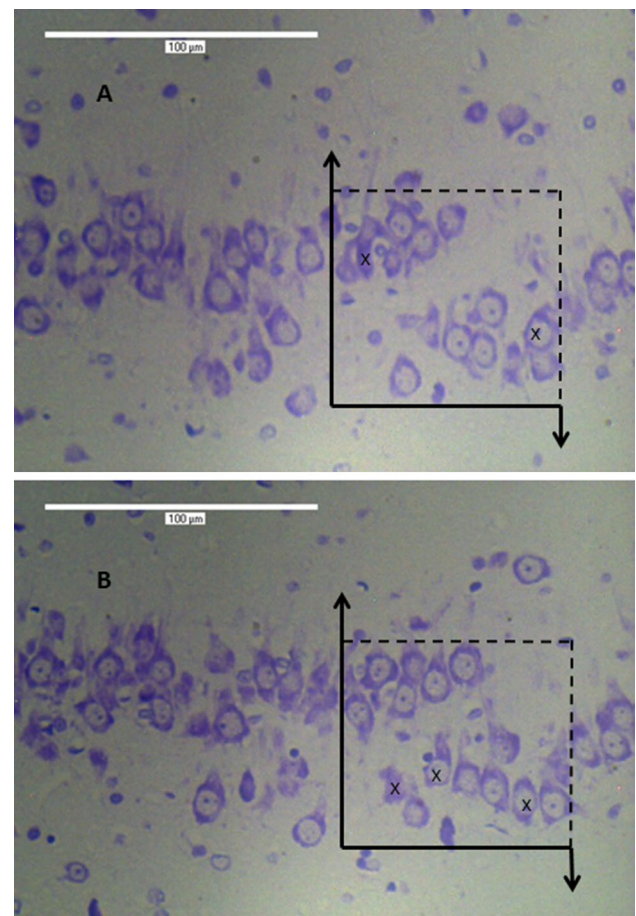


Fig. 3 Micrographs showing pyramidal cells of the CA region of the hippocampus counted using the physical disector probe. **a**, **b** Two consecutive sections of 3- μ m thickness representing test sections and look up sections to each other. Neurons showing a nuclear profile appear in the test section but not in the look-up section were counted (x)

had been dehydrated. The shrinkage factor was calculated by dividing the number of points on the hippocampal picture after the dehydration process to those prior to dehydration; the value obtained was subsequently subtracted from 1. The average shrinkage factor was found to be 12.52 %.

The estimated total number of pyramidal cells in the CA1 and CA2–CA3 regions of the hippocampus was calculated by multiplying the volume with the numerical densities. This estimate was not affected by the shrinkage factor, since the shrinkage factor correction incorporated into the volumes and numerical densities data cancelled each other out in the calculation of total number of cells.

Statistical analyses

The normality and variance of all data were tested using Saphiro-Wilk and Levene tests, respectively. Whenever the data were normally distributed and homogenous, the data were analysed using parametric test (one-way ANOVA). Otherwise, the data were transformed into log 10 data, and provided that the data became normal and homogenous, they were analysed using parametric test. Whenever the data were failed to be normalized, they were analysed using Kruskal-Wallis test. LSD post hoc tests were carried out whenever necessary. All data were analysed using SPSS software version 19. The significance level was set at $p < 0.05$.

The MWM test

The data on escape latencies and path lengths of escape acquisition and memory persistence tests did not pass the normality and variance tests. Following transformation, the data were still not normal and not homogenous and, consequently, could not be analysed using a parametric test [two-way analysis of variance (ANOVA)]. We therefore analysed the data on a per-trial basis. In trials in which data passed the normality and variance tests, one-way ANOVA was used; otherwise, the Kruskal–Wallis test was used to test the data. The data obtained from the sensorimotor test consisting of escape latencies and path lengths were analysed using the Friedman test. Statistical analyses were done on the escape latency data of all trials, but only significant results are shown in Table 1.

Estimated total number of hippocampal pyramidal cells

The data of estimated total number of pyramidal cells of hippocampal CA1 region were not normally distributed and not homogeneous. Therefore these data were transformed into log10 data prior to the one-way ANOVA procedure. On the other hand, the data of the estimated total number of

Table 1 The results of post hoc analyses using the least significant difference procedure on path length during the 3-day escape acquisition phase of the Morris Water Maze test

Trials	Groups	Post hoc p
Trial 2	C– vs. C+	0.035
	C+ vs. T5	0.003
	C+ vs. T10	0.034
	T2.5 vs. T5	0.017
Trial 13	C– vs. T5	0.033
	C– vs. T10	0.001
	C+ vs. T10	0.014
	T2.5 vs. T10	0.027
Trial 14	C– vs. C+	0.003
	C– vs. T5	0.006
Trial 17	C– vs. T2.5	0.003
	C+ vs. T2.5	0.036
	T2.5 vs. T5	0.039
Trial 18	C– vs. C+	0.020
	C+ vs. T2.5	0.041
	C+ vs. T5	0.004
	C+ vs. T10	0.003

C–, Negative control group, NaCl 0.9 % intraperitoneal (ip) + NaCl 0.9 % per oral (po); C+, positive control group, monosodium glutamate (MSG) 2 mg/g body weight (bw) (ip) + NaCl 0.9 % (po); T2.5, treatment group, 2.5 mg MSG/g bw (ip) + garlic (*Allium sativum*) 2.5 mg MSG/200 g bw (po); T5, treatment group, treatment of 2 mg MSG/g bw (ip) + *A. sativum* 5 mg/200 g bw (po); T10, treatment group, treatment of 2 mg MSG/g bw (ip) + *A. sativum* 10 mg/200 g bw (po)

pyramidal neurons of the CA2–CA3 region were normally distributed and homogenous, and hence these data were straightforwardly tested using one-way ANOVA.

Results

MWM test

Escape acquisition test

The data recorded for escape latency are presented in Fig. 4. It can be seen that following trial 4, the T10 group had the steadiest and flattest curve, while the C+ group had the most fluctuating one. A significant difference between groups can be seen for trial 13 ($p = 0.026$). The post hoc least significant difference (LSD) test of these data showed that the escape latency of the T10 group was significantly shorter than that of the C– ($p = 0.002$), C+ ($p = 0.027$) and T2.5 ($p = 0.034$) groups.

Figure 5 shows the data recorded on the path lengths of the escape acquisition test for all groups of rats. Similar to

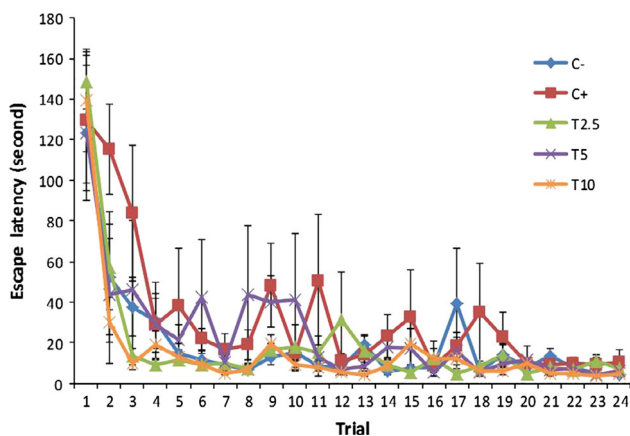


Fig. 4 Mean \pm standard error of the mean (SEM) values for escape latency during the 3-day escape acquisition phase of the Morris Water Maze (MWM) test for the five groups of rats. See footnote of Table 1 or caption of Fig. 1 for detailed description of the groups

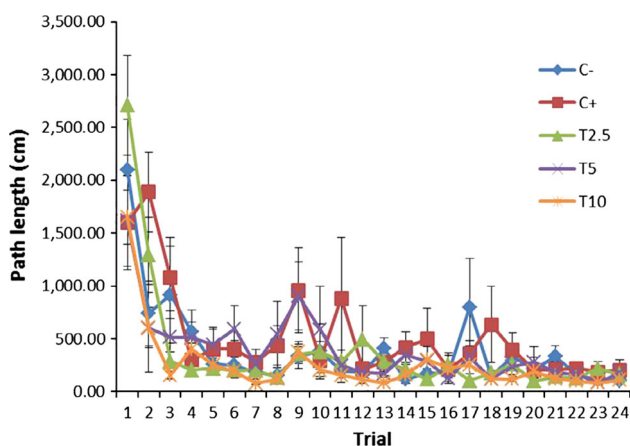


Fig. 5 Mean \pm SEM values for path length during the 3-day escape acquisition phase of the MWM test for the five groups of rats. See footnote of Table 1 or caption of Fig. 1 for detailed description of the groups

the plot of escape latency of the escape acquisition test, following trial 4, the T10 group had the steadiest and flattest curve, while the C+ group had the most fluctuating curve. One-way ANOVA showed that there were significant differences between groups in trials 2, 13, 14, 17 and 18. The results of post hoc LSD test of these trials are presented in Table 1 (only those with $p < 0.05$ are shown in the table for the sake of brevity).

In these trials, post hoc analysis showed that the groups given black garlic (T2.5, T5, T10) followed significantly shorter swimming trajectories than those of the control groups (C- and C+), with the MSG-treated group (C+) generally having the longest path lengths. Furthermore, in trials 2, 14, and 18, the C+ group had a significantly longer swimming path than the C- group. These results show that MSG at the dosage administered had some degree of detrimental effect on spatial memory acquisition.

Table 2 Mean values of the \log_{10} escape latency in the memory persistence test of the Morris Water Maze procedure

Groups	n	Trial day		
		Day 3	Day 10	Day 17
C-	5	1.29 \pm 0.22	1.25 \pm 0.24	1.53 \pm 0.12
C+	5	1.11 \pm 0.18	0.93 \pm 0.16	1.38 \pm 0.30
T2.5	5	0.66 \pm 0.09	1.05 \pm 0.19	1.26 \pm 0.09
T5	5	1.05 \pm 0.16	1.30 \pm 0.11	1.02 \pm 0.15
T10	5	0.95 \pm 0.16	1.18 \pm 0.19	1.27 \pm 0.24
One-way ANOVA		df = 4.20; F = 1.756; p = 0.178	df = 4.20; F = 0.621; p = 0.653	df = 4.20; F = 0.862; p = 0.503

ANOVA, Analysis of variance; n, number of animals; df, degrees of freedom

Data are presented as the mean \pm standard error of the mean (SEM); See footnote of Table 1 or caption of Fig. 1 for detailed description of the groups

Table 3 Mean values of the \log_{10} path length in the memory persistence test of the Morris Water Maze procedure

Groups	n	Trial day		
		Day 3	Day 10	Day 17
C-	5	2.69 \pm 0.19	2.55 \pm 0.26	2.82 \pm 0.13
C+	5	2.44 \pm 0.15	2.17 \pm 0.16	2.52 \pm 0.27
T2.5	5	1.97 \pm 0.08	2.30 \pm 0.17	2.45 \pm 0.10
T5	5	2.43 \pm 0.14	2.57 \pm 0.13	2.26 \pm 0.17
T10	5	2.30 \pm 0.14	2.41 \pm 0.19	2.53 \pm 0.22
		df = 4.20; F = 3.166; p = 0.036 ^a	df = 4; p = 0.416 ^b	df = 4.20; F = 1.076; p = 0.395 ^a

Data are presented as the mean \pm SEM; See footnote of Table 1 or caption of Fig. 1 for detailed description of the groups

^a One-way ANOVA

^b Kruskal-Wallis test

Memory persistence test

Tables 2 and 3 present the results of memory persistence test. In general, at the end of the persistence test (day 17), the black garlic-treated groups (T2.5, T5, and T10) had shorter escape latencies and followed shorter path lengths than the controls C- and C+, albeit no statistically significant differences were detected. There were significant differences between groups in path length ($p = 0.036$) in trial 17 (day 3). In this trial, the post hoc LSD test revealed that the T2.5 group travelled significantly shorter trajectories than the C- ($p = 0.003$), C+ ($p = 0.036$), and T5 ($p = 0.039$) groups.

Sensorimotor test

The Friedman test of the data of sensorimotor test revealed that there were no significant differences between groups in

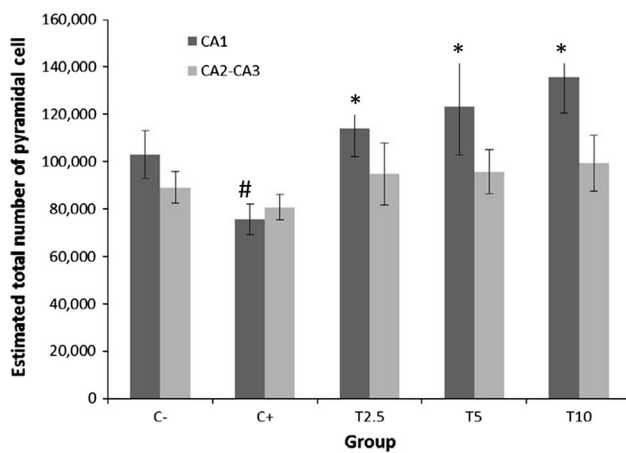


Fig. 6 Mean \pm SEM values for the estimated total number of pyramidal cells of the CA1 and CA2–CA3 regions of the hippocampus of the five groups of rats. * $p < 0.05$ compared with C+ (MSG treated group) # $p < 0.05$ compared with the T2.5, T5 and T10 groups. See footnote of Table 1 or caption of Fig. 1 for detailed description of the groups

escape latencies and path lengths, thereby indicating that there were no differences in sensorimotor capability between groups. Thus, the results of the MWM test may have purely reflected the individual spatial memory capability of each animal.

Estimated total number of hippocampal pyramidal cells

Data collected on the estimated total number of pyramidal cells of the hippocampus are presented in Fig. 6. One-way ANOVA of the log10 transformation data of the estimated total number of pyramidal cells in the CA1 region of hippocampus showed a significant difference between groups ($p = 0.027$). Post hoc LSD tests of these data revealed that the estimated total number of pyramidal neurons of the hippocampal CA1 region of the C+ group (75.471 ± 6.575) was significantly lower than that of the T2.5 (114.054 ± 11.929 ; $p = 0.024$), T5 (123.264 ± 20.666 ; $p = 0.013$), and T10 (135.705 ± 15.119 ; $p = 0.002$) groups. On the other hand, one-way ANOVA of the data of the estimated total number of pyramidal cells in the CA2–CA3 regions of hippocampus found no significant differences between groups ($p = 0.695$).

Discussion

The results of this study reveal that the black garlic-treated groups (groups T2.5, T5, T10) had significantly shorter escape latencies and path lengths than the control groups (C–, C+) in several trials of the non-visible platform test of the MWM procedure. In addition, the C+ group performed significantly worse than C– group in several trials

of the escape acquisition phase of MWM test. These differences likely stemmed from differences in memory ability since there were no significant differences between groups in the escape latencies and path lengths in the visible platform version of MWM test. In addition, the estimated total number of pyramidal cells of the CA1 region of the hippocampus was significantly higher in the black garlic-treated groups (T2.5, T5, T10) than in the MSG only-treated group C+). On the other hand, there was no significant difference between groups in the estimated total number of hippocampal pyramidal neurons of the CA2–CA3 region.

Several studies have reported that any disturbance or damage to the CA1 region due to focal lesion (Bartsch et al. 2010), two-vessel occlusion (Auer et al. 1989), four-vessel occlusion (Olsen et al. 1994) or NMDAR1 gene knock-out (Tsien et al. 1996) impairs spatial learning in the MWM task. However, Nakazawa et al. (2002) found that selective ablation of NMDAR1 in the CA3 region of the hippocampus did not interrupt the performance of mice in the hidden platform version of the MWM task. It has been argued that CA1 NMDARs are critically involved in the acquisition of spatial memory and that CA3 NMDARs are not—rather the latter contribute more to the retrieval of the memory using pattern completion and not spatial memory acquisition (Nakazawa et al. 2004). Our study shows that all black garlic-treated groups performed better than both control groups at several trials of the escape acquisition phase of the MWM test, but not in the memory persistence test, suggesting that this better performance may have been brought about by alterations in the CA1 regions of the rats.

To our knowledge, only few studies have focused on the effects of MSG on spatial learning and pyramidal cells of hippocampus of rats. These previous studies reported that the administration of low-dose MSG altered mice behaviour (Onaolopo and Onaolopo 2011) and caused neuronal apoptosis and necrosis (Blaylock 1997; Balazs et al. 2006; Wang and Qin 2010), swelling and degeneration of cells (Xiong et al. 2009), and damage to neuronal cells in the hypothalamus (Eleftheriou et al. 2003) and hippocampus (Puica et al. 2009). While our study extends the previous findings of Onaolopo and Onaolopo (2011), who reported on the effects of MSG on the behaviour of mice, we were unable to definitively confirm that MSG (at current dosage) reduces the number of hippocampal pyramidal cells. Nevertheless, we found that the estimated total number of pyramidal neurons of the CA1 region of black garlic-treated groups was significantly higher than that of MSG-treated group (C+), but was not different from that of the negative control group (C–). This result may indicate that MSG may cause reduction in the number of pyramidal cells and that black garlic may reverse this deficit. However, it is also plausible that MSG might have damaged the

pyramidal cells without reducing the number of the cells. Puica et al. (2009) noted some degeneration in the CA regions of the hippocampus of rabbits administered an oral dose of MSG lower than that used in our study on rats. In the same line as our findings, another study in our laboratory found that a specific dose of MSG did not cause deficits in terms of the number of Purkinje cells or motor coordination functions (unpublished results), whereas other studies using the same dose of MSG reported pathological changes in the Purkinje cells (Eweka and Om'Iniabohs 2006; Hashem et al. 2012). The precise reason for these discrepancies remains uncertain at the present time.

Our finding that there were significant differences in the estimated total number of pyramidal cells of the hippocampal CA1 region between the five groups, but not in that of the hippocampal CA2–CA3 region, is apparently based on the differential susceptibility [termed “selective neuronal vulnerability” (SNV); Wang and Michaelis 2010] of the cells of these two regions to neurotoxic or environmental insults. Previous studies have reported that the CA1 region is more vulnerable than the CA3 region to duroquinone (a superoxide anion generator) (Wilde et al. 1997), colchicine (Xavier et al. 1999), ischaemia (Bernaudin et al. 1998; Herguido et al. 1999; Rao et al. 1999; Cronberg et al. 2005; Gee et al. 2006), alcohol (Bonthius and West 1990, 1991; Bonthius et al. 2001; Tran and Kelly 2003), chronic mild stress (Ritchie et al. 2004), paraquat (Wang et al. 2009) and malnutrition (Lister et al. 2005). In contrast, compared to the CA1 region, it has been reported that the CA2–CA3 region suffers more from considerable cellular degeneration in response to toluene (Korbo et al. 1996), dexamethasone (Sousa et al. 1999), kainate (Ramsden et al. 2003), fluid percussion injury (Grady et al. 2003) and hydroxyl radical damage due to ferrous sulphate (Wilde et al. 1997). Such SNV to external manipulation may imply differential neuronal properties between these two regions (Matsuyama et al. 1994; Kato et al. 1995; Wilde et al. 1997; Lister et al. 2005). Studies on the distribution of glutamate receptors (Korbo et al. 1996; Fiacco et al. 2003) suggest that the CA3 region contains more kainate receptors than the CA1 region, whereas the CA1 region possesses more NMDA receptors. The activation of NMDARs results in elevation of the cytosolic Ca^{2+} level which eventually leads to mitochondrial damage, activation of mitochondrial permeability transition, release of pro-apoptotic proteins and production of reactive oxygen species. These effects are even more significant in the CA1 region than in the CA3 region since the activation of NMDARs induces a much higher increase of Ca^{2+} level in CA1 neurons than in CA3 neurons (Gee et al. 2006; Butler et al. 2010; Stanika et al. 2010; Wang and Michaelis 2010). Several investigations (Mattiasson et al. 2003; Wang et al.

2005, 2009; Wang and Michaelis 2010) have found that the gene expression levels of superoxide anion, pro-oxidant and anti-oxidant enzymes are normally higher in the CA1 region than in the CA3 region. At a physiologically high level, superoxide anion is involved in developing synaptic long-term potentiation in the CA1 region, but not in CA3 region (Wang et al. 2005). Superoxide anion is also well-known for its deleterious effect on cells (Fang et al. 2002). Since the oxidative redox balance of the hippocampus is readily shifted towards the pro-oxidant status, any additional exposure of the cells of the hippocampus to generators of free radical may easily induce cellular death (Wang et al. 2005). All of the above-mentioned factors may (partially) explain the MSG-induced selective damage of pyramidal cells in the CA1 region of the hippocampus, but not in the CA3 region (Gee et al. 2006; Butler et al. 2010; Wang and Michaelis 2010).

Black garlic extract may exert a possible preventive effect via its antioxidant properties. The main active substance in black garlic extract is *S*-allyl-cysteine (SAC) (Wang et al. 2010), which can act both directly and indirectly as an antioxidant. As a direct antioxidant, SAC scavenges free radicals (Borek 2001; Atif et al. 2009; Colin-Gonzalez et al. 2012). As an indirect antioxidant, SAC restores glutathione peroxidase, glutathione reductase and superoxide dismutase levels, activates the Nrf2 factor, which is a transcription factor that regulates the expression of antioxidant and cytoprotective genes and inhibits NF κ B activation (a transcription factor activated by oxidative stress) (Borek 2001; Ide and Lau 2001; Colin-Gonzalez et al. 2012). In addition, it has been shown that in rat hippocampal neuron culture, SAC increases neuron survival and axonal branching (Colin-Gonzalez et al. 2012).

Conclusions

In summary, the results presented here demonstrate that the combined administration of black garlic extract and MSG may have exerted interactive effects on both the structure and functioning of the hippocampus of rats. The dosage of MSG tested in this study may not have been adequate to effect a reduction in the number of pyramidal cells of the hippocampus. However, the dosage was sufficient to disrupt the spatial memory functioning of the rats, and the administration of black garlic extract seemed to prevent this deficit. Our results also suggest that the pyramidal cells of the CA1 region are more sensitive to concomitant administration of black garlic extract + MSG than those of CA2–CA3 region. The precise significance and mechanisms of our findings clearly merit further study.

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