Effects of caloric restriction on monoaminergic neurotransmission, peripheral hormones, and olfactory memory in aged rats

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Abstract

Aging is associated with a reduced ability to identify and discriminate scents, and olfactory dysfunction has been linked to preclinical stages of neurodegenerative diseases in humans. Moreover, emerging evidence suggests that smell-driven behaviors are regulated by hormones like insulin or leptin, and by metabolic parameters like glucose, which in turn may influence monoaminergic neurotransmission in brain areas related to cognition. Several studies have suggested that dietary interventions like caloric restriction (CR) can mitigate the age-induced decline in memory by modifying metabolic parameters and brain monoaminergic levels. The present study explored the effects of CR on age-dependent olfactory memory deficits, as well as their relationship with peripheral leptin, insulin and glucose levels, and brain monoamines. To this end, aged rats (24-months-old) fed on a CR diet or with *ad libitum* access to food, and adult rats (3-4 months), were trained in an odor discrimination task (ODT). The peripheral plasma levels of insulin, leptin, and glucose, and of monoamines and metabolites/precursors in brain areas related to olfactory learning and memory processes, such as the striatum and frontal cortex (FC), were determined. The data obtained indicated that CR attenuated the age-dependent decline in olfactory sensitivity in old animals fed *ad libitum*, which was correlated with the performance in ODT retention trial, as well as with leptin plasma levels. CR enhanced dopamine levels in the striatum, while it attenuated the age-related decline in serotonin levels in the striatum and FC. Such findings support a positive effect of CR on age-dependent olfactory sensitivity decline and dysfunctions in brain monoamine levels.

Keywords: Aging, striatum, frontal cortex, leptin, IGF-1, odor-reward association

1. Introduction

Olfactory dysfunction is a common feature in older individuals, its prevalence and severity increasing substantially with age [1]. Olfactory impairment is frequently associated with some neurodegenerative diseases and it is regarded as a clinical correlate in both Alzheimer's (AD) and Parkinson's disease (PD) [2–6]. Behavioral studies of olfactory function in both old rodents and aged humans, have indicated a decreased sensitivity and ability in terms of discriminating [7–9] and associating odors [10–13], as well as a disruption in performing odor-based learning tasks [14–16]. Multiple factors may contribute to such age-related functional impairment. For example, olfactory dysfunction and memory loss have been related to anatomical and biochemical alterations within the olfactory system [3,8], and also in specific brain networks in the frontal cortex (FC) [17–19] and striatum [20,21]. As the olfactory system (considered an indicator of the integrity of the aging brain) influences physical well-being and daily-life activities, preventing age-related functional olfactory decline is relevant to promote healthier aging.

A major tool available to prevent some negative consequences of aging is caloric restriction (CR) which consists of restricting food intake by organisms without causing malnutrition [22]. CR is the only dietary intervention shown to significantly increase longevity and improve health during the aging process in several species [23–26]. Such dietary interventions may regulate the neurotransmitter and neuromodulatory systems affected by aging [27,28]. In this regard, CR may also regulate peripheral hormones [29–31], such as insulin or leptin, and metabolic parameters like glucose [32,33], as well as influence monoaminergic neurotransmission in brain areas related to olfactory-driven behaviors in rodents [33–38]. Nevertheless, while the beneficial effects of CR on life expectancy and health are well known [23,39,40], it is less clear whether CR slows

age-dependent cognitive decline [36,41–43]. Hence, further studies are necessary to investigate whether CR can attenuate age-related olfactory memory decline.

Therefore, the objective of the current study was to explore whether a life-long CR diet was able to prevent the age-dependent deficits in an odor-association task [44], the odor discrimination task (ODT) performed by aged Wistar rats. In these animals, we also analyzed IGF-1 as this serves as a marker of cognitive deterioration in normal aging [45]. In the aged and adult animals tested, we examined plasma levels of insulin, leptin and glucose, and the levels of the monoamines noradrenaline (NA), dopamine (DA), and serotonin (5-HT) in the striatum and FC, as well as those of their metabolites and precursors 3,4-Dihydroxyphenylacetic (DOPAC), 3-Methoxytyramine (3MT), and 5-Hydroxyindoleacetic acid (5-HIAA). Accordingly, in the light of this study, life-long CR diets can attenuate age-related deterioration in olfactory sensitivity, probably by reducing high levels of leptin, and improve dysfunctions in brain monoamines. More studies are needed to see if these effects can help reduce olfactory memory deficits.

2. Materials and Methods

2.1. Subjects

In this experiment, 45 naive male Wistar rats from our laboratory breeding stock (Prolabor, Charles River Laboratories, Arbresle, France) were paired-housed in 50 x 22 x 14 cm transparent plastic cages with sawdust bedding, and they were maintained in a controlled environment of 60-70% humidity, at a temperature of 20 to 22 ºC and on a 12-hour light-dark cycle. Rats were fed on standard laboratory food (dry pellets obtained from Harlan Laboratories Inc., Madison, USA), containing wheat, maize, maize gluten, calcium carbonate, soybean oil, mineral dicalcium phosphate, corn gluten

feed, sodium chloride, magnesium oxide and additives (per kg): vitamin A (E672) 6000 I.U, vitamin D3 (E671) 600 I.U, Fe (E1) 50 mg, Mn (E5) 44 mg, Zn (E6) 31 mg, Cu (E4) 7mg, I (E2) 6.2mg, Co (3b302) 0.5mg and with the following analytical constituents: Moisture 12%, Crude protein 14.5%, Crude oils and fats 4%, Crude fiber 4.5%, Crude ash 4.7%. Food was distributed inside the cage to avoid competition among individuals. Animals were assigned to three experimental groups: a CR group (n = 19; age = 24-27 months; weight = 469.38 g, SEM = 6.85 g) that followed a 25-30% reduction in food intake from 4 months of age; an *Ad libitum* group (n = 11; age = 24-27 months; weight = 641.46 g, SEM = 29.72 g) composed by old animals that had unrestricted access to food and water; and the Adult group (n =15; age =3-4 months; weight = 433.66 g, SEM = 9.54 g). The amount of food consumed AL was approximately 25 grams/day by adult rats and 32 grams/day by old rats. Food was available once daily at 8 am. All groups had free access to water.

All the behavioral experiments were performed during the light cycle (lights on at 7 am). Moreover, all the 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

2.2.1. *Odor Discrimination Task*

The ODT requires the discrimination of three odors, one of which is associated with an edible reward [44]. The odor-reward associative task procedure is based on foraging for palatable food in a single, four-trial session, without the element of stress and fear [46] and it has been described in detail elsewhere [47]. The test was performed in a black square box (60 x 60 x 40 cm) that contained three yellow sponges (8.5 x 6.5 x 5.5 cm)

placed in a glass slide staining rack, with a 3 cm diameter and 2 cm deep hole in the center. The food reinforcement placed in the hole of the sponge was chocolate rice crispy breakfast food (Kellogg's, Spain), such that the rat had to put its head into the sponge (nose poke) to obtain the reward. Each sponge was infused with an odor, vanilla (0.3 ml), orange (0.6 ml) and anise (0.2 ml: Vahiné, Ducros S.A. Sabadell, Spain). In each trial, some cereal pieces were crumbled and scattered all over the box floor to avoid the animal being guided by the chocolate rice odor and ensure that was guided by the odor of the sponge. The odors used in the experiment were reinforced in a balanced way between the different experimental groups throughout the study. All groups were food deprived (12 g/day, to maintain body weight at 85% of freely feeding weight) 5 days prior to the ODT procedure, to ensure motivation to perform the task. The ODT procedure was conducted after the three habituation sessions over three consecutive days in which the animals were given free access to the food reinforcement in a plastic bottomed cage (50 x 22 x 14 cm) and the time to eat 10 pieces of cereal was recorded. After consuming the cereal, they were placed in the training box without the reinforcement and allowed to explore it for 15 minutes to get habituated to the training-test conditions.

One day after the last habituation session, ODT acquisition was assessed in a four-trial session. Reinforcement (unconditioned stimulus) was associated with the same odor (conditioned stimulus) in each of the trials for each rat, and the target odor was assigned randomly to the different animals. At the start of the trial, the rats were placed in the training box facing the corner without the sponge. An inter-trial interval of 1 min was maintained, and there was a 3 min limit for the rats to find and consume the reinforcement. After the rats had found and eaten the cereal, they remained in the box for a few seconds before being removed and placed in the intertrial cage. The rats were

142 tested for retention (first trial) and relearning (three trials) 72 hours after acquisition,
143 using the same procedure as that used for acquisition except the first test trial was not
144 reinforced to directly measure long-term olfactory memory.

145 The learning and memory variables scored were the latency to make a correct response
146 (nose-poking into the target sponge) and the combination of the two kinds of errors:
147 omission error (sniffing the rewarded sponge with no subsequent nose-poking) and
148 commission error (nose-poking into a non-rewarding sponge [48]. The sessions were
149 recorded with a video camera fixed above the apparatus using the SMART video
150 tracking system (SMART v3.0, Panlab, Spain).

151 *2.2.2. Buried Food Test*

152 An additional olfactory test was carried out 24 hours after finishing the ODT test. The
153 buried food test measures how quickly a rat can find a small piece of familiar palatable
154 food, such as a cookie, to discard deficits in olfactory abilities. The test was carried out
155 as described previously [47,48], having fed the rats with a butter-flavored cookie 24
156 hours before (Brambly Hedge, Denmark) to habituate them to the new flavor. The
157 following day a clean plastic cage was set-up and filled with 2 cm of sawdust, and a
158 piece of the cookie was buried in one of its corners. The rats were then placed in the
159 cage, and the latency to find the buried cookie and start eating it was measured.

160 2.3. Biochemical procedures

161 *2.3.1. Blood collection and sample acquisition*

162 After completing the behavioral tests, the animal's food was removed the day before
163 sacrifice [36] in order to minimize the possible differences due to the amount of food
164 consumed immediately before sacrifice [49] or to lipemia [50], in a subset of old and
165 adult rats (Old *Ad Libitum*, n= 6; Old CR, n= 10; Adult, n= 8). Blood samples were

collected in Heparin tubs (Sodium Heparin, 5000 USP/mL: Chiesi Spain, SA, Spain), centrifuged for 15 min at 4000 rpm and 4 ºC (RCF 2361 xg), and the supernatant was recovered and frozen. Subsequently, the rat's brain was removed rapidly, and the whole striatum and the whole FC were dissected out on ice. Both these brain regions were weighed, frozen and stored individually at -80 ºC in 1 ml Eppendorf tubes, to then be analyzed by HPLC-ED and in Western Blots.

2.3.2. Blood plasma analysis

Insulin, leptin and IGF-1 levels were measured by competitive ELISA on an EMS Reader MF V.2.9-0 using the following kit for each protein: Rat Insulin ELISA (Mercodia AB, Sweden; Quantikine®), Mouse/Rat leptin ELISA (R&D Systems, Inc. USA; Quantikine®); and Mouse/Rat IGF-1 ELISA (R&D Systems, Inc. USA). Glucose levels were detected using the Hexokinase method. All of the tests described above were carried out using the Olympus AU400 (Germany) analyzer and the reactive OSR (Olympus System Reagent, Beckman Coulter®, Ireland).

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

Brain samples were homogenized in buffer (perchloric acid 60% w/w 0.25M, sodium metabisulphite 100 µM, EDTA Na_2 $2H_2O$ 250 µM) at a 9/1 ratio (p/v; ml/mg). The tissue was rapidly disrupted in a polytron homogenizer, the homogenate was centrifuged (10 min at 4000 rpm, 4 ºC: RCF 1485 xg) and the supernatant filtered from the purified supernatant (50 µL aliquots) were analyzed by HPLC on a reverse phase 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 0.1M citric acid, 0.05M EDTA, 1.2M SOS, 10% acetonitrile (v/v) adjusted to pH 2.75 with tetraethylammonium

(TEA). 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). NA, DOPAC, DA, 3MT, 5-HIAA and 5-HT levels were detected electrochemically, and the current produced was monitored by an interface connected to a computer. The concentrations (ng/g) of the compounds in a given sample were calculated by interpolating the peak height into a standard calibration curve using the EZChrom Elite Software.

2.3.4. Western Blotting

The striatum and the FC tissue were collected separately in lysis buffer (0.15M NaCl, 1% Triton X-100, 10% Glycerol, 0.001M EDTA, 0.05M Tris pH 7.4) with phosphatase and protease inhibitor tablets (Roche, France). The lysates were homogenized and centrifuged, and the total amount of protein was quantified using a Pierce^{TM} BCA assay kit (Pierce Chemical Co., Thermo Fisher Scientific, USA). Equal amounts of protein (30 μg/well) were resolved on 8–12% polyacrylamide SDS-PAGE gels, then transferred to nitrocellulose membranes (Whatman, Dassel, Germany) for 90 min at 120 V on ice to avoid possible overheating, and for 70 min at 100 V. The membranes were then blocked for 1 hour at room temperature in 5 % non-fat milk in TBS-T buffer (75 mM NaCl, 1.5 mM KCl, 12.4 mM Tris-HCl pH 7.4 and 0.1% Tween-20), and they were subsequently incubated overnight with the primary antibodies (Sigma, USA) at 4 °C diluted in 5% (w/v) bovine serum albumin (BSA): mouse anti-tyrosine hydroxylase (TH, 1:1000, T2928) and mouse anti-tryptophan hydroxylase (TPH, 1:500, T-06781). A mouse anti-GAPDH antibody (1:10,000G8795) was also used to control for loading control and to normalize the levels of protein detected. The secondary antibody was anti-mouse-HRP (1:2,000: Dako Denmark, Glostrup, Denmark), which was visualized with enhanced

chemiluminescence. The chemiluminescence signals of the bands obtained were all within the linear range of the imaging system and were not saturated (ChemiDoc XRS+ System, Bio-Rad Laboratories). Densitometry and quantification were carried out using a ChemiDoc MP Imaging System, Image Lab program (Bio-Rad) and Microsoft Excel was used to determine the levels of the proteins, and the monoamine precursors TPH and TH.

2.3.5 Statistical analysis

The behavioral and biochemical data were analyzed using the Statistical Package for the Social Sciences (SPSS) v20 software and plotted as the mean ± SEM. A test 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.

To analyze the ODT acquisition, retention and relearning trials, a repeated measures ANOVA was carried out followed by *post hoc* contrast tests when necessary, and multiple comparisons were performed with the Bonferroni correction. A one-way analysis of variance (ANOVA) was carried out to determine the between-group differences in the retention trial, olfactory test, and for the monoamine, glucose and hormone levels. Moreover, Spearman Rank correlations were assessed to examine the relationship between behavioral performance and blood plasma parameters. A p-value of 5% or lower was considered to be statistically significant.

3. Results

3.1. Behavioral data

3.1.1. Odor discrimination task

In the ODT habituation, no significant between-group differences were detected in the latency to eat the ten pieces of cereal during the first ($F_{(2,42)} = 2.269$, $p = 0.116$) and the second ($F_{(2,42)} = 1.915$, $p = 0.160$) habituation sessions, but significant between-group differences were detected in the third session ($F_{(2,42)} = 3.877$, $p = 0.028$), specifically between the CR and Adult groups ($p=0.027$), indicating that CR rats took longer to eat the cereal. An ANOVA of the latencies to make the correct response in the acquisition, retention and relearning ODT trials failed to identify significant Group factor ($F_{(2,42)} = 2.184$, $p = 0.125$) or Group x Trial ($F_{(4,84)} = 1.552$, $p = 0.195$) interactions (Fig. 1A) . However, the Trial factor was significantly affected ($F_{(2,84)} = 4.759$, $p = 0.011$), indicating that the rats' performance generally differed across trials, with shorter latencies for CR and adult rats in the retention trials. Interestingly, the latency scores of a high percentage of CR aged rats were comparable those of Adult animals in the ODT retention trial (Fig. 2), while some extended beyond that range, indicating a dispersion effect of the measure that was more noticeable in the *Ad Libitum* group. Hence, CR might reduce the behavioral variability between aged animals, making their performance more like that of control animals. The statistical dispersion could explain the lack of significant between-group differences in the retention session, as observed in other studies analyzing rats' performance in probe trials of a spatial learning task [51,52]. In fact, when an ANOVA was applied to the retention trial, significant between-group differences were observed ($F_{(2,44)} = 4.616$, $p= 0.015$). Specifically, the *Ad libitum* group had a longer latency than the Adult group ($p = 0.014$), whose performance did not differ from that of the CR group ($p = 0.179$). An ANOVA analysis of the mean number of total errors (Fig. 1B) failed to detect significant differences for the three factors: Group ($F_{(2,42)} = 1322$, $p = 0.278$), Trial ($F_{(2,84)} = 2.856$, $p = 0.057$), and Group x Trial ($F_{(4,84)} = 0.970$, $p = 0.428$).

263 *3.1.2. Buried Food Test*

264

265 An ANOVA of the latencies to find the buried cookie in the olfactory test (Fig. 3)
266 indicated between-group differences ($F_{(2,42)} = 10.866$, $p < 0.001$), and a contrast analysis
267 showed that the *Ad Libitum* group performed significantly worse than both the Adult (p
268 < 0.001) and CR ($p = 0.001$) rats. A significant positive relationship was evident
269 through the Spearman correlation between the latency to find the reinforcement during
270 the ODT retention trial and the latency to find the buried cookie ($r = 0.445$, $p = 0.001$:
271 Fig. 4), suggesting that the rats that took the longest to find the buried cookie were those
272 with the highest latencies in the ODT retention test.

273 *3.2. Biochemical data*

274 *3.2.1. Blood plasma analysis*

275 The glucose levels obtained differed significantly between the groups ($F_{(2,21)} = 19.215$, p
276 < 0.001: Fig. 5A) and a contrast analysis showed that Adult rats had lower glucose
277 levels than CR ($p< 0.001$) and *Ad Libitum* rats ($p= 0.002$). In terms of leptin (Fig. 5B),
278 ANOVA also demonstrated between-group differences ($F_{(2,21)} = 21.536$, $p < 0.001$),
279 with the contrast analysis indicating there was more leptin in the *Ad Libitum* rats than in
280 the Adult ($p < 0.001$) and CR ($p < 0.001$) animals. Between-group differences in plasma
281 IGF-1 were also detected ($F_{(2,21)} = 8.630$, $p = 0.002$: Fig. 5D) and specifically, there
282 were higher plasma IGF-1 concentrations in Adult rats than in CR ($p = 0.005$) and *Ad*
283 *Libitum* ($p = 0.005$) rats. However, and somewhat unexpectedly, no between-group
284 differences in the insulin levels were evident ($F_{(2,21)} = 0.153$, $p = 0.859$: Fig. 5C).
285 Finally, the possible relationship between behavioral performance and blood plasma
286 parameters was evaluated by analyzing the blood parameters that show significant
287 between-group differences. Accordingly, only leptin levels showed a significant

correlation ($r = 0.518$, $p < 0.005$) with the latency to find the buried cookie in the olfactory test, with more leptin associated with a worse performance in the test (Fig. 6).

3.2.2. *Monoamine levels*

3.2.2.1. Striatum

Regarding the concentrations of monoamines, precursors and metabolites in the striatum (Fig. 7A), ANOVA showed significant between-group differences in DOPAC ($F_{(2,21)} = 19.764$, $p < 0.001$), DA ($F_{(2,19)} = 65.948$, $p < 0.001$), 3MT ($F_{(2,21)} = 30.595$, $p < 0.001$) and 5-HT ($F_{(2,21)} = 4.970$, $p = 0.017$). Contrast analyses indicated there were higher concentrations of DOPAC ($p = 0.007$, $p = 0.002$), DA ($p < 0.001$) and 3MT ($p < 0.001$) in the CR group than in the *Ad Libitum* and Adult groups. Spearman correlation demonstrated a significant relation between the levels of DA and the latency to find the reinforcement during the ODT acquisition ($r = 0.385$, $p = 0.039$) and the latency to find the buried cookie in the olfactory test ($r = 0{,}504$, $p = 0.008$), but not with the ODT retention. In terms of 5HT, there were higher levels in the CR group than in *Ad Libitum* animals ($p < 0.026$), while no between-group differences were found in NA and 5-HIAA in striatum.

3.2.2.2. Frontal cortex

In the FC (Fig. 7B), ANOVA revealed between-group differences in DOPAC ($F_{(2,21)} = 10.909$, $p = 0.001$), DA ($F_{(2,21)} = 27.537$, $p < 0.001$) and 5-HT levels ($F_{(2, 19)} = 3.931$, $p = 0.037$). The *post hoc* analysis indicated that there were higher levels of DOPAC ($p = 0.004$, $p = 0.0014$) and DA ($p = 0.035$, $p < 0.001$) in the Adult group than in the *Ad Libitum* and CR groups. By contrast, there were higher 5HT levels in the CR group than in *Ad Libitum* animals ($p = 0.038$). No between-group differences were found for NA and 5-HIAA.

3.2.3. Monoamine precursor levels

There were significant between-group differences in monoamine precursors levels when assessed by ANOVA, particularly of the TH levels in both the striatum ($F_{(2,12)} = 32.793$, $p < 0.001$) and FC ($F_{(2,14)} = 4.107$, $p = 0.040$: Fig. 8). In the striatum, more TH was detected in the CR than in the Adult ($p < 0.001$) and *Ad Libitum* ($p < 0.001$) rats, and there was a tendency towards significantly higher TH levels in the FC of the Adult rats than in the CR animals ($p = 0.051$). Regarding the TPH, no between-group differences were evident in the FC ($F_{(2,10)} = 0.917$, $p = 0.431$).

4. Discussion

The data obtained here indicate that a CR dietary intervention in rats from 4 months of age attenuates age-related decline in olfactory sensitivity, observed in the performance of the *Ad Libitum* old rats in the buried food test. Although the CR diet did not improve ODT acquisition, when latencies were assessed in the 72-hour ODT retention trial, the *Ad Libitum* group performed worse, which could be interpreted as if diet affected somehow odor sensitivity and/or memory encoding, since the ODT test cannot measure both processes separately. However, as such deficit was not observed in the number of errors, it cannot be discarded that differences in latencies could be explained by other factors, such as locomotor activity. Moreover, our results also confirm that the nutritional state influences odor sensitivity [53,54] given that higher levels of leptin were related to a worse performance in the buried food test, but not in the ODT. All animals were food-deprived 5 days before the ODT procedure, so the results can hardly be attributed to a differential acute effect of diet on motivation. Moreover, no significant between-group effects were detected in the first and second habituation ODT sessions,

when all rats were habituated to the edible reward. However, old CR rats were slower in consuming cereal compared to Adult rats in the last habituation session. This result also does not seem to indicate that CR rats were less motivated to eat as they performed similarly to the Adult animals in the ODT task and the buried food test.

In general, the data obtained are in agreement with previous experiments demonstrating that old rodents can discriminate between different odors [14,15,55,56], even though they experience mild olfactory dysfunction [8,56,57]. Furthermore, earlier experiments [12,51] also suggested that there is wide variability among old rodents, with only some individuals exhibiting significant olfactory learning deficits. Therefore, the greater variability of the scores in both the aged groups, but especially in the *Ad Libitum* group, could have prevented more statistically significant differences appearing in the data. In addition, the absence of general differences between the groups in the task might be due to the olfactory memory deficit being more evident in complex learning tasks, such as associative contextual and/or reversal learning [14,15]. In this regard, ODT could be considered a robust memory task involving a survival-based behavior to find food in conditions of deprivation [47]. As such, it might well be less sensitive to the effects of aging or other factors. Similarly, senescent rats were not only seen to learn conditioned taste aversion, which involved odor perception, but their performance was even better than that of younger rats [7]. In fact, a CR diet in old Fischer-344 rats failed to reduce age-related neophobic deficits to novel flavoring foods [58]. These data suggest that the harmful effects of aging may differ depending on the type of learning considered and its complexity or biological significance.

Our results suggest that nutritional status may influence odor sensitivity since higher leptin levels correlated with poorer performance on the buried food test, consistent with earlier studies suggesting that leptin and its receptors, present in the rat olfactory

mucosa, are modulated by the nutritional status [53] and modulate olfactory-mediated pre-ingestive behavior [54]. Indeed, the olfactory system is more reactive to odors in conditions of starvation and this activity diminishes after satiety [59]. In this regard, the olfactory bulb is thought to be a metabolic or nutrient sensor, and metabolic hormones like insulin and leptin can modulate olfactory-driven behaviors [38,60,61]. In general, these hormones tend to increase with age [29,30,33,62] and dietary interventions like CR could restore their levels to those of young animals [33]. Thus, as leptin modulates odor sensitivity, high levels of this hormone in the *Ad libitum* rats could account, at least in part, for the differences observed in the cookie test. Although our results confirm that a lifelong hypocaloric diet may reduce leptin levels, which could be related to olfactory sensitivity, insulin and glucose levels did not change here. These results do not fully match with those previously observed in which CR reduced high insulin levels in aged animals [33]. Therefore, further experiments are needed to confirm the capacity of a CR diet to restore glucose and insulin blood levels in old rats.

The data presented also show that regardless of diet, older animals had less IGF-1, considered a potential biomarker of cognitive decline in the aged brain [45,63,64]. Plasma IGF-1 levels generally change with aging and here, CR did not reverse the effects of aging on IGF-1 levels, consistent with our previous findings [33] but not with those showing that this dietary intervention decreases [65] or may even increase [62] IGF-1 levels. Therefore, more experiments should be carried out to assess the relevance of this hormone to the age-induced impairment in olfactory perception and memory decline, and how this may be affected by diet and other factors.

Regarding neurotransmitters, a 30% lifetime reduction in food intake enhanced DA levels in the striatum, as well as its DOPAC and 3MT metabolites, when compared to the *Ad Libitum* [33] and Adult rats. Comparably, more TH was also detected in the

striatum of CR rats. DA neurons in the midbrain provide rich innervation to the striatum and FC, and they play a fundamental role in a multitude of cognitive processes, including reward processing, learning, decision making and motivation to engage in goal-orientated behaviors (e.g., eating and drinking) [66,67]. Moreover, the olfactory bulb sends projections to the olfactory tubercle, that is considered a component of the ventral striatum closely related to the mesolimbic dopaminergic pathway involved in motivation and hedonic responses for foods (reviewed in [68]). In our experiment, DA levels were correlated with performance in ODT acquisition and olfactory sensitivity test, but not with ODT retention. Therefore, high levels of DA may increase motivation to eat while learning the task, but not during later phases, once the smell has been associated with the reward.

In addition, there was more 5-HT in the striatum of old CR rats, a neurotransmitter related to cognitive processes [69,70]. Indeed, there is evidence that selective activation of 5-HT4 receptors can improve memory performance in elderly rats in a task of olfactory associative discrimination [10]. Such findings suggest that 5-HT may be involved in olfactory memory and that its increase could enhance the olfactory performance of old rats. As to the effects of aging on monoaminergic neurotransmission in the striatum, we could not confirm that levels of DA decline with age, in agreement with some previous studies [28,71–75] but see also [76–83]. Furthermore, in aged rats there were no changes detected here in 5-HT [72,74,75,77,78] nor in NA [74,75,78–81,83].

The data regarding neurotransmission in the FC contrasts with that obtained in the striatum. As reported previously [73,83–85], the levels of DA and its metabolite decrease in aged rats irrespective of diet, as further supported by the low levels of TH. However, no such effect [28,74,79,80,82,86] or even an increase [72] in DA and

DOPAC have also been described. The CR diet was unable to attenuate such age-dependent decreases in the FC, unlike a previous report where a 60% reduction in the *ad libitum* diet increased DA in the FC [37]. Our results confirmed that aged animals experience an overall reduction of 5-HT in the FC [37,79,83,87] but see [28,72–75,77,80,82], which in this case was reversed by a CR diet. However, neither aging nor CR affected the levels of the TPH neurotransmitter precursor in the FC. Finally, no differences were found between the groups in terms of NA, as seen previously in some [28,37,74,79,84] but not all reports [73,75,80,82].

5. Conclusions

The results of our study suggest that in general, a lifetime CR diet may attenuate the age-related decline in olfactory sensitivity, observed in the *Ad Libitum* old rats. The specific mechanism through which CR may exert such beneficial effect is not completely determined, as a better locomotor activity could partially mediate the performance in the tests. However, it could also be linked to a reduction in the age-related increase in leptin, and enhancements in DA and 5-HT in the striatum, a region related to olfaction. Our study will serve as a foundation for a follow-up study that can address more causal contribution. Future research into the effects of CR on the aged brain will be of great interest to fully understand the effects of such dietary intervention on olfactory memory, and to favor behavioral habits that ensure a long and healthy life in humans.

Figure legends

Figure 1: (A) Latency (s) to make the correct response (± SEM) and (B) mean number of total errors (± SEM) in the acquisition, retention, and relearning trials of the ODT (Latency: Group $F_{(2,42)} = 2.184$, $p = 0.125$, Trial $F_{(2,84)} = 4.759$, $p = 0.01$, Group x Trial

435 $F_{(4,84)}$ = 1.552, p = 0.195; Error: Group $F_{(2,42)}$ = 1322, p = 0.278, Trial $F_{(2,84)}$ = 2.856, p =
436 0.057, Group x Trial $F_{(4,84)}$ 0.970, p = 0.428).

437 Figure 2: Dispersion cloud of the ODT latency in the retention trial. Each dot represents
438 a specific subject and the black line across the dispersion cloud depicts the mean latency
439 to find the reinforcement for each group (*p < 0.05). ($F_{(2,44)}$ = 4.616, p = 0.015, *Ad*
440 *Libitum* > Adult group (p = 0.014).

441 Figure 3: Latency (s) to find the buried cookie (± SEM) for each group of animals.
442 (***p < 0.001). ($F_{(2,42)}$ = 10.866, p < 0.001*; Ad Libitum* > Adult (p < 0.001) and CR (p =
443 0.001)).

444 Figure 4: Spearman's correlation between the latency to find the reinforcement in the
445 ODT retention trial and the latency to find the buried cookie in the olfactory test (r =
446 0.445, p = 0.001).

447 Figure 5. Blood plasma concentration of (A) Glucose, (B) Leptin, (C) Insulin, and (D)
448 IGF-1 in each group of animals (mean ± SEM). (** p < 0.01, *** p < 0.001). Glucose
449 ($F_{(2,21)}$ = 19.215, p < 0.001; Adult < CR (p < 0.001) and *Ad Libitum* (p = 0.002). Leptin
450 ($F_{(2,21)}$ = 21.536, p < 0.001); *Ad Libitum* > Adult (p < 0.001) and CR (p < 0.001). IGF-1
451 ($F_{(2,21)}$ = 8.630, p = 0.002); Adult > CR (p = 0.005) and *Ad Libitum* (p = 0.005). Insulin
452 ($F_{(2,21)}$ = 0.153, p = 0.859).

453 Figure 6. Spearman's correlation between the latency to find the buried cookie in the
454 olfactory test and the plasma leptin levels (r = 0.518 p < 0.005). The dispersion cloud is
455 milder for the CR and adult animals than the *Ad Libitum* subjects.

456 Figure 7. Concentration of monoamines, precursors, and metabolites, in the striatum (A)
457 and FC (B), as analyzed by HPLC-ED for each group (mean ± SEM) (*p < 0.05, ** p <
458 0.01, *** p < 0.001). DOPAC ($F_{(2,21)}$ = 19.764, p < 0.001, DA $F_{(2,19)}$ = 65.948, p <

0.001, 3MT $F_{(2,21)}$ = 30.595, p < 0.001); CR > *Ad Libitum* and Adult. DOPAC (p = 0.007, p = 0.002), DA (p < 0.001) and 3MT (p < 0.001) and 5-HT ($F_{(2,21)}$ = 4.970, p = 0.017, CR > *Ad Libitum* (p < 0.026)). FC (DOPAC $F_{(2,21)}$ = 10.909, p = 0.001), DA ($F_{(2,21)}$ = 27.537, p < 0.001; Adult > *Ad Libitum* and CR DOPAC (p = 0.004, p = 0.0014)) and DA (p = 0.035, p < 0.001). 5-HT ($F_{(2, 19)}$ = 3.931, p = 0.037, CR > *Ad Libitum* (p = 0.038)).

Fig. 8: Representative Western Blots and percentage change (mean ± SEM) in the intensity of the TPH and TH monoamine precursors in the striatum (A) and FC (B) of *Ad Libitum*, CR and Adult rats, taking the intensity in Adults rats as 100% (*p < 0.05, **p <0.01, *** p < 0.001); (C, D) Representative images of the integrated density bands of proteins for each group and brain region. TH striatum ($F_{(2,12)}$ = 32.793, p < 0.001 CR > Adult (p < 0.001) and *Ad Libitum* (p < 0.001) and FC $F_{(2,14)}$ = 4.107, p = 0.040), Adult > CR (p = 0.051)).

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Declaration of Competing Interest

The authors have no financial or potential conflict of interest to declare.

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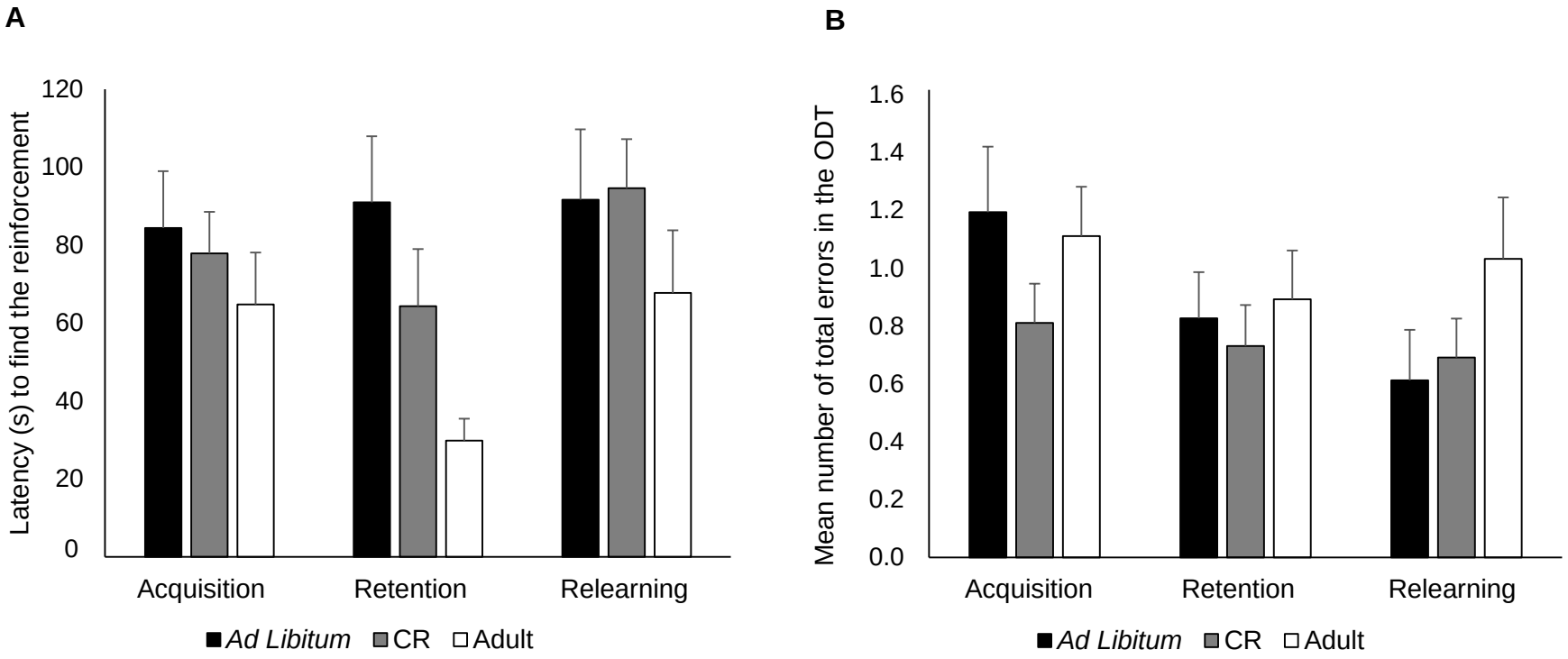

Figure 1: (A) Latency (s) to make the correct response (± SEM) and (B) mean number of total errors (± SEM) in the acquisition, retention, and relearning trials of the ODT (Latency: Group $F_{(2,42)} = 2.184$, $p = 0.125$, Trial $F_{(2,84)} = 4.759$, $p = 0.01$, Group x Trial $F_{(4,84)} = 1.552$, $p = 0.195$; Error: Group $F_{(2,42)} = 1322$, $p = 0.278$, Trial $F_{(2,84)} = 2.856$, $p = 0.057$, Group x Trial $F_{(4,84)}$ 0.970, $p = 0.428$).

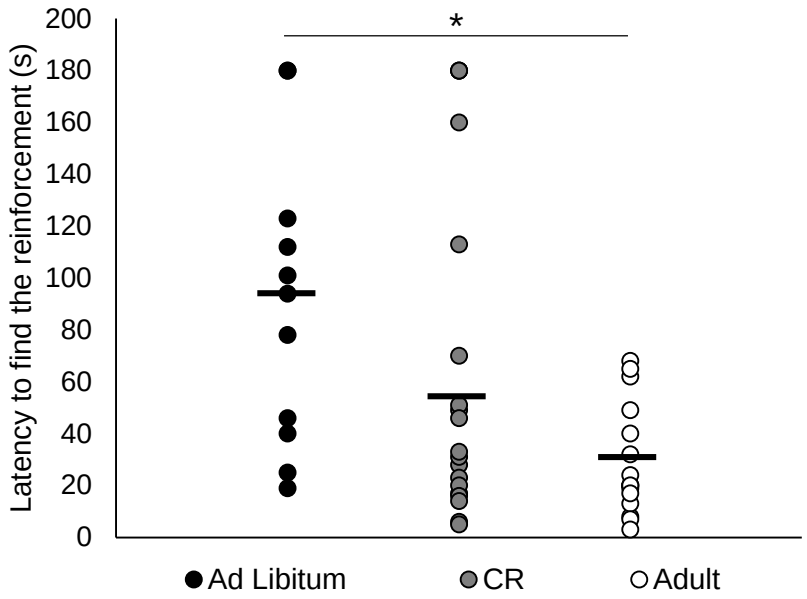

Figure 2: Dispersion cloud of the ODT latency in the retention trial. Each dot represents a specific subject and the black line across the dispersion cloud depicts the mean latency to find the reinforcement for each group (*$p < 0.05$) ($F_{(2,44)} = 4.616$, $p = 0.015$, *Ad Libitum* > Adult group ($p = 0.014$).

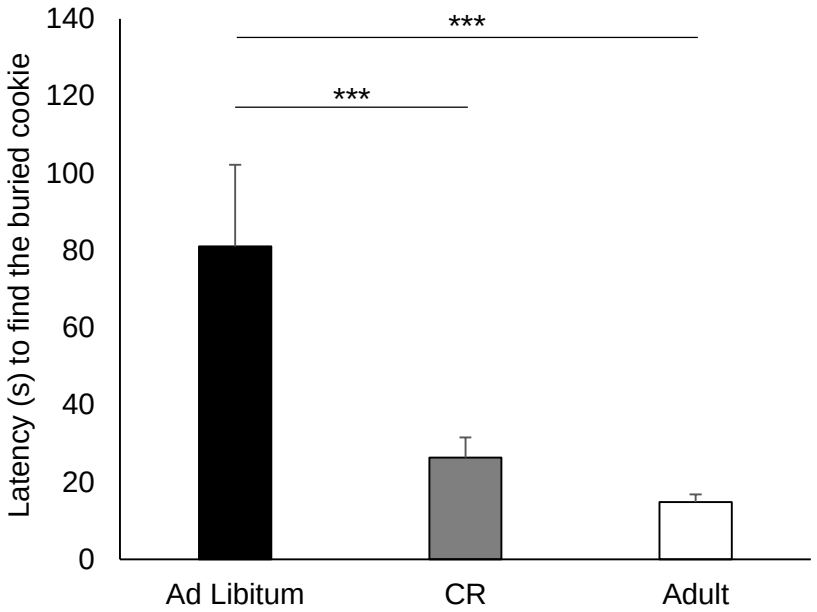

Figure 3: Latency (s) to find the buried cookie (± SEM) for each group of animals. (***$p < 0.001$). ($F_{(2,42)} = 10.866$, $p < 0.001$*; Ad Libitum* > Adult ($p < 0.001$) and CR ($p = 0.001$)).

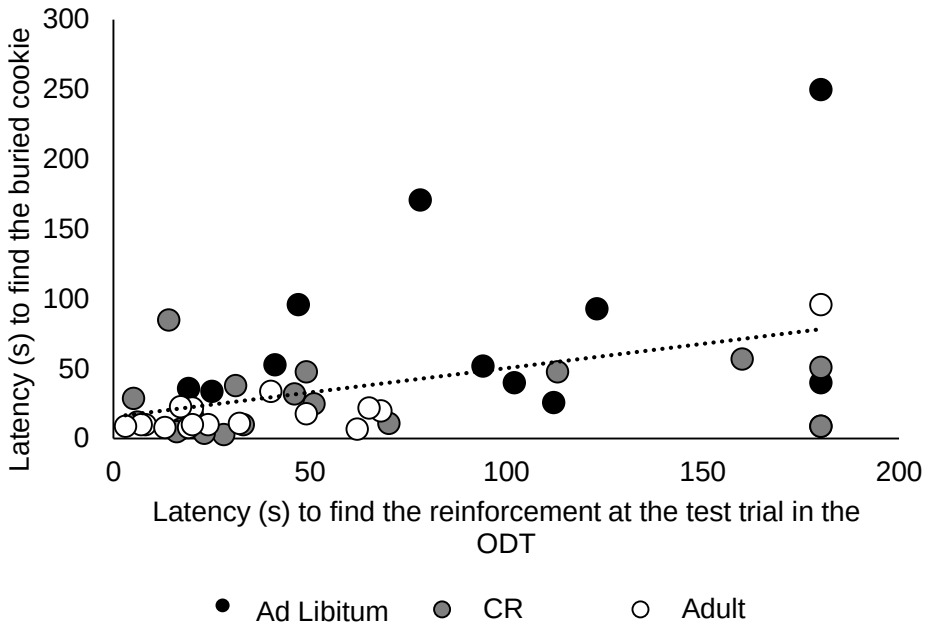

Figure 4: Spearman's correlation between the latency to find the reinforcement in the ODT retention trial and the latency to find the buried cookie in the olfactory test ($r = 0.445$, $p = 0.001$)

Figure 5

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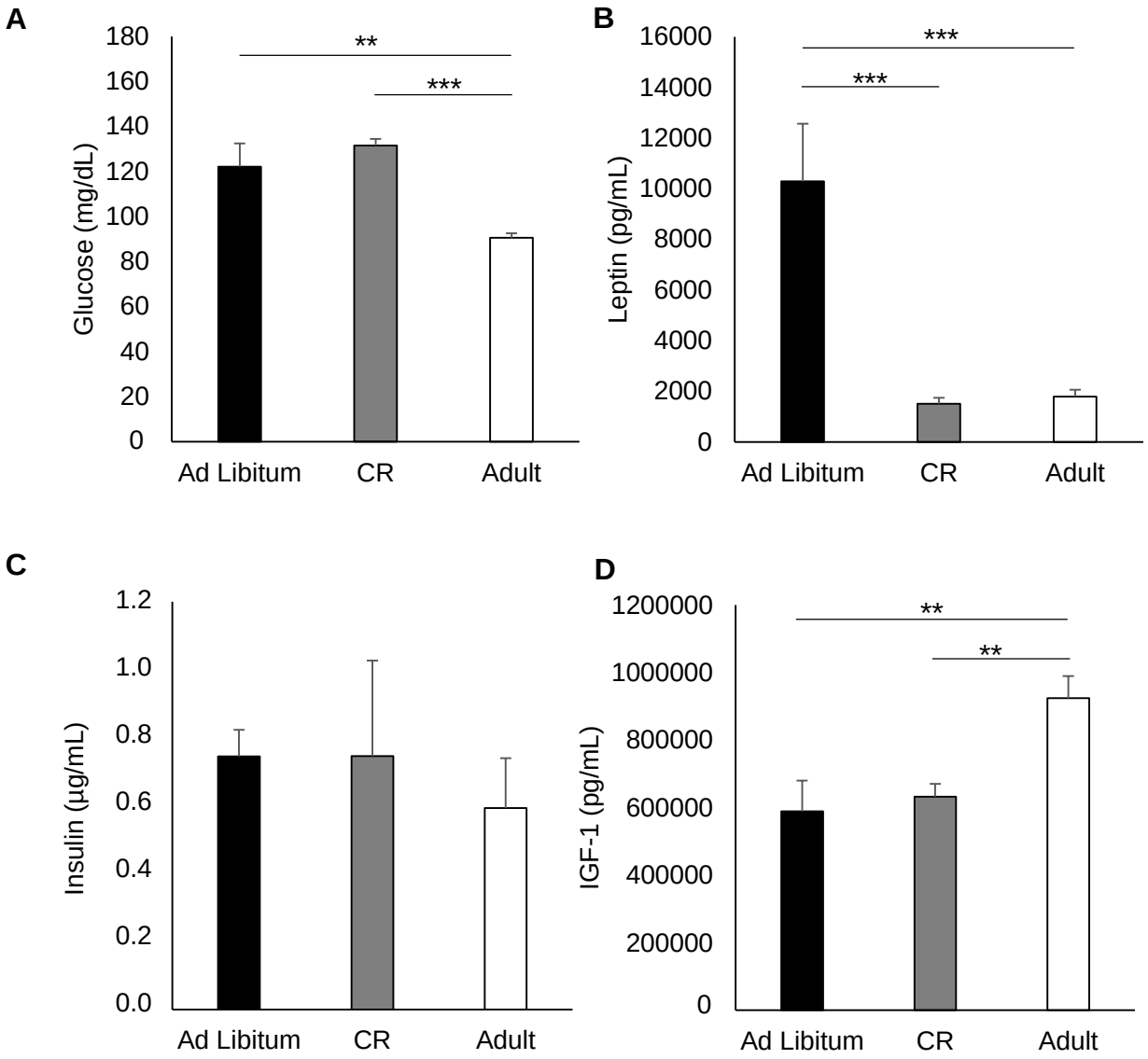

Figure 5. Blood plasma concentration of (A) Glucose, (B) Leptin, (C) Insulin, and (D) IGF-1 in each group of animals (mean ± SEM). (** $p < 0.01$, *** $p < 0.001$). Glucose ($F_{(2,21)} = 19.215$, $p < 0.001$; Adult < CR ($p < 0.001$) and *Ad Libitum* ($p = 0.002$). Leptin ($F_{(2,21)} = 21.536$, $p < 0.001$); *Ad Libitum* > Adult ($p < 0.001$) and CR ($p < 0.001$). IGF-1 ($F_{(2,21)} = 8.630$, $p = 0.002$); Adult > CR ($p = 0.005$) and *Ad Libitum* ($p = 0.005$). Insulin ($F_{(2,21)} = 0.153$, $p = 0.859$).

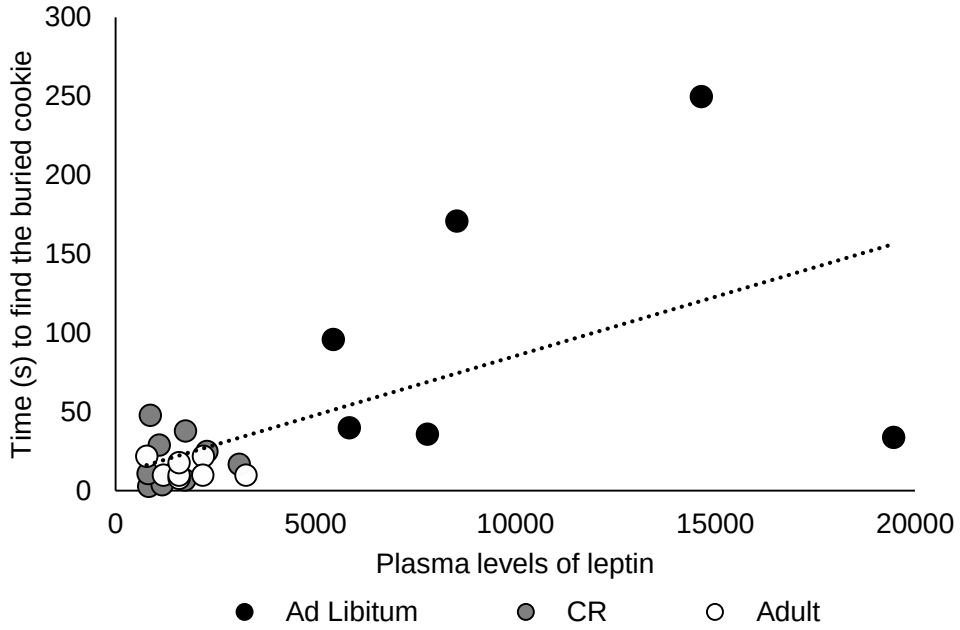

Figure 6. Spearman's correlation between the latency to find the buried cookie in the olfactory test and the plasma leptin levels ($r = 0.518$ $p < 0.005$). The dispersion cloud is milder for the CR and adult animals than the *Ad Libitum* subjects.

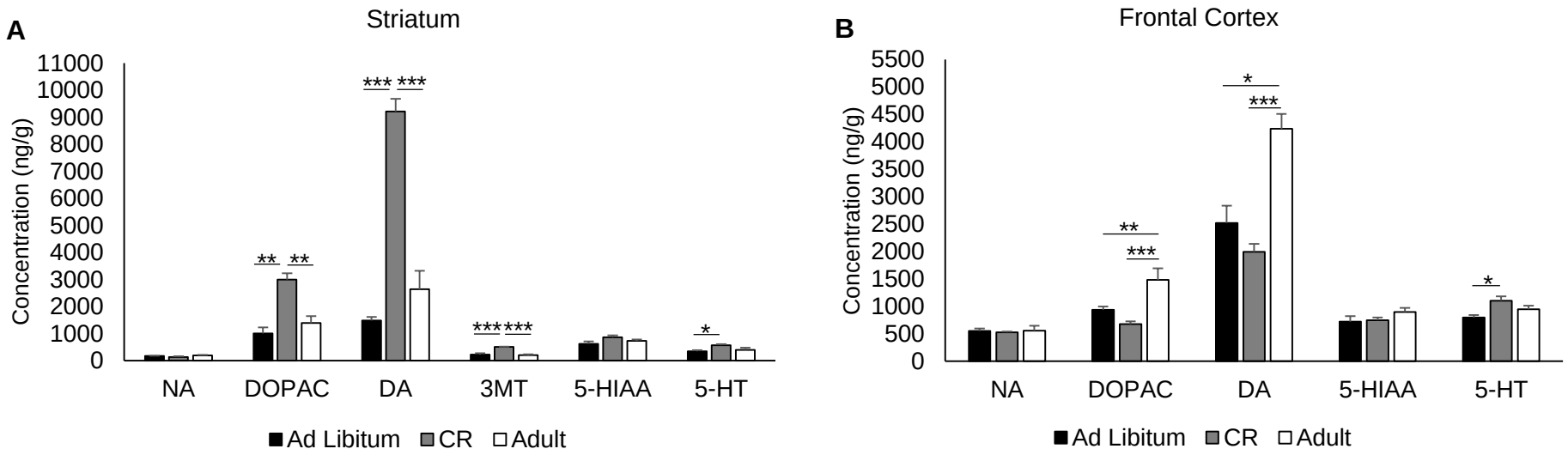

Figure 7. Concentration of monoamines, precursors, and metabolites, in the striatum (A) and FC (B), as analyzed by HPLC-ED for each group (mean ± SEM) (*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). DOPAC ($F_{(2,21)} = 19.764$, $p < 0.001$, DA $F_{(2,19)} = 65.948$, $p < 0.001$, 3MT $F_{(2,21)} = 30.595$, $p < 0.001$); CR > *Ad Libitum* and Adult. DOPAC ($p = 0.007$, $p = 0.002$), DA ($p < 0.001$) and 3MT ($p < 0.001$) and 5-HT ($F_{(2,21)} = 4.970$, $p = 0.017$, CR > *Ad Libitum* ($p < 0.026$)). FC (DOPAC $F_{(2,21)} = 10.909$, $p = 0.001$), DA ($F_{(2,21)} = 27.537$, $p < 0.001$; Adult > *Ad Libitum* and CR DOPAC ($p = 0.004$, $p = 0.0014$)) and DA ($p = 0.035$, $p < 0.001$). 5-HT ($F_{(2, 19)} = 3.931$, $p = 0.037$, CR > *Ad Libitum* ($p = 0.038$)).

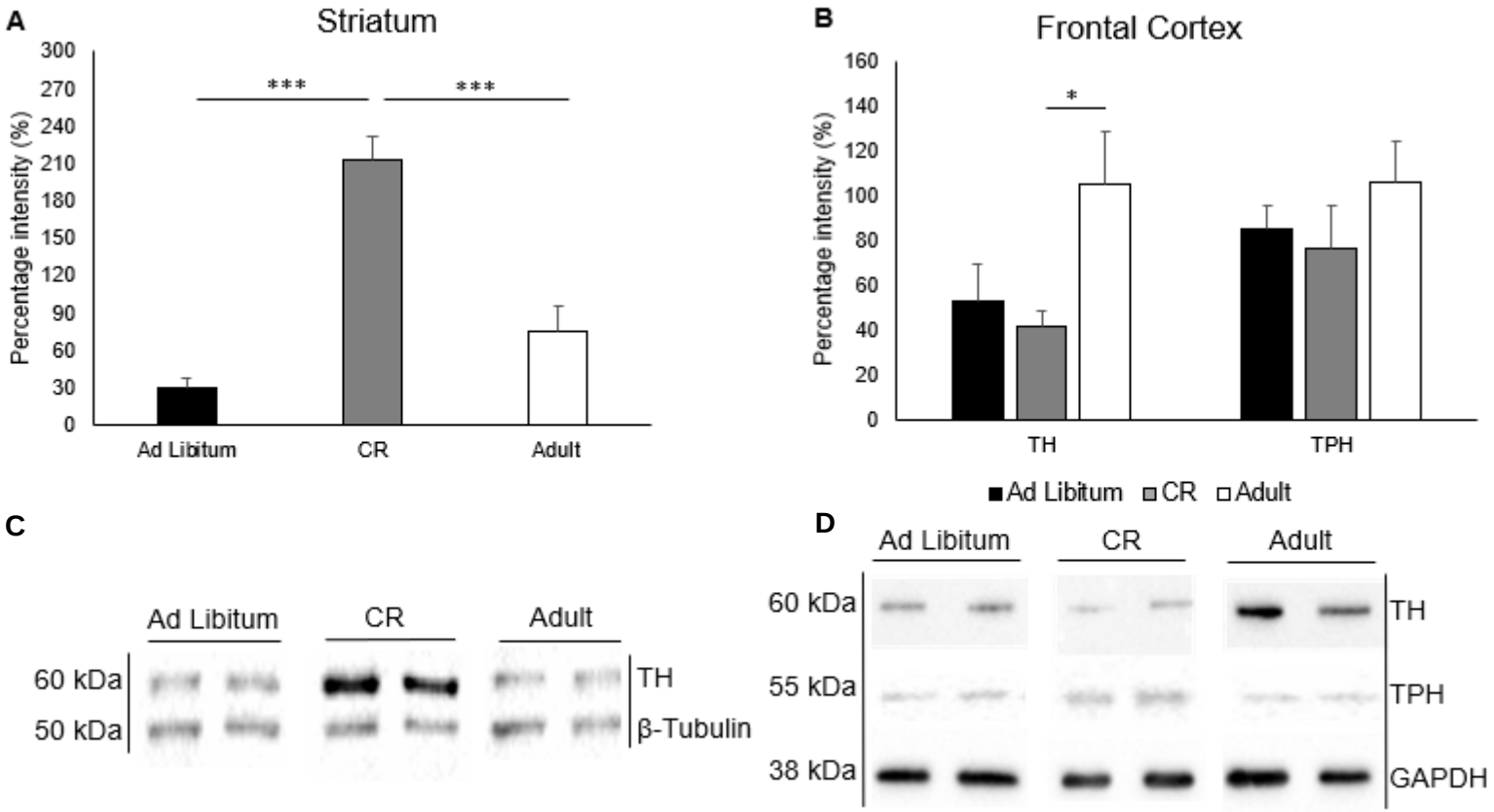

Fig. 8: Representative Western Blots and percentage change (mean ± SEM) in the intensity of the TPH and TH monoamine precursors in the striatum (A) and FC (B) of *Ad Libitum*, CR and Adult rats, taking the intensity in Adults rats as 100% (*p < 0.05, **p <0.01, *** p < 0.001); (C, D) Representative images of the integrated density bands of proteins for each group and brain region. TH striatum ($F_{(2,12)}$ = 32.793, p < 0.001 CR > Adult (p < 0.001) and *Ad Libitum* (p < 0.001) and FC $F_{(2,14)}$ = 4.107, p = 0.040), Adult > CR (p = 0.051))

Author Agreement Statement

We the undersigned declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We understand that the Corresponding Author is the sole contact for the Editorial process. She is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs

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