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Pulsed EMF stimulation increased BDNF and activated S6 levels in the hippocampus of senescent rats

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ORIGINAL ARTICLE

ABSTRACT

Purpose: Low-frequency electromagnetic field (EMF) exposure in rat has positive effects on neuronal processes *in vitro*. Moreover, EMF improves learning-memory and psychomotor activity during advanced ageing, but the underlying molecular mechanisms are not known in the brain. In the present study we aimed to investigate the molecular effects of chronic EMF stimulation in the hippocampus of senescent rats *in vivo*. *Materials/Methods:* Thirty months old rats were treated for six weeks with different EMF doses of 45, 95, and 1,250 μT. After sacrifice the levels of Brain Derived Neurotrophic Factor (BDNF) and activated ribosomal protein S6 as measures for protein synthesis intensity in the hippocampus were determined by Western blot analysis. *Results:* The results showed that chronic EMF exposure dose dependently increased BDNF and the amount of phosphorylated S6 protein at the highest dose. The effects on the two proteins positively correlated at individual level. The results indicate that EMF exposure may enhance neurotrophic processes indicated by increased BDNF expression in the hippocampus of senescent rats. Increased phosphorylated S6 protein suggests coupling to support molecular regulation of protein synthesis. *Conclusions:* In a broader perspective, these findings may support EMF as a beneficial alternative form of passive exercise in active, exercise-limited, aged individuals.

KEYWORDS

BDNF, S6, EMF stimulation, senescent age, hippocampus

INTRODUCTION

Recent *in vivo* studies have demonstrated that a number of biological processes can be modulated by the low frequency electromagnetic field (EMF) exposure [1]. For example, biological effects of EMF stimulation lead to gene upregulation, cell differentiation, molecular turnover, and regenerative tissue processes [2]. Regarding the so-called passive forms of physical exercise, the behavioural effects of low frequency electromagnetic field exposure in animals have recently gained increasing interest. The variability of the EMF stimulation parameters such as frequency and duration moves along a wide spectrum in previous studies dealing also with impact on brain and behaviour functions. In our preceding study we found that whole-body EMF stimulation improves learning-memory and psychomotor activity especially during the advanced ageing period of rats [3].

It was previously reported that exercise increases the synthesis and release of BDNF, associated with synaptic plasticity and cell survival [4, 5]. The effect of EMF stimulation to

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BDNF exposure remains to be established in aged rodents as well, since only one *in vivo* study is available on this topic and only in the neonatal rat in cultured dorsal root ganglion neurons [6]. The hippocampus, which plays a key role in learning and memory, represents the primary site of neurogenesis and exhibits the greatest potential for neuroplasticity in the adult brain. Moreover, it was reported that BDNF increases global protein synthesis by activating mTOR activation stimulated translation cascade involving p70S6 kinase/rpS6 in neuronal dendrites [7].

In the brain, the ribosomal protein S6 (rpS6) phosphorylation is evoked by a wide variety of pharmacological and physiological stimuli and is considered as a widely used marker to track changes in neuronal activity [8] and in synaptic plasticity [9]. In a mechanistic sense rpS6 phosphorylation has a primary stimulating effect on global protein synthesis in neurons.

In the current study we extended our study on the molecular effects of chronic EMF stimulation in the hippocampus, evoking cognitive support as indicated above, to senescent rats by measuring the levels of BDNF and activated S6 protein in this brain structure.

MATERIALS AND METHODS

Animals and the EMF exposure

Senescent, 30–32 months old male Wistar rats were selected for this study. Animals were housed in a room maintaining 22 ± 1 °C with a 12:12 h light/dark cycle with the light period starting at 7:00. Food and water were available *ad libitum* and two rats were housed per cage. The methodological details of pulsed EMF stimulation were previously described [3] including the instrument used (Sanza, Santerra MCR System, Piding, Germany). Briefly, EMF stimulations started at the age of 30 month and were continued for 6 weeks (Fig. 1). Each daily stimulation session lasted for 24 min. The animals were randomly divided into four experimental groups: control group ($n = 6$), 45 μT group (with 45 μT EMF exposure, $n = 6$), 95 μT group (with 95 μT EMF exposure, $n = 6$), 1,250 μT group (with 1,250 μT EMF exposure, $n = 6$).

Forty-eight hours after the last EMF exposure the animals were sacrificed by decapitation under light CO_2 anaesthesia and the brains were quickly removed on an ice-cooled glass plate. The hippocampus was excised and immediately frozen on dry ice. The samples were stored at −80 °C until processing.

Western blot analysis

The hippocampus of each animal was homogenised on ice and lysed in a lysis buffer containing 137 mM NaCl, 20 mM Tris-HCl pH 8.0, 1% Nonidet P-40, 10% glycerol, protease, and phosphatase inhibitors. Lysates was centrifuged for 15 min at 14.000 g at 4 °C. Protein concentration was measured using the Bradford assay. Protein (40 μg) was separated on 10–15% (v/v) SDS-PAGE (sodium dodecyl sulphate-polyacrylamide) gels at room temperature (RT) and transferred onto PVDF membrane (pore size: 0.2 and 0.4 μm) at 4 °C. The nonspecific binding of immune-proteins was blocked with 5% BSA (bovine serum albumin) dissolved in Tris-buffered saline Tween 20 (TBS-T) for 1 h at RT. After blocking, the membranes were incubated with primary rabbit BDNF antibody (1:1500; Alomone Labs), rabbit S6 Ribosomal Protein (1:5000; Cell Signaling), rabbit Phospho-S6 Ribosomal Protein (1:5000; $Ser^{235/236}$; Cell Signaling) and mouse α-Tubulin (1:60000; Sigma) overnight at 4 °C. Antibodies were dissolved in TBS-T containing 5% BSA. After overnight incubation the membranes were rinsed in TBS-T attended by 1 h incubation with HRP-conjugated secondary antibodies at RT. The secondary antibodies were: anti-rabbit and anti-mouse IgG in TBS-T containing 1% BSA (1:10000; Jackson Immunoresearch). Between incubation times, the membranes were washed repeatedly (3x15 min) and after the last wash we incubated with an enhanced chemiluminescent reagent (ECL Star Enhanced Chemiluminescent Substrate; Euroclone) for 1 min. The protein bands were visualised on X-ray film. Bands were quantified by ImageJ software and standardised to α-Tubulin.

Statistical analysis

For evaluation of the numerical results, we applied the Statistica 13.2 program. Following one-way ANOVA analysis the Tukey's post hoc *t*-test was used to compare two groups. Mean±SEMs are shown in the diagrams where post hoc *t*-test results are all depicted. For correlation analysis between BDNF versus p-S6/S6 ratio we used a parametric correlation test of Statistica 13.2. Statistical significance was established at $\alpha = 0.05$, $P < 0.05$.

RESULTS

EMF exposure increased BDNF in the hippocampus (ANOVA, $F(3,20) = 27.80$, $P < 0.001$) dose dependently (Fig. 2). *Post hoc t*-test revealed significant differences at the lower ($P < 0.001$), middle ($P = 0.004$), and highest ($P < 0.001$) doses as it is presented on the left side of Fig. 2. Regarding between groups effects, the two lower doses were not significantly different, but both of these intensities differed from the highest EMF dose (at 45 μT $P < 0.041$; at 95 μT $P < 0.001$). In other words, EMF increased the level of

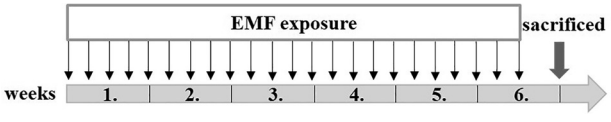

Fig. 1. Experimental design in the course of a 6week-period. The experimental groups were exposed to EMF stimulation of 24 min, 5 times per week. Three different doses of stimulation were used (45, 95, and 1,250 μT). Forty-eight hours after the last EMF session the animals were sacrificed

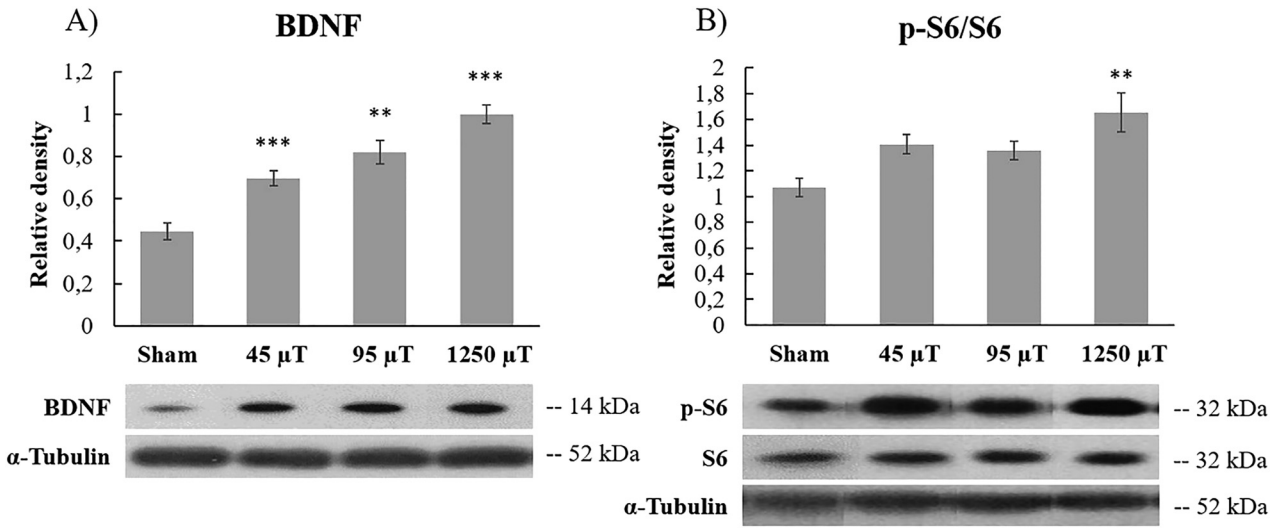

Fig. 2. Effects of EMF exposure on BDNF (A) and p-S6/S6 (B) levels in the hippocampus of senescent rats indicated by Western blot analysis. The EMF treatment was dose dependently effective to increase the levels of BDNF shown by columns (**$P < 0.01$, ***$P < 0.001$ vs. Sham control group, $n = 6$ in all groups). Activation of S6 protein was significantly enhanced by the highest dose of EMF stimulation (1,250 µT) compared to the sedentary control group (**$P < 0.01$, $n = 6$ in all groups). Lower panels: the representative Western blots show the immunoreactivities of BDNF, S6, p-S6 and α-Tubulin by a selected animal closest to the mean levels from each group

BDNF by 33% at the 45 µT, by 45% at the 95 µT, and by 55% in the 1,250 µT groups.

At the right side of Fig. 2 the phosphorylation of S6 protein was evaluated by dividing the phospho-specific form by the native S6 protein form while both forms were normalised to α-Tubulin. The result of ANOVA showed a significant overall difference ($F(3,20) = 6.038$, $P = 0.004$). The phosphorylation of S6 molecules increased significantly by the highest dose of EMF treatment ($P = 0.004$, 35% increment) compared to the Sham control group. Regarding the low and middle doses, the increment of phosphorylated S6 protein was 24 and 21% respectively, although these doses did not reach significance against controls by the post-hoc *t*-test. The lower part of the figure shows the densities of immune-reactive Western blot spots taken one animal from each group and the calculated densities were normalised to α–Tubulin.

Evaluating a possible interaction between BDNF vs. p-S6/S6 values in the individual cases a correlation analysis was applied. A positive correlation was found between the BDNF values compared to p-S6/S6 ones ($n = 24$; $r = 0.516$, $P = 0.010$) suggesting the presence of a possible positive impact on protein synthesis.

DISCUSSION

BDNF, a neurotrophin, plays an important regulatory role in the formation, growth, maintenance, survival, and regeneration of neurons. Previous studies showed that exercise enhances the synthesis and release of BDNF, associated with synaptic plasticity and cell survival [4, 5]. Importantly, the most consistent associations between BDNF and hippocampal function/dysfunction have emerged from research on BDNF protein expression in rodents and serum and plasma concentrations of BDNF in humans [10]. Furthermore, BDNF can increase resistance to neurodegenerative disorders and could promote healthy ageing [11, 12].

We have already confirmed the positive effect of different doses of EMF in the hippocampus-dependent cognitive and psychomotor activities in senescent rats [3]. In the present study we have discussed the molecular aspects of chronic EMF exposure on the hippocampus in very advanced aged rats (30–32 months) and have found a dose-related significant enhancement in the levels of BDNF and activated S6 proteins. These findings are partly similar to a previous report, which showed that 6 weeks long treadmill exercise increased the level of BDNF in the hippocampus of young and middle-aged rats [13]. In the present study it may be stressed that even passive type of exercise by EMF exposure may result in similar neurotrophic effect as the active type of treadmill training. This possibility is especially noticeable in the advanced age in which active physical training has limited applicability. This line of reasoning is corroborated by results published earlier [14], accordingly, the EMF treatment markedly enhanced the growth of longest neurites and simultaneously increased the BNDF expression in Neuroscreen-1 cells *in vitro*.

It has also been revealed that BDNF and the activated S6 can reflect further activation in the translation machinery of proteins in synaptoneurosomes [15], which principally can mediate neuroprotective effects [16]. Finally, our results in the interaction analysis of BDNF vs. p-S6/S6 ratio further suggest that BDNF may have modulatory action on the S6 phosphorylation. Recent studies in other laboratories demonstrated this type of modulating effect of BDNF as well, since BDNF increased the level of p-S6 in the hippocampus *ex vivo* [17, 18].

CONCLUSIONS

The impact of EMF, a passive type of exercise, on molecular regulation of protein synthesis in the hippocampus of senescent rats has not been addressed previously. Our data demonstrated that EMF stimulation could increase both BDNF and activated S6 protein levels in the hippocampus, one of the main regulatory brain regions for memory and learning processes, which is especially vulnerable in late advanced age.

Authors' contribution: TT was responsible for data extraction, statistical analysis and writing the article; CN and PGML were responsible for planning and guidance on this paper; RS and GD were responsible for correcting and writing the article.

Ethical approval: All experimental procedures which were carried out on the animals have been conducted in accordance with the Declaration of Helsinki and according to requirements of all applicable local and international standards, and had been approved by the Research Ethics Committee, University of Physical Education, Budapest, under the approval number: TE-KEB/No3/2020.

Conflicts of interest: The authors have no conflict of interest to declare.

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