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Calorie restriction changes age-related anxiety-like behaviour in male Wistar rats and modulates expression of dopamine D1R and D2R receptors

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Belgrade, November 17th, 2021.

Dear Professor Gonos,

Please find enclosed our manuscript entitled **"***"Calorie restriction changes age-related anxiety-like behaviour in male Wistar rats and modulates expression of dopamine D_1R and D_2R receptors"***"** by Prvulovic et al., to be considered for the publication in the ***Mechanisms of Ageing and Development***.

For decades dietary restriction has been considered as a universally beneficial dietary approach for preserving brain health. However, in our recent study (Todorovic et al., https://doi.org/10.1093/gerona/gly015), we have proposed the existence of a certain time window during the lifetime when dietary/food restriction should not be applied as it induces more adverse than positive effects.Herein, given the high incidence of anxiety in elderly, we further explore the potential of FR as an environmental paradigm to prevent/ameliorate age-associated anxiety-like behaviour. We demonstrate that FR introduced in later stages of life increases the likelihood of anxiety-like behaviour in aging rats and reduces the availability of dopamine receptors in a region-specific manner.These findings impute a great caution when choosing the appropriate age for the implementation of food restriction as an anti-ageing strategy.

We hope that submitted manuscript will meet the criteria for publication in the ***Mechanisms of Ageing and Development*** journal. The data submitted to the journal are not under consideration for publication elsewhere and submission is approved by all authors. We have no conflict of interest to declare.

We propose the following reviewers:

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Highlights

Aging is associated with increased anxiety-like behaviour in the open field and light-dark box tests

CR implemented at late age increases anxiety-like behaviour in male Wistar rats

Short-term late-onset CR decreases the availability of D_2 dopamine receptor levels in the cortex and mesencephalon

Abstract

Although initially recognized as a universally beneficial approach for prevention of age-related impairments, the outcome of calorie restriction (CR) is now known to depend on several factors, most notably the age of the subject at the CR commencement and CR duration. We aimed to examine if and how CR affects anxiety-like behaviour and the expression of dopamine receptors (D_1R, D_2R) in cortex, striatum, and mesencephalon of rats of varying age. The study was performed on male Wistar rats, fed ad libitum (AL) or exposed to calorie restriction (60% of AL intake) at middle age and late middle age. Open field and light-dark tests were used to study anxiety-like behaviour, while PCR and western blot were used to examine the expression of dopamine receptors. Calorie restriction implemented at middle-age led to variable outcomes on anxiety-like behaviour, while CR implemented at late middle age increased anxiety and decreased the availability of D_2R levels in the cortex and mesencephalon. Taken together, these results advice caution when implementing calorie restriction late in life.

Calorie restriction changes age-related anxiety-like behaviour in male Wistar rats and modulates expression of dopamine D_1R and D_2R receptors

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Abbreviations: LDF- limited daily feeding; mdDA - major dopaminergic neurons; LTCR- long-term calorie restriction; STCR- short-term calorie restriction; OF-open field; LDB- light/dark box; ECL-enhanced chemiluminescence

Abstract

Although initially recognized as a universally beneficial approach for prevention of age-related impairments, the outcome of calorie restriction (CR) is now known to depend on several factors, most notably the age of the subject at the CR commencement and CR duration. We aimed to examine if and how CR affects anxiety-like behaviour and the expression of dopamine receptors (D_1R, D_2R) in cortex, striatum, and mesencephalon of rats of varying age. The study was performed on male Wistar rats, fed ad libitum (AL) or exposed to calorie restriction (60% of AL intake) at middle age and late middle age. Open field and light-dark tests were used to study anxiety-like behaviour, while PCR and western blot were used to examine the expression of dopamine receptors. Calorie restriction implemented at middle-age led to variable outcomes on anxiety-like behaviour, while CR implemented at late middle age increased anxiety and decreased the availability of D_2R levels in the cortex and mesencephalon. Taken together, these results advice caution when implementing calorie restriction late in life.

Keywords: aging; calorie restriction; anxiety; dopamine receptors; behavior

1. Introduction

Nutrition strongly impacts body functions and can significantly affect incidence, onset and outcome of many age-related diseases. Among various environmental interventions, dietary modifications, especially calorie restriction (CR) continuously attracts attention as a potential anti-aging treatment (Heilbronn and Ravussin, 2003). Two most common forms of calorie restriction are limited daily feeding (LDF) when restricted amount of food is available to the experimental animals daily, usually 15-50% of an average food consumption, and intermittent fasting (IF), which involves 24 h of ad libitum food consumption alternated with 24 h of complete or partial food restriction.

Calorie intake also represents one of the major determinants of brain health and vulnerability to injury and disease. Reduction in calorie/calorie intake prevents age-related atrophy of the brain, ameliorates age-related decrement in long-term potentiation, preserves synaptic plasticity during aging, increases neurogenesis, may prevent neurodegeneration including age-related cognitive decline and improves mood disorders (Mattson, 2012).

Prevalence of mood disorders, anxiety and depression is a significant public health problem faced by the elderly. Estimates of the prevalence of anxiety in older age (mainly above 50 years of age) vary from 1.2–14% in general community to 1–28% in clinical studies, conducted mostly in Europe and North America (Remes et al., 2016). Emerging evidence suggests beneficial effects of various dietary interventions, including Mediterranean diet, fruit and vegetable-rich diet, caloric restriction and caloric restriction mimetic (like rapamycin) on symptoms of depression and anxiety in both human and animal models (Firth et al., 2019; Halloran et al., 2012; Parikh et al., 2016), recommending them as a promising non-pharmacological approach for amelioration of emotional distress.

Emotional processing in the brain, including anxiety, takes place in the limbic system that encompasses the amygdala, the prefrontal cortex and the hypothalamus, affect-related regions that are densely connected and modulated by the ventral hippocampus in rodents and humans (Loh et al., 2017; Wang et al., 2019). Though pharmacological interventions for anxiety primarily target serotonergic and GABAergic neurotransmitter system, dopamine has been

suggested as one of the main mediators in the effects of diet on anxiety-like behaviour, in addition to its well-established role in regulation of motoric activity, memory and learning, reward and depression (Ayano, 2016; Berry et al., 2019; Volkow et al., 2011; Zarrindast and Khakpai, 2015). During aging, level of dopamine changes; the most noticeable decline has been detected in the hippocampus, brain stem and striatum (Portero-Tresserra et al., 2020). However, both CR and CR-mimetic rapamycin, significantly restored level of dopamine and its metabolites in various brain regions (Halloran et al., 2012; Portero-Tresserra et al., 2020). This was specifically evident in the midbrain, which contains major dopaminergic neurons (mdDA). These neurons are critical for controlling voluntary movement, creating associations with re-warding stimuli, maintenance of working memory and for the regulation of emotions. mdDA neurons in the ventral tegmental area (VTA) innervate the ventral striatum and the prefrontal cortex, constituting the mesostriatal (involved in reward/motivation) and mesocortical DA pathways (which regulate arousal, affective and cognitive processes). Another population of mdDA neurons in the substantia nigra pars compacta (SNc) with projections to dorsal striatum is critical for the control of voluntary movement, via the nigostriatal pathway. VTA area is rich in dopamine D1 and D2 receptors; both types are important in mediating anxiety and CR-induced behavioural and molecular response (Carr et al., 2003), although most likely through different mechanisms (Berry et al., 2019; Zarrindast and Khakpai, 2015).

Reduction in calorie intake has been considered as beneficial for decades; however, few recent reports imposed the need for this claim to be reevaluated, especially having in mind potential unfavorable outcome in the elderly (Morgan et al., 2003; Yanai et al., 2004). In line with these studies, we have recently shown that CR can exert either positive or harmful impact on locomotion, learning and memory; the outcome was highly dependent on both the age of animals when CR was introduced and on CR duration (Todorovic et al., 2018). Considering this finding and previous contradictory data on the effect of dietary restriction on anxiety-like behaviour in young and adult animals (Genn et al., 2003; Jahng et al., 2007), we sought to examine the effects of CR on anxiety in aged animals.

We designed a cross-sectional study in which CR was implemented to Wistar rats at specific breakpoints through their adulthood: middle- (12 and 15 months of age), late middle- (18 months of age) and old age (21 months of age). Having in mind dopamine role in anxiety-like behaviour and its involvement in CR-induced effects (Jones et al., 2017; Zarrindast and

Khakpai, 2015) we examined expression of D1 and D2 receptors in the cortex, striatum and mesencephalon, structures rich in dopaminergic neurons and/or their projections.

Our hypothesis was that decreased amount of calorie, although being considered as a universally beneficial environmental strategy for improving brain health, could differently impact the age-related anxiety-like behaviour and in dependence of the onset and duration.

2. Materials and Methods

2.1. Animals and treatment

Male Wistar rats were used in this study (n=86). Based on years of work within this type of research and in accordance with the 3R's, we accounted for the minimal sufficient number of animals for statistical analysis of behavioural data, as well as expected loss of animals during aging. Before tissue harvesting al animals were anesthetized using general anesthetic Zoletil 100 (75mg/kg) prior to transcardial perfusion, in order to minimize suffering.

All animal handling procedures were described in detail in Todorovic et al. (2018). They complied with the EU Directive 2010/63/EU for animal experiments and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research „Sinisa Stankovic "- The National Institute of Republic of Serbia, University of Belgrade, and by The National ethic research committee (#323-07-13536/2020-05). Animals were maintained in a 12-h light/dark cycle with unlimited access to water.

Based on the feeding paradigm, rats were divided into two main groups: ad libitum (AL) group, with unlimited access to food, and calorie restricted (CR) group. Calorie restricted animals were exposed to limited daily feeding experimental paradigm (Vaughan et al., 2017), in which rats were receiving a daily allotment of food that represented a fixed percentage (60%) of the mean AL daily intake of a standard laboratory chow (manufacturer VZS Stocna hrana d.o.o, Subotica, Serbia). Such a limited dietary regimen was set either as long-term (6 months duration) started at 12 or 18 months of age and lasted until the animals were 18-, or 24-months-old (long-term calorie restriction, LTCR) or short-term (3 months duration), started at 15 and 21 months of age and lasted until 18 and 24 months of age, respectively (short-term calorie restriction, STCR) (Figure 1). General wellbeing of animals was monitored daily. AL rats were sacrificed at 6, 18 and 24 months of age, while CR groups were sacrificed at 18 and 24 months of age (Figure 1). The total number of animals per experimental group that passed behavioural testing was the

following: 8 animals in 6mAL, 10 animals in 18mAL, 9 animals in 24mAL, 10 animals in 18mLTCR, 8 animals in 24mLTCR, 9 animals in 18mSTCR group and 6 animals in 24mSTCR group. From each experimental group and in accordance with 3R's principle, 5 animals were sacrificed for further tissue isolation and molecular analysis. AL animals were used as controls for evaluating the effects of CR, while 6-months-old AL group served as a control group solely for the effects of aging.

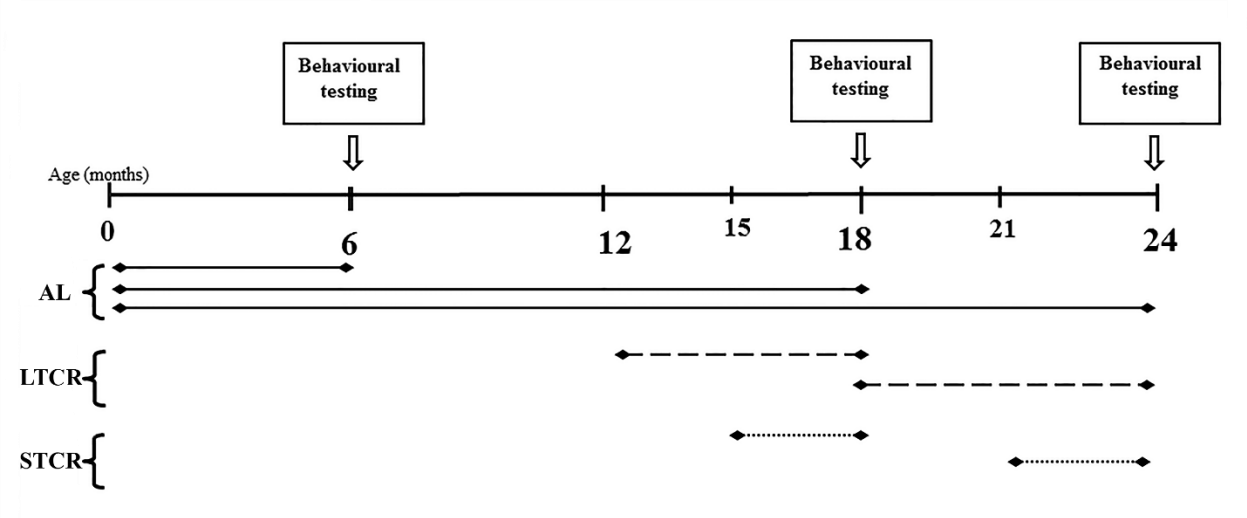

Figure 1. Schematic diagram of onsets and durations of AL and CR regimens. AL-ad libitum, CR-calorie restriction, LTCR- long-term calorie restriction, STCR- short-term calorie restriction. (2-column fitting image)

2.2. Behavioural testing

Behavioural testing was performed at 6 months of age (for the AL group) and at 18 or 24 months of age (for both AL and CR groups). Animals were allowed to acclimate to the testing room for 30 minutes prior to the commencement of experimentation. Open field test was performed first, followed by one day of rest and, afterwards, the light-dark box test.

2.3. Open field test

We used the open field (OF) test for the initial screening of the unconditioned anxiety-like behaviour in male Wistar rats during aging and under different dietary paradigms. Motor activity of rats was recorded in Opto-Varimex cages (Columbus Instruments, OH). Animals were placed in the middle of the arena and they were allowed to freely explore it for 10 minutes. The inner field of the OF was defined as the central area (20x20 cm). Total distance traveled, the number of entries into the central area and the time spent in the central area were calculated, and characteristic tracks of movement were captured.

2.4. Light-dark test

The light/dark box (LDB) test was used to further evaluate the effects of CR on anxiety-like behaviour of Wistar rats. A LD test is based on the natural conflict in rodents between the motivation to explore novel areas and an aversion to brightly lit, open spaces. The LDB apparatus was made of white and black opaque Plexiglas (31 × 31 × 36 cm light compartment, 20 × 31 × 36 cm dark compartment, 10 × 10 cm door in the middle of the wall separating the two chambers). Animals were placed in the middle of the light chamber and their behaviour was recorded by video camera for 10 min. Four parameters were measured: number of entries and time spent in the light compartment, and frequency and time spent in sticking out/stretching into the light chamber (not all four feet in the light chamber).

2.5. RT-PCR

RNA was isolated from 10 mg of tissue (striatum, cortex and mesencephalon) using Trizol Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacture instructions. RNA was reverse-transcribed using High-capacity cDNA RT-kit (Applied Biosystems, Carlsbad, CA, USA). For quantitative real-time PCR, assay-on-demand kits (Applied Biosystems, Carlsbad, CA, USA) were used: D_1 receptor (D_1R, Assay ID Rn03062203_s1) and dopamine D_2 receptor (D_2R, Assay ID Rn00561126_m1). TaqMan Universal PCR Master Mix (Applied Biosystems) was used for amplification in accordance with the manufacturer's instructions in an ABI Prism 7000 Sequence Detection System (Applied Biosystems). All samples were amplified in triplicate. Values were normalized to the housekeeping gene beta-actin (assay ID Rn00667869_m1) that did not show differences between groups. Quantification

was performed by the $2^{-\Delta\Delta Ct}$ method. The fold changes of mRNA levels in all samples were expressed relative to the calibrator (100%).

2.6. Western blot

For Western blot analysis proteins were isolated in RIPA buffer (50 mM Tris–Cl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% Triton X-100, 0.1% SDS, 1 mM EDTA, 1 mM EGTA) containing a complete protease and phosphatase inhibitor cocktail (Roche, Mannheim, Germany).) Total of 20 µg of proteins were loaded per lane. Proteins were separated by SDS-PAGE and blotted onto PVDF membranes. The membranes were blocked with 5% non-fat dry milk/Tris buffered saline with 0.05%Tween 20 (TBST) (150mM NaCl, 50 mMTris, pH 7.4, and 0.05 % Tween 20) for 1 h at room temperature (RT) and incubated with the following primary anti-bodies: anti-D_1 receptor (sc-31478, 1:500) and anti-D_2 receptor (sc-9113, 1:1000) overnight at +4 °C. The antibodies were diluted in TBST. Following several rinses in TBST, the membranes were incubated for 1h at RT with the appropriate horse radish peroxidase (HRP)-conjugated secondary antibodies (bovine anti-rabbit, 1:5000 sc-2370, donkey anti-goat, 1:5000 sc-2020) diluted in TBST. HRP-immunoreactive bands were visualized by enhanced chemiluminescence (ECL, GE Healthcare) using iBright Imaging System (Termo Fischer Scientific). Ponceau staining was used as loading control. Signals were quantified densitometrically using Image Quant software (v. 5.2, GE Healthcare) and expressed as relative values (i.e., normalized to the corresponding Ponceau staining). Changes in the levels of analyzed proteins were expressed as ratios to the appropriate control values. All values were expressed as the mean ± SEM.

2.7. Statistical analysis

Adult (6-month-old animals) group served as a control group for the effects of aging; thus, in order to determine age-related changes, we compared 6-month-old AL rats to 18- and 24-month-old rats also fed ad libitum. To determine the effects of calorie restriction, we always compared AL and CR groups of the same age, i.e. a 18-month-old AL group of rats served as an age matched control for 18-month-old LTCR and STCR groups, while a 24-month-old AL group served as an age-matched control group for the 24-month-old LTCR and STCR groups of rats.

D'Agostino's K2 normality test with a 95% confidence level was used to test the normal distribution of data. Data without normal distribution passed a normality test after transformation. For the effect of aging (6AL, 18AL and 24AL), one-way ANOVA was performed with Dunett's post hoc multiple analysis (95% confidence interval). For the effect of combined factors, aging (18 and 24 months) and reduction in calorie intake (AL, LTCR and STCR), two-way ANOVA with Tukey's post hoc multiple analysis (95% confidence level) was performed. All statistical measurement was performed using GraphPad Software (San Diego, CA). Statistical significance was set at $p<0.05$. RT PCR results were analyzed by RQ Study Add ON software with a confidence level of 95% ($P < 0.05$).

3. Results

3.1. Effect of age and CR on anxiety level in male Wistar rats

3.1.1. Open-field test

Open-field (OF) task was performed in order to examine general activity and anxiety-related behaviour in rodents. Since there is a natural tendency in rodents to stay in the periphery of the OF arena vs. tendency to explore the new environment, significantly longer time spent in the central zone of the open field may demonstrate reduced anxiety-like behaviour.

Although the travelled distance and the number of entries into the central zone remained mainly unchanged (Figure 2A and B, graphs on the left), time spent in the central area of the OF decreased significantly during aging (Figure 2C, graphs on the left), (one-way ANOVA, ($F_{(2,24)}=3.77$, $p=0.0391$)). Namely, Dunett's post hoc test revealed that 18- and 24-month-old AL rats spent significantly less time in the central square of the OF arena in comparison to the control, 6-month-old animals (50%, $p=0.0374$, and 36%, $p=0.0436$, respectively), indicating that aging brings on a higher anxiety level.

In groups exposed to CR, adverse effects on anxiety-like behaviour were detected. A general trend of reduction of all parameters measured in the open field arena was detected in LTCR and STCR animals in comparison to AL counterparts. Two-way ANOVA revealed a significant effect of age ($F_{(1,46)}=7.456$, $p=0.009$), diet ($F_{(2,46)}=10.82$, $p=0.0002$) and their interaction ($F_{(2,46)}=14.97$; $p<0.0001$) on the distance animals travelled, but the effect of diet was only observed in the number of entries ($F_{(2,46)}=34.38$, $p<0.0001$) and the time spent in central

area ($F_{(2,46)}$=27.53, p<0.0001). Post hoc test revealed that 18-month-old LTCR rats showed significantly reduced locomotor activity (p=0.0035) (Figure 2A, right graph, left panel, light-grey bar), and reduced number of central zone entries (p=0.0289), (Figure 2B, right graph, left panel light-grey bar). Despite the high locomotor activity (p=0.0426) in the 24-month-old group (LTCR), (Figure 2A, right graph, right panel, light-grey bar) number of entries (p=0.0003) and time spent in central area (p=0.0474) decreased more than 50% in comparison to age-matching control rats (Figure 2B and C, right graphs, right panels, light-grey bars).

Statistical analysis showed that the STCR diet significantly decreased all of the OF parameters in both age groups. This 3-month-long CR regimen decreased locomotor activity in 18- and 24-month-old rats (Figure 2A, black bars) (reduction by 40%, post hoc: p<0.0240; and 41%, post hoc: p<0.0481, respectively), as well as the number of entries in the OF central square (post hoc: p=0.0001, p<0.0001, respectively) (Figure 2B, black bars). Notably, in 18- and 24-month-old STCR males a 8- and 30-fold decrease in the time spent in the central square was detected (post hoc: p<0.0001, p<0.0001, respectively) (Figure 2C, black bars) in comparison to the age-matched AL control. Reduced distance travelled, together with the decreased number of entries and the time spent in the central area indicated high anxiety levels in parallel to low general physical activity in STCR rats.

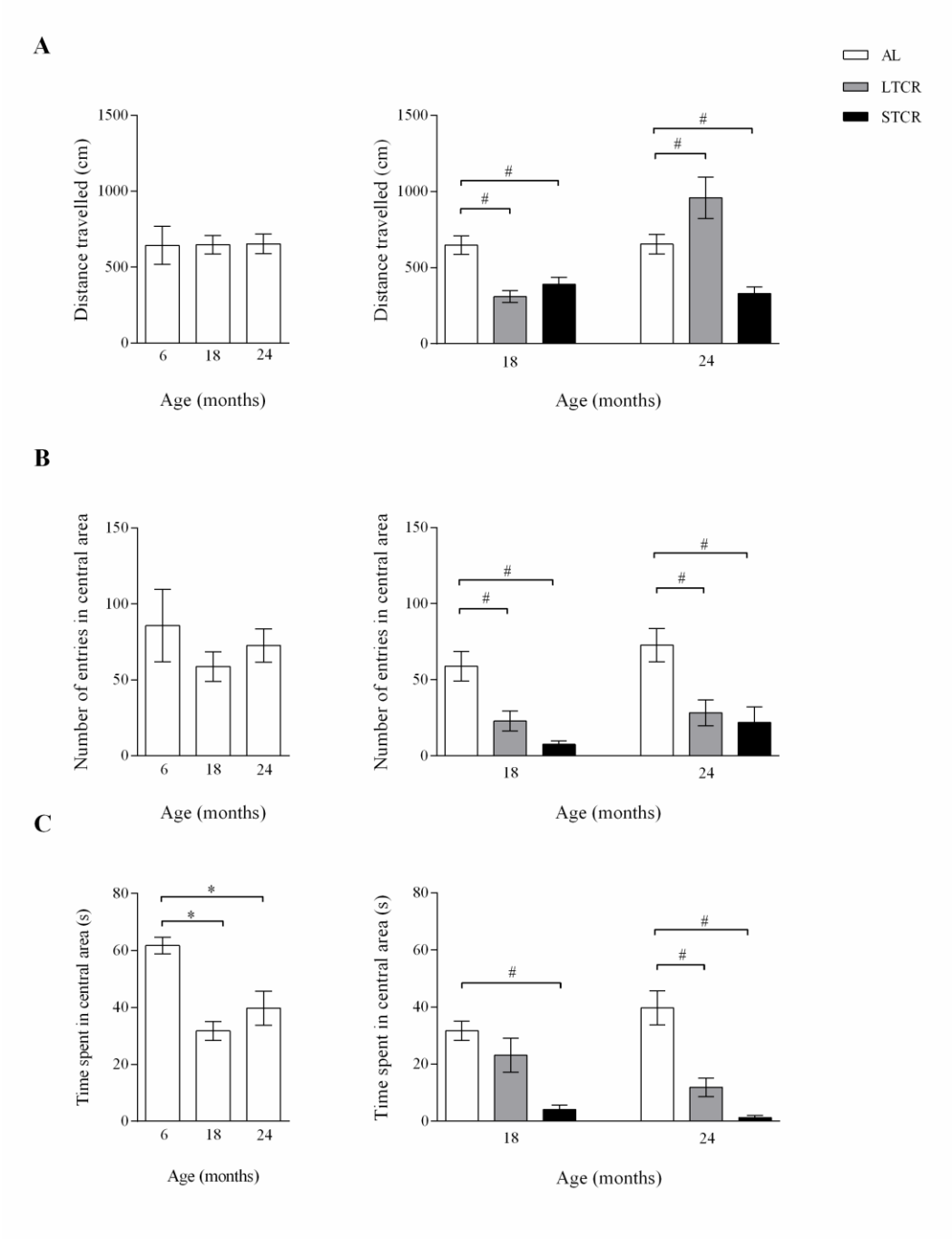

Figure 2. The behavioural profile of 6-, 18-, and 24-month-old ad libitum (AL) and calorie-restricted (LTCR and STCR) groups of animals in the open-field (OF) test. Distance travelled (A), number of entries (B) and time spent in the central area (C) of the OF. Results are expressed as mean ± SEM for 10-minute period. *$p < .05$ versus 6 AL group for the effect of aging, #$p < .05$ versus age-matched AL control for the effect of food regimen. LTCR- long-term calorie restriction, STCR- short-term calorie restriction. (1.5-column fitting image)

Representative trajectories of exploration made by AL (A) and CR (B-C) Wistar male rats during aging are shown in Figure 3. While there are no obvious changes in the trajectories with aging (Figure 3A), it was evident that CR induced specific movement patterns. Both LTCR and STCR rats entered this central zone less frequently than their AL counterparts (Figure 3B and C). Rats exposed to late onset CR showed a very characteristic pattern of movement, exclusively next to the walls of the OF and with almost no entries into central area, which, along with significantly reduced total activity in comparison to all other groups, indicated a high level of anxiety in this experimental group.

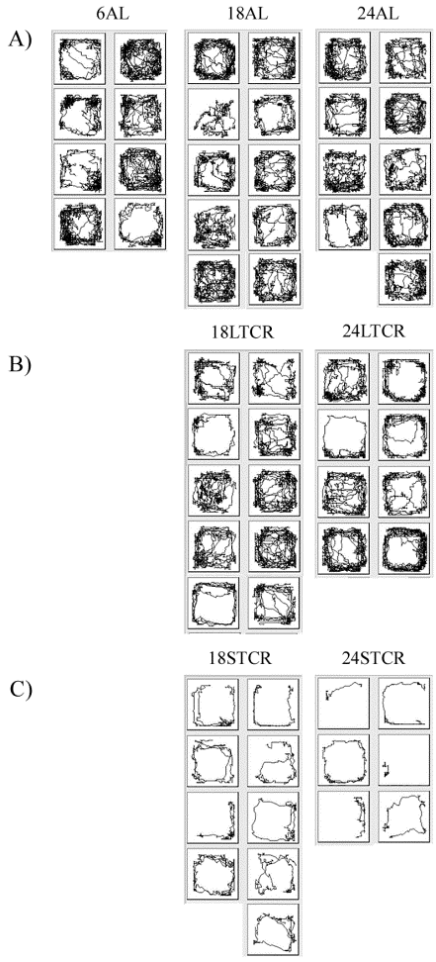

Figure 3. Representative trajectories of AL (ad libitum) and CR (calorie restricted) groups in the open field. During the task animals leave characteristic patterns of movements which indicate anxiety level. Columns of panels correspond to different feeding regimens: trajectories of 6-, 18- and 24-months-old AL rats are presented in the column A, while LTCR and STCR group of rats are presented in the column B and C respectively. The age of the animals is indicated above the columns. LTCR- long-term calorie restriction, STCR- short-term calorie restriction. (1-column fitting image)

3.1.2. Light-dark box test

As the OF test indicated potentially adverse effect of calorie restriction on anxiety in both CR groups we performed the light-dark exploration test (LDB) to further evaluate these results. Though rodents prefer the protected, dark area of the LDB, the unbalanced lack of innate, spontaneous exploratory behaviour in the unprotected light area could indicate increased anxiety levels. The results are presented in the Figure 4.

For AL fed animals, statistical analysis showed unfavorable influence of age for almost all parameters analyzed herein (Figure 4A-D, graphs on the left). One-way ANOVA indicated that aging significantly decreased the number of entries to the light area of the box ($F_{(2,24)}$=11.98, p=0.0002, post hoc: 18AL: p=0.0113, 24AL: p=0.0001) (Figure 4B, graph on the left) and the frequency of sticking out into the light chamber ($F_{(2,24)}$=24.95, p<0.0001, post hoc: 18AL: p<0.0001, 24AL: p<0.0001) (Figure 4D, graph on the left). In addition, the oldest animals spent significantly less time in the light area of the test-box ($F_{(2,24)}$=5.942, p=0.0080) (Figure 4A, graph on the left). Namely, 24-month-old animals spent 56 sec in the light compartment od LDB, which was about twofold reduction in comparison with 18-month-olds (114 sec), and about threefold when compared with 6-month-olds (160 sec) (post hoc: p=0.0067). Similarly, 24-month-old animals showed a decreased tendency for sticking out/stretching, regarding the time spent in the door area ($F_{(2,24)}$=8.837, p=0.0013). Post hoc test showed that 24-month-old AL animals had a 3.5-fold reduction of the time spent in the door area in comparison to 6-month-old animals (p=0.0040) (Figure 4C, graph on the left). All this data indicates a higher level of anxiety in aged animals.

While analyzing the impact of CR on behaviour in LDB, two-way ANOVA showed significant effects of diet alone ($F_{(2,46)}$= 29.67, p<0.0001), and interaction of diet and age ($F_{(2,46)}$=18.87, p<0.0001) regarding time spent in the light compartment. The only significant change in 18-month-old animals was observed under the effect of STCR, since those animals spent less time in the light compartment (2.5-fold decrease post hoc: p=0.0002), (Figure 4A, right graph, left panel, black bar) compared to age-matched controls.

Significant differences between LTCR and STCR groups of rats (presented by light-grey and black bars, respectively) were more evident in the oldest animals, where three out of four measured parameters were significantly improved in LTCR group (Figure 4A-C, light-grey bars, graphs on the right) in comparison to AL controls. Two-way ANOVA revealed that diet

($F_{(2,46)}$=29.67, p<0.0001) and age x diet (($F_{(2,46)}$=18.87, p<0.0001) had a significant effect on the time animals spent in light compartment. Namely, at the age of 24 months LTCR increased the time spent in the bright area (Figure 4A, right graph, right panel, light-grey bar), (post hoc: p<0.0001). Also, this type of diet doubled the number of transitions into the light compartment (effect of age ($F_{(1,46)}$=4.061, p=0.0498); diet x age: ($F_{(2,46)}$=11.20, p=0.0001), post hoc: p=0.0103) (Figure 4B, right graph, right panel, light-grey bar), and increased time spent at the door (age ($F_{(1,46)}$=5.174, p=0.0276, diet x age: ($F_{(2,46)}$=11.93, p<0.0001), post hoc: p=0.0054) (Figure 4C, right graph, right panel, light-grey bar) compared to AL group of corresponding age.

In contrast to LTCR, in 24-month-old animals STCR feeding regimen led to fourfold decrease regarding time spent in the light zone of the apparatus when compared to the age matched AL control group (post hoc: p=0.0320) (Figure 4A, right graph, right panel, black bar).

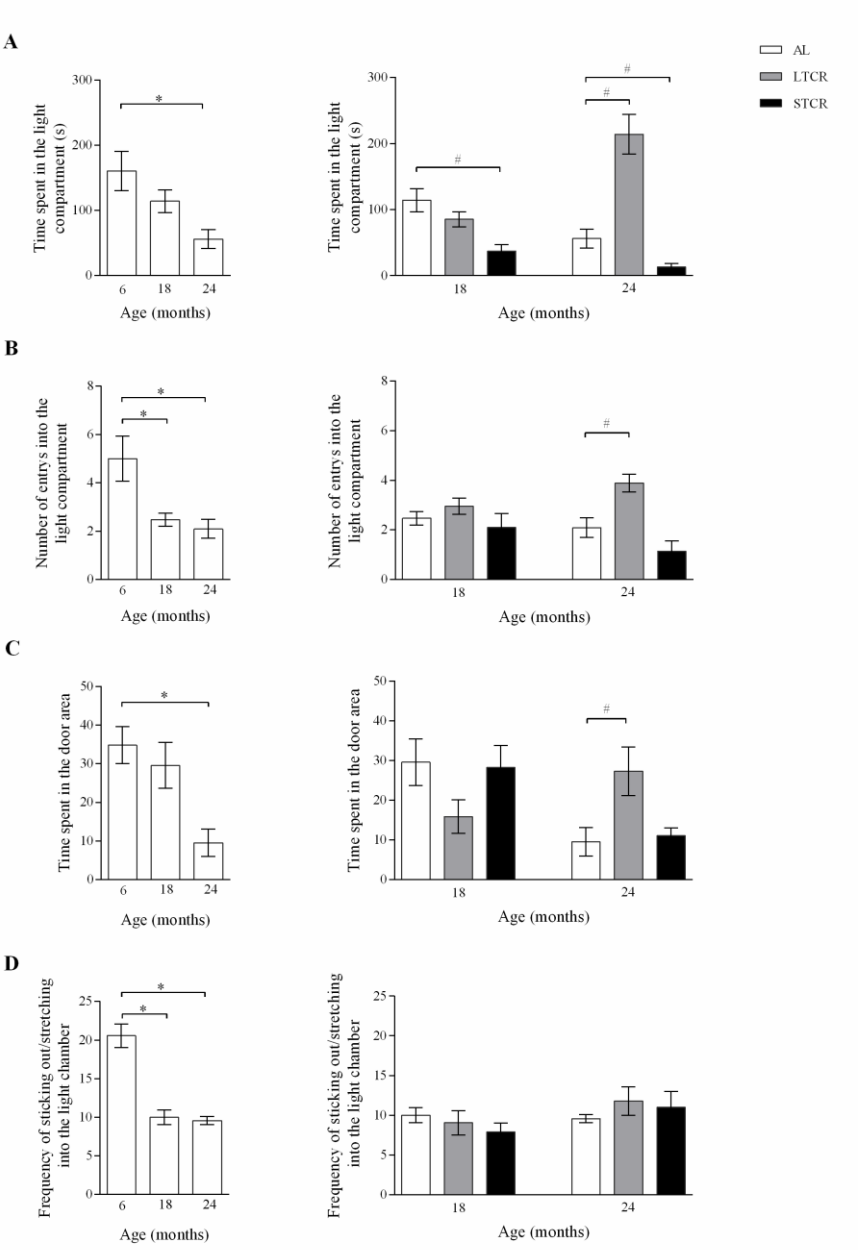

Figure 4. The behavioural profile of 6-, 18-, and 24-month-old ad libitum (AL) and calorie-restricted (LTCR and STCR) group of animals in light-dark maze task. Time spent (A), and number of entries in the light compartment (B), time spent in the door area (C), and frequency of sticking out/stretching into the light compartment (D). Results are expressed as mean ± SEM .. *$p < .05$ versus 6 AL group for the effect of aging, #$p < .05$ versus age-matched AL control for the effect of food regimen. LTCR- long-term calorie restriction, STCR- short-term calorie restriction. (1.5-column fitting image)

3.2. Effect of age and CR on the mRNA expression level of dopamine receptors

Physiological aging differentially affected the expression of mRNA for dopamine receptors in the distinct brain regions. Namely, while there was a 3-fold increase in the cortical D_1R mRNA level of 24-month-old AL rats in comparison to those 6-month-old (Figure 5A, graph on the left), D_1R mRNA expression in the striatum and mesencephalon appeared to be unaffected by aging (Figure 5C and E, graphs on the left). On the other hand, D_2R mRNA expression was increased in the cortex (1.6- and 2.6-fold in 18 and 24 months of age, respectively) and mesencephalon (1.7-fold for both time points analyzed) (Figure 5B, and 5F, graphs on the left). Slightly different pattern was observed in the striatum, where 1.6-fold increase in mRNA D_2R levels was observed only in the 24-month-old rats (Figure 5D, graph on the left).

Following exposure to different feeding regimens, a regional- and receptor type-specificity was detected.

LTCR type of calorie restriction led to receptor- and region-specific changes. While increasing the level of D_1R mRNA in the mesencephalon (1.6- and 1.4-fold at 18 and 24 months, respectively; Figure 5E, light-grey bars), LTCR mainly decreased the level of D_2R mRNA in striatum (Figure 5D, right graph, right panel, light-grey bar) and cortex (Figure 5B, right graph, right panel, light-grey bar) for about 2-fold.

STCR feeding type caused a continuous increase in the expression of D_1R mRNA in all regions at 18 months, and in a range of 1.5-2.3-fold; however, the only statistically significant increase was detected in the cortex of the 18-month-old group (Figure 5A, right graph, left panel, black bar). A different pattern of changes was observed for the D_2R mRNA; namely, a significant reduction in the level of D_2R mRNA was measured in the cortex at 24 months of age (Figure 5B, right graph, right panel, black bar).

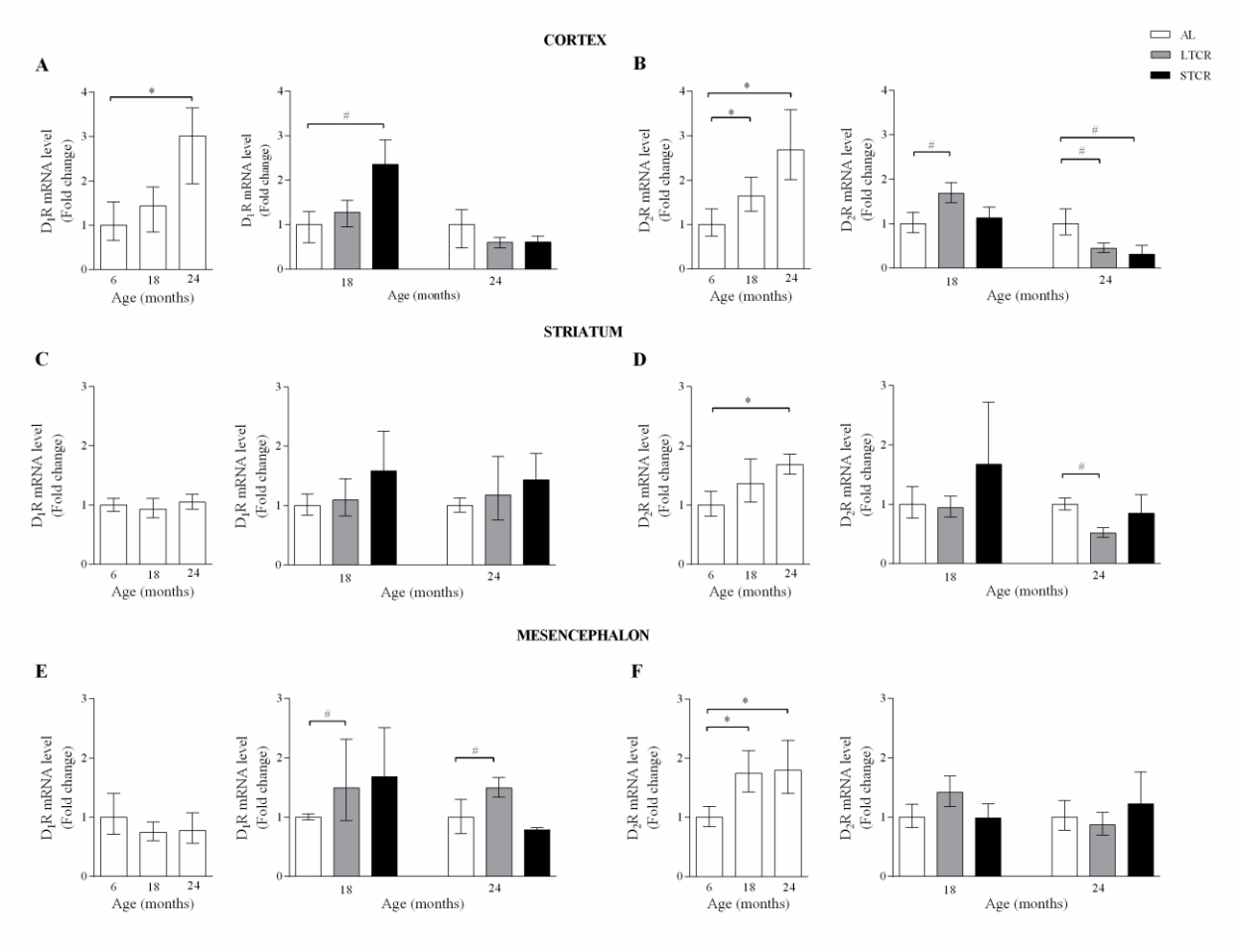

Figure 5. mRNA levels of D_1R and D_2R in cortex (A and B), striatum (C and D), and mesencephalon (E and F) of 6-, 18-, and 24-month-old ad libitum (AL), (graphs on the left) and calorie-restricted (LTCR and STCR) groups of animals (graphs on the right). *$p < .05$ versus 6 AL group for the effect of aging, #$p < .05$ versus age-matched AL control for the effect of food regimen. Data are expressed as the mean ± SEM. LTCR- long-term calorie restriction, STCR- short-term calorie restriction. (2-column fitting image)

3.3. Effect of age and CR on the protein expression level for dopamine receptors

Following age-related increase in the mRNA expression, D_1 and D_2 receptor protein levels were also significantly increased in the cortex of both 18- and 24-month old AL animals [($F_{(2,12)}$=7.863, p=0.0106), ($F_{(2,12)}$=9.802, p=0.0055)], respectively. (Figure 6A and B, graphs on the left). D_1R protein level increased by 50 % at 18- and 24-month time points (post hoc: p=0.0143, p=0.0130, respectively), while D_2R protein level increased by ~40% (post hoc: p=0.0033, p=0.0364, respectively). Similar was detected in the striatum, where D_1 and D_2 protein

levels were continually rising during aging [($F_{(2,12)}$=10.69, p=0.002, post hoc: 24AL: p=0.0012), ($F_{(2,12)}$=10.71, p=0.0018, post hoc: 24AL: p=0.0009)], respectively (Figure 6C and D, graphs on the left). In contrast to the cortex and striatum, no major changes were detected in the mesencephalon, except a decrease (40%) in D_1R protein expression in the oldest group (Figure 6E, graph on the left), ($F_{(2,12)}$=13.65, p=0.0008, post hoc: p=0.004).

The protein levels of dopamine receptors 1 and 2 in the cortex remained rather insensitive to reduced calorie intake. The only modification was detected in 24-month-old STCR males, where significant effect of age: ($F_{(1,24)}$=5.290, p=0.0336); diet: ($F_{(2,24)}$=6.066, p=0.0097) and their interaction: ($F_{(2,24)}$=7.632, p=0.0040) was detected by two-way ANOVA. These animals had a 30% lower level of D_2R protein in comparison to age matched AL fed rats (Figure 6B, right graph, right panel, black bar), (post hoc: p=0.0297).

In contrast to the cortex, two other brain regions analyzed herein have undergone significant alterations in protein expression depending on the onset and duration of restricted calorie intake. LTCR either had no effects or induced an increase in the level of both D_1 and D_2 receptors. In striatum, LTCR type of calorie restriction induced an increase in dopamine receptors abundance of 18-month-old animals (Figure 6C and D, right graphs, left panels, light-grey bars). Two-way ANOVA revealed significant effects of diet: ($F_{(2,24)}$=11.20, p=0.0002) and age x diet interaction: ($F_{(2,24)}$=4.300, p=0.0232) regarding D_1R expression (65%, increase), (post hoc: p=0.0242), and effect of diet: ($F_{(2,24)}$=4.433, p=0.0241) and age x diet interaction: ($F_{(2,24)}$=4.107, p=0.0305), post hoc: p=0.0469) for D_2R expression (increase for 31%). In mesencephalon, LTCR led to the increase of both D_1 and D_2 receptors in 24-month-old animals (Figure 6E and F, right graphs, right panels, light-grey bars). D_1R expression was increased for 145%, and two-way ANOVA proved the effect of diet alone: ($F_{(2,24)}$=6.322, p=0.0058), (post hoc: p=0.0085); while diet: ($F_{(2,24)}$=34.55, p<0.0001) increased D_2R expression for 25%, (post hoc: p=0.0205). Additionally, in the striatum LTCR led to 34% increase of D_1R expression in the oldest group (Figure 6C, right graph, right panel, light-grey bar). Two-way ANOVA revealed effect of diet: ($F_{(2,24)}$=11.20, p=0.0002) and interaction of age and diet: ($F_{(2,24)}$=4.300, p=0.0232, post hoc: p=0.0258).

Shorter duration CR regimen with a late onset (STCR) caused inconsistent changes in protein expression. While increasing the expression of D_1 and D_2 receptors in striatum at 18 months (Figure 6C and D, right graphs, left panels, black bars), (post hoc: p=0.0333, p=0.0219, respectively), STCR decreased significantly the D_1R level in mesencephalon of the oldest group (post hoc: p=0.0498) (Figure 6E, right graph, right panel, black bar) and D_2R level in both age groups (post hoc: 18 STCR: p=0.0367, 24 STCR: p=0.0333) (Figure F, black bars).

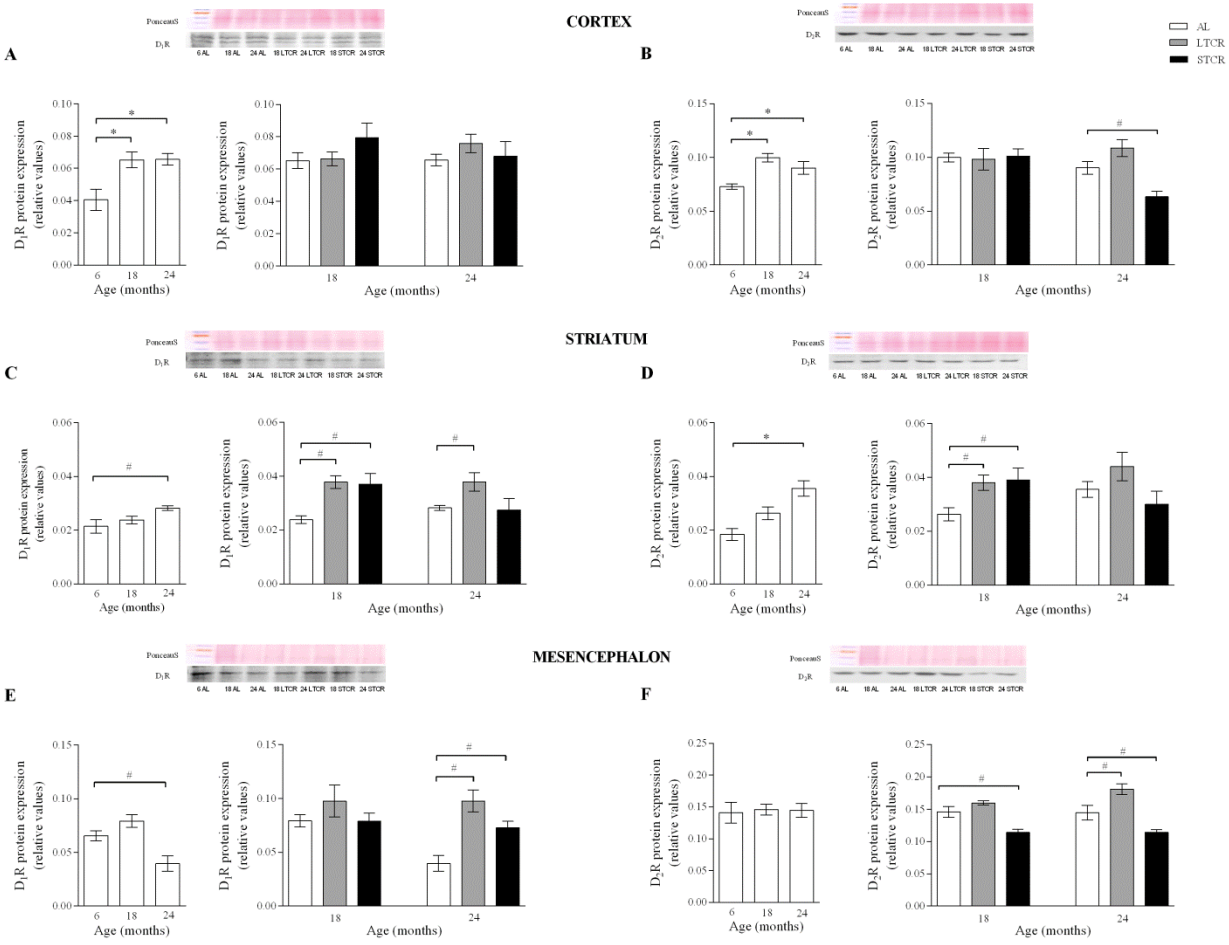

Figure 6. Protein levels of D1R and D2R in cortex (A and B), striatum (C and D), and mesencephalon (E and F) of 6-, 18-, and 24-month-old ad libitum (AL) and calorie-restricted (LTCR and STCR) groups of animals. Each graph is accompanied by representative immunoblots. Ponceau S staining of the membranes served as a control protein loading. Data are expressed as the mean ± SEM. *p < .05 versus 6 AL group for the effect of aging, #p < .05 versus age-matched AL control for the effect of food regimen. LTCR- long-term calorie restriction, STCR- short-term calorie restriction. (2-column fitting image)

4. Discussion

While our previous research has shown that long-term calorie restriction implemented at a young adult stage attenuates age-related decline in synaptic plasticity (Mladenovic Djordjevic et al., 2010; Mladenovic Djordjevic et al., 2014) and increases expression of neurotrophic factors in the aging brain (Smiljanic et al., 2015), our recent study demonstrated that short-term late-onset CR worsens the frailty status of male Wistar rats (Todorovic et al., 2018).

Scarce reports published so far about the impact of CR on anxiety showed contradictory findings. Both 1 day and 10 days of restrictive feeding (2 hours of calorie access per day) in adult male rats resulted in reduction of anxiety, with persisting effect even after normalization of calorie intake (Inoue et al., 2004). Others showed that an acute episode (7 days) of CR has sex-dependent effects on anxiety, inducing anxiolytic effects in adult males, but not in females (Genn et al., 2003). The outcome of chronic CR varies significantly depending on the onset (Jahng et al., 2007; Levay et al., 2007). Namely, when implemented in young rats (from postnatal day 28), CR led to the development of anxiety (Jahng et al., 2007) while it diminished anxiety-like behaviour when CR started in the adult stage (3 months) (Levay et al., 2007).

This study is the first one trying to look into the above-mentioned inconsistency by comparing the effects of CR of various duration and onset during aging. The study confirmed that anxiety-like behaviour increases as animals grow older. This was underpinned by the observed decrease in OF and LDB parameters. Furthermore, we found that 3 months of CR have not only failed to attenuate age-related changes but have additionally worsened anxiety-like behaviour. Namely, the level of anxiety consistently increased in this (STCR) experimental group and was particularly high when CR was implemented at 21 months of age. A somewhat different outcome was noticed with a longer duration (6 months) of calorie restriction. Interestingly, when this type of feeding regimen was introduced at 12 months of age, it had no effect on majority of behavioural parameters, apart from the decrease in general activity in the OF. However, when it was implemented at 18 months of age, it improved behavioural performances in both tests used. These results indicate that the duration of CR has a major impact and that in order to induce some beneficial effect calorie reduction needs to last for at least 6 months. Namely, 24-month-old animals had significantly decreased anxiety parameters when exposed to CR for 6, but not for 3 months. In addition, shorter duration of CR mainly worsened anxiety-like behaviour regardless of the age of the subject.

Mechanisms underlying the effect of the CR on the anxiety-like behaviour are not fully understood. Dopaminergic system, together with glutamatergic and GABAergic systems, has been considered to have a significant modulatory role on anxiety (Zarrindast and Khakpai, 2015). Significant alterations of the components of the dopaminergic system in calorie-restricted rats have been reported (Carr, 2007; Zhen et al., 2006).

In our study, aging was characterized with stable or even increased D_1R and D_2R expression mainly in the cortex and striatum. LTCR was also primarily associated with increased expression of dopamine receptors. In contrast to LTCR, STCR induced inconsistent changes in the level of dopamine receptors, increasing the level of D_1R and decreasing the level of D_2R. This is in alignment with a previous report from Keeler et al. (Keeler et al., 2016) where increased levels of D_1R expression were detected in striatum and lumbar spinal cord of old mice. Since decreased dopamine levels go along with the aging process (Haycock et al., 2003) as well as with calorie restriction (Carr et al., 2003), the increase in D_1 receptor expression we observed in both AL and CR groups might represent a compensatory mechanism of the system trying to utilize available DA. In addition, as it has been suggested by Carr et al. (Carr et al., 2003), it could be that CR causes an imbalance between presynaptic and postsynaptic D_2 receptors, without necessarily changing the total D_2R expression, leading to increased motility, as observed in old LTCR rats.

Regional specificity was noticed in response to CR in this and in our previous studies (Mladenovic Djordjevic et al., 2010; Smiljanic et al., 2015). All three of the investigated brain regions are rich in dopaminergic terminals but are differentially involved in dopamine-regulated functions, including anxiolytic behaviour (Baik, 2020). Herein, while the cortex remained rather unaffected at the receptor protein level, the majority of changes induced by calorie reduction were observed in the striatum and mesencephalon. Ventral tegmental area (VTA) and nucleus accumbens (NAc), parts of mesencephalon and striatum, are the core components of the dopaminergic reward system which could be the reason why they underwent major changes. Further, dopaminergic projections going from mesencephalon to cortex end up mainly in the prefrontal area of the cortex; thus, subtle changes in the expression of DA receptors could remain undetected given that in our study the whole cortex was analyzed.

In addition, the neural circuits and behaviour controlled by dopamine are particularly complex: reciprocal connections link the mesencephalon, striatum and cortex, while significant

differences in anatomical wiring, molecular features, electrophysiological characteristics, and DA receptors localization/co-localization exist. These differences lead to distinct functions of subpopulations of DA neurons and their circuits. For example, while the nigrostriatal pathway is involved primarily in the control of motor function and goal-directed behaviour, including reward-related cognition and learning, mesocortical pathways are involved in reward-related positive and negative reinforcement (Baik, 2020). Both dopamine receptors, D_1R and D_2R are important in mediating anxiety, albeit with different mechanisms (Bananej et al., 2012; de la Mora et al., 2010; Matsuda et al., 2012; Perez de la Mora et al., 2012). Given that synergistic interaction between these two types of receptors is needed for the expression of the majority of dopamine-controlled behaviour (Zarrindast and Khakpai, 2015) it is expected that D_1R and D_2R undergo different types of changes.

The majority of elderly experience anxiety and common treatments are often less effective in older persons (Andreescu and Lee, 2020) imposing the need for discovering novel, effective methods for anxiety treatment. While the capacity of long-term CR and /or its early onset to improve consequences of aging was broadly investigated, the potency of CR when implemented in mid-adulthood and older age has largely been ignored. Our results clearly showed that calorie restriction can have an adverse outcome and that expected beneficial effects are highly dependent on the onset and duration of CR. We have proposed the existence of a certain time window during the lifetime when calorie restriction should not be applied as it induces more adverse than positive effects.

The complete molecular link between anxiety and calorie restriction remains to be elucidated and for a full picture it would be necessary to explore changes in both serotonin and noradrenaline receptors (Portero-Tresserra et al., 2020) and interactions of dopaminergic system with other neurotransmitters in modulation of anxiety-like behaviour (Zarrindast and Khakpai, 2015) in various brain regions. The role of hippocampus especially remains to be revealed within this frame (Jimenez et al., 2018; Loh et al., 2017; Wang et al., 2019). Putting aside this limitation of our study, results presented herein show that CR affects differently both the anxiety level and the expression of DA receptors in the age-, duration- and region- specific manner.

5. Conclusion

Although CR holds great potential in the modification of anxiety-related behaviour, it remains to be precisely established how the DA pathways are involved in these processes and

whether there is potential to use these pathways to identify dietary approaches which may be beneficial in promoting healthy aging. In alignment with our previous report on this ground (Todorovic et al., 2018) restricted calorie regimen perseveres as one of the most promising nutritional strategies for the optimization of cognitive functions during aging, but it should be implemented with caution.

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Figure 1

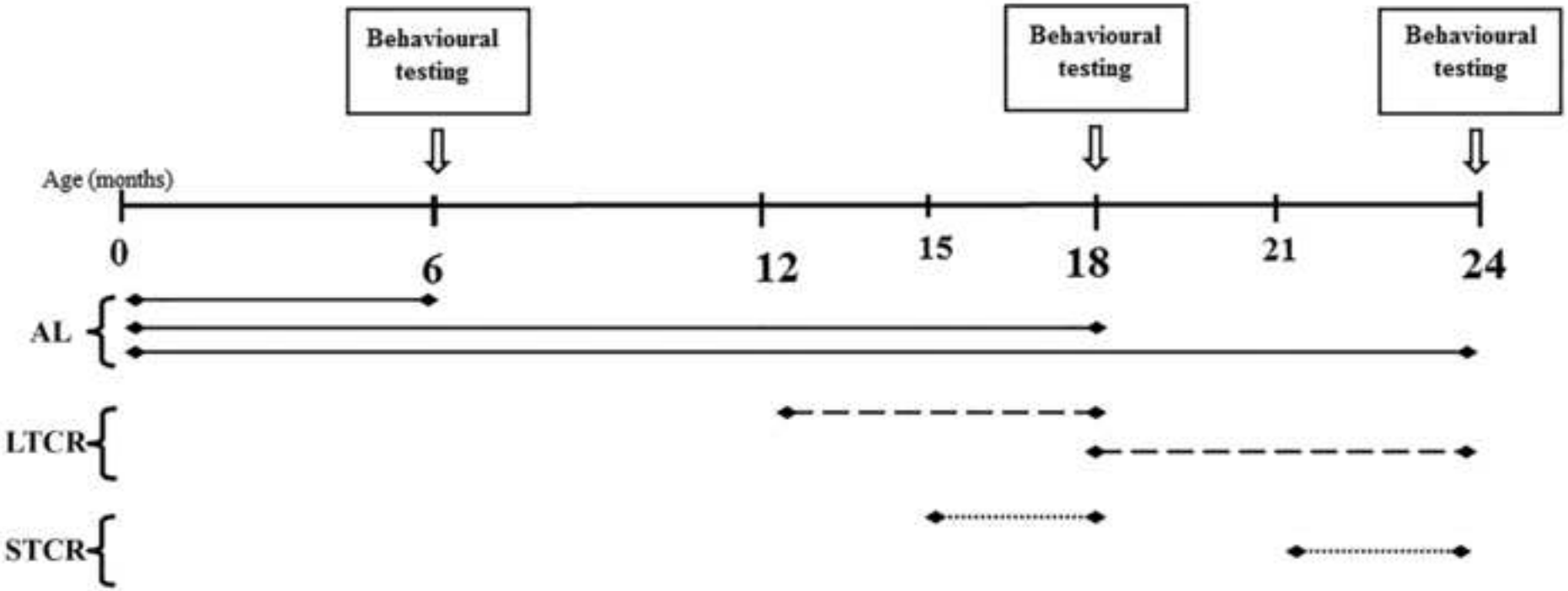


Figure 2

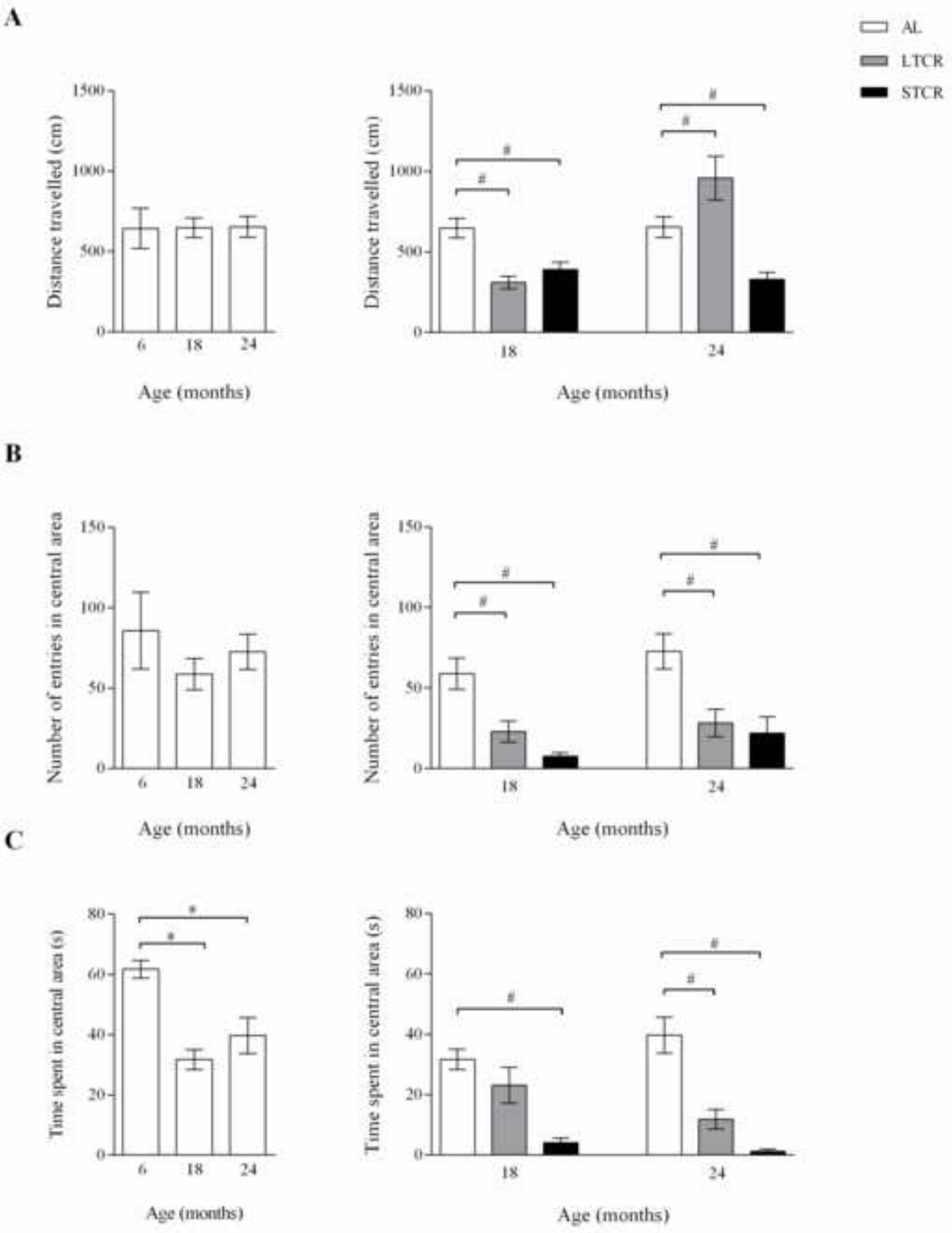


Figure 3

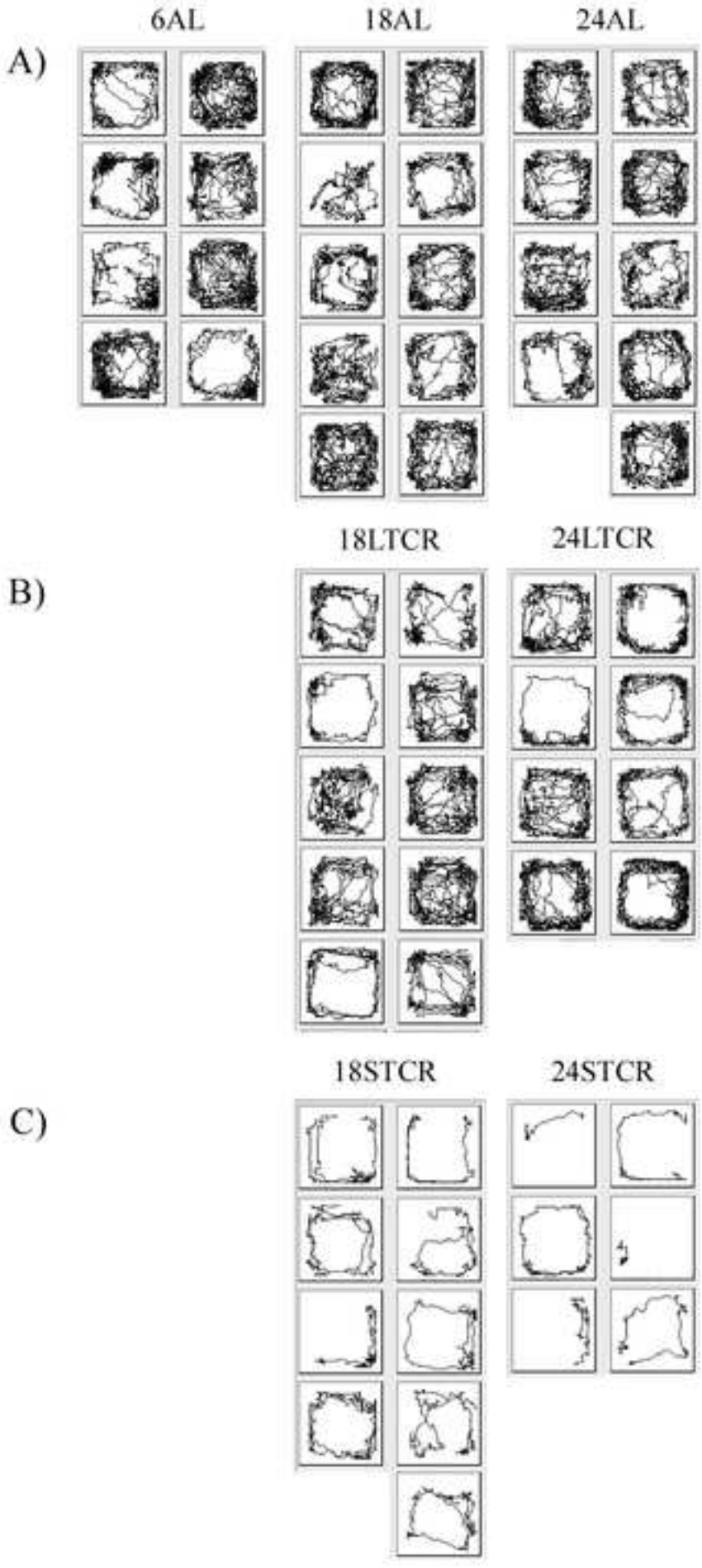


Figure 4

Click here to access/download;Figure;FIGURE 4.tif

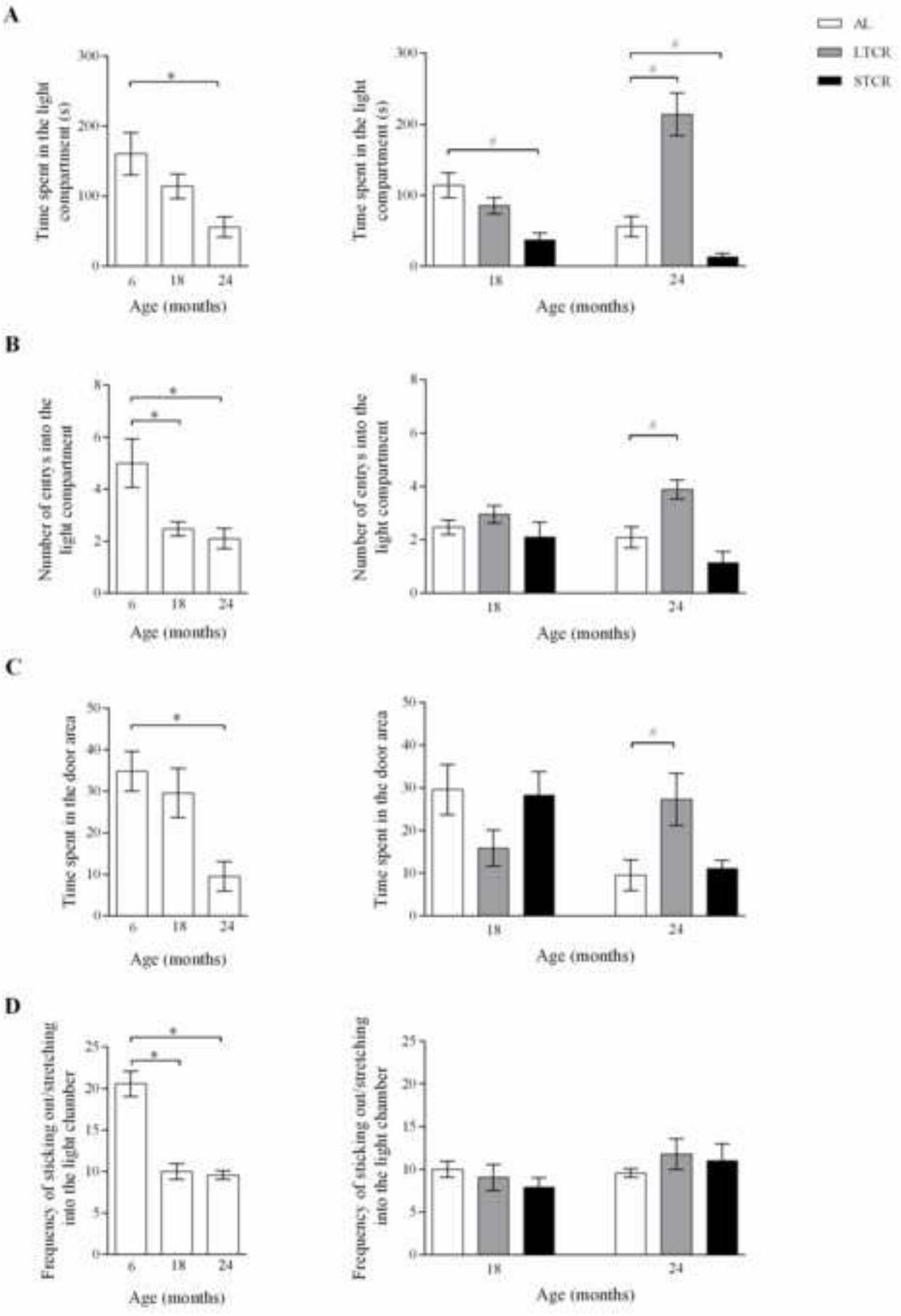


Figure 5

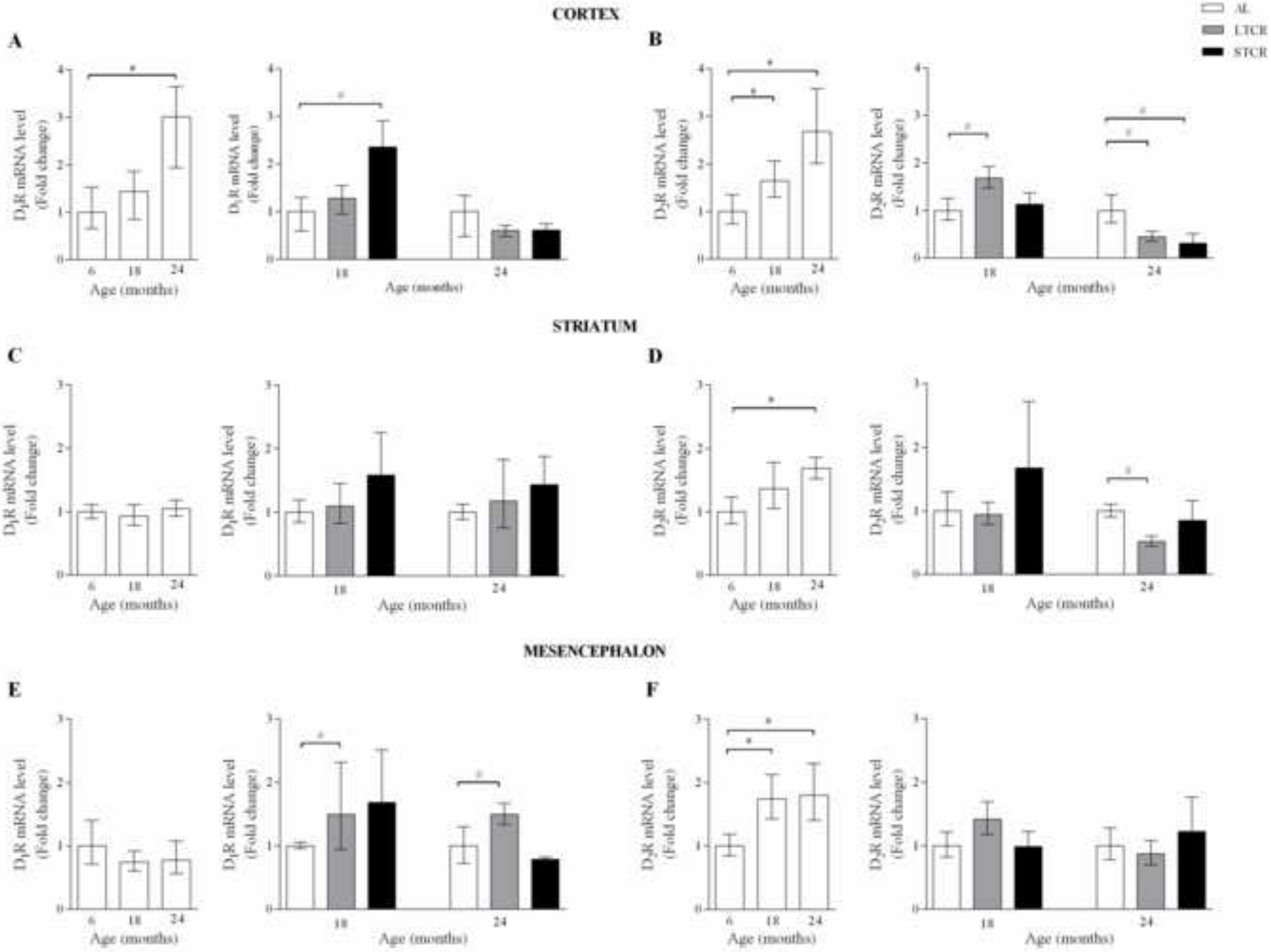
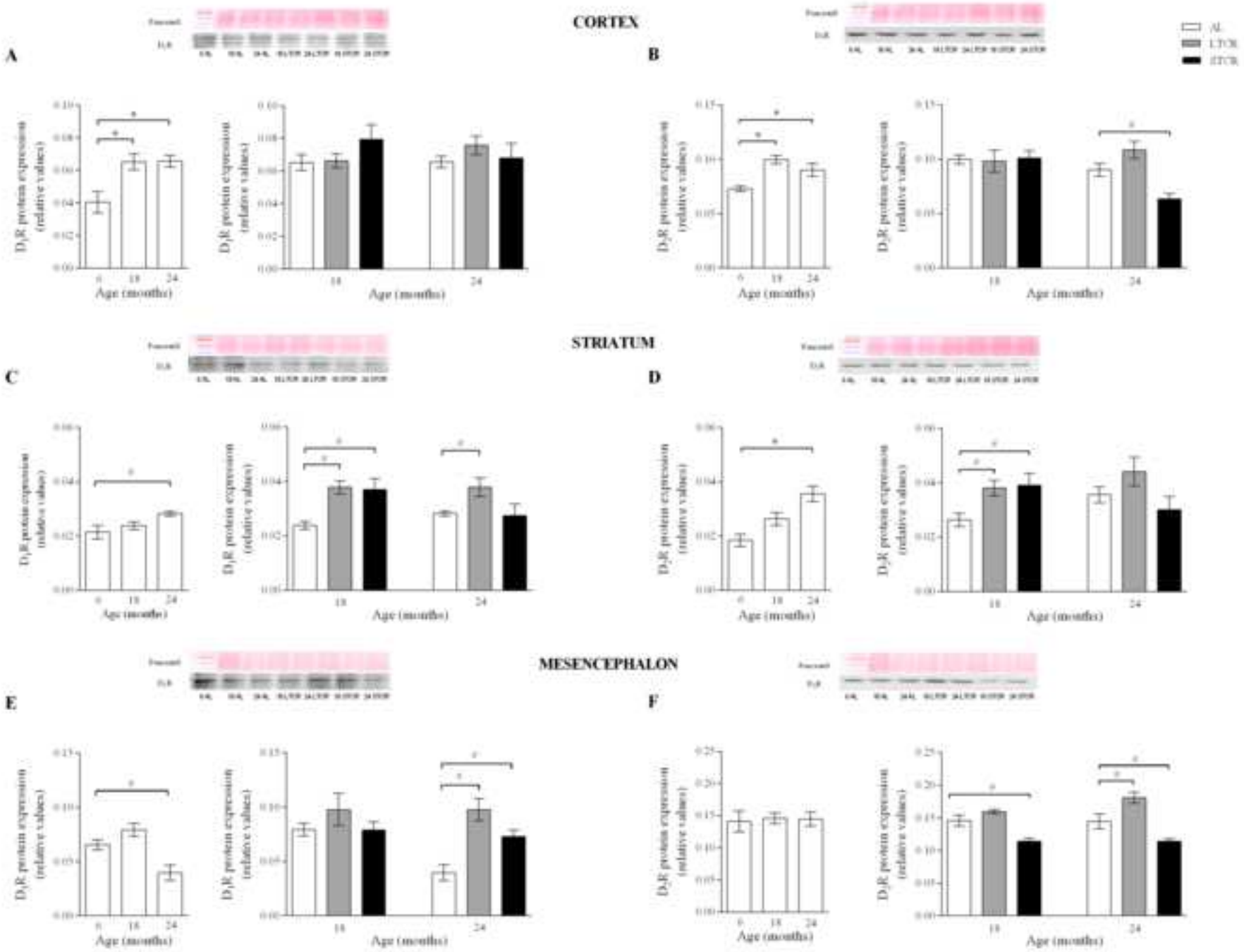


Figure 6

Click here to access/download;Figure;FIGURE 6.tif



Calorie restriction changes age-related anxiety-like behaviour in male Wistar rats and modulates expression of dopamine D_1R and D_2R receptors

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