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Exercise increases leptin levels correlated with IGF-1 in hippocampus and prefrontal cortex of adolescent male and female rats

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ABSTRACT

It is known that regular aerobic exercise has positive effects on hippocampus and prefrontal cortex. We have previously have been able to demonstrate that aerobic exercise increased IGF-1 in hippocampus. Leptin, which is associated with cognitive functions, is also involved in fat metabolism and stimulates energy consumption. While it is known that leptin stimulates IGF-1 production in hepatocytes, little known is on the link between IGF-1 and leptin in brain during aerobic exercise.

In this study, we investigated the effects of regular aerobic exercise on leptin, leptin receptor expression levels in hippocampus and prefrontal cortex. Additionally, we investigated the correlation of IGF-1 levels with leptin and leptin receptor expression. During the experiment, exercise group was run on a treadmill for 30 min per session at a speed of 8m/min and 0° slope, five times a week for 6 weeks. Leptin, leptin expression, IGF-1 levels and cell numbers increased in prefrontal cortex and hippocampus of exercise groups. Blood leptin levels increased in female rats in exercise group; whereas it did not change in male rats; blood IGF-1 levels were found to be increased in exercised male rats. There was a strong positive correlation between hippocampal leptin levels and hippocampal IGF-1 levels; also a strong positive correlation between hippocampal leptin receptor expression and hippocampal IGF-1.

These results indicate that, increased leptin and leptin receptor expression are correlated with IGF-1 in regular aerobic exercised rats. Blood leptin and IGF-1 levels were also found to be associated with gender. Females had high blood leptin levels and males had high blood IGF-1 levels in the exercise groups.

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1. Introduction

It is known that regular aerobic exercise improves cognitive functions in animals and humans (Cetinkaya et al., 2013; Uysal et al., 2005; Jedrziewski et al., 2010; Knöchel et al., 2012). In our previous two studies, we demonstrated that regular aerobic exercise could improve learning and memory in correlation with increased hippocampal neuron density. These study findings also supported that regular aerobic exercise resulted in increase of blood and hippocampal IGF-1 (Cetinkaya et al., 2013; Uysal et al., 2005).

IGF-1 is an essential regulator for brain development, maturation and neuronal survival (Aksu et al., 2012; Aleman and Torres-Alemán, 2009; Ozdemir et al., 2012). Many tissues in the body such as liver, brain, muscles and skin produce IGF-1. Liver produces approximately 70% of the total serum IGF-1 in response to pituitary growth hormone (Torres-Aleman, 2010). IGF-1 mediates the action of growth hormone, which controls IGF-1 production (Frank, 2001). IGF-1 passes the blood-brain barrier (Reinhardt and Bondy, 1994), and has important neurocognitive functions in adults (Aksu et al., 2012; Ozdemir et al., 2012; Trejo et al., 2008). In adolescent rat brain, serum IGF-1 directly regulates hippocampal IGF-1 and modifies gene expression (Yan et al., 2011).

Leptin is an anorexigenic (appetite suppressant) peptide hormone that is involved in brain development by regulating appetite. Leptin (an adipokine) is expressed in both adipose tissue

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and central nervous system. It also can pass through the blood brain barrier (Ahima et al., 1999). Leptin receptor mRNA is expressed in hypothalamus, hippocampus and prefrontal cortex (PFC) of humans and rodents (Wada et al., 2014; Couce et al., 1997; Dayi et al., 2012; Ates et al., 2014; Elmquist et al., 1998). The brain weight and myelin content were shown to be decreased in leptin deficient mice (Bereiter and Jeanrenaud, 1979; Sena et al., 1985), and intraperitoneal leptin treatment increased cortical volume and brain size in these mice (Steppan and Swick, 1999). Leptin stimulates neurogenesis, synaptogenesis, axon and dendrite morphology and growth (Paz-Filho, 2016). Leptin exhibits neuroprotective effects by inhibiting apoptotic cell enzymes, which as a result supports cell survival (Morrison, 2009; Teryaeva, 2015.). It also has a role in increasing synaptic transmission by enhancing excitability of neurons (Harvey, 2007) and increasing synaptic motility and density; particularly in hippocampal neurons (O'Malley et al., 2007).

Leptin and IGF-I have roles in signaling the energy status between brain and body (Li et al., 2005). IGF-1 increases leptin expression and leptin regulates back IGF-1 expression (Li et al., 2005). Recently, Won et al. demonstrated that, leptin has also a role in modulating endocrine growth by enhancing hepatic IGF-1 gene expression in liver cells (Won et al., 2016). In this study, we aimed to investigate the effects of regular aerobic exercise on leptin level, leptin receptor expression and their correlation with IGF-1 levels in hippocampus and PFC in male and female rats.

2. Materials and methods

All experiments were performed in accordance with the guidelines provided by the Experimental Animal Laboratory and approved by the Animal Care and Use Committee of Dokuz Eylul University, School of Medicine (ethic approve number 32/2012). Twenty-five-day old male and female Wistar Albino rats were kept in individual cages with free access to water and laboratory chow. Cages were kept at a 12-h-light/dark cycle at constant room temperature (21 °C), and humidity (60%). Thirty-two rats were used, which were divided into four groups; sedentary females, sedentary males, treadmill exercised females, treadmill exercised males.

Exercised groups were acclimated to the treadmill training at 5 m/min 0° slope, for 10 min/day, 5 days for a week. Exercise groups performed treadmill training that rats were run on the treadmill at a speed of 8 m/min, 30 min every day for, 5 consecutive days each week for a total period of 6 weeks (Uysal et al., 2005; Cetinkaya et al., 2013). Control group was taken to the training room and subjected to identical handling.

Following exercise period, one week later, all animals were euthanized by decapitation under ether anesthesia. Brain tissue was removed and one hemisphere was fixed in 10% formalin in phosphate-buffer for histological-examination. Hippocampus and prefrontal cortex tissues were extracted from the contralateral hemisphere for biochemical-analysis.

Tissue samples were fixed in 10% formalin in phosphate buffer overnight, then dehydrated through a series of increasing concentration of ethanol (60%, 70%, 80%, 90%, absolute ethanol) and xylene and then, and embedded in paraffin blocks. Brain was sliced into sequential 5 μm coronal sections using a microtome (Thermo Finesse M+, USA). Immunohistochemical staining procedure was performed using the streptavidin/biotin method (85-9043, Invitrogen, Camarillo, CA). The immunohistochemistry procedure for anti-leptin (1:100, ob, sc-842, Santa Cruz Biotech., CA, USA), leptin receptor (1:100, OB-R, sc-1834, Santa Cruz Biotech., CA, USA) and IGF-1 (1:100, bs-0014R, Bioss, USA) antibodies was performed. Briefly; the sections were incubated overnight with anti-leptin, leptin receptor and IGF-1 antibodies and then for another 30 min with the biotinylated mouse secondary antibody. The bound secondary antibody was then amplified with Histostain® plus bulk kit (Invitrogen, 85-9043). The antibody-biotin-avidin-peroxidase complexes were visualized using DAB. The sections were finally mounted onto lysine-coated slides.

The images were analyzed by using a computer-assisted image analyzer system, consisting of a microscope (Olympus CX41, Japan) equipped with a high-resolution video camera (Olympus DP25, Japan). Immunohistochemical evaluation was performed based on the intensity of leptin immunoreactivity in the rat hippocampus and prefrontal cortex (Paxinos and Watson, 1982). Immunoreactivity was graded as follows: More than 10% of the cells staining were graded as positive. No detectable staining or <10% of cells staining was graded as negative. The semiquantative intensity of staining for IGF-1 was assessed using a scale from "0" to "+++", where (0), none; (+), mild (1); (++), moderate (2); and (+++) represents the strongest (3) stain (Kiray et al., 2014; Cetinkaya et al., 2013).

Serum leptin levels were determined by a radioimmunoassay method (sensitivity limits: 0.63 ng/ml, 50 ng/ml, 2.0–4.6%, 3.0–5.7%)(RL–83 K, Millipore, Billerica, MA). Prefrontal cortex and hippocampus tissue homogenate was analyzed by enzyme immunoassay for leptin (Catalog Number EK0438, Boster Immunoleader, Wuhan, China; with assay sensitivity <10 pg/ml and range 62.5–4000 pg/ml) and IGF-1 (Catalog Number EK0377, Boster Immunoleader, Wuhan, with assay sensitivity <5 pg/ml and range 62.5 pg/ml–4000 pg/ml). Tissue protein contents were determined using a total protein kit (Randox Labs., Crumlin, UK).

PFC and hippocampus tissues were homogenized individually with Qiagen TissueLyser-II homogenizer in 1 mL QIAzol lysis reagent and total RNA was isolated using the RNeasy lipid tissue mini kit (Qiagen, GmbH, Germany) according to the manufacturer's instructions. DNA contamination was removed by optional on-column DNase-digestion using the RNase-Free DNase Set (Qiagen, GmbH, Germany). The quantity of total RNA was measured by the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The integrity of total RNA was analyzed in a MOPS-buffered 1.2% agarose gel containing formaldehyde and confirmed by visualization with EtBr-staining and UV illumination. The absence of contaminating genomic DNA was also confirmed by setting reverse transcriptase minus (RT-) negative control reaction. The cDNA synthesis was performed with a RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific Inc.) using 2 μg of total RNA according to the manufacturer's instructions.

Specific rat primer pairs and probes for reference and target genes for real-time polymerase chain reaction (PCR) analysis were designed using the ProbeFinder Software, which is available online (www.universalprobelibrary.com). β-actin was used as the reference gene. The UPL (universal probe library) probe number for the leptin receptor gene is #118 and the primer sequences were as follows: Forward: 5′-TGTCAGAAATTCTATGTGGTTTTGT–3′, Reverse:5′-TTGGATAGGCCAGGTTAAGTG (76 bp). The UPL probe number for β-actin gene was #115 and the primer sequences were as follows: Forward: 5′-CTAAGGCCAACCGTGAAAAG-3′; Reverse: 5′-GCCTGGATGGCTACGTACA-3′ (79 bp). All PCR reactions were performed using LightCycler®480.

Probes Master in a 20 μL reaction mixture (2 μL cDNA; 10 μL LightCycler® 480 Probes Master; 2 μL (10 mM) each specific gene primer pairs and upl probes) in a LightCycler®480 Instrument, 96-well format (Roche Diagnostics, Indianapolis, Ind.). The PCR program was initiated at 95 °C for 10 min followed by 40 cycles, each for 10 s at 95 °C, 30 s at 60 °C, 1 s at 70 °C and 30 s at 40 °C for cooling. Negative control, consisting of real time RT PCR reaction mixture using sterile distilled water instead of template RNA, was included in each batch of PCR. In addition, repeatability and

reproducibility of the assays were assessed by performing 3 replicas. Each amplification cycle was analyzed with the Light-Cycler®480 software.

Statistical analysis performed by using SPSS 15.0. Differences between groups were analyzed using one-way ANOVA, post hoc Bonforroni comparison. Also, Man Whitney-*U* test was used for analyzing sex differences. Correlations among groups were assessed by using Pearson correlation analysis. Results are presented as mean ± S.E.M, where $p < 0.05$ is considered statistically significant.

3. Results and discussion

Initial body weight was same for all groups (females, 35.45 ± 1,7; males, 37.28 ± 1,4) and no statistical difference in weight was found at the end of the experiment ($p > 0.05$; exercised females, 164.23 ± 2.6; control females, 178.07 ± 3.1; exercised males, 175.86 ± 2.8; control males, 187.52 ± 3.6)

In PFC and hippocampus, leptin, leptin receptor and IGF-1 levels were increased in both exercise groups in comparison to the sedentary group of same sex (all regions in all groups; leptin $p < 0.001$, leptin receptor $p < 0.0001$, IGF-1 $p < 0.0001$) (Fig. 1A, B, C, D, F and G). While blood leptin level was increased in exercised female group, serum IGF-1 level was increased in exercised male group (serum leptin $p < 0.0001$; serum IGF-1 $p < 0.003$) (Fig. 1E and H). Additionally, statistical difference was found between exercised males and females in PFC leptin and leptin receptor levels as well as hippocampal leptin receptor levels (all comparisons, $p < 0.05$). In addition, there was a significant difference between control males and females in PFC leptin levels and leptin receptor levels and serum leptin levels (PFC leptin and leptin receptor levels $p < 0.008$; blood leptin levels $p < 0.05$).

In immunohistological examination, leptin, leptin receptor and IGF-1 reactivity were found to be increased in the exercise groups (Fig. 2A, B and C) (Table 1). The cell numbers were found to be increased in CA1 and dentate gyrus in hippocampus; and prefrontal cortex of exercised animals (hippocampus, $p < 0.003$ and prefrontal cortex, $p < 0.002$ both group of).

In correlation analysis; a very strong positive correlation between hippocampal leptin and hippocampal IGF-1 levels ($r = 0.785$, $p = 0.0001$); a strong positive correlation between hippocampal leptin receptor expression and hippocampal IGF-1 levels ($r = 0.519$, $p = 0.001$); a strong positive correlation between hippocampal leptin receptor expression and blood IGF-1 levels ($r = 0.572$, $p = 0.0001$); finally, a strong positive correlation between blood and hippocampal leptin levels ($r = 0.614$, $p = 0.001$) were found.

Our findings strongly suggest that regular aerobic exercise increases leptin level and leptin receptor activity in PFC and hippocampus. Results not only further support the positive correlation between aerobic exercise and IGF-1, but they also provide new evidence for positive correlations between leptin levels and IGF-1 levels in these brain areas. We believe that this result could help us further understand the complicated physiological workings of IGF-1 and leptin. Our study supports that hippocampal leptin and leptin receptor expression levels are correlated with hippocampal IGF1 levels. To our knowledge, no prior study has yet investigated the effects of regular exercise on leptin, leptin receptor and IGF-1.

Leptin receptors, which are expressed in hippocampus (Shanley et al., 2001; Li et al., 2002), modulate presynaptic neurotransmitter release in hippocampus and increase the neurotransmitter sensitivity of postsynaptic neurons (Oomura et al., 2006). It was actually shown that leptin has a very favorable effect in long term potentiation (LTP) by improving hippocampal functions and synaptic plasticity (Shioda et al., 1998; Shanley et al., 2001; Wayner et al., 2004). It involves in the process of conversion of LTP to spatial memory within hippocampus by up-regulating N-methyl-D-aspartate (NMDA) receptors (Bliss and Collingridge, 1993). In conjunction with these findings, hippocampal leptin injection was shown to facilitate LTP, which was found to be correlated with improved learning and memory performance (Farr et al., 2006; Dayi et al., 2012; Wayner et al., 2004). In our study, regular aerobic exercise increased leptin, as well as leptin receptor expression in hippocampus and prefrontal cortex of both male and female rats. It should be also mentioned here that, in our previous studies, regular aerobic exercise was found to improve hippocampus-dependent learning and memory (Uysal et al., 2005; Cetinkaya et al., 2013). It is a well-established fact that prefrontal cortex and hippocampus are essentially important for learning-memory formation and executive function (Kupfermann, 1991; Koechlin et al., 2003).

IGF-1 is important in brain development and neuronal viability (Aleman and Torres-Alemán, 2009). IGF-1 increases both neurogenesis and synaptogenesis in the brain (O'Kusky et al., 2000). It is known that IGF-1 reduces G1 phase length and accelerates cell cycle process, increases cell cycle re-entry and promotes neuronal proliferation (Hodge et al., 2004). IGF-1 levels can be affected easily by various factors, such as brain hypoxia/ischemia injury (Guan et al., 2003), traumatic brain injury (Ozdemir et al., 2012) and exercise (Cetinkaya et al., 2013; Uysal et al., 2014). In our study, regular aerobic exercise increased IGF-1 levels in both hippocampus and prefrontal cortex of rats.

We have found that IGF-1 increased the expression of leptin in hippocampal slices. This finding seems to be important because it is known that leptin is a compound involved in hippocampal synaptic function and endocrine neurobiology (Marwarha et al., 2011). Additionally, IGF-1 is known to activate mammalian target of rapamycin complex 1 (mTORC1) which functions as an integrative protein that modulates synaptic information and plays important roles in cognitive processing (Grimberg, 2003). It is known that mTORC1 increases leptin expression activity and there is also evidence to suggest that leptin may be involved in IGF-1 expression (Li et al., 2005). It is shown that, IGF-1 treatment increased leptin expression in rabbit hippocampus (Marwarha et al., 2011). Consistent with these previous findings, in the current study, our findings also support a positive correlation between hippocampal leptin and IGF1 levels; in addition a positive correlation between hippocampal leptin receptor expression and IGF1 levels.

It is known that, blood IGF1 and leptin levels are affected by many factors such as gender, weight, nutrition and exercise (type and intensity) (Balaskó et al., 2014; Eliakim et al., 1998; Gomes et al., 2006; Leme et al., 2007; Rosendal et al., 2002; Koehler et al., 2013; Caton et al., 2012; Xie and Wang, 2013). According to a previous study, plasma leptin concentration was high ($p < 0.05$) and IGF-I concentration was low with low carbohydrate-high fat diet, ($p < 0.01$) (Caton et al., 2012). It was found that during carcinogenesis induced weight loss, blood IGF-1 and leptin levels decreased significantly (Xie and Wang, 2013). In another study it was found that acute strenuous exercise in human males decreased plasma leptin levels and increased plasma IGF-1 levels (Elias et al., 2000). Additionally, it was found that more than 5 weeks of mild exercise increased blood IGF-1 levels; whereas shorter than 5 weeks of exercise decreased blood IGF-1 levels in adolescent human males (Eliakim et al., 1998; Gomes et al., 2006; Leme et al., 2007; Rosendal et al., 2002). Similarly, in another study, it was also found that, physical exercise did increase serum IGF-1 levels in human males (Eliakim et al., 1998), whereas it did not change blood IGF-1 levels in females (Eliakim et al., 2013). Consistent with human studies, in our study, serum IGF-1 levels increased in exercised male rats, but unchanged in female rats.

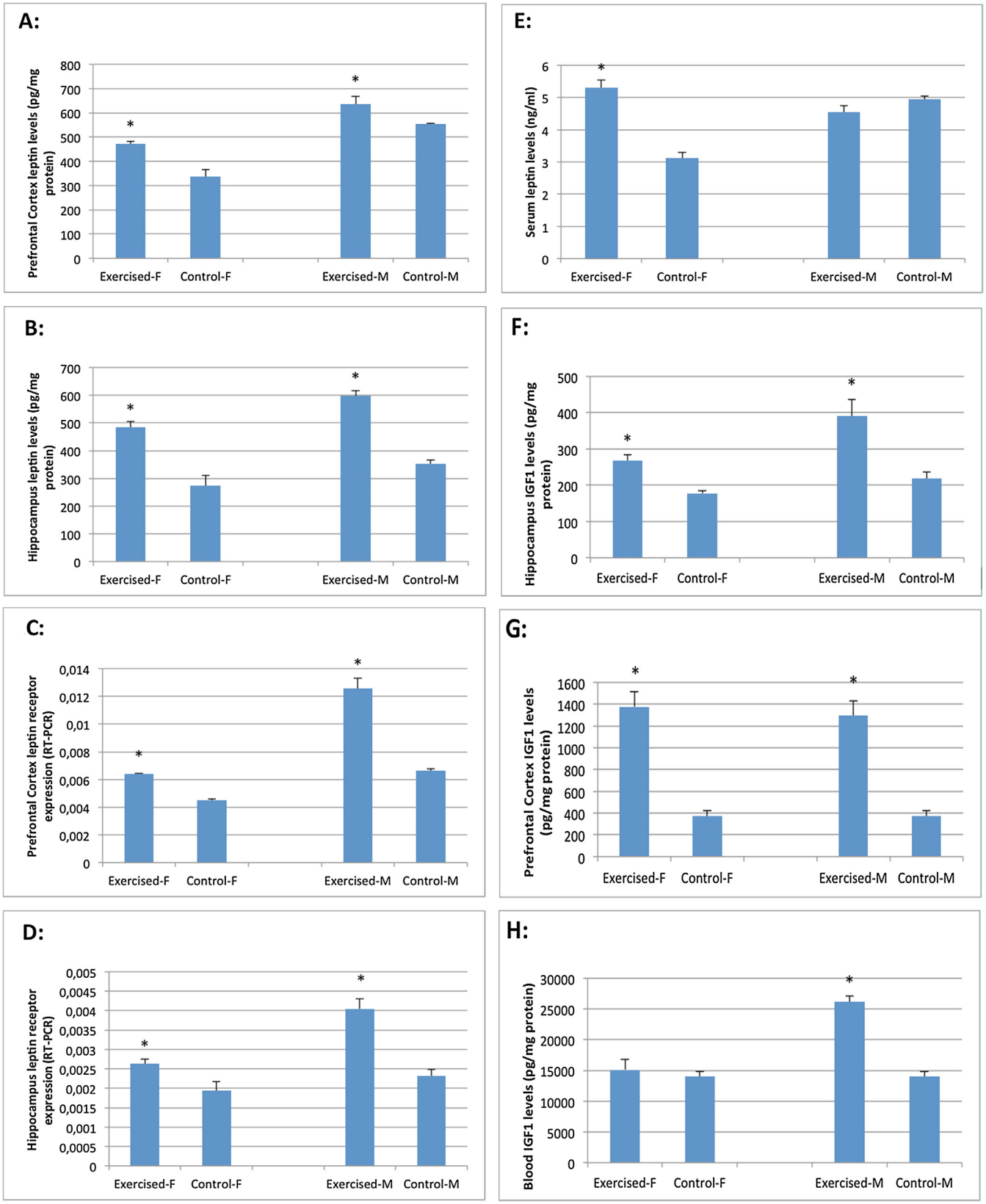


Fig 1. Leptin and leptin receptor results. (A) Prefrontal cortex leptin levels, (B) Hippocampal leptin results, (C) Prefrontal cortex leptin receptor expression levels, (D) Hippocampal leptin receptor expression levels, (E) Serum leptin levels. *$p < 0.05$ compared with control group of same sex, (F) Hippocampal IGF1 levels, (G) Prefrontal cortex IGF1 levels, (H) Serum IGF1 levels. *$p < 0.05$ compared with control group of same sex.

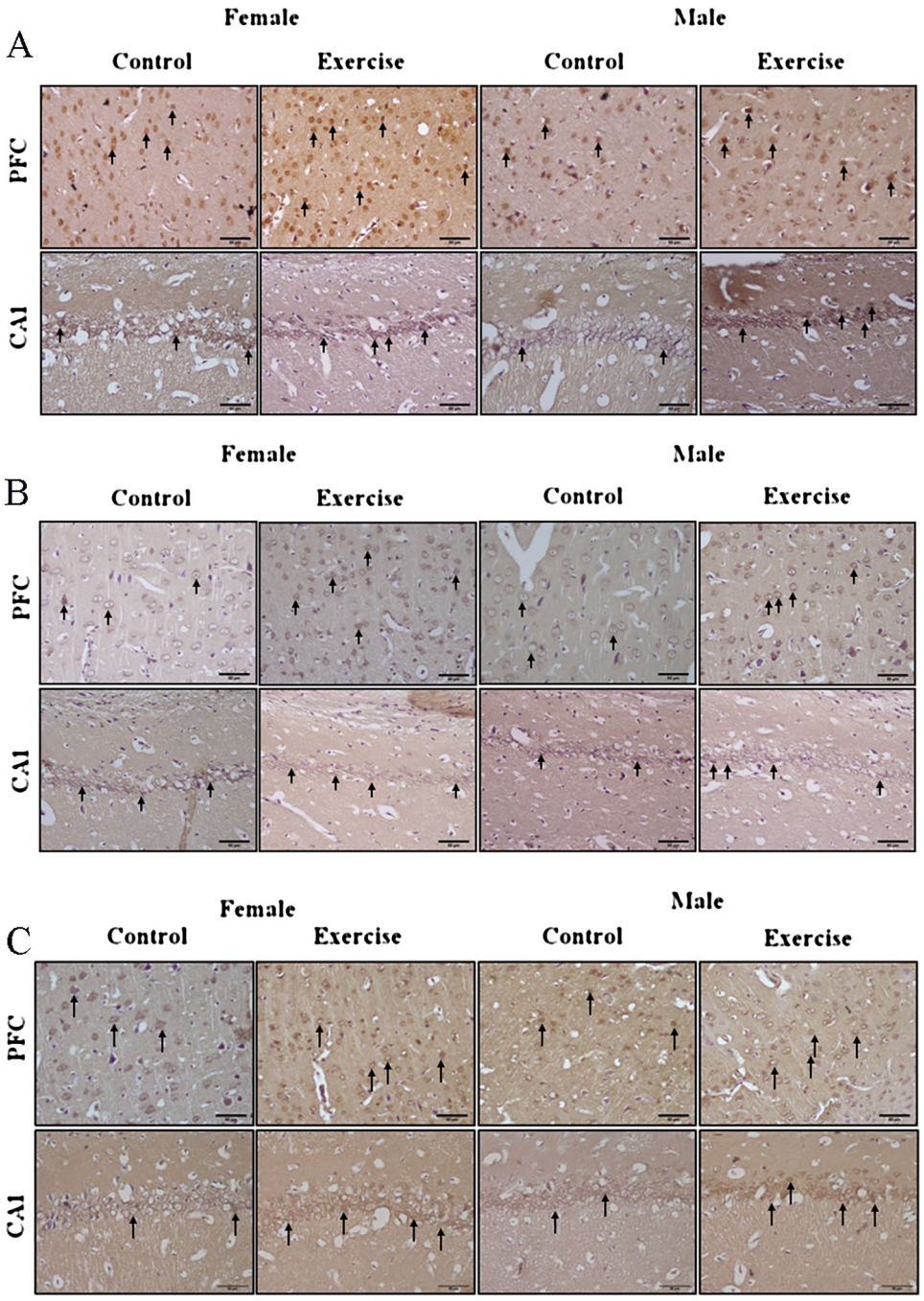


Fig. 2. Histologic evaluation results. (A) Representative photomicrographs of leptin (+) immune staining cells in hippocampus CA1 region and prefrontal cortex (B) Representative photomicrographs of leptin receptor (+) immune staining cells in hippocampus CA1 region and prefrontal cortex, (C) Representative photomicrographs of IGF-1(+) immune staining cells in hippocampus CA1 region and prefrontal cortex (arrows), Scale bar: 50 μm.

Table 1
Quantitative evaluation of IGF-1, leptin and leptin receptor immunostaining. Leptin, leptin receptor and IGF-1 immunostaining qualitative intensity of staining were also assessed using a scale between 0 and +++. With 0 representing no detectable stain and +++ representing strongest stain. $^{*}p < 0.05$ compared with control of same sex.

	Prefrontal Cortex						Hippocampus					
	Leptin		Leptin receptor		IGF-1		Leptin		Leptin receptor		IGF-1	
Exercised-F	2,8 ± 0,2*	+++	2,6 ± 0,2*	+++	2,6 ± 0,2*	+++	2,8 ± 0,2*	+++	2,8 ± 0,2*	+++	2,8 ± 0,2*	+++
Control-F	2 ± 0,3	++	1,6 ± 0,2	+	1,6 ± 0,2	+	2,2 ± 0,2	++	1,8 ± 0,4	+	1,8 ± 0,2	+
Exercised-M	2,8 ± 0,2*	+++	2,8 ± 0,2*	+++	2,8 ± 0,2*	+++	2,8 ± 0,2*	+++	2,8 ± 0,2*	+++	2,8 ± 0,2*	+++
Control-M	1,8 ± 0,2	+	1,8 ± 0,2	+	1,8 ± 0,2	+	2 ± 0,3	++	2 ± 0,3	++	1,6 ± 0,2	+

As previously shown, plasma leptin levels are higher in women than in men, because of they had higher adipose mass (Baumgartner et al., 1999). High blood leptin levels caused burning of fats (Lafontan and Langin, 2009). Serum leptin levels may be more sensitive from external or internal exposures in females. In our study, serum leptin levels were increased with exercise in female rats, but unchanged in male rats.

4. Conclusion

This study not only demonstrated that regular physical aerobic exercise increased IGF-1, leptin and leptin receptor expression in PFC and hippocampus of male and female rats but it also revealed a very strong positive correlation between hippocampal leptin/leptin receptor expression and IGF-1. This finding may be important, since it could provide the link between complicated interactions of IGF-1 and leptin neurobiology in hippocampus and PFC, the areas known for their important roles in memory, learning and executive function. Evidence supporting the role of aerobic exercise in IGF-1 physiology is growing and our findings confirmed the positive correlation of exercise on hippocampal and cortical IGF-1. We also revealed the positive correlation between aerobic exercise and leptin, suggesting that leptin levels could be modified with aerobic exercise, which could be an area of human research. In addition this study showed that serum IGF-1 and leptin levels of gender are different with exercise. The serum leptin levels high in trained female rats. Leptin signaling continues resting period, so that trained female rats continue fat burning. Further animal and human studies are needed to clarify the complex workings of leptin and IGF-1 in the higher cortical areas and hippocampus, their involvement in important cognitive functions and their relationship with aerobic exercise.

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