Concentrates of buttermilk and krill oil phospholipids moderately improve cognition in aged rats

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Abstract

Cognitive decline is one of the hallmarks of aging and can vary from mild cognitive impairment to dementia to Alzheimer's disease. In addition to some lifestyle interventions, there is room for the use of nutraceuticals/functional food, as pharma-nutritional tools to lessen the burden of cognitive decline before it worsens. We previously reported the promising molecular actions of milk fat globule membranes and krill oil's concentrates in a rat model of aging. In this study, we concentrated on the activities on cognition, using an array of validated tests. We also performed lipidomic analyses of plasma, erythrocytes, and different brain areas. We report lower emotional memory (contextual fear conditioning) in aged rats supplemented with polar lipids' concentrates from buttermilk or krill oil at doses that approximate human consumption. No other behavioral parameter was significantly influenced by the supplements calling for further research to confirm or not the purported salubrious activities of polar lipids, namely those rich in n-3 long-chain polyunsaturated fatty acids on cognitive decline.

Keywords: aging; cognitive decline; phospholipids; milk; omega 3 fatty acids; behavior; nutraceuticals; functional foods.

Introduction

According to the latest Global Burden of Disease, the world population enjoys better overall health [1]. However, the number of years spent with disability is increasing [1]. The reason is that, thanks to better hygiene, diet, and medicine, we live longer. Aging is the most important risk factor for cardiovascular diseases and neurodegeneration, due to declines in cellular function and resistance to stress [2]. Many of the risk factors for ageing-related pathologies are preventable and include obesity, poor diet, and physical inactivity. Cognitive decline is one of the hallmarks of aging and can vary from mild cognitive impairment to dementia to Alzheimer's disease [3]. As of today, there is no effective pharmacological intervention to treat advanced cognitive decline and dementia [3]. Hence, in addition to the aforementioned lifestyle interventions, there is room for the use of nutraceuticals/functional food, as pharma-nutritional tools to lessen the burden of cognitive decline before it worsens [3, 4].

Among the many purportedly bioactive ingredients of functional foods targeted at cognitive decline, polar lipids are most interesting because of the abundance in the brain, mostly as phospholipids such as phosphatidylcholine (PC) [5, 6]. In addition, during aging, the central nervous system becomes depleted of the polyunsaturated fatty acid (PUFA) docosahexaenoic acid (DHA) [7]. Hence, the rationale for using PUFAs to restore their proper cerebral concentrations. One interesting source of polar lipids is the milk fat globule membrane (MFGM), which is made of a core, mainly composed of triacylglycerols (TAG; 98%–99%), and different concentrations of other compounds such as diacylglycerols, monoacylglycerols, free fatty acids, and cholesterol. Buttermilk (BM) is a by-product of butter manufacturing with a high content of MFGM; it is particularly rich, i.e. up to 20% of total fat, in polar lipids [5, 8], namely phosphatidylserine (PS) and sphingomyelin (SM). In summary, BM appears to be a suitable candidate for the preparation of nutraceuticals to be employed in the cognitive decline arena [6]. Krill oil (KO) has also been suggested as a convenient

source of neuroactive n-3 FAs, because a large proportion of them is incorporated into PC (potentially increasing docosahexaenoic acid's bioavailability [9]), and because some preliminary data suggest their beneficial effects on elderly's cognition [10].

We previously reported the promising molecular actions of MFGMs and krill oil's concentrates in a rat model of aging [11, 12]. In this study, we concentrated on the activities on cognition, using an array of validated tests. We also performed lipidomic analyses of plasma, erythrocytes, and different brain areas.

Materials and methods

Preparation of polar lipid-rich supplements

We prepared jelly lollipops as previously described [11, 12]. Briefly, we followed the procedure described by Castro-Gomez et al. [8], in which BM fat was extracted by pressurized liquid extraction (PLE) using an accelerated solid ASE-200 extractor (Dionex Corp. Sunnyvale, CA). Then, 15 grams of powdered BM was mixed with sand (1:1, by weight) and loaded into a stainless-steel extraction cell. KO concentrate was obtained by fractionation with ethanol, added to obtain an upper polar phase at -35 °C for 24 hours [8]. The lipid extracts were stored at − 35 °C.

Animals and experimental design

Animals

All animal protocols were approved by the Ethics' Committee of the UNED, followed the "Principles of laboratory animal care", and were carried out in accordance to the European Union Directive (2010/63/EU). Nine-month old male Wistar rats (n= 46) were purchased from Charles River Laboratories (Barcelona, Spain) and were kept pair-housed in transparent Plexiglas cages, on a 12-hour light/dark cycle, with free access to chow and water.

Experimental design

When the animals reached 18 months of age, they were fed a EURodent Diet 22% (LabDiet), selected because of its lipid composition, which was the most appropriate for our study due to its low polar lipids (PL) and eicosapentaenoic (EPA) + DHA content [12]. Water was still supplied *ad libitum*.

The rats were then randomly allocated to four groups and their diets were daily supplemented with PL in form of a jelly lollipop. Briefly, control diet with refined olive oil (C) and supplementation diet with phospho- and sphingolipids concentrates from buttermilk fat (BMFC) and krill oil (KOC), or a combination of both (BMFC+KOC) were produced as previously described [11, 12]. After three months of this regimen, behavioral tests were initiated. Prior to the beginning of the tests, the animals were handled by the experimenter for two minutes daily, for three days.

Behavioral and cognitive tests

Elevated Plus Maze (EPM)

Anxiety-related behavior was evaluated using the EPM, which is a validated test for the study of anxiety in rodents. It is based on the aversion they experience to height and open spaces [13]. The EPM consists of two opposing open arms (45 x10 cm) and two enclosed arms (45x 10x 50 cm) that extend from a central platform (10x 10 cm), elevated 65 cm above the floor. The rats were placed individually on the central platform facing an enclosed arm and were allowed to freely explore the maze for 5 min. Entry into an arm was defined as entry of all four paws into one arm. The time spent in the open and closed arms, as well as the number of times the animal entered each type of arm and the latency before entering an open arm were recorded. The behavior of each rat was monitored using a video

122 camera, and the movements of the rats were automatically registered and analyzed with a
123 computerized tracking system (Ethovision 1.90, Noldus IT, The Netherlands).

124

125 *Spatial learning procedure*

126 The water maze was a black circular pool (2 m diameter, 45 cm high) filled with water
127 (30 cm depth) at 24 ± 1 °C. The pool was divided into four quadrants of equal size. An
128 invisible escape platform (11 cm diameter) was placed in the middle of one of the quadrants
129 (1.5 cm below the water surface) equidistant from the sidewall and middle of the pool. The
130 testing room contained numerous extra-maze cues. The behavior of the animal was
131 monitored by a video camera, mounted in the ceiling above the center of the pool, and a
132 computerized tracking system (Ethovision 1.90, Noldus IT, The Netherlands). Four different
133 starting positions were equally spaced around the perimeter of the pool. On each day, all
134 four start positions were used once in a random sequence equal for every rat. A trial began
135 by placing the rat into the water facing the wall of the pool at one of the starting points. If the
136 rat failed to escape within 120 sec, it was guided to the platform by the experimenter. Once
137 the rat reached the platform, it was allowed to stay there for 30 sec and, then, placed in a
138 holding cage for an inter-trial interval of 30 sec. After the last trial of each day, the rats were
139 dried off by placing them in a waiting cage for 30 min, in a room heated to 30 °C.
140 Subsequently, rats were returned to their home cages. The acquisition phase consisted of
141 a block of six trials on the first day, and blocks of four trials on the two consecutive days.
142 Recall of the platform location was tested 24 h after the last training session, by giving rats
143 a 60 sec transfer test (free swim without platform). Different parameters of rats' performance
144 were analyzed, i.e. latency or total time that rats need to find the platform (sec); total distance
145 swam to reach the platform (cm); swim speed (cm/sec); and, during the transfer test, also
146 the percentage of time spent by the rat swimming in the place where it was placed at training.

147

148 *Fear conditioning*

149 Conditioning and testing took place in a rodent observation cage using a shock
150 generator (model LI100-26 Shocker, LETICA I.C., Madrid, Spain). The observation cage (30
151 x 37 x 25 cm) was placed in a sound-attenuating chamber. The side walls of the observation
152 cage were constructed of stainless steel and the back walls and doors were constructed of
153 clear Plexiglass. The floor consisted of 20 steel rods through which a scrambled shock from
154 a LETICA I.C. (Spain) shock generator (Model LI100-26 Shocker) could be delivered. The
155 observation cage was cleaned with a 0.1% acetic acid solution before and after each
156 session. Ventilation fans provided a background noise of 68 dB and a 20W white light bulb
157 illuminated the chamber. On the conditioning day each animal was transported from the
158 colony room to the laboratory (situated in adjacent rooms) and placed in the conditioning
159 chamber. After 160 s, three tone-shock pairings were delivered with an inter-shock interval
160 of 60 s. The tone (85 dB sound at 1000 Hz) sounded for 20 s and at the end of each tone
161 an electric foot shock was delivered (1 s, 0.4 mA, constant current). The rodents were
162 removed from the conditioning chambers 30 s after the final shock presentation and returned
163 to their home cages. Thus, a conditioning session lasted 330 s. Testing for contextual fear
164 conditioning was performed one day after conditioning. At testing, rats were placed back in
165 the same chamber as used for conditioning but in the absence of shock or tone, for an 8
166 min context test. Testing for auditory fear conditioning was performed two days after
167 conditioning. Animals were placed in the absence of shock in a novel context (same cages
168 but with different walls, floor and background odor) in the absence of the conditioning tone
169 (3 min; pre-tone period) and then re-exposed to the tone (5 min; tone period). Using a time-
170 sampling procedure the behavior was evaluated in each experimental session and each
171 animal was scored blindly as either freezing or active every 2 s. Freezing was defined as
172 behavioral immobility except for movement required for breathing. This freezing response is
173 considered as a fear index [14]. At conditioning, behavioral scores were noted for the 3 min

period prior to shock (pre-shock period) and for the 2.5 min period starting immediately after presentation of the first shock (post-shock period). Scores for each of these periods were analyzed separately. At testing for contextual fear conditioning (24 h after training), the scores for the total 8 min context test were analyzed. At testing for auditory fear conditioning (48 h after training), the scores for the pre-tone and tone periods were also considered separately.

Foot shock sensitivity test

To assess whether social isolation modified the sensitivity to foot shocks in our experimental conditions, each animal was placed individually in a conditioning chamber different to that used for conditioning. After 120 s each rat received an ascending series of 1 s foot shocks, separated by 20 s, in 0.05 mA increments from 0 mA until the animal showed the first signs of discomfort and pain (defined as the animal's paws leaving from the grid floor, jumping and vocalization, scored as jump). The shock intensity that elicited this reaction was assessed.

Lipidomics

Following behavioral tests and a 12-hour fast, rats were sacrificed by decapitation. Blood was collected in heparinized tubes and plasma and erythrocytes were separated by centrifugation. Hippocampus; frontal (FC), occipital (OC), and temporal cortex (TC); and cerebellum were quickly separated, washed in PBS, snap-frozen in liquid nitrogen, and stored at -80 ˚C.

Plasma, erythrocytes, and tissue lipids were extracted using the Löfgren et al. [15] with slight modifications as described by Crespo et al. [11]. Briefly, samples were dissolved in methanol and were sonicated. Then, 1:2 methanol/dichloromethane (v/v) was added and the sample was mixed for 20 min. After, acetic acid 20 mM (1:3 acetic acid/dichloromethane)

was added, the sample was mixed for 20 min and centrifuged at 2100 rpm for 5 min at 4 °C. The upper methanol phase was re-extracted twice and the bottom organic phases were collected, mixed, and filtered through a 0.45 µm filter. The extract was evaporated under nitrogen and weighted. Lipids extracts were maintained at −35 °C until they were submitted to exhaustive lipidomic characterization. Briefly, lipid classes were analyzed by HPLC-ELSD, fatty acids methyl esters (FAMEs) by GC-MS, triacylglycerols (TAGs) and cholesterol (Chol) by GC-FID and phospholipid and sphingolipid molecular species by UPLC-QToF-MS as previously described [16]. All assays were carried out in triplicate.

Statistical Analyses

All results are expressed as mean ± S.E.M. and analyzed using analyses of variance (ANOVA) or with repeated measures (including, treatment and 'training day' in the water maze as a repeated measure). Dunnett's Multiple Comparison Test was used for post-hoc analyses. The data were analyzed using the SPSS package (version 22.0 for Windows, SPSS Inc. IBM, Armark, New York, USA).

Results

The FAME composition of plasma and erythrocytes are shown in **Tables 1 and 2**, respectively. Whereas plasma lipids are considered as short-term indicators of dietary intakes, red blood cell lipids are a stable indicator of the overall fatty acid status [17].

The plasma FAMEs profiles of the control group showed significantly lower concentrations of n-6 fatty acids as compared with the others, namely 18:3 (gamma-linolenic acid; GLA) and 20:4 (arachidonic acid; AA). In the KOC-supplemented group, the concentration of 20:5 (eicosapentaenoic acid) was approximately thrice of that of the C and BMFC groups ($p < 0.05$). This is very likely due to the provision of EPA via KO, even though we did not record a parallel increase in docosahexaenoic concentrations (**Table 1**). This

agrees with Vigerust et al. [18], who also reported higher increases in EPA than DHA concentrations after the administration of KO. Finally, even though the experimental diets increased caloric intakes, we did not record significant increases in HDL- and LDL-cholesterol (data not shown).

The only significant change we recorded in the FAMEs profile of erythrocytes was a significantly lower concentration of 20:3n-6 in the treated groups and a non-significant increase in EPA (**Table 2**).

Brain tissues

The three cortical areas (FC, TC and OC) we analyzed exhibited fatty acids and phospho- and sphingolipids contents that agreed with a recent review [19]; the total polar lipid content of the brain areas was approximately 50% of total lipids, of which ~40-48% phosphatidylethanolamine (PE), ~45% PC, and ~7% PS. Phospholipids' distribution among the three areas was similar and independent of diets. However, we recorded a higher ganglioside and ceramide concentration in TC and OC after administration of the PL-enriched diets as compared with FC regions and controls (~11% vs. ~9%, respectively) (**Figure 1**).

We did not record significant increases in LC-PUFAs of the n-3 series in any of the brain areas we analyzed (**Table 3**). Albeit surprising, this result agrees with that of Chen et al. [20], who analyzed the same regions after administration of fish oil.

The FA composition of the HP showed a similar pattern as for FC, TC and OC, where most FAs were C16, C18, C18: 1 n-9, C20: 4 (n-6) and C22: 6 (n-3) (**Table 4**). Compared to controls, a lower content of C17:1 odd chain FAs was found in the BMFC + KOC group. Odd chain FAs are found in minor amounts in body tissues (approximately 1% of total fat), although they are preferably incorporated in brain SL [21, 22]. The roles of odd FAs in cognition have been poorly investigated. Recently, Fonteh et al. [22] observed lower levels

252 of certain FAs in patients with AD, including n-3 PUFAs and odd chain FAs (mainly saturated
253 C15 and C17), compared to healthy individuals.
254
255 Cerebellum
256 The distribution of FAs obtained in CB of the supplemented animals was mainly
257 composed of C16:0, C18:0, C18:1 ω9, C20:4 ω6 and C22:6 ω3 (**Table 5**). These data agree
258 with those reported by Rahman et al. [23], who analyzed, in male mice, the profile of FAs in
259 the cerebral cortex, CB, and HP and reported higher contents of the above-mentioned FAs.
260 In another study, conducted in male mice by Valenzuela et al. [24], cerebral FAs distribution
261 was as follows: C16:0, C18:1ω9, AA and DHA 26%, 22%, 8% and 3% g/100 g FAMEs,
262 respectively, comprising a total of 48% SFA, 31% MUFAs and 21% PUFAs, i.e. figures that
263 are similar to ours.
264 In this brain region no significant differences were found between the supplemented
265 groups as regards ceramides and gangliosides, although, as was the case for CT, CF or
266 HP, animals supplemented with BMFC and KOC jointly showed a trend for increased levels
267 of gangliosides and decreased levels of ceramides compared to the control diet (data not
268 shown).
269 To evaluate the anxiety state of the animals, we calculated the percentage of time
270 the rats remained in the open arms of the elevated plus-maze. The statistical analysis
271 indicated that there were no significant differences between the four groups (**Figure 2**; p>
272 0.295).
273 The spatial learning abilities of the animals were evaluated by measuring the distance
274 swam to find the hidden submerged platform in the Morris water maze (MWM). Acquisition
275 of spatial learning occurred through the training procedure (**Figure 2**; $F(15)=6.01$; $P<0.001$).
276 However, no significant differences were observed after analysis of the effects of treatment
277 ($F(3)=1.85$; $P<0.153$) or interaction of time x treatment $F(3,45)= 0.88$; $P>0.695$).

The spatial memory was evaluated 24 hours after the fourth day of training by removing the platform from the pool and allowing the rat to search for it. The time that the animals stayed in the zone where the hidden platform was previously located during the 30 first seconds of the memory probe test, as well as the number of crossings in this area were used as measures of spatial memory. The results obtained showed no significant differences between experimental groups in spatial memory (**Figures 3 and 4**; all $p > 0.05$).

During the training phase of fear conditioning, the different experimental groups showed similar freezing behavior after the tone fear conditioning training ($p > 0.56$) (**Figure 5**). However, in the contextual memory test, performed 24 hours after training, significant differences were observed between groups ($p < 0.037$). Post-hoc analyses indicated that animals that only received the buttermilk supplement and the group that received krill oil showed lower freezing levels compared to control group (**Figure 6a**; $p < 0.05$). A tone-cued fear conditioning test was performed 48 h after training. Analysis of freezing behavior indicated that there were no significant differences between the different groups (**Figure 6b**; $p > 0.319$).

One day after the tone-cued fear conditioning test was completed, an electrical shock sensitivity test was performed. Analysis of the data indicated that there were no significant differences in the response to electrical shock sensitivity between the different experimental groups ($p > 0.05$; **Figure 7**).

Discussion

We wanted to assay the behavioral effects of polar lipid concentrates from BM and KO in an animal model of aging, providing low, nutritional doses and performing validated tests. Indeed, molecular biology data were supportive of our rationale [11, 12] and data are accumulating on the effects of MFGMs on behavior and cognition [6, 25, 26]. We actually

304 report minimal effects of those two formulations, whose doses were chosen to approximate
305 human consumption [27].

306 As the population ages the prevalence of many age-related diseases increases [1].
307 There are several contributors to neurodegeneration, impaired cognitive decline and AD,
308 many of which are still unknown. Common features are increased inflammation
309 (inflammaging) [28] and a loss of PUFAs, namely long-chain omega 3 FA [7, 29]. Hence,
310 there is justification for the use of supplements, either in the form of nutraceuticals or
311 functional foods, to slow cognitive decline [2]. Some products are, indeed, available in the
312 market and have been tested with equivocal results [30]. It must be underscored that there
313 are several hurdles to overcome when fatty acids are tested in the cardiovascular or
314 neurological arenas [31]. Time of administration, e.g. at breakfast or dinner, doses, and lack
315 of lipidomic analyses often impede drawing firm conclusions [31].

316 Lipidomic analyses revealed that the supplementations did not significantly alter
317 circulating and cerebral n-3 LC-PUFAs, which could largely explain the modest effects on
318 cognitive behavior. It is worth underscoring that n-3 fatty acids do not incorporate into the
319 different brain areas in a random fashion [32] and that EPA is poorly uptaken and rapidly
320 metabolized once it crosses the blood-brain barrier [33, 34]. In short, physiological and
321 metabolic constraints might prevent large accumulations of n-3 LC-PUFAs in the brain, in
322 turn limiting their potential therapeutic actions. Future research should possibly concentrate
323 on their peripheral actions and the formation of metabolites [33].

324 Even though our study is the first one to test PL, the lack of effect of BM and KO
325 concentrates on anxiety, as assessed by the elevated plus-maze test, fits with other
326 investigations that employed omega 3 fatty acids. For example, Song et al. studied EPA and
327 compared it to a 1) palm oil control diet, 2) 0.5% arachidonic acid diet, and 3) gamma
328 linolenic acid-enriched diet [35]. Other experimental approaches yielded similar results [36,
329 37], contradicting some – admittedly limited – human data [38, 39].

Spatial learning was also uninfluenced by BM and KO supplementation, in agreement with Gustavsson et al. [40]. In a rat model of αβ1-42-induced AD, Zhang et al. [41] did report positive effects of phosphatidylserine, which fits with data by Lee et al. [42]. Conceivably, the administration of large doses of phosphatidylserine to diseased rats might, indeed, lessen the neurological effects of AD, whereas our model and our low, physiological doses would be ineffective in asymptomatic aged rats.

We observed a significantly lower contextual freezing behavior in rats supplemented with BMFC or KOC as compared with the control group, but not when both supplements were given together. This effect cannot be attributed to an altered nociception or to a deficit in amygdala functioning in BMFC and KOC groups since foot-shock sensitivity and tone-cued fear memory was similar in all the groups. Therefore, our results in the contextual fear conditioning could suggest the existence of a synergic effect between the BM and KO concentrates supplemented to the diet, as occurred in the animals of this group regarding lipid profile modifications. Few studies evaluated the effect of PL supplementation on associative fear memory. As mentioned, the age-associated decrease of cerebral n-3 LC-PUFAs is being implicated in altered cognitive state and neuronal disorders frequently seen in the elderly [29]. Three-month-old rats reared on n-3 PUFA deficient diets during the postnatal period did not exhibit altered auditory-cued fear conditioning, but did show increased freezing behavior in combination with early maternal separation [43]. Also, Cutuli et al. [44] reported greater associative memory in the contextual fear conditioning test of rats supplemented with n-3 LC-PUFAs, in agreement with Yamada et al. who used krill oil as did we [45]. The authors attributed this effect to an antagonistic effect on the cannabinoid CB1 receptor, similar to that of rimonabant. Subsequently, the same group reported that mice fed with a high (0.97) ω3 to ω6 PUFA ratio diet, showed reduced auditory-cued fear responses compared with mice fed with a low (0.14) ω3 to ω6 PUFA ratio diet [46]. Conversely,

intranasal krill oil administered two hours after fear conditioning did not affect contextual fear memory in mice [47].

In conclusion, we report lower emotional memory (contextual fear conditioning) in aged rats supplemented with PL concentrates from buttermilk or krill oil. No other behavioral parameter was influenced by the supplements calling for further research to confirm or not the purported salubrious activities of polar lipids, namely those rich in n-3 LC-PUFAs on cognitive decline.

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Table 1. PUFAs composition of plasma.

	C	BMFC	KOC	BMFC+KOC
C18:2 n6	29.04 ± 4.90	27.39 ± 8.60	26.23 ± 6.57	27.92 ± 7.64
C18:3 n6	0.268 ± 0.15	0.161 ± 0.07 *	0.158 ± 0.07 *	0.144 ± 0.06 *
C18:3 n3	0.58 ± 0.24	0.51 ± 0.38	0.47 ± 0.26	0.56 ± 0.30
C20:3 n6	0.39 ± 0.20	0.25 ± 0.12	0.27 ± 0.22	0.19 ± 0.22
C20:4 n6	13.93 ± 6.76	8.838 ± 4.1 *	9.382 ± 5.6 *	7.588 ± 4.83 *
C20:5 n3	0.366 ± 0.19	0.279 ± 0.11	1.092 ± 0.83 *§	0.738 ± 0.46
C22:5 n3	0.32 ± 0.18	0.28 ± 0.12	0.45 ± 0.38	0.41 ± 0.34
C22:6 n3	1.09 ± 0.75	0.57 ± 0.31	1.09 ± 0.95	0.88 ± 0.86

Data are mean percentages ± SD. * Different from control ($p < 0.05$). § Different from BMFC ($p < 0.05$).

521 **Table 2**. PUFAs composition of erythrocytes.

	C	BMFC	KOC	BMFC+KOC
C18:2 n6	9.52 ± 8.06	14.94 ± 4.69	11.31 ± 6.21	12.88 ± 6.21
C18:3 n6	-	-	-	-
C18:3 n3	0.847 ± 0.67	0.453 ± 0.37	0.939 ± 0.7	0.231 ± 0.11
C20:3 n6	0.378 ± 0.17	0.123 ± 0.05 *	0.136 ± 0.08 *	0.189 ± 0.08 *
C20:4 n6	8.141 ± 4.27	8.729 ± 7.32	3.905 ± 5.94	3.695 ± 3.01
C20:5 n3	-	-	-	-
C22:5 n3	0.218 ± 0.22	0.317 ± 0.31	0.671 ± 0.7	0.359 ± 0.36
C22:6 n3	4.536 ± 5.04	0.669 ± 0.64	1.223 ± 1.35	0.711 ± 0.8

522

523 Data are mean percentages ± SD. * Different from control ($p < 0.05$).

524

525 **Table 3**. Fatty acid composition of frontal, temporal, and occipital cortexes.

		C	BMFC	KOC	BMFC+KOC	
Frontal cortex	*C14*	0.28 ± 0.23	0.27 ± 0.17	0.38 ± 0.41	0.25 ± 0.14	
	DMA16	2.32 ± 0.56	1.95 ± 0.26	1.76 ± 0.56	1.46 ± 0.61	*§†
	C16	25.91 ± 3.50	29.76 ± 3.44	27.24 ± 4.35	28.50 ± 6.54	
	C16:1	0.42 ± 0.16	0.50 ± 0.27	0.51 ± 0.29	0.97 ± 1.68	
	DMA18	3.96 ± 0.81	3.47 ± 0.53	3.08 ± 1.10	2.73 ± 1.33	*§†
	C18i	1.21 ± 0.53	0.80 ± 0.21	0.84 ± 0.27	0.66 ± 0.50	*§†
	C17:1	1.62 ± 0.63	1.12 ± 0.30	1.19 ± 0.43	0.95 ± 0.63	*§†
	C18	20.73 ± 2.79	22.31 ± 2.74	21.14 ± 3.73	23.13 ± 6.62	
	C18:1c9	18.40 ± 2.23	19.28 ± 1.22	19.79 ± 2.80	20.69 ± 3.31	
	C18:1c11	3.42 ± 0.43	3.83 ± 0.36	3.64 ± 0.61	4.19 ± 0.99	*§†
	C18:2 n6	0.51 ± 0.16	0.75 ± 0.82	1.06 ± 1.38	1.97 ± 5.26	
	C18:3 n3	1.44 ± 0.58	1.03 ± 0.24	1.36 ± 0.34	1.29 ± 0.35	
	C20:1	0.30 ± 0.06	0.21 ± 0.08	0.29 ± 0.14	0.31 ± 0.14	
	C20:3 n6	0.24 ± 0.12	0.14 ± 0.05	0.21 ± 0.12	0.20 ± 0.09	
	C20:4 n3	8.20 ± 2.74	6.88 ± 2.46	7.74 ± 3.67	5.31 ± 5.20	
	C24	0.35 ± 0.54	0.20 ± 0.07	0.23 ± 0.10	0.22 ± 0.07	
	C22:6 n3	8.54 ± 3.69	5.75 ± 2.45	7.56 ± 5.37	5.54 ± 6.29	
	SFA	54.28 ± 4.98	57.97 ± 4.81	53.78 ± 6.56	56.32 ± 11.65	
	MUFA	24.07 ± 2.50	24.90 ± 1.22	25.42 ± 3.21	27.10 ± 4.62	
	PUFA	21.09 ± 6.74	16.33 ± 5.60	19.96 ± 9.44	15.92 ± 13.32	
	n6	8.91 ± 2.68	7.76 ± 2.59	9.01 ± 3.57	7.48 ± 7.42	
	n3	10.02 ± 3.81	6.79 ± 2.53	9.01 ± 5.32	6.90 ± 6.27	
	DMA	6.29 ± 1.30	5.43 ± 0.75	4.84 ± 1.64	4.20 ± 1.90	*
Temporal cortex	*C14*	0.27 ± 0.19	0.19 ± 0.15	0.16 ± 0.06	0.17 ± 0.08	
	DMA16	2.45 ± 0.25	2.37 ± 0.42	2.31 ± 0.25	2.14 ± 0.47	
	C16	23.44 ± 2.72	27.00 ± 4.60	24.39 ± 2.66	22.27 ± 8.52	
	C16:1	0.42 ± 0.13	0.38 ± 0.06	0.41 ± 0.11	0.75 ± 0.86	
	DMA18	4.11 ± 0.52	3.96 ± 0.57	3.86 ± 0.44	3.26 ± 0.72	
	C18i	1.50 ± 0.44	1.21 ± 0.23	1.13 ± 0.47	0.93 ± 0.63	*§†
	C17:1	2.09 ± 0.53	1.65 ± 0.47	1.81 ± 0.39	1.55 ± 0.65	
	C18	18.11 ± 2.00	19.33 ± 2.73	19.10 ± 2.58	21.48 ± 7.65	
	C18:1c9	17.61 ± 1.71	18.89 ± 1.52	19.11 ± 1.97	21.68 ± 5.39	*§†
	C18:1c11	3.35 ± 0.46	3.66 ± 0.34	3.54 ± 0.45	4.31 ± 1.37	
	C18:2 n6	0.60 ± 0.20	0.48 ± 0.11	0.64 ± 0.16	1.49 ± 3.22	
	C18:3 n3	1.41 ± 0.43	1.34 ± 0.38	1.60 ± 0.32	1.90 ± 0.83	
	C20:1	0.41 ± 0.22	0.27 ± 0.11	0.30 ± 0.09	0.45 ± 0.26	
	C20:3 n6	0.16 ± 0.05	0.15 ± 0.08	0.17 ± 0.07	0.27 ± 0.09	*§†
	C20:4 n3	10.71 ± 2.52	8.70 ± 2.46	9.33 ± 2.70	7.76 ± 4.64	
	C24	0.26 ± 0.06	0.23 ± 0.08	0.24 ± 0.07	0.39 ± 0.41	
	C22:6 n3	10.01 ± 2.64	8.16 ± 3.53	9.73 ± 3.56	7.42 ± 4.85	
	SFA	50.52 ± 4.33	54.23 ± 5.72	51.11 ± 4.56	50.86 ± 7.00	
	MUFAs	23.92 ± 2.18	24.83 ± 1.58	25.17 ± 2.61	28.70 ± 6.75	*§†
	PUFAs	25.66 ± 5.93	20.94 ± 6.79	23.72 ± 6.78	20.44 ± 10.26	
	n6	11.60 ± 2.47	9.33 ± 2.53	10.13 ± 2.63	9.49 ± 5.73	
	n3	11.69 ± 3.04	9.62 ± 3.76	11.44 ± 3.61	9.41 ± 4.44	
	DMA	6.73 ± 0.78	6.11 ± 1.25	5.91 ± 0.83	5.42 ± 1.15	*§†
Occi[illegible]	*C14*	0.25 ± 0.13	0.16 ± 0.05	0.21 ± 0.13	0.29 ± 0.36	
	DMA16	2.23 ± 0.25	2.13 ± 0.40	1.75 ± 0.27 *§	1.72 ± 0.53	*§

C16	22.64	±	3.00	25.41	±	3.94	24.07	±	5.03		25.86	±	5.46	
C16:1	0.42	±	0.12	0.40	±	0.08	0.40	±	0.08		0.49	±	0.13	
DMA18	4.17	±	0.47	4.05	±	0.37	3.49	±	0.58	*§	3.19	±	1.07	*§
C18i														
C17:1	2.03	±	0.42	1.68	±	0.36	1.49	±	0.37		1.38	±	0.66	
C18	19.09	±	2.45	20.04	±	2.66	20.18	±	3.98		22.28	±	4.58	
C18:1c9	19.22	±	1.93	19.78	±	1.60	20.05	±	3.01		21.79	±	3.22	
C18:1c11	3.45	±	0.35	3.69	±	0.35	3.44	±	0.58		3.86	±	0.75	
C18:2 n6	0.61	±	0.23	0.62	±	0.21	0.60	±	0.12		0.55	±	0.29	
C18:3 n3	1.43	±	0.36	1.30	±	0.39	1.48	±	0.35		1.64	±	0.33	
C20:1	0.39	±	0.21	0.25	±	0.10	0.28	±	0.07		0.33	±	0.14	
C20:3 n6	0.34	±	0.27	0.19	±	0.09	0.20	±	0.08		0.27	±	0.14	
C20:4 n3	9.76	±	2.78	8.58	±	1.95	7.91	±	2.74		6.64	±	4.95	
C24	0.33	±	0.11	0.29	±	0.10	0.31	±	0.10		0.33	±	0.09	
C22:6 n3	9.55	±	3.42	7.95	±	2.26	10.98	±	8.11		7.22	±	5.28	
SFA	50.50	±	5.20	53.50	±	4.75	51.29	±	8.25		54.92	±	8.05	
MUFAs	25.48	±	1.93	25.81	±	1.68	25.66	±	3.66		27.81	±	3.69	
PUFAs	24.01	±	7.00	20.69	±	5.02	23.06	±	11.20		17.27	±	11.54	
n6	10.68	±	2.84	9.39	±	2.02	8.70	±	2.64		7.47	±	5.20	
n3	11.08	±	3.55	9.31	±	2.48	12.53	±	8.12		8.33	±	5.37	
DMA	6.41	±	0.69	6.18	±	0.74	5.24	±	0.80	*§	4.91	±	1.58	*§

Data are mean percentages ± SD. * Different from control ($p< 0.05$). § Different from BMFC ($p< 0.05$). † Different from KOC ($p< 0.05$). MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; SFA, saturated fatty acids; DMA: dimethyl acetals.

534 **Table 4**. Fatty acid composition of hippocampus.

	C	BMFC	KOC	BMFC+KOC	
C14	0.57 ± 0.70	0.60 ± 0.62	0.39 ± 0.31	0.25 ± 0.12	
DMA16	2.30 ± 0.32	2.00 ± 0.50	2.12 ± 0.48	1.78 ± 0.64	
C16	26.96 ± 4.54	31.19 ± 4.13	29.14 ± 4.11	30.73 ± 5.47	
C16:1	0.47 ± 0.21	0.44 ± 0.22	0.34 ± 0.12	0.32 ± 0.11	
DMA18	3.49 ± 0.70	3.00 ± 1.01	3.21 ± 0.67	2.75 ± 1.09	
C18i	1.13 ± 0.45	0.86 ± 0.62	0.91 ± 0.27	0.62 ± 0.28	
C17:1	20.78 ± 2.80	22.44 ± 4.19	22.05 ± 3.48	24.74 ± 4.68	*
C18	17.54 ± 2.29	18.24 ± 1.68	18.43 ± 2.04	19.23 ± 2.97	
C18:1c9	2.88 ± 0.43	2.91 ± 0.66	2.95 ± 0.35	3.10 ± 0.68	
C18:1c11	0.75 ± 0.54	1.02 ± 1.43	0.68 ± 0.53	0.46 ± 0.28	
C18:2 n6	0.48 ± 0.63	0.10 ± 0.00	0.06 ± 0.01	0.10 ± 0.02	
C18:3 n3	0.99 ± 0.39	0.84 ± 0.36	0.77 ± 0.22	0.69 ± 0.30	
C20:1	0.30 ± 0.23	0.16 ± 0.02	0.07 ± 0.03	0.21 ± 0.16	
C20:3 n6	0.38 ± 0.31	0.12 ± 0.07	0.19 ± 0.14	0.17 ± 0.06	
C20:4 n6	10.27 ± 3.56	8.18 ± 3.22	8.90 ± 4.01	6.87 ± 5.52	
C24	0.26 ± 0.05	0.18 ± 0.05	0.21 ± 0.04	0.22 ± 0.15	
C22:6 n3	7.81 ± 2.98	5.93 ± 2.54	7.07 ± 4.01	6.02 ± 5.45	
SFA	55.43 ± 6.09	60.09 ± 5.91	57.74 ± 6.25	60.91 ± 8.37	
MUFAs	22.13 ± 2.41	22.49 ± 2.39	22.65 ± 2.45	23.32 ± 3.49	
PUFAs	22.51 ± 7.63	17.42 ± 6.30	19.61 ± 8.34	15.77 ± 11.61	
n6	11.16 ± 3.87	8.93 ± 3.55	9.64 ± 3.73	7.32 ± 5.46	
n3	8.66 ± 3.08	6.60 ± 2.54	7.89 ± 3.98	6.73 ± 5.30	
DMA	5.79 ± 0.98	4.99 ± 1.42	5.33 ± 1.13	4.53 ± 1.73	

535
536 Data are mean percentages ± SD. * Different from control ($p < 0.05$). MUFAs,
537 monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; SFA, saturated fatty
538 acids; DMA: dimethyl acetals.
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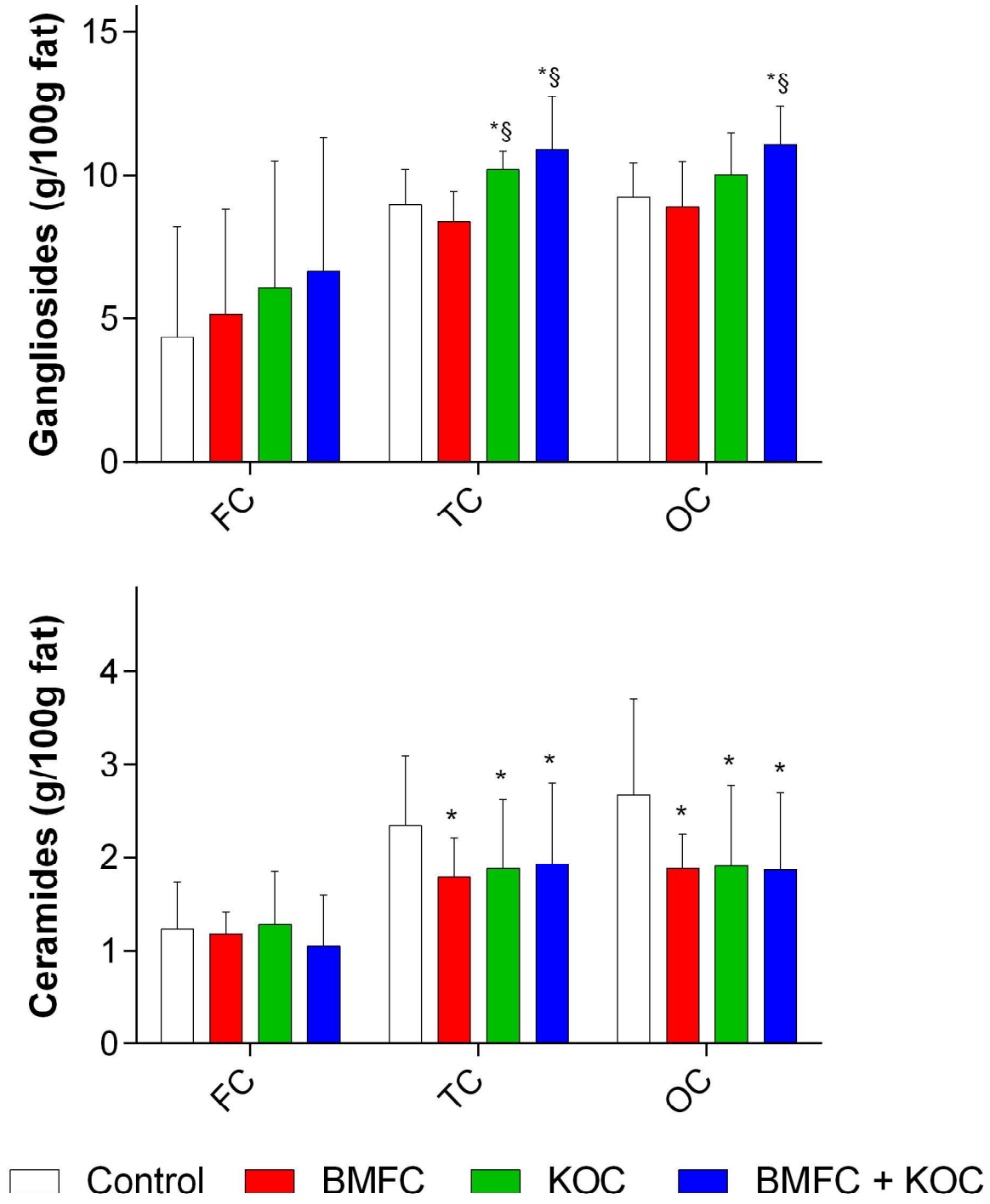
542 **Table 5**. Fatty acid composition of cerebellum.

	C		BMFC		KOC		BMFC+KOC	
C14	0.25 ± 0.15		0.18 ± 0.09		0.24 ± 0.26		0.16 ± 0.08	
DMA16	2.69 ± 0.51		2.01 ± 0.77		2.40 ± 0.43		1.82 ± 1.22	
C16	23.98 ± 3.41		25.66 ± 2.92		22.77 ± 3.39		24.33 ± 5.27	
C16:1	0.47 ± 0.14		0.54 ± 0.38		0.47 ± 0.11		0.50 ± 0.14	
DMA18	3.32 ± 0.68		3.06 ± 0.57		3.47 ± 0.77		2.50 ± 1.33	
C17:1	3.24 ± 0.85		2.66 ± 0.36	*	2.93 ± 0.44		2.33 ± 1.09	*
C18:1 c9	17.59 ± 2.23		18.20 ± 1.80		16.24 ± 1.93		18.34 ± 4.04	
C18:1 c11	21.90 ± 2.21		23.19 ± 2.00		21.04 ± 1.95		23.35 ± 4.32	
C18:1c9	6.10 ± 0.39		6.52 ± 0.82		5.65 ± 0.63		6.54 ± 1.34	
C18:2 n6	0.94 ± 0.24		0.82 ± 0.21	*	1.11 ± 0.25		0.77 ± 0.29	*
C20	0.35 ± 0.12		0.21 ± 0.05		0.22 ± 0.03		0.24 ± 0.07	
C18:3 n3	3.55 ± 0.94		3.66 ± 0.80		3.58 ± 0.60		3.82 ± 1.25	
C20:1	1.04 ± 0.46		0.82 ± 0.34		0.82 ± 0.15		1.02 ± 0.29	
C20:3 n6	0.36 ± 0.22		0.18 ± 0.07		0.25 ± 0.15		0.34 ± 0.11	
C20:4 n3	5.48 ± 2.55		4.46 ± 2.06		6.41 ± 1.79		4.61 ± 3.92	
C24	0.29 ± 0.07		0.27 ± 0.09		0.29 ± 0.08		0.32 ± 0.08	
C22:6 n3	5.35 ± 3.81		5.30 ± 3.19		9.29 ± 3.39		6.68 ± 6.35	
SFA	50.15 ± 4.32		50.81 ± 3.10		46.96 ± 4.19		48.90 ± 6.83	
MUFAs	32.51 ± 1.54		33.20 ± 2.70		30.43 ± 2.33		33.24 ± 4.79	
PUFAs	17.69 ± 5.82		15.45 ± 5.50		22.14 ± 6.14		17.40 ± 11.34	
n6	6.78 ± 2.60		5.46 ± 2.09		7.77 ± 1.86		5.72 ± 4.14	
n3	8.93 ± 3.43		8.98 ± 2.99		12.93 ± 3.94		10.55 ± 6.57	

543

544 Data represent mean percentages ± SD. * Different from control ($p < 0.05$). MUFAs,
545 monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; SFA, saturated fatty
546 acids; DMA: dimethyl acetals.
547

548 Figure 1



549
550 Data are mean percentages ± SD. For each brain tissue under analysis: * Different from
551 control ($p < 0.05$), § Different from BMFC ($p < 0.05$).

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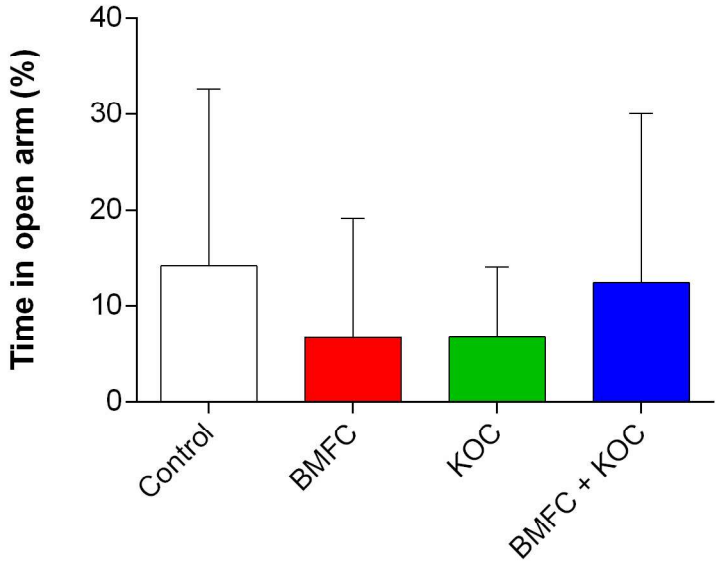
553

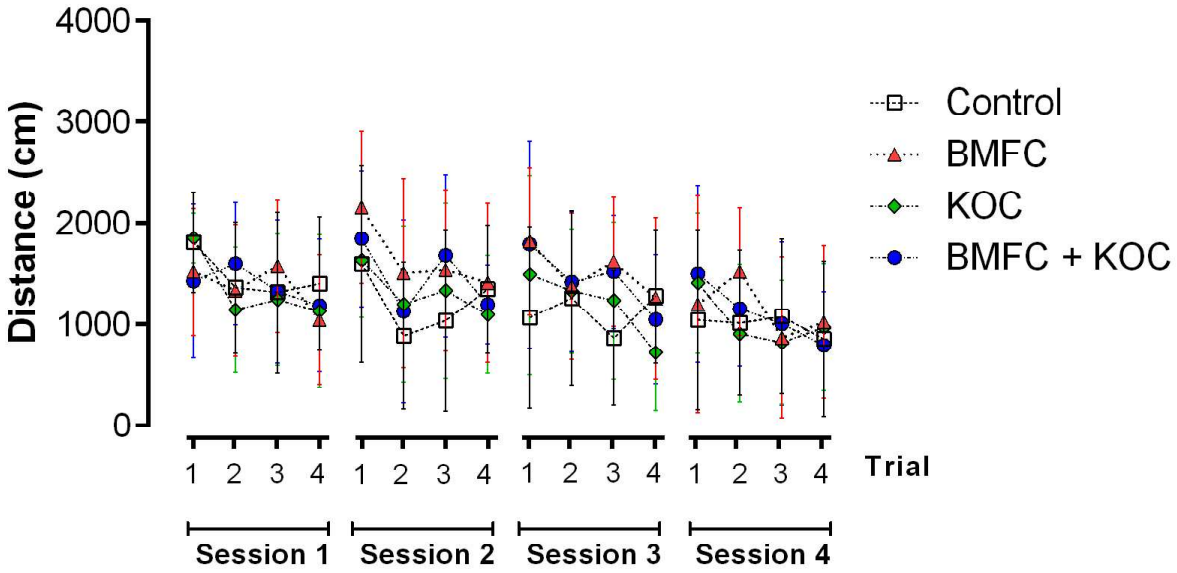
554 **Figure 2**. Anxiety levels evaluated in the elevated plus-maze test.



555
556 Data are mean percentages ± SD.

557
558

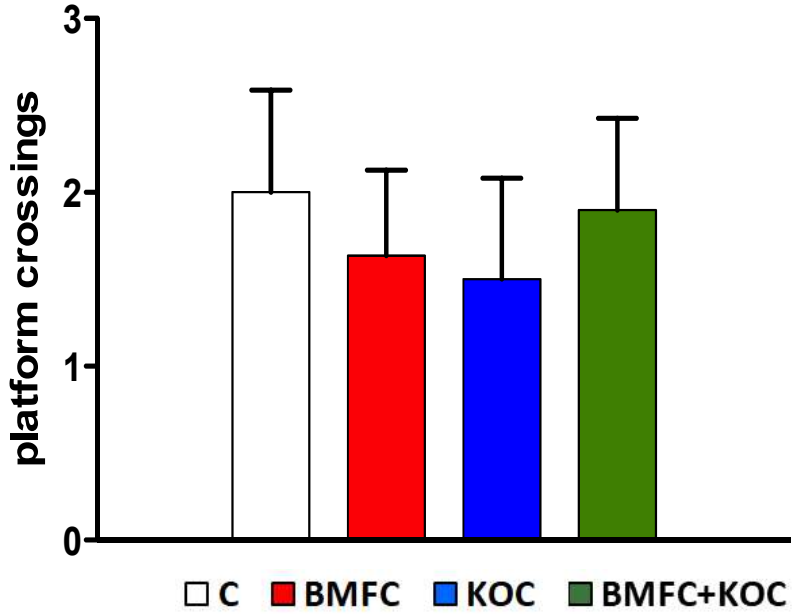
559 **Figure 3**. Spatial learning test.
560



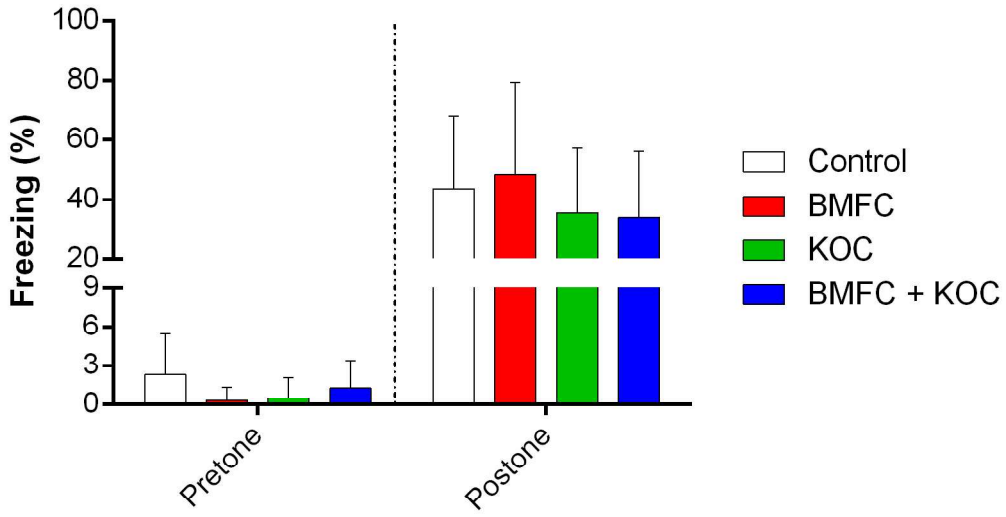
561
562 Data are mean percentages (SD values are not represented for simplification purposes).

563
564

Figure 4. Spatial memory test. Number of times that rats crossed the exact location of the platform during the spatial memory probe test



588 **Figure 5**. Percentage of freezing behavior during tone fear conditioning training
589

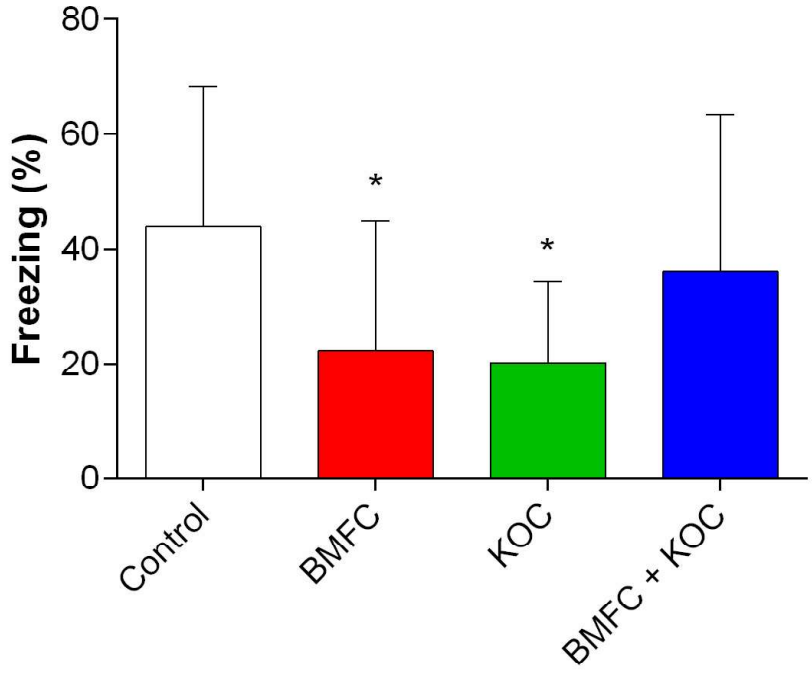


590
591 Data are mean percentages ± SD.

592
593

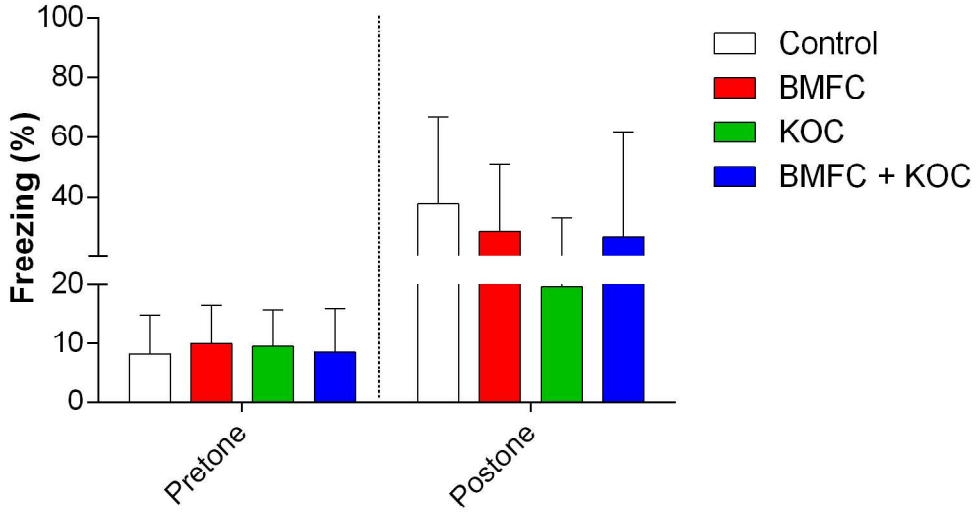
Figure 6.

A. Percentage of freezing behavior in the contextual fear conditioning test.



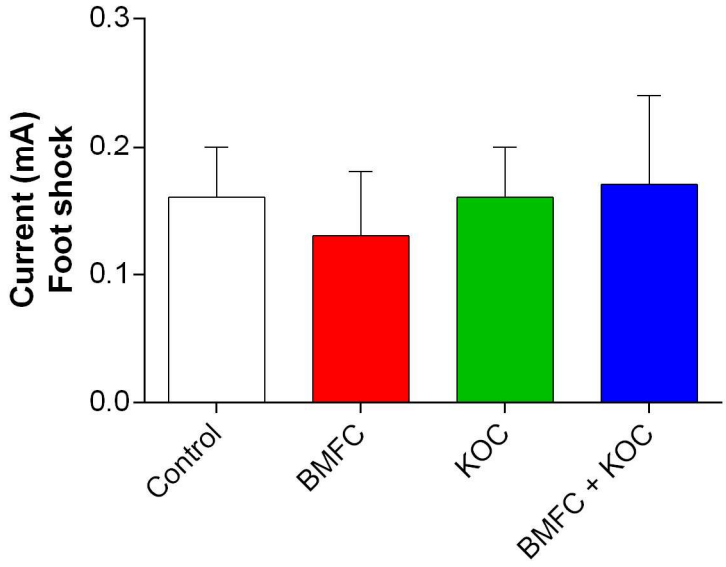
Data are mean percentages ± SD. * Different from control ($p < 0.05$).

B. Freezing behavior during the auditory-cued fear conditioning test.



Data are mean percentages ± SD.

Figure 7. Electrical foot-shock sensitivity



Data are mean percentages ± SD.