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Abstract: Exercise capacity and dietary restriction (DR) are linked to improved quality of life, including enhanced brain function and neuro-protection. Brain derived neurotrophic factor (BDNF) is one of the key proteins involved in the beneficial effects of exercise on brain. Low capacity runner (LCR) and high capacity runner (HCR) rats were subjected to DR in order to investigate the regulation of BDNF. HCR-DR rats out-performed other groups in a passive avoidance test. BDNF content increased significantly in the hippocampus of HCR-DR groups compared to control groups ($p<0.05$). The acetylation of H3 increased significantly only in the LCR-DR group. However, Chip-assay revealed that the specific binding between acetylated histone H3 and BNDF promoter was increased in both LCR-DR and HCR-DR groups. In spite of these increases in binding, at the transcriptional level only, the LCR-DR group showed an increase in BDNF mRNA content. Additionally, DR also induced the activity of cAMP response element-binding protein (CREB), while the content of SIRT1 was not altered. Peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) was elevated in HCR-DR groups. But, based on the levels of nuclear respiratory factor-1 and cytocrome c oxidase, it appears that DR did not cause mitochondrial biogenesis. The data suggest that DR-mediated induction of BDNF levels includes chromatin remodeling. Moreover, DR does not induce mitochondrial biogenesis in the hippocampus of LCR/HCR rats. DR results in different responses to a passive avoidance test, and BDNF regulation in LCR and HCR rats.

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Eating habits modulate short term memory and epigenetical regulation of brain derived neurotrophic factor in hippocampus of low-and high running capacity rats

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Abstract

Exercise capacity and dietary restriction (DR) are linked to improved quality of life, including enhanced brain function and neuro-protection. Brain derived neurotrophic factor (BDNF) is one of the key proteins involved in the beneficial effects of exercise on brain. Low capacity runner (LCR) and high capacity runner (HCR) rats were subjected to DR in order to investigate the regulation of BDNF. HCR-DR rats out-performed other groups in a passive avoidance test. BDNF content increased significantly in the hippocampus of HCR-DR groups compared to control groups ($p<0.05$). The acetylation of H3 increased significantly only in the LCR-DR group. However, Chip-assay revealed that the specific binding between acetylated histone H3 and BNDF promoter was increased in both LCR-DR and HCR-DR groups. In spite of these increases in binding, at the transcriptional level only, the LCR-DR group showed an increase in BDNF mRNA content. Additionally, DR also induced the activity of cAMP response element-binding protein (CREB), while the content of SIRT1 was not altered. Peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) was elevated in HCR-DR groups. But, based on the levels of nuclear respiratory factor-1 and cytocrome c oxidase, it appears that DR did not cause mitochondrial biogenesis. The data suggest that DR-mediated induction of BDNF levels includes chromatin remodeling. Moreover, DR does not induce mitochondrial biogenesis in the hippocampus of LCR/HCR rats. DR results in different responses to a passive avoidance test, and BDNF regulation in LCR and HCR rats.

Keywords: Brain-derived neurotrophic factor, histone, epigenetics, dietary restriction, exercise capacity

1. Introduction

It has been postulated that aerobic running could be important in the evolution of homo sapiens(Bramble and Lieberman, 2004; Dumas et al., 2007; Lieberman and Bramble, 2007) A life-style associated with physical activity provides excellent conditions for the regulation of brain derived neurotrophic factor (BDNF)(Mattson, 2012). Early in the evolution of humans it would have been difficult to have consistent, daily nutrition. Eating on alternate days during the hunting/gathering period would be considered normal. Therefore, genes apparently developed to store energy, based on irregular eating conditions (Chakravarthy and Booth, 2004).Alternate day eating has been used as a tool to study the effects of mild dietary restriction (DR)(Brown-Borg and Rakoczy, 2013; Pesic et al., 2010; Rodriguez-Bies et al., 2010; Terzibasi et al., 2009), and found to result in decreases in body mass and cardio protection(Garcia Ramos, 1980), and enhanced muscle performance, and increased lipid metabolism (Rodriguez-Bies et al., 2010). DR is well known to increase life expectancy (Sohal and Weindruch, 1996; Weindruch and May, 1995). Moreover, it also has been reported that DR can promote brain function (Qiu et al., 2012; Quintas et al., 2012; Singh et al., 2012) as it has been suggested that DR can mediate BDNF signaling and consequently play a role in memory neuro-protection, synaptic plasticity, and neurogenesis (Kumar et al., 2009; Radak et al., 2013b; Rich et al., 2010; Rothman et al., 2012).

The neuro-protective effects of physical exercise are well demonstrated (Barrientos et al., 2011; Radak et al., 2010; Radak et al., 2013a; Stranahan and Mattson, 2011) and include up-regulation of BDNF levels as well as improved brain function as measured by the Morris maze and passive avoidance tests (Griesbach et al., 2009; Radak et al., 2006; Sarga et al., 2013; Wu et al., 2013).

An experimental model was created for the running capacity of rats (Koch and Britton, 2001). Low capacity runners (LCR) and high capacity runners (HCR) were indentified. LCR rats

were found to have shorter life-spans and decreased resistance to oxidative stress (Hart et al., 2013; Koch et al., 2012; Koch et al., 2011b). LCR rats also readily develop cardiovascular disorders, and markers of metabolic syndrome, such as visceral adiposity, increased blood pressure, dyslipidemia and insulin resistance when compared to HCR rats (Koch et al., 2011a). Therefore, due to the shorter life span, adiposity, and impaired brain function of LCR rats, this appears to be an excellent model to study the effects of DR. HCR rats also have been shown to outperform LCR rats in cognitive tests (Wikgren et al., 2012).

Regular exercise and nutrition could have an effect of epigenetics (Barnes and Ozanne, 2011; Milagro et al., 2011; Radak et al., 2012; Vaquero and Reinberg, 2009). Reversible modification of lysine residues of histone proteins, such as acetylation and deacetylation, can readily alter the gene expression levels of proteins(Kaelin and McKnight, 2013; Sanders et al., 2013; Shankar et al., 2013), and these modifications can be inherited. It would be interesting to know whether DR can alter the level of histone acetylation at the promoter regions of BDNF, and hence, have a long term effect on BDNF signaling. Therefore, the present investigation was carried out to study the effects of alternate day eating on the epigenetical modulation of BDNF in hippocampus of low and high running capacity rats.

2. Materials and methods

2.1. Animals

Selectively bred rat strains differing in intrinsic aerobic capacity – low capacity runners (LCR) and high capacity runners (HCR) – were used in this study(Koch and Britton, 2001). Endurance running capacity was assessed on a treadmill and the total distance run during a speed-ramped exercise test was used as a measure of maximal aerobic capacity. Rats with the highest running capacity were bred to produce the HCR strain and rats with poor running

capacity were bred to generate LCR rats. A subgroup of male rats from generation 22 was phenotyped for intrinsic treadmill running capacity when11 weeks old, at the University of Michigan (Ann Arbor, USA) and then shipped via air freight to Semmelweis University (Budapest, Hungary) for further study. Investigations were carried out according to the requirements of The Guiding Principles for Care and Use of Animals, EU, and approved by The Ethics Committee of Semmelweis University.

2.2. Animal setting and Dietary restriction (DR)

LCR and HCR male rats, aged 13 months, were assigned to control LCR (LCR-C), dietary restricted LCR (LCR-DR), control HCR (HCR-C) and dietary restricted HCR (HCR-DR) groups (*n* = six rats per group). Dietary restriction was performed by feeding the animal every other day for 16 weeks (**Fig. 1**).

2.3. Tissue samples

After decapitation, brains were rapidly removed, hippocampi from both hemispheres were dissected and flash frozen in liquid nitrogen. All samples were kept at −80°C until homogenized. Samples thawed on ice were quickly pre-homogenized for 10 s at medium speed (IKA's Ultra-Turrax homogenizer) in two volume of PBS (pH 7.4) to gain a crude homogenate. Crude bilateral hippocampal homogenates were divided into four aliquots for ChIP, qRT-PCR, Western blot, and ELISA assays. Aliquots for ChIP assay were processed immediately, whereas the others were stored at −80°C awaiting further processing.

2.4. Passive avoidance test

The test was performed according to the step-through method as described earlier (Sarga et al., 2013). The apparatus consists of a two-compartment acrylic box with a lighted chamber

connected to a darkened chamber by a guillotine door. As soon as the rats entered the dark chamber, they received an electrical shock (0.5 mA, 1 sec). The latency times for entering the dark chamber were measured in the training test, after 24h and ten days later in the retention test.

2.5. Assays

2.5.1. AcH3-ChIP

This assay was based on the Acetyl-Histone H3 Immunoprecipitation Assay Kit (Cat# 17-245Upstate/Millipore). In brief, DNA formaldehyde was added directly to 100 µl aliquots of total pre-homogenate (preH) representing about 30 mg of hippocampus in a final concentration of 1% and incubated for 15 minutes at 37ºC. Formaldehyde was removed by washing of the pre-homogenate two times with ice cold PBS containing protease inhibitor (1mM phenylmethylsulfonyl fluoride (PMSF)), and pelleted for four minutes at 700 x g at 4ºC.

Supernatants were diluted tenfold in ChIP Dilution Buffer (0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl, pH 8.1, 167mM NaCl) supplemented with protease inhibitor PMSF. An aliquot of this chromatin solution was stored at -20°C to quantify the amount of DNA present in different samples before immunoprecipitation (Input).

Anti-acetyl histone H3 (AcH3) specific antibody (Catalog # 06-599, Upstate/Millipore) was added to 2µg of supernatant fraction (chromatin solution) and incubated overnight on ice with constant agitation. The other 1ml of chromatin solution was saved for a normal rabbit serum IgG (sIgG) (Cat# I5006, Sigma-Aldrich, St. Louis, MO, USA) no-antibody control. An immune complex of antigen-antibody-chromatin was collected by adding 60µl of salmon sperm DNA/Protein agarose slurry for one hour at 4ºC with rotation. A beads complex was collected by centrifugation for 1 min at 700 x g at 4°C in a microcentrifuge.

Beads were washed for 3-5 minutes at 4ºC with rotation with 1ml of each of the buffers: Low Salt Immune Complex Wash Buffer (0.1% SDS, 1%Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 150mM NaCl), High Salt Immune Complex Wash Buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 500mM NaCl), LiCl Immune Complex Wash Buffer (0.25M LiCl, 1% NP40, 1% deoxycholate, 1mM EDTA, 10mM Tris-HCl, pH 8.1); and 2x1mL 1X TE (10mM Tris-HCl, 1mM EDTA, pH 8.0). Immune complexes were eluted by adding 250µl of freshly prepared elution buffer (1%SDS, 0.1M NaHC03) to pelleted beads. After vortexing, and brief centrifugation, the supernatant fraction was carefully transferred to another tube and elution was repeated, combining the two eluates. Protein-DNA crosslinks were reversed by adding 20µl of 5M NaCl to the combined eluates and incubated at 65ºC for four hours. Proteins were digested by adding 10µl of 0.5M EDTA, 20µl 1M Tris-HCl (pH 6.5), and 2µl of 10mg/ml proteinase K to the eluate and incubated for one hour at 45ºC. DNA was purified by phenol/chloroform extraction and ethanol precipitation. Before precipitation 20µg of glycogen were added to improve DNA yield. DNA pellets were washed with 70% ethanol, allowed to air dry, and resuspended in DNase/RNase-free water.

2.5.2. AcH3-ChIP – BDNF-qPCR

To quantify BDNF promoter-specific sequences, DNA samples obtained before and after immunoprecipitation, with AcH3 or sIgG, were subjected to quantitative real-time PCR (qPCR). PCR was performed using a primer pair specific for rat BDNF exon IV promoter (from -73 to +14 bp) forward: TCTATTTCGAGGCAGAGGAGGTATC, reverse: AATGGGAAAGTGGGTGGGAG; as described by(Chen et al., 2003; Gomez-Pinilla et al., 2011)

PCR quantification

The threshold cycle (Ct) value was normalized by subtracting the negative control value for each corresponding sample.

2.5.3.RNA isolation

This assay was based on theNucleospin® RNA II kit (Macherey-Nagel, Düren, Germany).

Aliquots of crude bilateral hippocampal homogenates for mRNA quantification assay were stored at -80°C.Pre-homogenate samples were thawed quickly on ice and added to buffer R1, supplemented with β-mercaptoethanol and disrupted with Ultra Turrax® (IKA®-Werke, Staufen, Germany) for 60s at high speed. RNA isolation from the pre-homogenate was performed in accordance with the manufacturer's instructions. RNA was eluted by adding 60µl RNase-free water to columns. After brief centrifugation the flow-out fraction was applied to the eluate and placed onto the column for reelution.

RNA concentration, purity and integrity

The absorbance ratio of the RNA solution was measured at wavelengths 260nm/280nm ranging from 1.71 to 1.97 and at wavelengths 260nm/230nm ranging from 1.79 to 1.88. To check the integrity of RNA, aliquots were run on etidium bromide-stained agarose gel.

RNA samples were stored at -80°C.

2.5.4. cDNA synthesis

cDNA was synthesized using a TetrocDNA Synthesis kit (Cat# BIO-65026 Bioline, Luckenwalde, Germany) in accordance with the manufactures' instructions. Briefly, the reaction conditions were as follows: 1 µg of RNA, 1 µl of random primers, 1 µl of 10 mMdNTP, 1 µl of RNase inhibitor, and 0.25 µl of 200 µ/µL reverse transcriptase in a final volume of 20 µl, and the solution was incubated for ten minutes at 25°C, primer annealing,

followed by 42°C for 60 minutes, primer elongation, and followed by 80°C for five min termination. cDNA samples were stored at -20°C.

2.5.5. Real time quantitative RT-PCR (qRT-PCR) of BDNF mRNA

Based on the principle of the Sybr Green detection method, EvaGreen® dye (Biotium, Hayward, CA, USA) was used to detect PCR products. The PCR was performed using a primer pair specific for rat BDNF mRNA, forward: CCATGAAAGAAGCAAACGT, reverse: CTCCAGCAGAAAGAGCAGA. PCR amplifications consisted of equal amounts of template DNA, 10 μL of ImmoMix™ complete ready-to-use heat-activated 2× reaction mix (Bioline GmbH, Luckenwalde, Germany), 1 μL of 20xEvaGreen (Biotium, Hayward, CA, U.S.A.), 2.5 μL of 10 nmol/L forward and reverse primer (IBAGmbH, Göttingen, Germany) and water to a final volume of 20 μL. Amplifications were performed in a Rotor-Gene 6000 thermal cycler (Corbett Life Science/Qiagen, London, UK) at 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec, 60°C for 20 sec and 72°C for 30 sec. Melting analysis was performed as a final step after the PCR amplification process. The validity of the signal was evaluated by melting analysis and agarose gel electrophoresis. The melting analysis was accepted if an appropriate melting point value of the product was detected (neither other products nor primer dimer were detected). The gel analysis was accepted if only one band was visible with an appropriate size (neither other products nor primer dimer were visible). To eliminate the differences in signal between different PCR runs, three control cDNA's were selected to validate the samples of each run. Rat β-actin gene served as an endogenous control gene.

2.5.6. Western blot and ELISA analysis of BDNF

Aliquots of crude bilateral hippocampal homogenates for Western blot assay were stored at -80°C. The Western blot was carried out as described earlier (Radak et al., 2006). Samples

were incubated in block solution supplemented with primary antibody against BDNF, diluted 1:5000 (polyclonal rabbit anti-BDNF Santa Cruz SC-546) overnight at 4°C with constant agitation. To normalize the expression of BDNF, the blots were re-incubated with a mouse anti-GAPDH antibody. Antibody anti β-actin (monoclonal mouse Cat# sc-8035, Santa Cruz) was added to the incubation solution for endogenous control. The concentration of BDNF was determined from the hippocampal section of the brain, using the E-Max ImmunoAssay System (Promega, Madison, WI) as described previously (Radak et al., 2013b).

2.6. Statistics

The results were compared with a Kruskal-Wallis analysis of variance (ANOVA) using Mann-Whitney post-hoc analyses. Results were expressed as means ± SD. Significance level was set at $p < 0.05$.

3. Results

At the end of the study, the body masses of the DR animals were significantly lower than those of the LCR-C and HCR-C groups (LCR-C:510+/-42g, HCR-C:475+/-24g, LCR-DR:430+/-21g, HCR-DR:415+/-17g).

The average VO2max of the LCR animals was 40ml/kg/min while 65ml/kg/min, a significantly higher ($p<0.05$) value, was measured in the HCR rats. The effects of DR were not significant for VO2max.

The passive avoidance test is a fear-motivated test to assess memory of laboratory rodents. Our data revealed that in short term memory HCR-DR animals had better results than all of the other groups ($p<0.05$, **Fig.2**). All of HCR-DR animal stayed the maximal time (5 min) in the lighted section of the chamber of the box.

DR has been used to increase the activity/content of histone deacetylase SIRT1, but differences amongst the groups were not detected at the protein levels of SIRT1 (**Fig.3**). Peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) is a down-stream transcription factor of SIRT1, and increased levels of PGC-1αwere observed in the HCR-DR group (**Fig. 4.A**). However, significant differences in the down-stream protein of PGC-1α, nuclear respiratory factor 1 (NRF1) (**Fig.4B**) were not detected. COX4 content was measured to assess mitochondrial content. Neither the running capacity nor DR altered the mitochondrial content in the hippocampus (**Fig. 4C**).

The activity of cAMP response element-binding protein (CREB) transcription factor regulates the expression of BDNF, and DR increased the phosphorylation activity of CREB in both groups (**Fig. 5A**).

BDNF content was measured by ELISA and the content increased significantly in the hippocampus of HCR-DR groups compared to other experimental groups (**Fig.5B**). The protein levels of BDNF were also measured by Western blots (**Fig 6A**) and in order to know the epigenetical regulation of BDNF, the global acetylation level of histone H3were measured and significantly increased in the LCR-DR group (**Fig 6B**). The specific binding between acetylated histone H3 and BNDF promoter IV region was detected and was increased by alternate day eating in the LCR and HCR groups (**Fig. 6C**). However, the increases in these bindings did not result in enhanced transcriptional levels of mRNA of BDNF in the HCR group (**Fig. 6D**), indicating the complexity of BDNF regulation.

4. Discussion

Results revealed that alternate day eating can improve memory in rats with high capacity running, while the effects of DR are not significant in low capacity runners. The levels of

BDNF changed in a similar fashion as memory. Gomez-Pinilla and co-workers (Gomez-Pinilla et al., 2011) have shown that voluntary exercise causes increased acetylation of H3 and remodels the chromatin at the promoter region of BDNF.

DR has been shown to alter H3 acetylation in aging liver(Kawakami et al., 2012), and it has been suggested that DR can cause epigenetical regulation of BDNF(Rosas-Vargas et al., 2011). BDNF is a neurotrophin and its gene contains a single coding exon with multiple promoter and alternative slicing transcripts (Bertaux et al., 2004).The binding between acetylated histone H3 and BDNF promoter IV was higher in the HC-DR group, although no changes in the mRNA levels were found. This seems to be controversial, but in the present study we focused on the BDNF promoter region IV, because it has been shown to be sensitive to exercise intervention (Gomez-Pinilla et al., 2011). The changes in mRNA or even protein levels could have originated from other promoter regions. BDNF is crucial for a variety of cellular functions in the nervous system, including proliferation, differentiation, synaptic activity, survival and neurotransmission (Gomez-Pinilla, 2008a, b; Lu et al., 2013).Moreover, BDNF is involved in the regulation of energy metabolism from appetite suppression, to glucose and lipid metabolism (Gomez-Pinilla, 2008a). BDNF can also be activated by the products of heme oxygenase-1 (HO-1), such as bilirubin and CO (Hung et al., 2010) and HO-1 can be activated by acute exercise (Niess et al., 2000). However, it must be noted that regular exercise down-regulates the level of HO-1. Thus, in the present experimental conditions, the possible role of HO-1 in the regulation of BDNF is uncertain.

It has been demonstrated that DR can elevate BDNF levels, in accordance with earlier observations (Duan et al., 2003; Lee et al., 2002) and the data from the present study revealed that the increases in DR-associated BDNF induction are dependent on the posttranslational modification of histones. Therefore, it cannot be ruled out that DR-dependent induction of BDNF is genetically controlled.

DR resulted in phosphorylation of the serine residue (S133) of the kinase-inducable domain of CREB, which initiates transcriptional activity, including the gene for BDNF (Tyagi et al., 2013). It is important to note that CREB can also regulate the transcription of SIRT1 in low nutrient conditions in skeletal muscle, liver and adipose tissue (Noriega et al., 2011). SIRT1 can also regulate the activation of CREB (Monteserin-Garcia et al., 2013). Under the present experimental conditions, SIRT1 protein content was not significantly altered in the hippocampus of rats by DR or running capacity .It has been suggested that energy deprivation, such as DR, can lead to increased AMP/ATP and NAD^+/NADH ratios leading to AMPK mediated phosphorylation and SIRT1 mediated deacetylation of PGC-1α (Scarpulla, 2011). PGC-1α activation then can lead to increased mitochondrial biogenesis. However, it has been questioned whether caloric restriction can cause mitochondrial biogenesis(Hancock et al., 2011). Moreover, there are conflicting data on whether or not SIRT1 mediated deacetylation can result in PGC-1α induced mitochondrial biogenesis (Gurd, 2011; Higashida et al., 2013; Lagouge et al., 2006; Philp et al., 2011; Rodgers et al., 2005). The present investigation did not aim to study the interaction of PGC-1α and SIRT1, but based on the COX4 and NRF1 data, it is suggested that DR does not result in increased mitochondrial biogenesis in the hippocampus of rats.

In summary, it is concluded that DR improves memory of rats with high capacity running, which is associated with enhanced BDNF content due to the acetylation of H3 at the promoter region of the BDNF gene. This observation suggests that, as a consequence of epigenetical regulation, DR induced BDNF levels can be inherited. Overall, our data confirm that the response of low and high running capacity runner rats to DR is different, emphasizing the importance of exercise and caloric control on brain health.

Acknowledgements

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Figure legends:

Fig. 1. Experimental setting.

Low capacity runner (LCR) and high capacity runner (HCR) male rats, aged 13 months, were assigned to control LCR (LCR-C), dietary restricted LCR (LCR-DR), control HCR (HCR-C) and dietary restricted HCR (HCR-DR) groups (*n* = six rats per group). Dietary restriction was performed by feeding the animal every other day for 16 weeks

Fig. 2. Passive avoidance test

Fear-motivated memory was tested by a passive avoidance test, and significant differences were detected between high running capacity-dietary restricted (HCR-DR) and low running capacity-control (LCR-C), and low running capacity-dietary restricted (LCR-DR) and high running capacity-control (HCR-C) groups. Values are expressed as means +/- SD for six animals per group (*P<0.05).

Fig. 3. SIRT1 levels in rat hippocampus

The levels of SIRT1 content were evaluated by a SIRT1 antibody. Values are expressed as means +/- SD for six animals per group (*P<0.05).

Fig. 4. The levels of mitochondrial biogenesis- associated proteins

PGC1a, (**A**) NRF1 (**B**) and COXIV (**C**) levels show how dietary restriction (DR) affected these proteins in the hippocampus of LCR and HCR rats. Values are expressed as means +/- SD for six animals per group (*P<0.05).

Fig. 5.pCREB/CREB ratio and BDNF levels in hippocampus

cAMP response element-binding protein (CREB) activities are shown on the figure as a ratio of phosphorylated and total protein content of CREB (**A**). BDNF levels were evaluated by ELISA (**B**). Values are expressed as means +/- SD for six animals per group (*P<0.05).

Fig. 6. BDNF protein, promoters, and mRNA levels and H3K14 contents in brain

BDNF (**A**), and H3K14 levels (**B**) were evaluated by Western blots, while the quantitative-DNA PCR for BDNF promoter IV region was performed on AcH3 histone immunoprecipitated ChIP samples (**C**). The mRNA levels of this samples are shown in panel **D**. Values are expressed as means +/- SD for six animals per group (*P<0.05).

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Eating habits modulate short term memory and epigenetical regulation of brain derived neurotrophic factor in hippocampus of low-and high running capacity rats

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Abstract

Exercise capacity and dietary restriction (DR) are linked to improved quality of life, including enhanced brain function and neuro-protection. Brain derived neurotrophic factor (BDNF) is one of the key proteins involved in the beneficial effects of exercise on brain. Low capacity runner (LCR) and high capacity runner (HCR) rats were subjected to DR in order to investigate the regulation of BDNF. HCR-DR rats out-performed other groups in a passive avoidance test. BDNF content increased significantly in the hippocampus of HCR-DR groups compared to control groups ($p<0.05$). The acetylation of H3 increased significantly only in the LCR-DR group. However, Chip-assay revealed that the specific binding between acetylated histone H3 and BNDF promoter was increased in both LCR-DR and HCR-DR groups. In spite of these increases in binding, at the transcriptional level only, the LCR-DR group showed an increase in BDNF mRNA content. Additionally, DR also induced the activity of cAMP response element-binding protein (CREB), while the content of SIRT1 was not altered. Peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) was elevated in HCR-DR groups. But, based on the levels of nuclear respiratory factor-1 and cytocrome c oxidase, it appears that DR did not cause mitochondrial biogenesis. The data suggest that DR-mediated induction of BDNF levels includes chromatin remodeling. Moreover, DR does not induce mitochondrial biogenesis in the hippocampus of LCR/HCR rats. DR results in different responses to a passive avoidance test, and BDNF regulation in LCR and HCR rats.

Keywords: Brain-derived neurotrophic factor, histone, epigenetics, dietary restriction, exercise capacity

1. Introduction

It has been postulated that aerobic running could be important in the evolution of homo sapiens(Bramble and Lieberman, 2004; Dumas et al., 2007; Lieberman and Bramble, 2007) A life-style associated with physical activity provides excellent conditions for the regulation of brain derived neurotrophic factor (BDNF)(Mattson, 2012). Early in the evolution of humans it would have been difficult to have consistent, daily nutrition. Eating on alternate days during the hunting/gathering period would be considered normal. Therefore, genes apparently developed to store energy, based on irregular eating conditions (Chakravarthy and Booth, 2004).Alternate day eating has been used as a tool to study the effects of mild dietary restriction (DR)(Brown-Borg and Rakoczy, 2013; Pesic et al., 2010; Rodriguez-Bies et al., 2010; Terzibasi et al., 2009), and found to result in decreases in body mass and cardio protection(Garcia Ramos, 1980), and enhanced muscle performance, and increased lipid metabolism (Rodriguez-Bies et al., 2010). DR is well known to increase life expectancy (Sohal and Weindruch, 1996; Weindruch and May, 1995). Moreover, it also has been reported that DR can promote brain function (Qiu et al., 2012; Quintas et al., 2012; Singh et al., 2012) as it has been suggested that DR can mediate BDNF signaling and consequently play a role in memory neuro-protection, synaptic plasticity, and neurogenesis (Kumar et al., 2009; Radak et al., 2013b; Rich et al., 2010; Rothman et al., 2012).

The neuro-protective effects of physical exercise are well demonstrated (Barrientos et al., 2011; Radak et al., 2010; Radak et al., 2013a; Stranahan and Mattson, 2011) and include up-regulation of BDNF levels as well as improved brain function as measured by the Morris maze and passive avoidance tests (Griesbach et al., 2009; Radak et al., 2006; Sarga et al., 2013; Wu et al., 2013).

An experimental model was created for the running capacity of rats (Koch and Britton, 2001). Low capacity runners (LCR) and high capacity runners (HCR) were indentified. LCR rats

were found to have shorter life-spans and decreased resistance to oxidative stress (Hart et al., 2013; Koch et al., 2012; Koch et al., 2011b). LCR rats also readily develop cardiovascular disorders, and markers of metabolic syndrome, such as visceral adiposity, increased blood pressure, dyslipidemia and insulin resistance when compared to HCR rats (Koch et al., 2011a). Therefore, due to the shorter life span, adiposity, and impaired brain function of LCR rats, this appears to be an excellent model to study the effects of DR. HCR rats also have been shown to outperform LCR rats in cognitive tests (Wikgren et al., 2012).

Regular exercise and nutrition could have an effect of epigenetics (Barnes and Ozanne, 2011; Milagro et al., 2011; Radak et al., 2012; Vaquero and Reinberg, 2009). Reversible modification of lysine residues of histone proteins, such as acetylation and deacetylation, can readily alter the gene expression levels of proteins(Kaelin and McKnight, 2013; Sanders et al., 2013; Shankar et al., 2013), and these modifications can be inherited. It would be interesting to know whether DR can alter the level of histone acetylation at the promoter regions of BDNF, and hence, have a long term effect on BDNF signaling. Therefore, the present investigation was carried out to study the effects of alternate day eating on the epigenetical modulation of BDNF in hippocampus of low and high running capacity rats.

2. Materials and methods

2.1. Animals

Selectively bred rat strains differing in intrinsic aerobic capacity – low capacity runners (LCR) and high capacity runners (HCR) – were used in this study(Koch and Britton, 2001). Endurance running capacity was assessed on a treadmill and the total distance run during a speed-ramped exercise test was used as a measure of maximal aerobic capacity. Rats with the highest running capacity were bred to produce the HCR strain and rats with poor running

capacity were bred to generate LCR rats. A subgroup of male rats from generation 22 was phenotyped for intrinsic treadmill running capacity when11 weeks old, at the University of Michigan (Ann Arbor, USA) and then shipped via air freight to Semmelweis University (Budapest, Hungary) for further study. Investigations were carried out according to the requirements of The Guiding Principles for Care and Use of Animals, EU, and approved by The Ethics Committee of Semmelweis University.

2.2. Animal setting and Dietary restriction (DR)

LCR and HCR male rats, aged 13 months, were assigned to control LCR (LCR-C), dietary restricted LCR (LCR-DR), control HCR (HCR-C) and dietary restricted HCR (HCR-DR) groups (*n* = six rats per group). Dietary restriction was performed by feeding the animal every other day for 16 weeks (**Fig. 1**).

2.3. Tissue samples

After decapitation, brains were rapidly removed, hippocampi from both hemispheres were dissected and flash frozen in liquid nitrogen. All samples were kept at −80°C until homogenized. Samples thawed on ice were quickly pre-homogenized for 10 s at medium speed (IKA's Ultra-Turrax homogenizer) in two volume of PBS (pH 7.4) to gain a crude homogenate. Crude bilateral hippocampal homogenates were divided into four aliquots for ChIP, qRT-PCR, Western blot, and ELISA assays. Aliquots for ChIP assay were processed immediately, whereas the others were stored at −80°C awaiting further processing.

2.4. Passive avoidance test

The test was performed according to the step-through method as described earlier (Sarga et al., 2013). The apparatus consists of a two-compartment acrylic box with a lighted chamber

connected to a darkened chamber by a guillotine door. As soon as the rats entered the dark chamber, they received an electrical shock (0.5 mA, 1 sec). The latency times for entering the dark chamber were measured in the training test, after 24h and ten days later in the retention test.

2.5. Assays

2.5.1. AcH3-ChIP

This assay was based on the Acetyl-Histone H3 Immunoprecipitation Assay Kit (Cat# 17-245Upstate/Millipore). In brief, DNA formaldehyde was added directly to 100 µl aliquots of total pre-homogenate (preH) representing about 30 mg of hippocampus in a final concentration of 1% and incubated for 15 minutes at 37ºC. Formaldehyde was removed by washing of the pre-homogenate two times with ice cold PBS containing protease inhibitor (1mM phenylmethylsulfonyl fluoride (PMSF)), and pelleted for four minutes at 700 x g at 4ºC.

Supernatants were diluted tenfold in ChIP Dilution Buffer (0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl, pH 8.1, 167mM NaCl) supplemented with protease inhibitor PMSF. An aliquot of this chromatin solution was stored at -20°C to quantify the amount of DNA present in different samples before immunoprecipitation (Input).

Anti-acetyl histone H3 (AcH3) specific antibody (Catalog # 06-599, Upstate/Millipore) was added to 2µg of supernatant fraction (chromatin solution) and incubated overnight on ice with constant agitation. The other 1ml of chromatin solution was saved for a normal rabbit serum IgG (sIgG) (Cat# I5006, Sigma-Aldrich, St. Louis, MO, USA) no-antibody control. An immune complex of antigen-antibody-chromatin was collected by adding 60µl of salmon sperm DNA/Protein agarose slurry for one hour at 4ºC with rotation. A beads complex was collected by centrifugation for 1 min at 700 x g at 4°C in a microcentrifuge.

Beads were washed for 3-5 minutes at 4ºC with rotation with 1ml of each of the buffers: Low Salt Immune Complex Wash Buffer (0.1% SDS, 1%Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 150mM NaCl), High Salt Immune Complex Wash Buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 500mM NaCl), LiCl Immune Complex Wash Buffer (0.25M LiCl, 1% NP40, 1% deoxycholate, 1mM EDTA, 10mM Tris-HCl, pH 8.1); and 2x1mL 1X TE (10mM Tris-HCl, 1mM EDTA, pH 8.0). Immune complexes were eluted by adding 250µl of freshly prepared elution buffer (1%SDS, 0.1M NaHC03) to pelleted beads. After vortexing, and brief centrifugation, the supernatant fraction was carefully transferred to another tube and elution was repeated, combining the two eluates. Protein-DNA crosslinks were reversed by adding 20µl of 5M NaCl to the combined eluates and incubated at 65ºC for four hours. Proteins were digested by adding 10µl of 0.5M EDTA, 20µl 1M Tris-HCl (pH 6.5), and 2µl of 10mg/ml proteinase K to the eluate and incubated for one hour at 45ºC. DNA was purified by phenol/chloroform extraction and ethanol precipitation. Before precipitation 20µg of glycogen were added to improve DNA yield. DNA pellets were washed with 70% ethanol, allowed to air dry, and resuspended in DNase/RNase-free water.

2.5.2. AcH3-ChIP – BDNF-qPCR

To quantify BDNF promoter-specific sequences, DNA samples obtained before and after immunoprecipitation, with AcH3 or sIgG, were subjected to quantitative real-time PCR (qPCR). PCR was performed using a primer pair specific for rat BDNF exon IV promoter (from -73 to +14 bp) forward: TCTATTTCGAGGCAGAGGAGGTATC, reverse: AATGGGAAAGTGGGTGGGAG; as described by(Chen et al., 2003; Gomez-Pinilla et al., 2011)

PCR quantification

The threshold cycle (Ct) value was normalized by subtracting the negative control value for each corresponding sample.

2.5.3.RNA isolation

This assay was based on theNucleospin® RNA II kit (Macherey-Nagel, Düren, Germany).

Aliquots of crude bilateral hippocampal homogenates for mRNA quantification assay were stored at -80°C.Pre-homogenate samples were thawed quickly on ice and added to buffer R1, supplemented with β-mercaptoethanol and disrupted with Ultra Turrax® (IKA®-Werke, Staufen, Germany) for 60s at high speed. RNA isolation from the pre-homogenate was performed in accordance with the manufacturer's instructions. RNA was eluted by adding 60µl RNase-free water to columns. After brief centrifugation the flow-out fraction was applied to the eluate and placed onto the column for reelution.

RNA concentration, purity and integrity

The absorbance ratio of the RNA solution was measured at wavelengths 260nm/280nm ranging from 1.71 to 1.97 and at wavelengths 260nm/230nm ranging from 1.79 to 1.88. To check the integrity of RNA, aliquots were run on etidium bromide-stained agarose gel.

RNA samples were stored at -80°C.

2.5.4. cDNA synthesis

cDNA was synthesized using a TetrocDNA Synthesis kit (Cat# BIO-65026 Bioline, Luckenwalde, Germany) in accordance with the manufactures' instructions. Briefly, the reaction conditions were as follows: 1 µg of RNA, 1 µl of random primers, 1 µl of 10 mMdNTP, 1 µl of RNase inhibitor, and 0.25 µl of 200 µ/µL reverse transcriptase in a final volume of 20 µl, and the solution was incubated for ten minutes at 25°C, primer annealing,

followed by 42°C for 60 minutes, primer elongation, and followed by 80°C for five min termination. cDNA samples were stored at -20°C.

2.5.5. Real time quantitative RT-PCR (qRT-PCR) of BDNF mRNA

Based on the principle of the Sybr Green detection method, EvaGreen® dye (Biotium, Hayward, CA, USA) was used to detect PCR products. The PCR was performed using a primer pair specific for rat BDNF mRNA, forward: CCATGAAAGAAGCAAACGT, reverse: CTCCAGCAGAAAGAGCAGA. PCR amplifications consisted of equal amounts of template DNA, 10 μL of ImmoMix™ complete ready-to-use heat-activated 2× reaction mix (Bioline GmbH, Luckenwalde, Germany), 1 μL of 20xEvaGreen (Biotium, Hayward, CA, U.S.A.), 2.5 μL of 10 nmol/L forward and reverse primer (IBAGmbH, Göttingen, Germany) and water to a final volume of 20 μL. Amplifications were performed in a Rotor-Gene 6000 thermal cycler (Corbett Life Science/Qiagen, London, UK) at 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec, 60°C for 20 sec and 72°C for 30 sec. Melting analysis was performed as a final step after the PCR amplification process. The validity of the signal was evaluated by melting analysis and agarose gel electrophoresis. The melting analysis was accepted if an appropriate melting point value of the product was detected (neither other products nor primer dimer were detected). The gel analysis was accepted if only one band was visible with an appropriate size (neither other products nor primer dimer were visible). To eliminate the differences in signal between different PCR runs, three control cDNA's were selected to validate the samples of each run. Rat β-actin gene served as an endogenous control gene.

2.5.6. Western blot and ELISA analysis of BDNF

Aliquots of crude bilateral hippocampal homogenates for Western blot assay were stored at -80°C. The Western blot was carried out as described earlier (Radak et al., 2006). Samples

were incubated in block solution supplemented with primary antibody against BDNF, diluted 1:5000 (polyclonal rabbit anti-BDNF Santa Cruz SC-546) overnight at 4°C with constant agitation. To normalize the expression of BDNF, the blots were re-incubated with a mouse anti-GAPDH antibody. Antibody anti β-actin (monoclonal mouse Cat# sc-8035, Santa Cruz) was added to the incubation solution for endogenous control. The concentration of BDNF was determined from the hippocampal section of the brain, using the E-Max ImmunoAssay System (Promega, Madison, WI) as described previously (Radak et al., 2013b).

2.6. Statistics

The results were compared with a Kruskal-Wallis analysis of variance (ANOVA) using Mann-Whitney post-hoc analyses. Results were expressed as means ± SD. Significance level was set at $p < 0.05$.

3. Results

At the end of the study, the body masses of the DR animals were significantly lower than those of the LCR-C and HCR-C groups (LCR-C:510+/-42g, HCR-C:475+/-24g, LCR-DR:430+/-21g, HCR-DR:415+/-17g).

The average VO2max of the LCR animals was 40ml/kg/min while 65ml/kg/min, a significantly higher ($p<0.05$) value, was measured in the HCR rats. The effects of DR were not significant for VO2max.

The passive avoidance test is a fear-motivated test to assess memory of laboratory rodents. Our data revealed that in short term memory HCR-DR animals had better results than all of the other groups ($p<0.05$, **Fig.2**). All of HCR-DR animal stayed the maximal time (5 min) in the lighted section of the chamber of the box.

DR has been used to increase the activity/content of histone deacetylase SIRT1, but differences amongst the groups were not detected at the protein levels of SIRT1 (**Fig.3**). Peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) is a down-stream transcription factor of SIRT1, and increased levels of PGC-1αwere observed in the HCR-DR group (**Fig. 4.A**). However, significant differences in the down-stream protein of PGC-1α, nuclear respiratory factor 1 (NRF1) (**Fig.4B**) were not detected. COX4 content was measured to assess mitochondrial content. Neither the running capacity nor DR altered the mitochondrial content in the hippocampus (**Fig. 4C**).

The activity of cAMP response element-binding protein (CREB) transcription factor regulates the expression of BDNF, and DR increased the phosphorylation activity of CREB in both groups (**Fig. 5A**).

BDNF content was measured by ELISA and the content increased significantly in the hippocampus of HCR-DR groups compared to other experimental groups (**Fig.5B**). The protein levels of BDNF were also measured by Western blots (**Fig 6A**) and in order to know the epigenetical regulation of BDNF, the global acetylation level of histone H3were measured and significantly increased in the LCR-DR group (**Fig 6B**). The specific binding between acetylated histone H3 and BNDF promoter IV region was detected and was increased by alternate day eating in the LCR and HCR groups (**Fig. 6C**). However, the increases in these bindings did not result in enhanced transcriptional levels of mRNA of BDNF in the HCR group (**Fig. 6D**), indicating the complexity of BDNF regulation.

4. Discussion

Results revealed that alternate day eating can improve memory in rats with high capacity running, while the effects of DR are not significant in low capacity runners. The levels of

BDNF changed in a similar fashion as memory. Gomez-Pinilla and co-workers (Gomez-Pinilla et al., 2011) have shown that voluntary exercise causes increased acetylation of H3 and remodels the chromatin at the promoter region of BDNF.

DR has been shown to alter H3 acetylation in aging liver(Kawakami et al., 2012), and it has been suggested that DR can cause epigenetical regulation of BDNF(Rosas-Vargas et al., 2011). BDNF is a neurotrophin and its gene contains a single coding exon with multiple promoter and alternative slicing transcripts (Bertaux et al., 2004).The binding between acetylated histone H3 and BDNF promoter IV was higher in the HC-DR group, although no changes in the mRNA levels were found. This seems to be controversial, but in the present study we focused on the BDNF promoter region IV, because it has been shown to be sensitive to exercise intervention (Gomez-Pinilla et al., 2011). The changes in mRNA or even protein levels could have originated from other promoter regions. BDNF is crucial for a variety of cellular functions in the nervous system, including proliferation, differentiation, synaptic activity, survival and neurotransmission (Gomez-Pinilla, 2008a, b; Lu et al., 2013).Moreover, BDNF is involved in the regulation of energy metabolism from appetite suppression, to glucose and lipid metabolism (Gomez-Pinilla, 2008a). BDNF can also be activated by the products of heme oxygenase-1 (HO-1), such as bilirubin and CO (Hung et al., 2010) and HO-1 can be activated by acute exercise (Niess et al., 2000). However, it must be noted that regular exercise down-regulates the level of HO-1. Thus, in the present experimental conditions, the possible role of HO-1 in the regulation of BDNF is uncertain.

It has been demonstrated that DR can elevate BDNF levels, in accordance with earlier observations (Duan et al., 2003; Lee et al., 2002) and the data from the present study revealed that the increases in DR-associated BDNF induction are dependent on the posttranslational modification of histones. Therefore, it cannot be ruled out that DR-dependent induction of BDNF is genetically controlled.

DR resulted in phosphorylation of the serine residue (S133) of the kinase-inducable domain of CREB, which initiates transcriptional activity, including the gene for BDNF (Tyagi et al., 2013). It is important to note that CREB can also regulate the transcription of SIRT1 in low nutrient conditions in skeletal muscle, liver and adipose tissue (Noriega et al., 2011). SIRT1 can also regulate the activation of CREB (Monteserin-Garcia et al., 2013). Under the present experimental conditions, SIRT1 protein content was not significantly altered in the hippocampus of rats by DR or running capacity .It has been suggested that energy deprivation, such as DR, can lead to increased AMP/ATP and NAD^+/NADH ratios leading to AMPK mediated phosphorylation and SIRT1 mediated deacetylation of PGC-1α (Scarpulla, 2011). PGC-1α activation then can lead to increased mitochondrial biogenesis. However, it has been questioned whether caloric restriction can cause mitochondrial biogenesis(Hancock et al., 2011). Moreover, there are conflicting data on whether or not SIRT1 mediated deacetylation can result in PGC-1α induced mitochondrial biogenesis (Gurd, 2011; Higashida et al., 2013; Lagouge et al., 2006; Philp et al., 2011; Rodgers et al., 2005). The present investigation did not aim to study the interaction of PGC-1α and SIRT1, but based on the COX4 and NRF1 data, it is suggested that DR does not result in increased mitochondrial biogenesis in the hippocampus of rats.

In summary, it is concluded that DR improves memory of rats with high capacity running, which is associated with enhanced BDNF content due to the acetylation of H3 at the promoter region of the BDNF gene. This observation suggests that, as a consequence of epigenetical regulation, DR induced BDNF levels can be inherited. Overall, our data confirm that the response of low and high running capacity runner rats to DR is different, emphasizing the importance of exercise and caloric control on brain health.

Acknowledgements

The LCR HCR rat model system was supported by grant R24 RR017718 from the National Center for Research Resources of the National Institutes of Health (to L.G.K. and S.L.B.), and National Institutes of Health grant RO1 DK077200 (to S.L.B.). The LCR and HCR model can be made available for collaborative study (contact: brittons@umich.edu or lgkoch@umich.edu).

Figure legends:

Fig. 1. Experimental setting.

Low capacity runner (LCR) and high capacity runner (HCR) male rats, aged 13 months, were assigned to control LCR (LCR-C), dietary restricted LCR (LCR-DR), control HCR (HCR-C) and dietary restricted HCR (HCR-DR) groups (*n* = six rats per group). Dietary restriction was performed by feeding the animal every other day for 16 weeks

Fig. 2. Passive avoidance test

Fear-motivated memory was tested by a passive avoidance test, and significant differences were detected between high running capacity-dietary restricted (HCR-DR) and low running capacity-control (LCR-C), and low running capacity-dietary restricted (LCR-DR) and high running capacity-control (HCR-C) groups. Values are expressed as means +/- SD for six animals per group (*P<0.05).

Fig. 3. SIRT1 levels in rat hippocampus

The levels of SIRT1 content were evaluated by a SIRT1 antibody. Values are expressed as means +/- SD for six animals per group (*P<0.05).

Fig. 4. The levels of mitochondrial biogenesis- associated proteins

PGC1a, (**A**) NRF1 (**B**) and COXIV (**C**) levels show how dietary restriction (DR) affected these proteins in the hippocampus of LCR and HCR rats. Values are expressed as means +/- SD for six animals per group (*P<0.05).

Fig. 5.pCREB/CREB ratio and BDNF levels in hippocampus

cAMP response element-binding protein (CREB) activities are shown on the figure as a ratio of phosphorylated and total protein content of CREB (**A**). BDNF levels were evaluated by ELISA (**B**). Values are expressed as means +/- SD for six animals per group (*P<0.05).

Fig. 6. BDNF protein, promoters, and mRNA levels and H3K14 contents in brain

BDNF (**A**), and H3K14 levels (**B**) were evaluated by Western blots, while the quantitative-DNA PCR for BDNF promoter IV region was performed on AcH3 histone immunoprecipitated ChIP samples (**C**). The mRNA levels of this samples are shown in panel **D**. Values are expressed as means +/- SD for six animals per group (*P<0.05).

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High lights

Dietary restriction improves memory of rats with high capacity running.

Dietary restriction shapes the chromatin at BDNF promoter region.

Different running capacity alters the regulation of BDNF.

***Response to Reviewers (without author identifiers)**

Dear Professor Vizi E. Szilvester

Editor of *Brain Research Bulletin*

Thank you very much for your kind evaluation on our manuscript. We accepted all of the comments and suggestions given by the reviewers and we extensively revised the manuscript accordingly. Please, see our detailed response below. We are very thankful to the reviewers and we feel that the quality of the paper increased significantly after the revision. We are very much looking forward to your evaluation.

Sincerely yours,

Zsolt RADAK, Ph.D.

Reviewers' comments:

Reviewer #1: I find the paper very straight-forward, but I also think there are parts that have not been written very carefully. In fact, the manuscript lacks some critical information needed to evaluate it.

I am not a native English speaker myself, but I found the wording a bit clumsy here and there. Please have the text checked.

The paper has been edited by an English speaking scientist and the appropriate corrections have been made.

In the beginning of the experiment, the animals were 13 months old. Dietary restriction took 16 weeks, so at the time of the behavioral experimentation and tissue sampling the animals were about 16-17 months old. For a rat, this is quite an old age. Would the authors predict to have the same effects with younger rats? Please discuss this.

This is an important point, the maximal life span of LCR rats is 31 and HCR is around 43 month (PMID:21921265). However, the results are applicable to rats of this age group.

There is a subtitle "exercise protocol and DR treatment". However, there is nothing about exercise in the text afterwards. Apart from phenotyping the animals, was there some kind of exercise involved? Exercise is also involved in the key words. Should it really be there?

Thank you very much, we changed the exercise to “exercise capacity” as we believe that this would be a more appropriate term.

The passive avoidance test is mentioned in the abstract and indirectly even in the

title, but there is no description of the protocol in the methods or any reference to this issue in the introduction.

The passive avoidance test is now described in the Methods section and mentioned in the Introduction as well.

Reviewer #2: Report to Author- Eating habits modulate short term memory and epigenetical regulation of brain derived neurotrophic factor in hippocampus of low-and high running capacity rats

The authors investigated the effect of alternate day eating on the epigenetical modulation of BDNF in hippocampus. They used LCR and HCR rats as experimental animal model. They concluded that dietary restriction (DR) improves memory of rats with high capacity of running, which is associated with enhanced BDNF content

This is an interesting study, however, the reviewer have some important comments which should be addressed in detail.

I have a few minor comments to improve the papers.

1. What kind of metabolic diifferences are between LCR and HCR rats? Please, clarify and conclude.

Thank you for this important question, we have added

It has been also shown that LCR rats readily develop cardiovascular disorders, and markers of metabolic syndrome, such as visceral adiposity, increased blood pressure, dyslipidemia and insulin resistance compared to HCR rats (PMID:21921265).

2. In the Abstract, the results are poorly concluded. Please, correct them.

We have added the following:

Moreover, DR does not induce mitochondrial biogenesis in the hippocampus of LCR/HCR rats. DR results in different responses to the passive avoidance test and BDNF regulation in LCR and HCR rats.

3. Why LCR and HCR used animal model for the testing of DR? Please, write a new paragraph in more detail. give a short introduction about it.

We have added the following:

Therefore, due to the shorter life span, adiposity and impaired brain function of LCR rats, this appears to be an excellent model to study the effects of DR.

4. This reviewer suggests to the authors preparing a new protocol figure to illustrate the methods in their experiments to avoid confusion of the readers.

Fig 1 now shows the experimental setting.

5. Why the 16 week DR period were determined in this study?

Sixteen weeks was selected because this duration has been shown to be effective for changing body mass, and the activity main metabolic pathways (PMID:16455763, PMID:24772421, PMID:22569236)

6. Please conclude the other mechanisms in the manuscript (for example BDNF/HO pathway, sexual dimorfism).

The following section has been added:

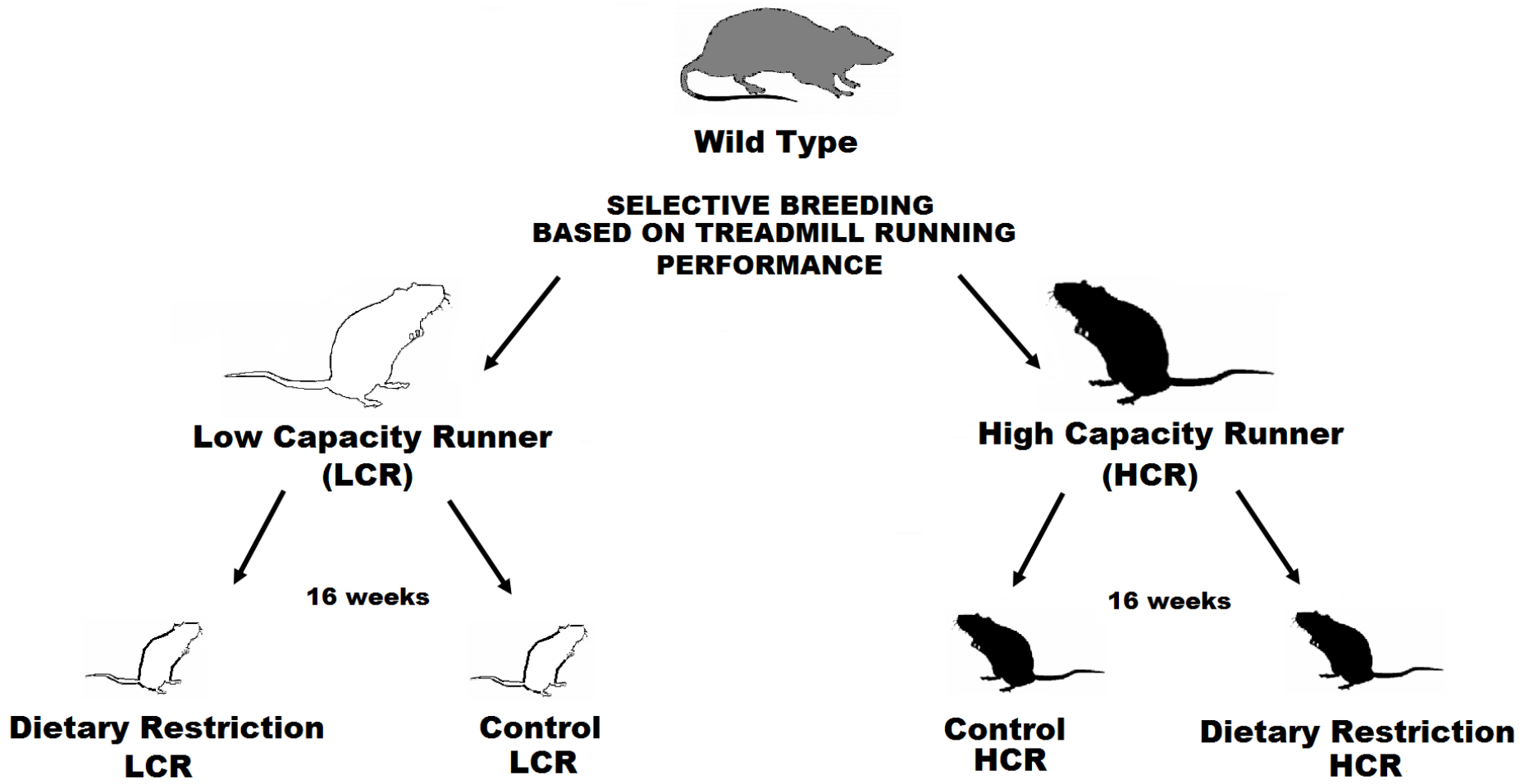
BDNF can also be activated by the products of heme oxygenase-1, such as bilirubin and CO (PMID:19925812) and HO-1 can be activated by exercise (PMID:11232592). However, it

must be noted that regular exercise down-regulates the level of HO-1. Thus, in the present experimental conditions the possible role of HO-1 in the regulation of BDNF is uncertain.

Dear Reviewers, thank you very much for your important and supporting comments.

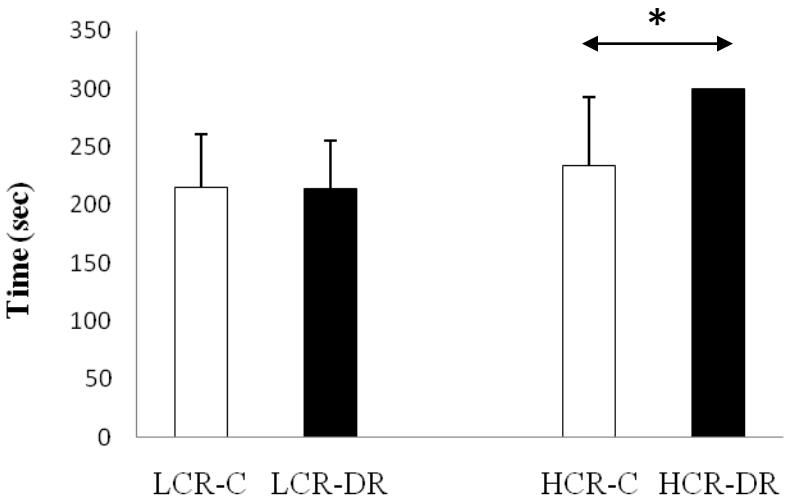
Figure(s)

Fig 1. Experimental setting



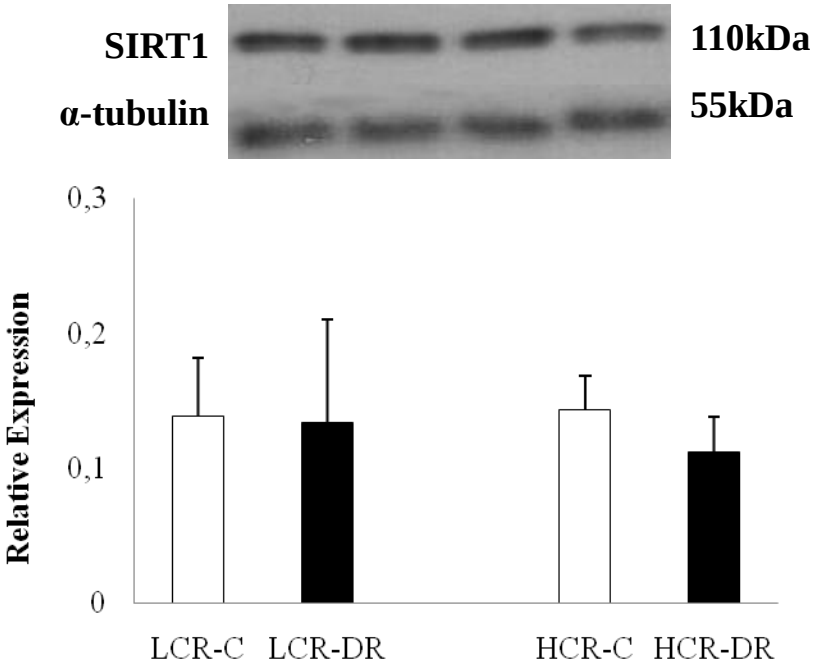
Figure(s)

Fig 2. Passive avoidance test



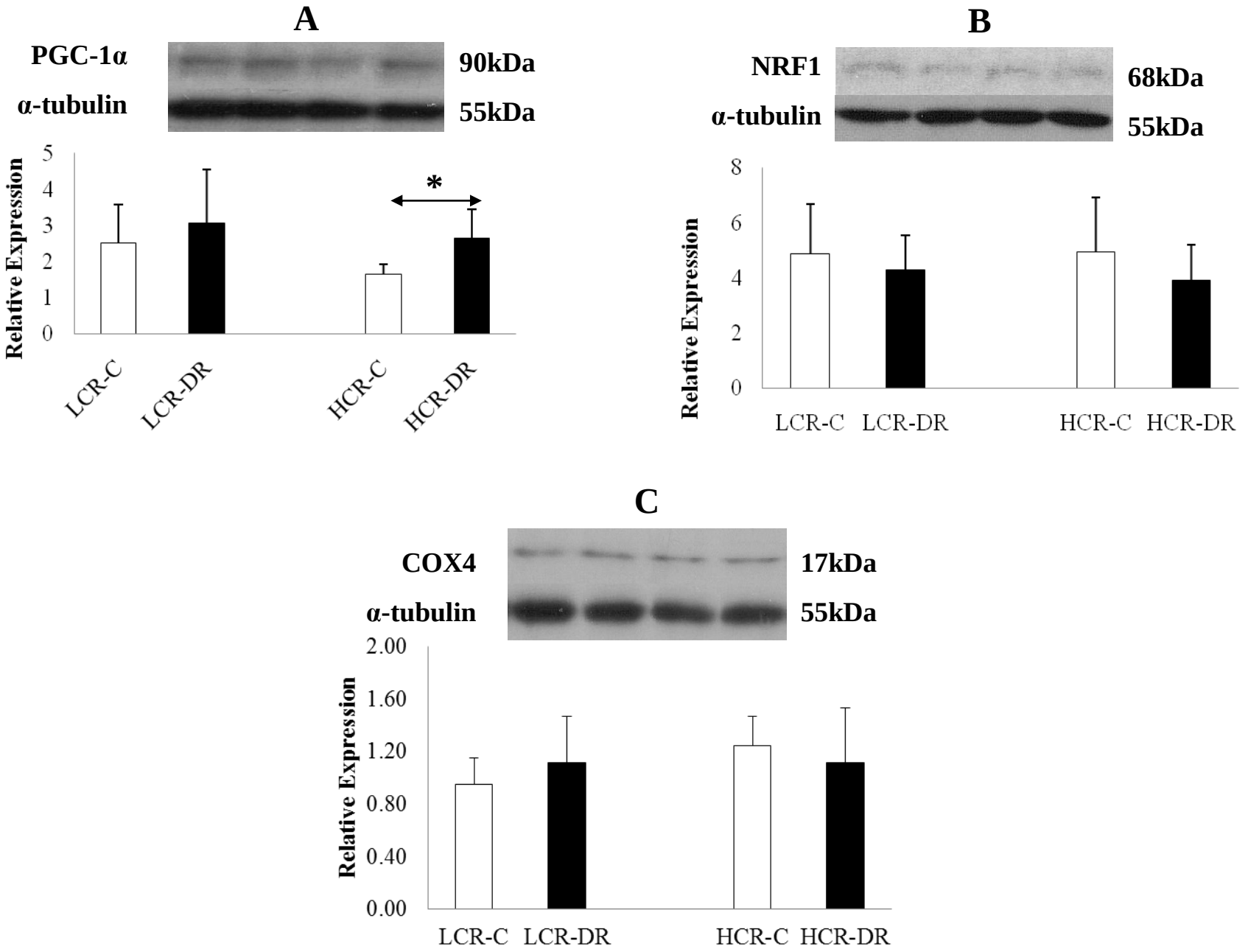
Figure(s)

Fig 3. SIRT-1 levels of rat hippocampus



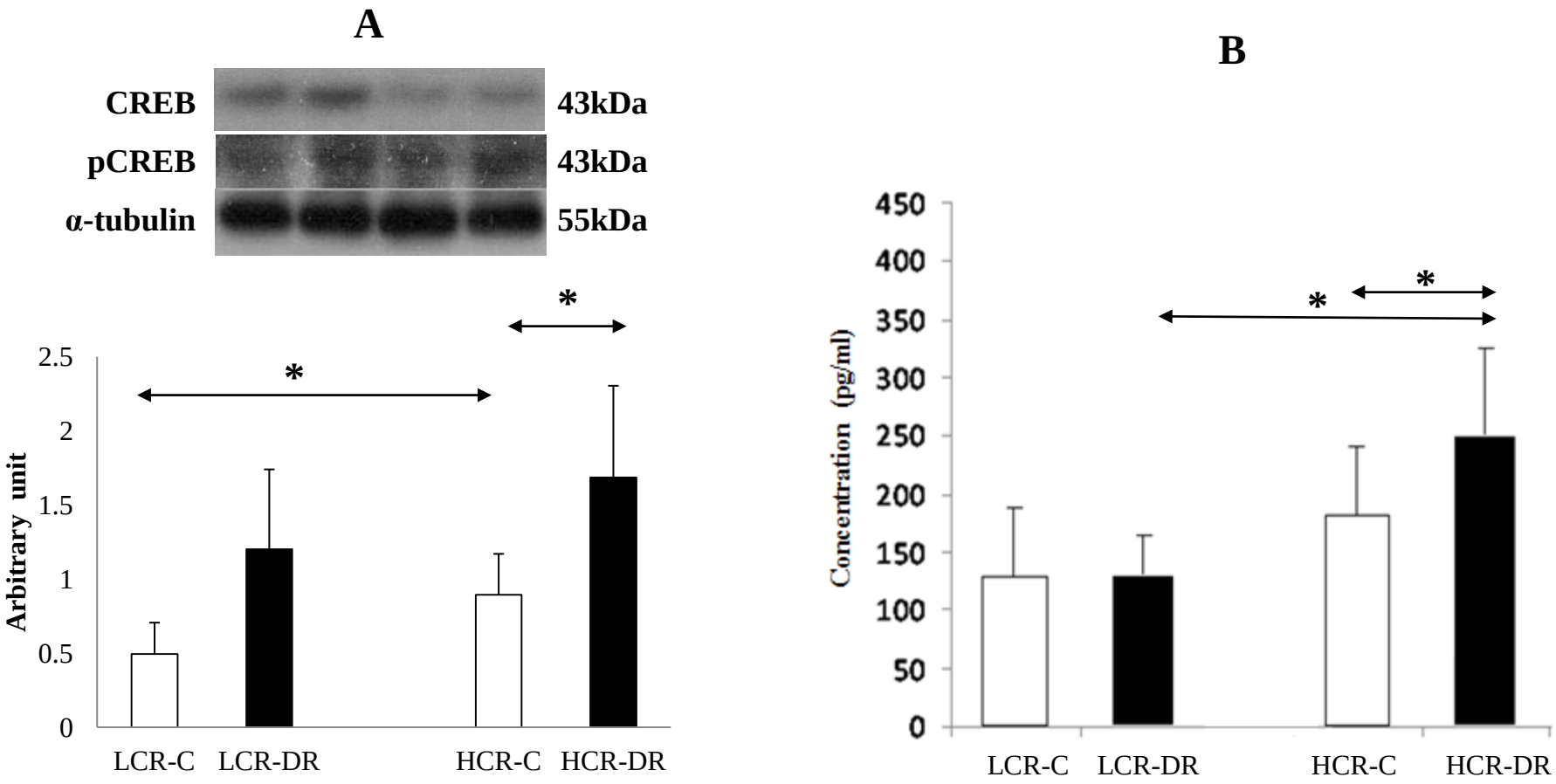
Figure(s)

Fig 4. PGC-1α (A), NRF1 (B), COX4 (C) Western blot



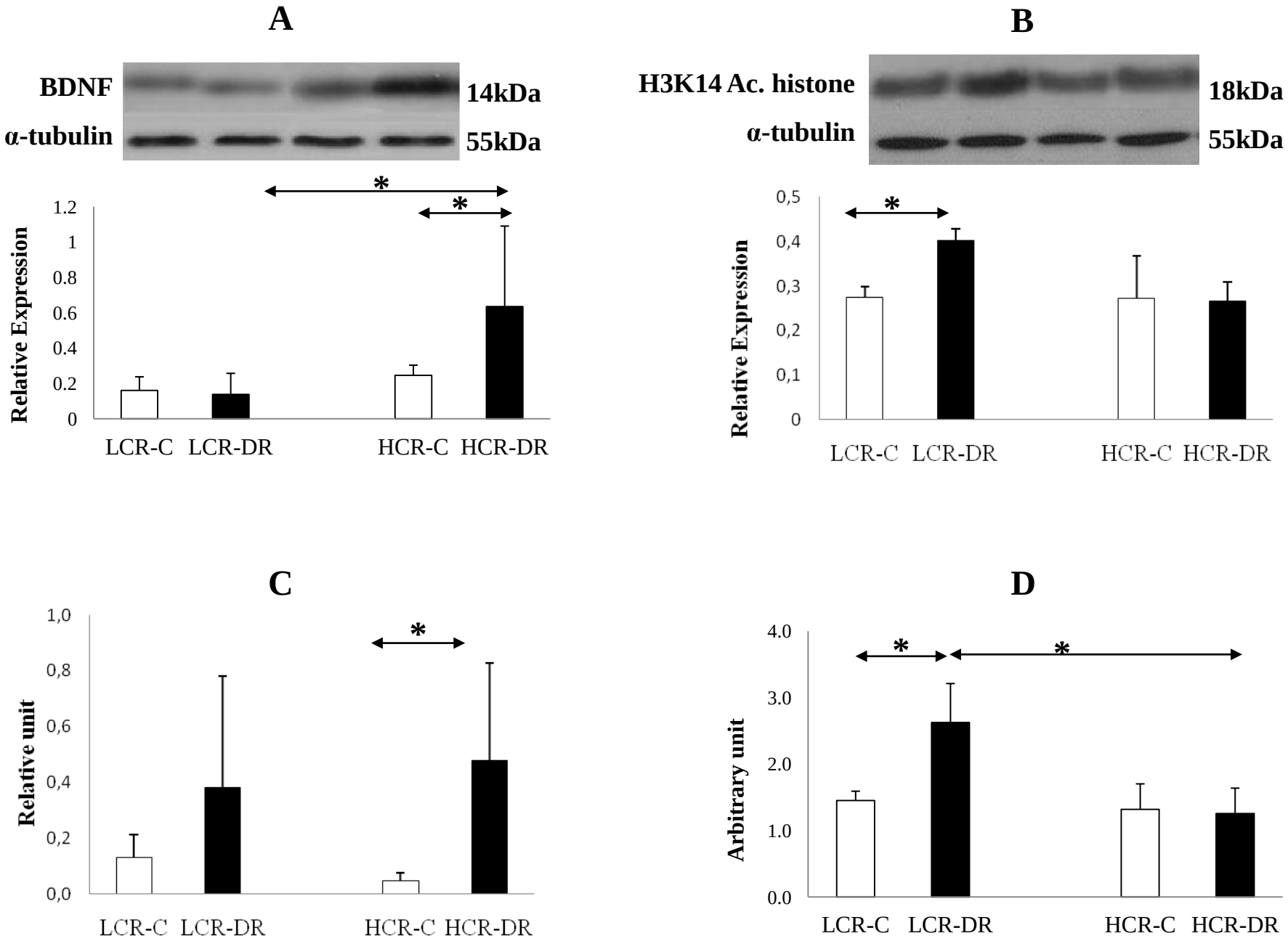
Figure(s)

Fig 5. pCREB/CREB ratio and BDNF levels



Figure(s)

Fig 6. BDNF (A), H3K14 Acetilated (B), BDNF ChIP (C), BDNF mRNA (D)



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