Caloric restriction modulates the monoaminergic and glutamatergic systems in the hippocampus, and attenuates age-dependent spatial memory decline

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Abstract

The beneficial effects of caloric restriction (CR) on health and life expectancy are well documented, although its ability to slow down age-dependent cognitive decline and the underlying biochemical changes remains unclear. Therefore, the aim of this study was to investigate the effects of CR on spatial memory in aged Wistar rats, as well as on monoaminergic and glutamatergic neurotransmission in the hippocampus (HPC). As such, animals maintained on different dietary regimes were trained in the Morris Water Maze (MWM): old rats (24-27 months) maintained on a 30% CR diet from four months of age, old rats (24-27 months) with unrestricted access to food (*Ad Libitum*); and adult rats (3-4 months) with *Ad Libitum* access to food. As well as their performance in the spatial memory task, monoamine levels, and NMDA and AMPA receptor subunit expression in the HPC were also assessed in these rats, as was the plasma corticosterone as a measure of the pituitary-adrenal response to stress. Accordingly, it appears that CR attenuates the spatial memory decline in aged rats and the age-associated decrease in the serotonin metabolite 5-HIAA, as well as the expression of the GluA1 and GluA2 AMPA receptor subunits in the HPC. In addition, CR augments the noradrenaline in this structure, although it did not modify the age-associated increase in plasma corticosterone levels. These findings support the positive effect of CR on spatial memory, suggesting that enhancing monoaminergic and glutamatergic neurotransmission in the HPC may help improve learning and memory in aged animals.

Keywords:

Aging; corticosterone; dietary intervention; glutamatergic receptors; monoamines metabolites; Morris water maze.

1. Introduction

Over recent years, healthy lifestyles that include regular physical exercise and dietary interventions like intermittent fasting (IF) or caloric restriction (CR) have been seen to potentially retard the progression of age-related diseases (Phillips, 2017). CR is defined as a reduction in the caloric intake without causing malnutrition, and maintaining a normal consumption of vitamins, minerals and essential biomolecules (Ribarič, 2012). Indeed, CR is considered one of the most effective dietary interventions to increase longevity and improve health during aging (Gillespie et al., 2016; López-Lluch and Navas, 2016). In fact, a reduction in food intake was originally reported to improve life expectancy in laboratory animals a century ago (Osborne et al., 1917). Since then, CR has been used as a robust research paradigm in studies of aging in rodents (Minor et al., 2010), predominately suggesting that CR has beneficial effects on general health (Gillespie et al., 2016; Fontana and Partridge, 2015; Speakman and Mitchell, 2011; Stranahan and Matsson, 2012; Van Cauwenberghe et al., 2016) while overconsumption of energy-rich foods negatively impacts health and cognition (Mattson, 2019).

Aging is defined as a time-related functional deterioration that affects most living organisms and that involves physical and cognitive decline (López-Otín, et al., 2013). Although there is significant heterogeneity (Sun et al., 2016), the cognitive functions most commonly affected by aging are attention, cognitive flexibility, and short- and long-term memory (Morrison and Baxter, 2012; Tromp et al., 2015). Some of these age-related cognitive changes have been correlated to structural and functional alterations in the hippocampus (HPC) (Bettio et al., 2017; Leal and Yassa, 2015; Portero-Tresserra et al., 2018), such as increased cell oxidative stress and neuroinflammation, or dampened neurogenesis and synaptic plasticity (Apple et al., 2017; Zeng et al., 2011). Moreover, cognition, and specifically memory, is also in part regulated by the monoaminergic

system (González-Burgos and Feria-Velasco, 2008; Kemp and Manahan-Vaughan, 2008; Leal and Yassa, 2015), with serotonin (5-HT), dopamine (DA) and noradrenaline (NA) exhibiting marked declines in different regions of the brain of aged individuals (Leal and Yassa, 2015; Stemmelin et al., 2000). Hence, alterations to these neurotransmitters might contribute to age-related cognitive impairment (Koprowska et al., 2004; Von Linstow et al., 2017).

Aging is also associated with more general alterations to glutamatergic (Kumar and Foster, 2019) and cholinergic (Bartus et al., 1982) neurotransmitter systems. Moreover, both these systems have also been implicated in prodromal or early stages of Alzheimer's disease (Petrasek et al., 2016). Specifically, it has been shown an age-related reduction in the *N*-methyl-d-aspartate receptors (NMDARs) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid ionotropic glutamatergic receptors (AMPARs) throughout the brain, and in particular in the HPC (Adams et al., 2008; Bolognin et al., 2014; Liu et al., 2008; Newton et al, 2008; Shi et al., 2007a). NMDA and AMPA receptors are associated with synaptic plasticity (Shi et al., 2007b) influencing synapse formation, maintenance and remodeling (Fischer et al., 2000; Lüscher et al., 2000), and they are essential for the induction and maintenance of long term potentiation (LTP) (Eckles-Smith et al., 2000; Lin et al., 2002). Accordingly, the decrease in both receptors is thought to participate in age-related alterations in hippocampal glutamatergic neurotransmission (Newton et al., 2008), consequently impairing learning and memory (Ménard et al., 2015). Studies in old rats have also revealed increases in blood plasma corticosterone (Montaron et al., 2006), considered a marker of the stress response (Barzilai and Bartke, 2009). Such increase in corticosterone may interfere with memory consolidation in aged animals, as increased hypothalamic-pituitary adrenal (HPA) activity has been implicated in pathological

changes in the HPC of cognitively impaired old rats (Alfarez et al., 2006; Yau and Seckl, 2012).

As indicated above, CR improves health, yet its ability to protect against the cognitive decline that accompanies aging remains unclear (Gallagher et al., 2011; Ingram and de Cabo, 2017). While several studies have shown that CR attenuates cognitive-decline in aged rats (Adams et al., 2008; Carter et al., 2009; Fitting et al., 2008; Geng et al., 2007; Goodrick, 1984; Gyger et al., 1992; Markowska and Savonenko, 2002; Pitsikas et al., 1991; Pitsikas and Algeri, 1992, 1990; Stewart et al., 1989), this was not necessarily the case elsewhere (Beatty et al., 1987; Bond et al., 1989; Del Arco et al., 2011; Hansalik et al., 2006; Markowska, 1999; Stewart et al., 1989; Yanai et al., 2004). Such differences may be due to the use of different rat strains and ages, the relative food reduction, the type of dietary intervention, and/or the learning models used (Gallagher et al., 2011; Ingram and de Cabo, 2017; Martí-Nicolovius and Arévalo-García, 2018). For instance, CR enhances memory in Sprague-Dawley aged rats, whereas it produces inconsistent effects on Wistar and Fischer 344 Brown-Norway rats (reviewed in Martí-Nicolovius and Arévalo-García, 2018). Hence, the strain of rat might be an important variable in explaining some contradictory results in the field. In addition, only a few studies have focused on how CR affects HPC function (biochemical changes) or on memory (behavioral changes) in aged rats, centering mainly on glutamatergic neurotransmission (Adams et al., 2008; Singh et al., 2015). Therefore, the capacity of CR to prevent age-dependent memory deficits and the underlying biochemical disturbances requires further study, especially the involvement of monoaminergic systems. For this reason, here we analyzed the behavioral effects of long-life CR in adult and old Wistar rats, a strain in which the effects of such dietary intervention on spatial memory have been scarcely studied (Bond et al., 1989; Del Arco et al., 2011; Goodrick, 1984; Yanai et al.,

2004) and in which we previously detected an age-related decline in spatial learning (Portero-Tresserra et al., 2018). For this purpose, we used a hippocampal-dependent memory task and assessed the biochemical effects in the HPC. Animals were trained in the spatial Morris Water Maze (MWM) task, thereafter assessing memory 72 h later in a probe test, and evaluating monoamine levels by HPLC-ED, or NMDAR and AMPAR subunits in Western blots. In addition, plasma corticosterone levels were examined to control the possible effects of age on the stress response and its role in memory consolidation by old rats.

2. Materials and Methods

2.1. Subjects

In this study, 46 naive male Wistar rats from our laboratory breeding stock were used (Prolabor, Charles River Laboratories, Arbresle, France). The CR group of old rats (n=19; age=24-27 months; weight=475.23 gr, SEM=6.70 gr) followed a food restriction protocol that involved a 30% reduction in food intake (15 to 18 gr/day) from the age of 4 months (Ingram et al., 2004). Another group of old rats (n=11; age=24-27 months; weight=637.95 gr, SEM=27.68 gr) had *Ad Libitum* access to food and water, as did a further control group of adult animals (n=16; age=3-4 months; weight=445.76 gr, SEM=8.19 gr). The animals were fed dry pellets (obtained from Harlan Laboratories Inc., Madison, USA) produced and packed by Mucedola, Sri. (MI, Italy). Throughout the experiment the animals were paired-housed in 50 x 22 x 14 cm transparent plastic cages with sawdust bedding, and they were maintained in a controlled environment: humidity (60-70%), temperature (20 to 22 ºC), and on a 12-hour light-dark cycle (lights on at 7 am). The behavioral tests were carried out during the light phase of the cycle. All procedures were performed following the EU Directive on the protection of animals used for experimental and other scientific purposes (2010/63/EU), and with the

authorization of the *Generalitat de Catalunya* (DOGC 2450 7/8/1997, DARP number 3866).

2.2. Behavioral studies

The MWM consisted of a circular, black wall pool (2 m diameter, 60 cm high wall) filled with 45 cm of water at 22 (± 2) ºC. The pool was surrounded by a black curtain that formed a circular enclosure of 2.4 m diameter and visual cues were present on the inner side of the curtain that faced the pool, helping the rats to locate the platform (Morris, 1984). The animals had to escape from the water onto a small black platform (11 cm in diameter) hidden 2 cm below the surface, which was accessed by both adult and old rats without any problem. Indeed, a similar sized platform has been used in previous studies (Portero-Tresserra et al, 2018). The platform could only be located by searching for the distal visual cues surrounding the pool (a colored plastic beach ball, a white box with horizontal black stripes, a brown teddy bear and a light facing the curtain). An automated tracking system monitored the animals' performance in the maze (Smart 2.5, PanLab, Barcelona).

The acquisition phase involved five consecutive sessions (1 each day), each consisting of four 90s trials and with an inter-trial interval of 120s. In each trial, the rats were placed in the pool facing one of the four cardinal points on the wall (randomly N, S, E or W) and they were required to find the platform, which was always located in the SE quadrant. Once the rodents reached the platform they were allowed to remain on it for 15s and if they failed to locate it, the experimenter guided the animal to the platform. During the inter-trial interval, the animals were towel-dried and placed inside a heated metal box to prevent hypothermia. A probe memory test was performed 72 hours after the last acquisition session, in which the platform was removed from the pool and the

animals were introduced into the water from the E cardinal point, and they were allowed to swim freely for 60s.

2.3. Biochemical studies

2.3.1. Blood plasma and tissue collection

After completing the behavioral procedures, food was removed the day before sacrifice to minimize the biochemical and hormonal differences between the groups due to the amount of food consumed immediately before (Kmiec et al., 2005) or due to lipidemia (Nikolac, 2014), and cohorts of old and adult rats were used for each biochemical study. Blood samples were collected in Heparin tubes (Sodium Heparin, 5000 USP/mL; Chiesi Spain, SA, Spain), centrifuged for 15 min at 4,000 rpm and at 4 ºC, and the supernatant was recovered and frozen at -20 ºC. Subsequently, the brain was removed rapidly and the HPC was dissected out on ice, and separated into two identical parts for HPLC-ED and Western Blot analysis, weighed, frozen and stored at -80 ºC.

2.3.2. Plasma corticosterone analysis

The final sample of plasma analysis consisted of: old CR group, n=10; old *Ad libitum* group, n=11; Adult group, n=8. The analysis was carried out at the *Servei de Bioquímica, Clínica Veterinària* (Universitat Autònoma de Barcelona), and measured corticosterone levels by competitive ELISA, using a EMS Reader MF V.2.9-0 and a Corticosterone EIA (Immunodiagnostic Systems Ltd, IDS Ltd, Boldon, United Kingdom).

2.3.3. *Monoamine quantification by High Performance Liquid chromatography (HPLC-ED)*

The cohort used for HPLC was: old CR group, n=4-10, old *Ad libitum group n*=6-8 and Adult group n=4-8 (depending on the neurotransmitter analyzed). Brain samples were homogenized in buffer (perchloric acid 60% w/w 0.25 M, sodium metabisulfite 100 µM, EDTA EDTA Na_2 $2H_2O$ 250 µM) at a 9/1 ratio (w/v; mg/ml). The tissue was rapidly disrupted in a polytron homogenizer and the homogenate was centrifuged for 10 min at 4000 rpm and 4 ºC. The supernatant recovered was filtered and 50 µL aliquots were analyzed by HPLC-ED on a phase reverse column (Cromolith Performance, 4.6 mm internal diameter x 10 cm length) coupled to a pre-column (4.6 mm x 5 cm). The mobile phase consisted of 0.1 M citric acid, 0.05 M EDTA, 1.2 mM SOS, 10% acetonitrile (v/v), adjusted to pH 2.75 with tetraethylammonium. The column was eluted at a flow rate of 0.8 mL/min and the HPLC apparatus (LaChrom Elite) was coupled to an electrochemical detector (ED: ESA Coulochem 5100A) with an ESA analytical dual electrode cell 5011A (detection potential for electrodes 1 and 2 was set at 70.05 and +0.4 V). The NA, DA and 5-HT monoamines, and the metabolites DOPAC, 5-HIAA and homovanillic acid (HVA), were all detected electrochemically, the current produced monitored by an interface connected to a PC. EZChrom Elite Software was used to determine the appropriate concentrations, expressed as ng/g tissue.

2.3.4. Western Blotting

The final sample of WB consisted of: old CR group, n=5-6, old *Ad libitum, n*=5-6 and Adult group, n=4-6 (depending on the specific receptor). HPC tissue was collected in lysis buffer (0.15 M NaCl, 1% Triton X-100, 10% Glycerol, $C_3H_8O_3$, 0.001 M EDTA, 0.05 M TRIS [pH 7.4]) containing phosphatase and protease inhibitors (Roche, France). The lysates were homogenized manually in Wheaton™ Dounce Tissue Grinders (ThermoFisher Scientific, USA) and the total amount of protein was quantified with a

BCA assay (Pierce Chemical Co., Thermo Fisher, USA). Equal amounts of protein (30 µg/well) were resolved on 8-12% polyacrylamide SDS-PAGE gels and transferred to nitrocellulose membranes (Whatman, Dassel, Germany) for 90 min at 120V on ice to avoid possible overheating, and for 70 min at 100V. The membranes were blocked for 1 h at 20 to 25 °C with 5% dry non-fat milk in TBS-T (75 mM NaCl, 1.5 mM KCl, 12.4 mM Tris-HCl and 0.1% Tween-20 [pH 7.4]), and they were then incubated overnight at 4 °C with the corresponding primary antibody diluted in 5% (w/v) bovine serum albumin (BSA), with shaking. The following primary antibodies were used: rabbit anti-GluA1 (AB1504, Merck, Germany), mouse anti-GluA2 (MAB397, Merck, Germany), mouse anti-GluN1 (556308, BD Biosciences), rabbit anti-GluN2A (AB1555, Merck, Germany), and mouse anti-β-Tubulin (556321, BD Biosciences). The membranes were then incubated with the corresponding secondary antibody, anti-mouse-HRP (Dako Denmark, Glostrup, Denmark) or anti-rabbit-IgG (H+L: Pierce, Thermo Fisher Scientific, USA), and antibody binding was visualized by ECL. All the samples to be compared were processed at the same time, transferred simultaneously to a membrane and probed with the same antibody dilution. Image processing was carried out using the ChemiDoc XRS+ System (Bio-Rad Laboratories), and the densitometry and quantification were carried out using the ChemiDoc MP Imaging System, Image Lab software (Bio-Rad).

2.4. Statistical analysis

Biochemical and behavioral data were analyzed using the SPSS v20 and plotted as the mean ± SEM. Tests of normality and of the homoscedasticity of variances (Levene's test) were applied to each data set before all the variables were compared among the three experimental groups (*Ad Libitum,* CR and Adult). For the analysis of the MWM five-day acquisition phase, a repeated measures ANOVA was carried out followed by

post hoc contrasts when necessary (multiple comparisons were performed with the Bonferroni correction). In addition, in the test phase of MWM, a one-sample t-test against a constant (25) was used for each group to determine whether the time in the target quadrant or the target annulus (a pre-defined circle surrounding the platform) was different to that of chance (25%). ANOVAs were also carried out to determine the between-group differences in hippocampal monoamine levels, receptor subunit expression and corticosterone levels. Moreover, Spearman Rank correlations were established to examine the relationship between different behavioral and biochemical variables. A p-value of 5% or lower was considered to be statistically significant.

3. Results

3.1. Behavioral data: Morris Water Maze

In general, the adult rats performed better in the MWM acquisition phase than the old rats, and the type of diet (i.e.: CR or *Ad Libitum*) did not appear to produce any differences or improve spatial learning. Analyzing the latencies to find the hidden platform in the MWM acquisition period (Figure 1A) indicated that although there was a progressive reduction in the latency in each group over the 5-day training period, both old groups took longer to find the platform than the adult rats: group ($F_{(2,43)}$=14.512, p=0.001), session ($F_{(4,172)}$=19.137, p<0.001), and group x session interaction ($F_{(8,172)}$=2.428, p=0.016). The contrast analysis confirmed the significant differences between the *Ad Libitum* and Adult groups on day one (p=0.016), three (p=0.001), four (p=0.002) and five (p< 0.001), and between the CR and Adult groups on day two (p=0.016), three (p=0.001), four (p=0.006) and five (p<0.001). A similar global pattern was evident for the total distance travelled in the five acquisition sessions, with adult animals travelling shorter distances to find the escape platform than both the old groups (Figure 1B). An ANOVA showed that the three factors were significantly different:

group ($F_{(2,43)}$=6.023, p=0.005), session ($F_{(4,172)}$=15.218, p=0.001) and interaction group x session ($F_{(8,172)}$=3.616, p=0.001). More specifically, significant differences were found between the Adult and old groups (*Ad Libitum* and CR) on day three (p=0.010, p=0.004), four (p=0.019, p=0.017) and five (p=0.001, p=0.001). When the time spent in the quadrant where the platform was placed was considered (Figure 1C), it was a parameter that increased in all three groups as the training progressed [session ($F_{(4,172)}$=14.58, p=0.001)]. The adult rats spent more time in the target quadrant than either of the aged groups, with no differences associated with the specific days [Group ($F_{(2,43)}$ =23.913, p=0.001), group x session ($F_{(8,172)}$=1.587, p=0.132)].

The animal's swim speed (mean velocity) was analyzed during the acquisition period to control for motor activity and no overall between-group differences were detected ($F_{(2,43)}$=1.276, p=0.290). By contrast, the session ($F_{(4,172)=}$8.041, p< 0.001) and group x session interactions ($F_{(8,172)}$=4.808, p=0.001) did display significant differences between the CR and Adult groups (p=0.003) or between the *Ad Libitum* and Adult groups (p=0.008) on day one, as well as between the *Ad Libitum* and Adult groups on day two (p=0.003). However, in the later sessions, from day three to five, all the groups displayed comparable motor activity in the pool. Furthermore, *thigmotaxis* was assessed as an index of anxiety, defined as the total time that the animal swam or stayed near the walls (Huang et al., 2012). An ANOVA only showed session to be a significant factor ($F_{(4,172)}$=51.559, p=0.001), suggesting that all groups of animals displayed a similar level of anxiety during the acquisition phase, which decreased as the training progressed.

Regarding the 72 h MWM test session, the time spent by the animals in the target quadrant from which the platform had been removed (Figure 2A) and the time spent specifically in the target annulus (Figure 2B) indicated that the long-term memory of

CR old animals was similar to that of Adult rats, and better than that of *Ad Libitum* old rats. ANOVA confirmed the significant between-group differences ($F_{(2,43)}$=4.985, p=0.011), and the differences between the Adult and *Ad Libitum* groups (p=0.009). The poor performance of the *Ad libitum* old rats was evident when assessed by an one-sample t-test (constant value 25), with values similar to the levels of chance ($t_{(10)}$=0.351, p=0.733). This contrasted with the adult ($t_{(15)}$=3.636, p=0.002) and CR old rats ($t_{(18)}$=4.824, p< 0.001), which spent a considerable amount of time in the target quadrant. The *Ad libitum* old group again seemed to exhibit worse memory when the time spent in the target annulus was considered (Figure 2B), an ANOVA showing between-group differences ($F_{(2,43)}$=4.897, p=0.012), and the contrast analysis confirming the significant differences between the *Ad Libitum* and Adult groups (p=0.011). As for the control variables, such as distance travelled, mean swim speed and thigmotaxis, an ANOVA failed to identify between-group differences ($F_{(2,43)}$=1.273, p=0.290, $F_{(2,43)}$=1.416, p=0,254, and $F_{(2,43)}$=0.548, p=0.582, respectively), suggesting that locomotion was not impaired and nor were there any differences in anxiety between the animals during the execution of the probe memory test.

3.2. Biochemical data

3.2.1. Monoamine levels

In general, the hippocampal concentrations of monoamines and metabolites (NA, DOPAC, DA, HVA, 5-HT and 5-HIAA) differed between old and adult rats (Figure 3). CR rats expressed higher levels of NA than the Adult and the old *Ad Libitum* animals when assessed by ANOVA ($F_{(2,22)}$=9.303, p=0.001). However, while there were no significant between-group differences in terms of DA and DOPAC, (ANOVA: $F_{(2,10)}$=2.073, p=0.177; $F_{(2,18)}$=0.459, p=0.639 respectively), differences in the DOPAC/DA ratio were detected ($F_{(2,9)}$=16.615, p=0.001) between Adult and old

animals (*Ad libitum* p=0.008 and CR p=0.001), with a lower DA turnover in old rats. There were also between-group differences in 5-HT ($F_{(2,22)}$=3.792, p=0.038), although there was only a tendency towards significantly higher levels between the Adult and old *Ad Libitum* rats (p=0.057). Between-group differences were also evident for 5-HIAA (ANOVA: $F_{(2,22)}$=4.443, p=0.024), as the old *Ad Libitum* group exhibited lower concentrations than the Adult rats (p=0.040). As for DA, 5-HT turnover seemed to decrease in aged rats as a difference in the 5-HIAA/5-HT ratio was detected ($F_{(2,21)}$ =13.657, p<0.001) between the old and Adult rats (*Ad libitum* p=0.002, CR p=0.001).

3.2.2. Subunit-receptor expression

When the presence of AMPAR subunits was assessed (Figures 4A-B), significant between-group differences were evident for GluA1 ($F_{(2,11)}$=5.387, p=0.023) and GluA2 ($F_{(2,13)}$=4.345, p=0.036), with the expression of AMPAR subunits more compromised in the *Ad Libitum* old rats than in the Adult group (GluA1 p=0.028, GluA2 p=0.05). By contrast, no between-group differences were detected for the GluN1 ($F_{(2,15)}$=0.496, p=0.618) and GluN2A ($F_{(2,15)}$=0.428, p=0.660) NMDAR subunits.

3.2.3. Corticosterone levels

When plasma corticosterone concentrations were evaluated (Figure 5), they were somewhat higher in both groups of old rats than in the adults. Overall between-group differences were detected (ANOVA $F_{(2,22)}$=5.170, p=0.014), with significant differences between the CR and Adult groups (p=0.021), and a tendency towards statistical significance between the old *Ad Libitum* and Adult groups (p=0.054).

3.3. Correlations between biochemical and behavioral data

The possible relationships between the biochemical variables and behavioral performance were assessed by studying the correlations between the measures showing

significant between-group differences (NA, DOPAC/DA, 5-HIAA, 5-HIAA/5-HT, GluA1, GluA2 and corticosterone) and the significant variables in the MWM acquisition and probe test. With regards the 5-HT system (Figure 6A-D), the time spent in the platform quadrant during the 5th acquisition session was positively correlated to both the levels of 5-HIAA and the 5-HIAA/5-HT ratio (metabolite r=0.385, p=0.03; ratio r=0.46, p=0.011), as was the case during the memory test (metabolite r=0.526, p=0.041) and in terms of the time in the annulus during that test (metabolite r=0.448, p=0.014; ratio r=0.0448, p=0.0139). Regarding AMPAR subunit expression (Figure 7A-D), GluA1 (r=-0.528, p=0.032) and GluA2 (r=-0.426, p=0.056) expression was negatively correlated to the latency to find the platform on the 5th acquisition day. By contrast, the time in the annulus zone and GluA1 expression (r=0.470, p=0.052) were positively correlated in the memory test, as were the time in the target quadrant and the amount of GluA2 (r=0.577, p=0.019).

4. Discussion

In this study, a CR diet from 4 months of age was seen to attenuate the memory decline evident in the MWM, although it did not improve task acquisition in old Wistar rats. This effect did not appear to be attributed to changes in locomotion or anxiety, as no between-group differences in swim speed or *thigmotaxis* were seen. Although CR did not affect MWM acquisition, an effect of age was detected. Thus, while the latency to locate the platform diminished gradually in all animals over the 5-day MWM acquisition period, reflecting spatial learning (Gallagher and Nicolle, 1993), this learning was slower in the two aged groups as it diminished more slowly and they travelled further than the Adult rats. A mild age-related reduction in swim speed cannot be ruled out during the first two days of acquisition, consistent with previous studies (Carter et al., 2002; Leite-Almeida et al., 2009; Portero-Tresserra et al., 2018) and in

agreement with the age-related changes reported in old rats (Adams et al., 2008; Carter et al., 2009; Geng et al., 2007; Nakamura and Ohno, 1995; Portero-Tresserra et al., 2018; Stewart et al., 1989; Wang et al., 2007). The fact that all animals swam over shorter distances and with a shorter latency over time makes it more difficult to find differences in the swim speed during the last trials.

In relation to MWM memory, the CR and Adult rats learned correctly and/or recalled the platform location, and in contrast to the old *Ad libitum* rats, they both performed above the level of chance in the 72 h memory test. This suggests that a life-long CR diet may improve long-term spatial memory in aged Wistar rats, as seen in other rat strains (Algeri et al., 1991; Gyger et al., 1992; Kuhla et al., 2013; Parikh et al., 2016; Pitsikas and Algeri, 1990; Stewart et al., 1989), although it does not seem to enhance performance in immediate (Fitting et al., 2008) or 24 h memory tests (Adams et al., 2008; Carter et al., 2009; Geng et al., 2007). Nevertheless, both the old groups of rats tested here seem to use a similar spatial strategy to search for the platform, as there were no differences in the total distance travelled during the test (Cardoso et al., 2016). Unfortunately, our results in Wistar rats cannot be easily compared with others. As far as we know there is only one previous study that explored the effects of CR on a set of different cognitive tasks in 24-month old Wistar rats (Yanai et al., 2004), including the use of a Morris-type maze, in which detrimental effects of diet were observed on the performance of this behavioral task. However, unlike our study, in this experiment the CR protocol was more severe, the evaluated task was different and cognitive deficits were also observed when the animals were young.

Moreover, changes in peripheral hormones like corticosterone produced by dysregulation of the HPA axis (Garrido et al., 2012) may contribute to the impaired MWM performance in old rats. It is well known that corticosterone levels increase

strongly in old animals (Moneo et al., 2017), and, the HPC is a prime target for glucocorticoids and a brain structure particularly vulnerable to aging (Yau and Seckl, 2012).

The positive effect of CR on MWM spatial memory may be related, at least in part, to biochemical changes in the HPC (e.g., enhanced monoaminergic and glutamatergic neurotransmission). Together with central neuromodulators like acetylcholine (Petrasek et al., 2016), which also influences cognitive performance, monoamines contribute to enhanced vigilance, alertness and attention. The HPC receives terminals from different brain regions that drive the release of noradrenaline, serotonin and dopamine (Del Arco and Mora, 2009), and it contains intrinsic glutamatergic neurons (Mora et al., 2012). Disturbances in these neurotransmitter systems may partially account for age-related changes in memory and attention (Von Linstow et al., 2017). In this context, the increase in NA associated with CR here may have contributed to attenuate memory decline in aged CR rats (Coradazzi et al., 2016; Pyrzanowska et al., 2012), although there were no differences in hippocampal NA between old *Ad libitum* and Adult rats, consistent with earlier studies (Lee et al., 1994; Luine et al., 1990; Míguez et al., 1999; Pyrzanowska et al., 2012) but not with the lower levels reported in old animals elsewhere (Koprowska et al., 2004; Stemmelin et al., 2000). In terms of the dopaminergic system, no changes were evident in hippocampal DA and DOPAC in old *Ad Libitum* rats, as described previously (Lee et al., 1994; Nakamura and Ohno, 1995; van Luijtelaar et al., 1992; Yurek et al., 1998). However, there was a lower DOPAC/DA ratio in the old rats, irrespective of their diet. These results may reflect a poorer turnover of this neurotransmitter, in agreement with the age-induced deficits in the dopaminergic system indicated elsewhere (Godefroy et al., 1989; Koprowska et al., 2004; Luine et al., 1990; Míguez et al., 1999; Stemmelin et al., 2000). Lower 5-HIAA and 5-HIAA/5-HT

ratios were also detected in old rats, again indicating an age-related decline in 5-HT synthesis and/or an enhanced metabolism of this monoamine (Miura et al., 2002). Moreover, there was a positive relationship between spatial MWM performance and both the 5-HIAA levels and the 5-HIAA/5-HT ratio, whereby animals with more of this metabolite or enhanced 5-HT turnover tend to acquire and recall the task better than animals with less. Similar results were reported previously in adult Wistar rats (Oliveira et al., 2010), indicating that the higher the hippocampal serotonergic activity, the better the animal's MWM acquisition performance. In addition, recent work on mice showed that optogenetic activation of serotonergic terminals in the hippocampal CA1 region improved spatial memory formation (Texeira et al., 2018). The data presented here confirm that such relationships might also be detected in old rats, confirming a role for this monoamine system in spatial learning.

Glutamatergic neurotransmission is also compromised with age (Shi et al., 2007a), as supported by the data regarding AMPAR subunit expression (Henley and Wilkinson, 2013), and the weaker hippocampal GluA1 and GluA2 subunit expression in the *Ad Libitum* rats. While these findings are in accordance with other studies into the GluA1 subunit, they only partially reflect earlier studies on the GluA2 subunit (Adams et al., 2008; Eckles-Smith et al., 2000; Newton et al., 2008; Shi et al., 2007b). Therefore, weaker AMPAR subunit expression may have contributed to the poor performance of *Ad libitum* old animals and indeed, the GluA1 and GluA2 levels were positively correlated with some variables measured in the MWM acquisition and test, as suggested previously (Henley and Wilkinson, 2013). Indeed, there is evidence that aging is associated with impaired hippocampal LTP in rodents (Izquierdo et al., 2008), which is related to deteriorated long-term memory consolidation (Eckles-Smith et al., 2000; Fischer et al., 2000; Lüscher et al., 2000). Conversely, neither age nor dietary

intervention had a clear effect on NMDAR subunit expression (Kumar et al., 2018), in contrast to earlier reports of an age-dependent decrease in GluN1 subunits (Adams et al., 2008; Eckles-Smith et al., 2000; Monti et al., 2004; Shi et al., 2007b; Portero-Tresserra et al., 2018) and/or GluN2A (Adams et al., 2008; Shi et al., 2007b), which may be attenuated by CR (Adams et al., 2008; Eckles-Smith et al., 2000; Monti et al., 2004; Shi et al., 2007b).

In conclusion, the data presented here confirm that, in general, a lifelong CR diet can attenuate the age-related spatial memory decline detected in old Wistar rats. Moreover, the beneficial effects of CR on memory may be related to an improvement in monoaminergic and glutamatergic neurotransmission in the aged brain. Indeed, dietary restriction enhanced the levels of NA and attenuated the age-associated decrease in the serotonin metabolite 5-HIAA, as well as that of GluA1 and GluA2 subunit expression in the HPC. Studying the effects of CR on memory decline are of particular interest when considering the effects of CR on the aging brain, and to search for new behavioral habits that assure a long and healthy life in humans.

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Disclosure statement

The authors have no actual or potential conflicts of interest to declare.

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Figure Legends

Figure 1. MWM acquisition. (A) Latency (sec) to find the hidden platform (mean ± SEM), (B) Total length (cm) travelled (mean ± SEM), and (C) Time spent in the platform quadrant (mean ± SEM) during the five sessions: *p<0.05, **p<0.01, ***p<0.001 Adult vs CR group; ## p<0.01, ###p<0.001 Adult vs *Ad Libitum* group.

Figure 2. MWM 72 h memory test. (A) Time spent in the platform quadrant (mean ± SEM) and (B) Time spent in the target annulus (mean ± SEM): the dotted line indicates 25% of time (random performance). Differences from chance level: **p<0.01, ***p<0.001; between-group differences ##p <0.01.

Figure 3. Concentration of monoamines, precursors and metabolites in the hippocampus (mean ± SEM), as analyzed for each group by HPLC-ED: *p< 0.05, ***p< 0.001.

Figure 4. Representative Western Blots of the hippocampus from aged *Ad Libitum*, CR and Adult rats probed for glutamatergic GluN2A, GluN1, GluA1, GluA2 subunits. β-tubulin was used as a loading control. (A) Representative images of the integrated density bands for each proteins from each group. (B) Change (mean ± SEM) in the intensity of the receptor subunits, taking the intensity in Adult rats as 100%: *p < 0.05.

Figure 5. Plasma corticosterone levels (mean ± SEM) for each group: *p < 0.05.

Figure 6. Scatter-plot for the one-tailed Spearman correlation of the 5-HIAA/5-HT ratio with the time spent in the target quadrant in the last acquisition session (A) or the time spent in the annulus during the probe test (B). Each point represents one subject.

Figure 7. Scatter-plot for the one-tailed Spearman correlations: (A) Between GluA1 receptor intensity in the hippocampus and the latency to find the platform in the last MWM acquisition session; (B) between GluA1 receptor intensity and the time spent in the target annulus during the MWM test; (C) between GluA2 receptor intensity and the

latency to find the platform in the last acquisition session of the MWM; and (D) between GluA2 receptor intensity and the time spent in the target quadrant during the MWM. Each point represents one subject.

Figure 1

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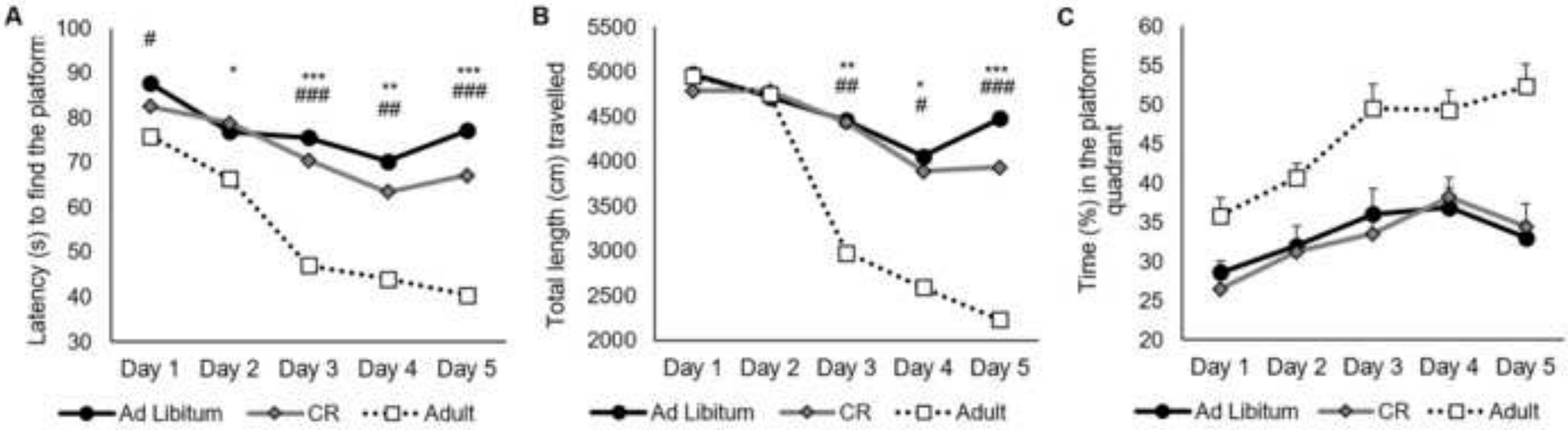

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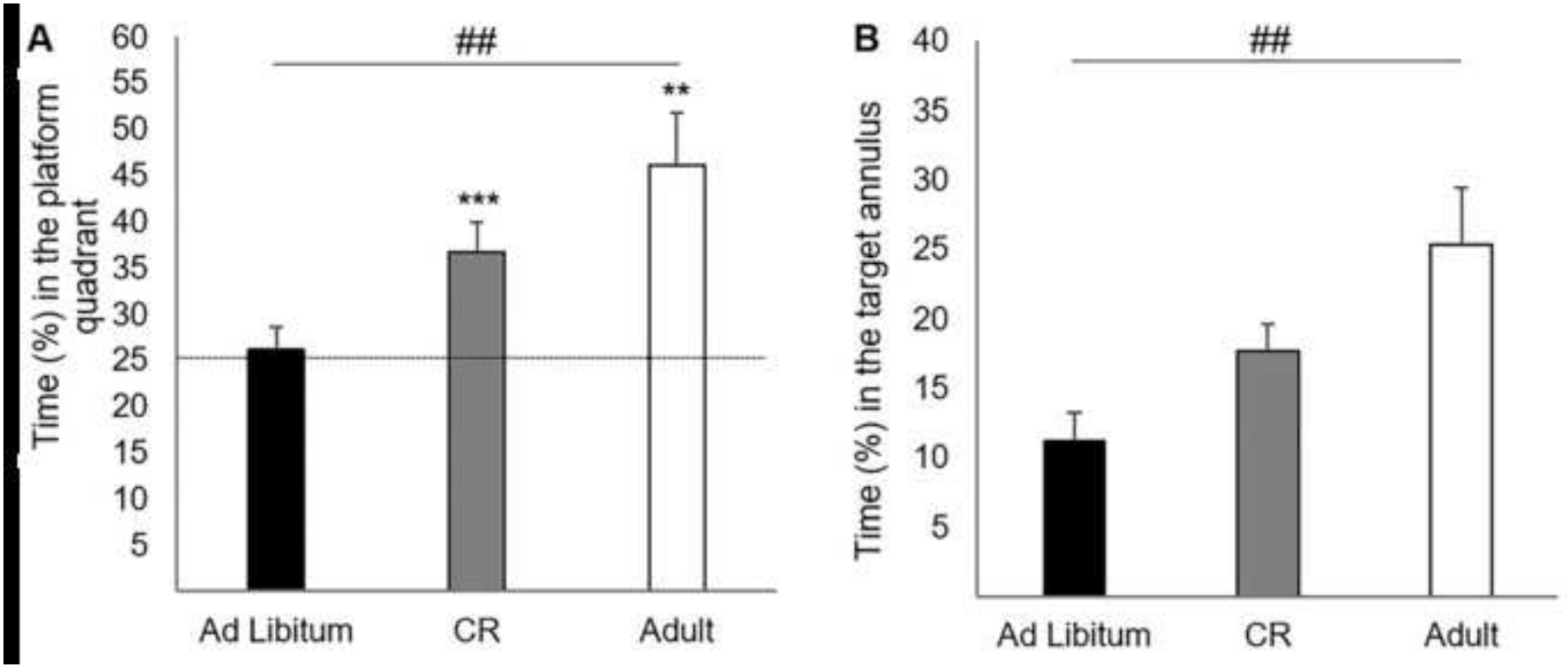

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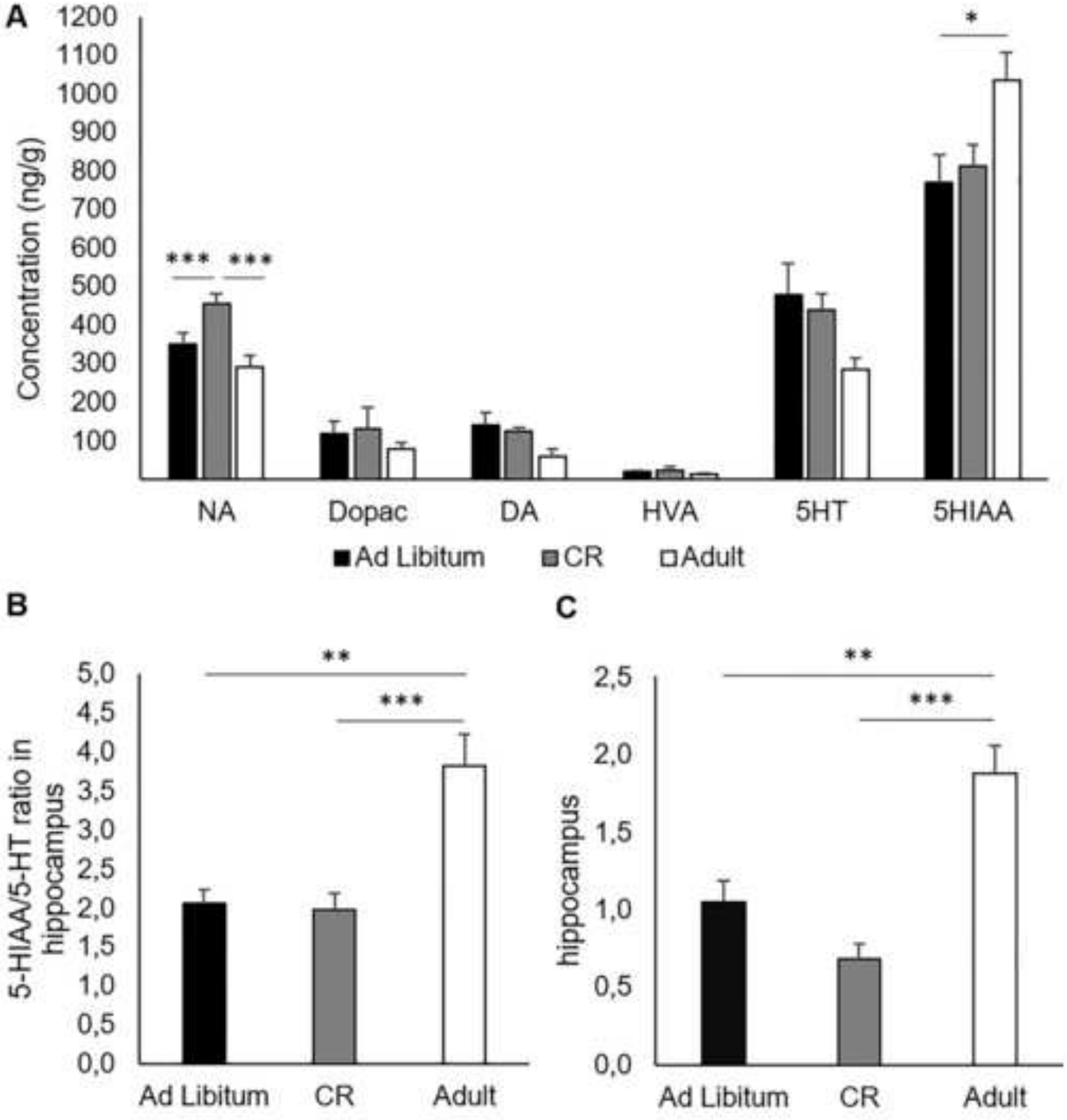

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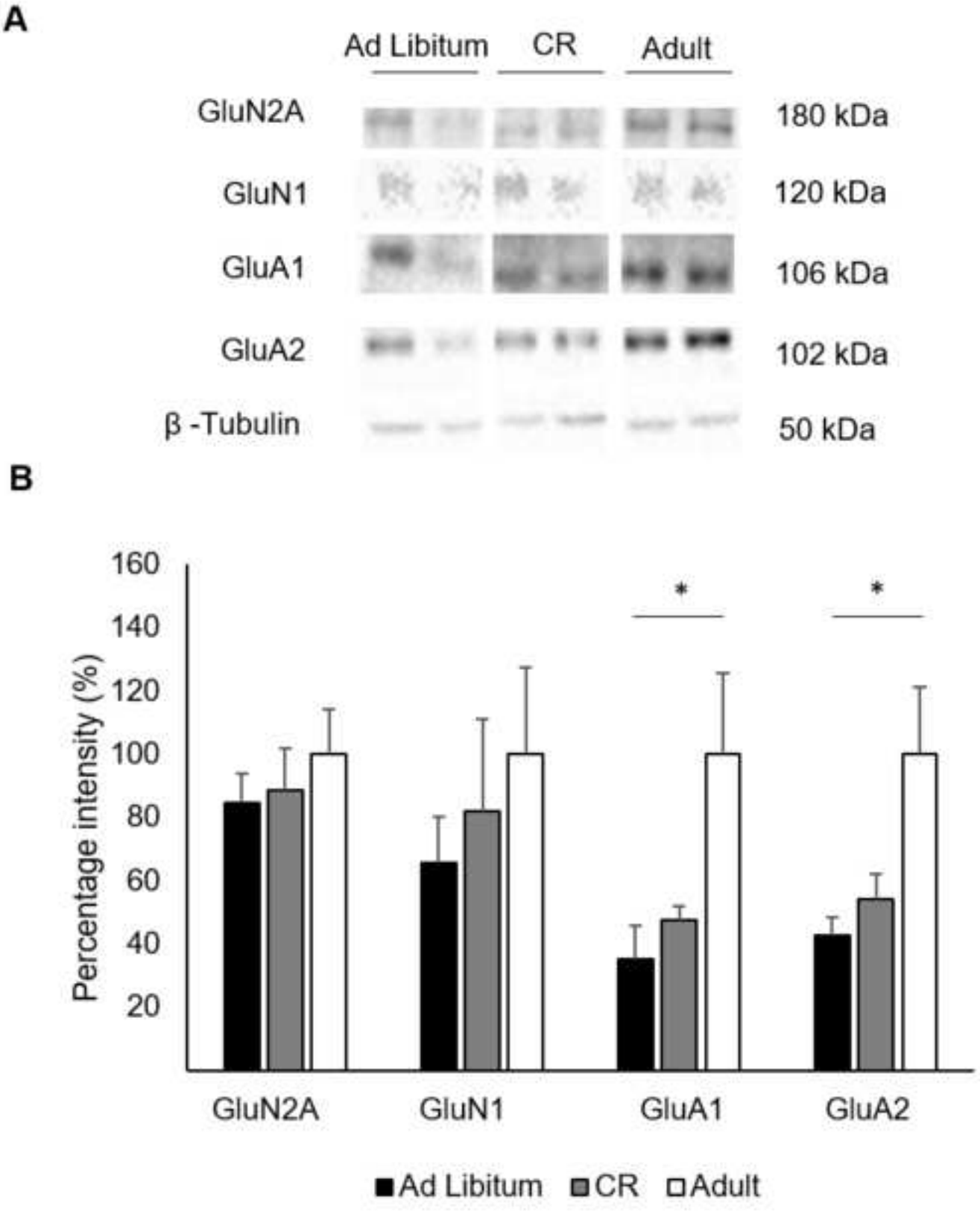

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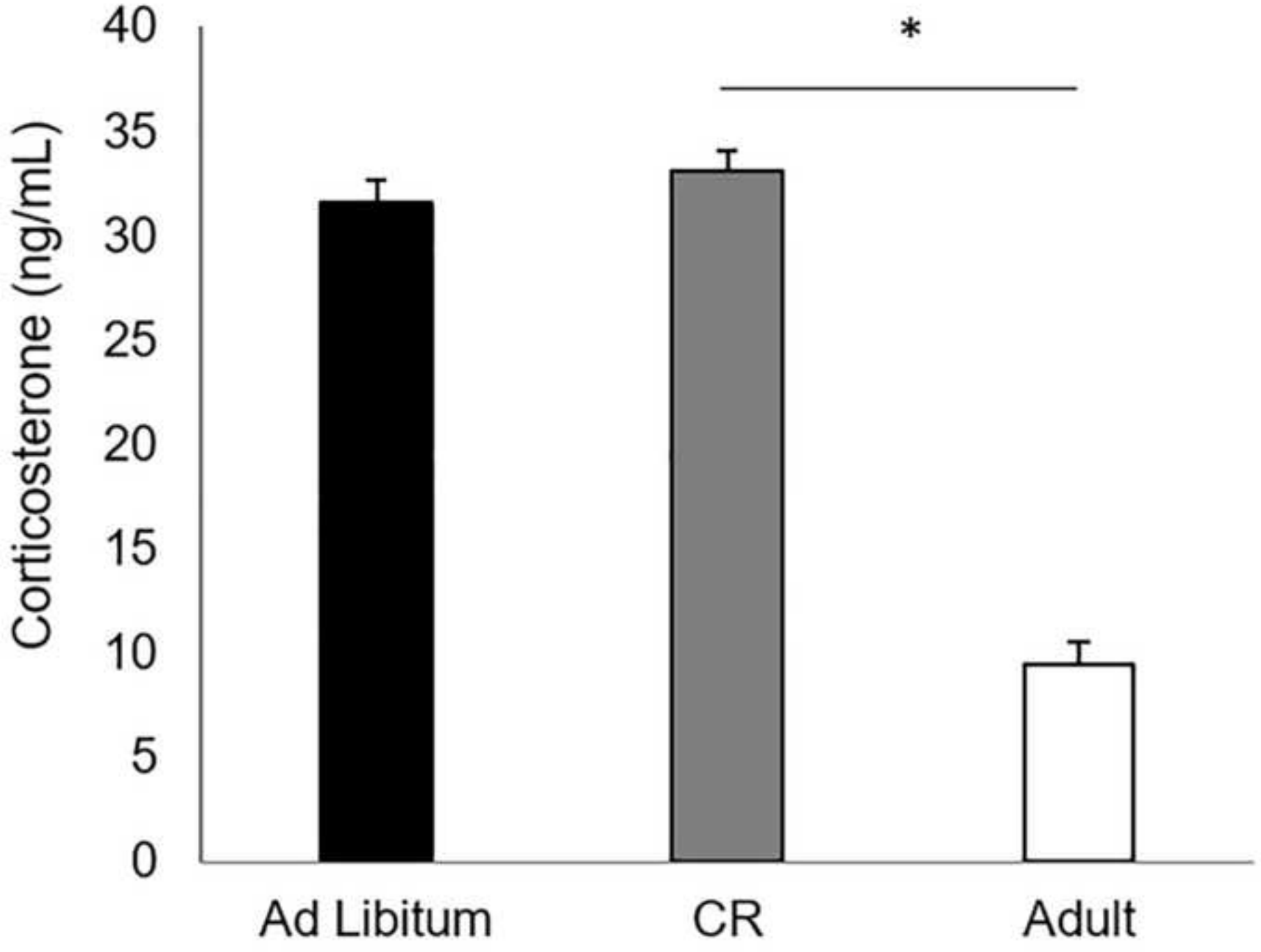

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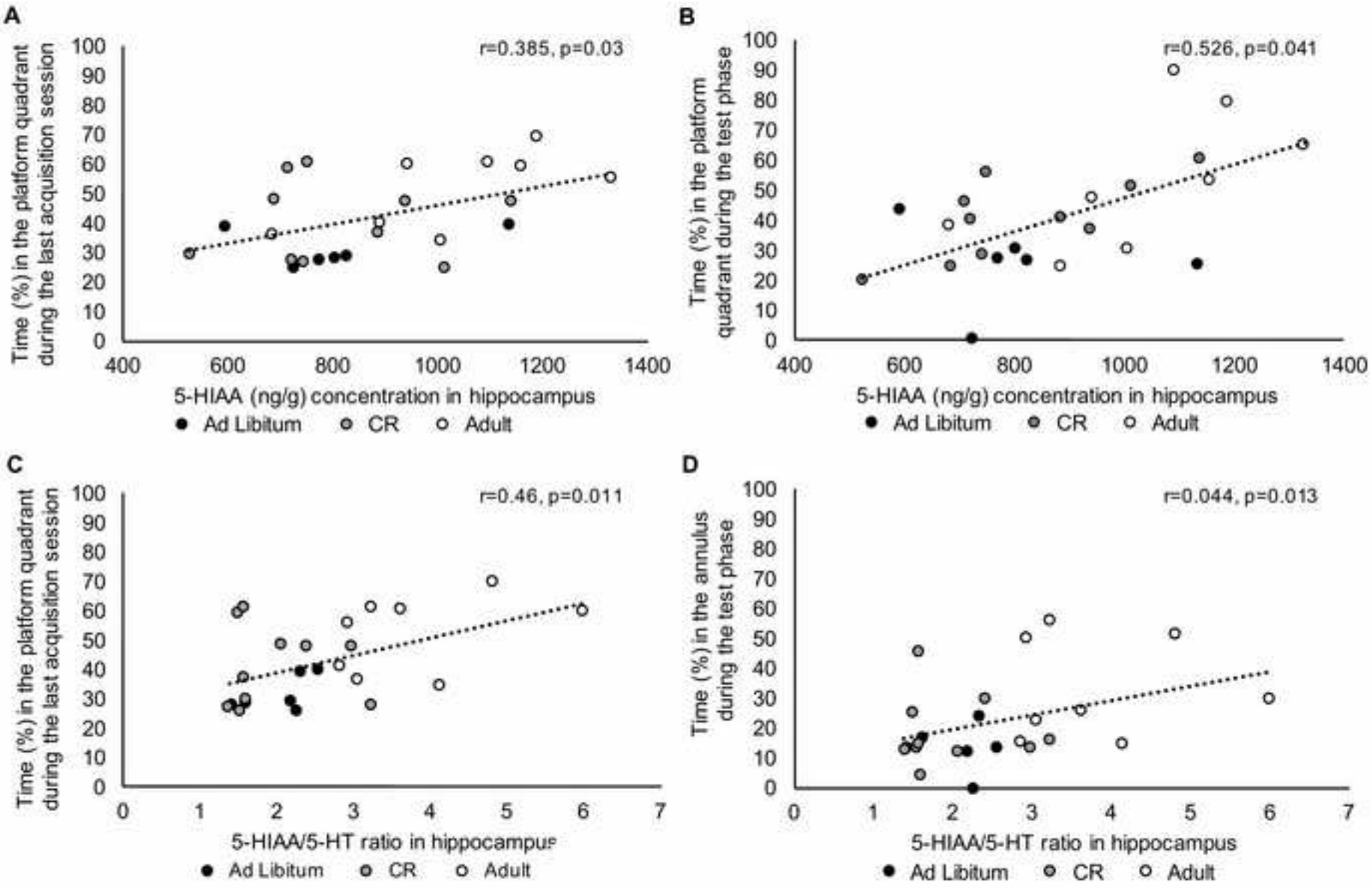

Figure 7

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