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Effects of chronic forced swim stress on hippocampal brain-derived neutrophic factor (BDNF) and its receptor (TrkB) immunoreactive cells in juvenile and aged rats

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A type of stress stimulation and age are claimed to affect the expression of brain-derived neurotrophic factor (BDNF) and its receptor - tyrosine kinase B (TrkB) in the hippocampal regions differentially. This study aimed to explore the influence of chronic (15 min daily for 21 days) forced swim stress (FS) exposure on the BDNF and TrkB containing neurons in the hippocampal CA1, CA3 pyramidal cell layers and dentate gyrus (DG) granule cell layer in juvenile (P28) and aged (P360) rats. An immunofluorescence (-ir) method was used to detect BDNF-ir and TrkB-ir cells. Under chronic FS exposure, in the group of juvenile rats a significant decrease in the density of BDNF immunoreactive neurons was observed in CA1 and DG ($p<0.001$), unlike CA3, where it remained unaltered just as the density of TrkB-ir cells in CA1 and DG, but in CA3 the number of TrkB-ir cells was found to grow ($p<0.05$) in comparison with control groups. After chronic FS exposure of aged (P360) rats, the density of BDNF-ir and TrkB-ir cells did not decline in any of the subregions of the hippocampus. In all subfields of the hippocampus, the denseness of BDNF-positive neurons was significantly higher in P360 stressed group, compared with P28 stressed group, but the density of TrkB-ir fell more markedly in P360 than in P28. In conclusion, chronic FS stress influenced the number of BDNF and TrkB immunoreactive neurons only in juvenile animals. The age of rats tested in the chronic forced swim test was a decisive factor determining changes in the density of BDNF-ir and TrkB-ir in the hippocampal structures.

Key words: BDNF, forced swim test, hippocampus, stress, TrkB

INTRODUCTION

The hippocampus, which plays an instrumental role in learning, memory and emotions (Bremner 1999, Lisman 1999, McEwen and Magarinos 2001, Bartolomucci et al. 2002, Richter-Levin 2004, Aggleton and Brown 2005, McEwen and Gianaros 2010), is also engaged in the regulation and control of anxiety response and conditioned fear (Yee et al. 2007). Additionally, this highly sensitive component of the brain possesses a remarkable degree of plasticity (McEwen and Gianaros 2010) and is a key element in the neuroendocrine response (Yan et al. 1997).

The pyramidal layer, located in the CA1-CA3 subfields, and the granular layer, situated in the dentate gyrus (DG) constitute the principal neuronal cell types in the hippocampus. Neurons in CA3 project directly to CA1, while CA3 pyramidal cells receive input from the DG (Amaral and Witter 1989). Extrinsic hippocampal connections with some of the subcortical (e.g., amygdala, hypothalamus) and cortical regions reach the CA3 and CA1 fields, the latter of which surpasses the former in the number of these links (Amaral and Witter 1989, Pikkarainen et al. 1999).

By participating in the termination of hypothalamo-pituitary-adrenocortical (HPA) axis responses to stress (Pizarro et al. 2004, McCormick et al. 2010), the hippocampal formation is one of the main constituents of stress circuit (Herman et al. 1995, Matsuda et al. 1996, Kim and Yoon 1998, Bartolomucci et al. 2002). Previous studies have demonstrated that this structure regulates the HPA axis through inhibiting its activity

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(Jacobson and Sapolsky 1991, Herman et al. 1996) and that hippocampal neurons are hypersensitive to stress (Rot et al. 2009), especially to long-term damaging influence of the glucocorticoids (McEwen and Magarinos 2001, Bartolomucci et al. 2002, Murakami et al. 2005). Long-lasting exposure to stress has been found to remodel hippocampal cells and result in a dysfunction of this brain component (Diamond et al. 1996, Bremner 1999, Sousa et al. 2000, Garcia 2002, Bridges et al. 2008, McEwen and Gianaros 2010).

A significant job in hippocampal biology, entailing interaction with extracellular high-affinity tyrosine kinase receptor B (TrkB), is performed by brain-derived neurotrophic factor (BDNF) (Alleva and Santucci 2001, Huang and Reichardt 2003, Silhol et al. 2005, Takeda et al. 2006, Tapia-Arancibia et al. 2008, Greenberg et al. 2009), a protein, besides TrkB, highly expressed in the hippocampus (Yan et al. 1997, McAllister et al. 1999, Tapia-Arancibia et al. 2004). Due to a diversity of physiological functions that BDNF and TrkB carry out these factors play a major regulatory part in hippocampal neurons throughout one's lifetime (Smith et al. 1995, Sato et al. 2001, Cheng et al. 2003, Mattson et al. 2004, Tapia-Arancibia et al. 2004). Based on their neurotrophic ability, BDNF and TrkB may be important for structural and functional aspects of hippocampal neural adaptive plasticity (Smith et al. 1995, McAllister et al. 1999, Duman et al. 2000, Poo 2001, Sato et al. 2001). In addition, BDNF and TrkB could participate in the stress response in hippampal neurons (Smith et al. 1995, Schaaf et al.2000, Tapia-Arancibia et al. 2004, Pardon et al. 2005, Sirianni et al. 2010).

Earlier researches on the role of BDNF and TrkB in the hippocampus under stress conditions relied mainly on the observations of alterations in the content of these proteins in adult animals during immobilization stress (Smith et al. 1995, Givalois et al. 2001, Rage et al. 2002, Marmigère et al. 2003, Reagan 2007), whereas only a few concerned BDNF or TrkB changes in other ontogenetic stages, or in response to other stressors than the forced swim test (FS) (Murakami et al. 2005). Moreover, to date no reports have been issued into chronic FS stress-induced responsiveness of BDNF and TrkB and their role in juvenile and aged animals exposed to prolonged FS stress. These two ontogenetic stages are critical in respect of morphologic and functional transformations which occur then in different brain structures (Lupien 2009).

Chronic FS was the subject of the present research on the grounds that the test, as a model of depression, combines psychological (novelty, water) and physiological (exercice) stressors, and is easy to repeat several times, not to mention the fact that swimming might be a frequent cause of stress for rats in their natural environment. Our previous study, designed to investigate the influence of chronic FS exposure on the density of BDNF-containing neurons in the hypothalamic paraventricular and supraoptic nuclei of juvenile and aged rats (under preparation for publishing), demonstrated a decrease in BDNF cells in these structures of juvenile rats, but no changes were noted in aged individuals. The findings induce further research into a similar possible influence of FS on the number of BDNF and TrkB – containg cells in another important structure of the limbic system, namely the hippocampus.

Taking the above into consideration, the aim of the present study was to investigate the influence of chronic FS stress on the density of BDNF and TrkB –immunoreactive (ir) cells in the pyramidal layer of hippocampal subregions - CA1, CA3 and granular cells of dentate gyrus (DG) in two age groups, namely in juvenile (P28) and aged (P360) rats.

METHODS

Animals

Twenty male Wistar Han rats from two age groups described as juvenile - P28 (P-postnatal day) and aged - P360 (body weight 75 - 100g and 380 - 450g, respectively) were housed in plastic cages (three rats per cage) for at least 1 week prior to the experiment. The animals, kept under constant temperature (21±1ºC) and lighting (light on from 7:00AM to 7:00PM) regimens in plastic cages with free access to water and food pellets. The care and treatment of the rats were in accordance with the guidelines for laboratory animals established by the National Institute of Health as well as by the Local Ethical Committee of the Medical University of Gdańsk. P28 and P360 animals were divided into experimental groups (n=5), exposed to chronic forced swim stress, and control non-stressed groups (n=5) , which remained in their home cages until perfusion. Control animals were handled for a few minutes daily by the same operator. At the outset of chronic stress stimulation, P28 rats were seven days old (P-7).

Experimental procedure

All tests were conducted once a day in 15-minute sessions for 21 consecutive days at the same time, between 9:00AM and 2:00PM.

Forced swim test (FS)

We used a chronic forced swim stress protocol (Porsolt et al. 1977). The forced swim test, also known as the behavioural despair test, has been proved to be an effective tool for assessing depression in laboratory rodents exposed to inescapable aversive stimulation (Porsolt et al. 1977, Cryan et al. 2002). Chronic FS test procedure applied to this experiment was used in our previous studies (Badowska-Szalewska et al. 2009). The rats were placed in a glass cylinder (45 cm high, 20 cm in diameter) filled with clear, fresh water at 22ºC up to a height of 30 cm. After each test trial the rats were dried before returning to their respective home cages. If some of the youngest individuals were not able to complete the 15 min FS test, that is when the tip of their nose did not remain above the water level, the experimental time was reduced.

Tissue collection and preparation

Experimental and control animals were sacrificed on postnatal day 28 (P28) and 360 (P360). Ninety minutes after the final exposure to FS, all rats were deeply anesthetized with a lethal dose of Nembutal (80 mg/kg body weight), and then perfused transcardially with 0.9% saline solution with heparin, followed by 4% paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.4). The brains were removed, postfixed in 4% paraformaldehyde for 3-4 hours, and kept overnight in 15% and 30% sucrose in 0.1 M phosphate buffer (all solutions at 4°C) until they sunk. Serial coronal sections of brain (40-µm-thick) were cut with a JUNG 1800 cryostat (Leica, Germany).

Immunohistochemistry

Adjacent sections were processed for BDNF and TrkB immunohistochemistry. Free-floating sections were blocked in 10% Normal Goat Serum (NGS) for 2 hours, and then incubated at 4ºC for 3 days with the primary polyclonal rabbit anti-BDNF antibody (Chemicon; dilution 1:300) or primary polyclonal rabbit anti-TrkB antibody (Chemicon; dilution 1:500) containing 0.3% Triton X-100. After multiple rinses in phosphate buffered saline (PBS), the sections were incubated (2-3 hours, room temperature) with an appropriate secondary antibody, namely Cy3-conjugated goat anti-rabbit (Jackson ImmunoResearch; dilution 1:600). Controls for the immunohistochemical procedures were obtained running some slides through the entire procedure with the omission of the primary or secondary antibodies. No staining was observed in these control slides. Immunohistochemically stained slides were examined under a Nikon Eclipse-TE300 fluorescence microscope equipped with Color View III camera (Olympus, Japan). Microscopy images were obtained using 10× objective lenses, and the optimal iris was used for magnification purposes.

Quantitative analysis

An image analysis system C.A.S.T. Grid (Olympus, Denmark) and 40× objective lens were employed to assess the number of BDNF-ir and TrkB-ir positive cells in the hippocampus, divided into three (counted separately) areas: CA1 subfield, CA3 subfield and dentate gyrus (DG). The regions of interest were outlined and computer-aided estimation was used to calculate the number of BDNF-ir cells or TrkB-ir cells in the pyramidal layer of CA1 and CA3, and in the granular layer of DG. Due to fact that biological variability of given parameter within group of animals is influenced mainly by differences between subjects, and, in much smaller extent, by differences between sections in subject, one representative section of the hippocampus was taken (between Bregma -2.30 and Bregma -3.60) (Paxinos and Watson 1998). Hemisphere was chosen randomly. Standard technique was used to estimate the number of BDNF-ir or TrkB-ir cells' profiles per unit area for each investigated hippocampal structure. Grid size was either 4067 µm sq. or 14913 µm sq depending on the amount of labeled cells. At least half of the area of given section was taken to the analysis; first test area was chosen randomly, remaining ones were selected by systematic random sampling. The data were analyzed by a two-way analysis of variance (ANOVA) for the factor groups (intact vs. chronic FS stress) and age groups (juvenile-P28 and aged-P360), followed by

Kruskall-Wallis post hoc test with significant values set at $p<0.05$. The results of the investigation are expressed as mean density (number/mm^2) ± standard deviation (SD). All data were presented on a graphs.

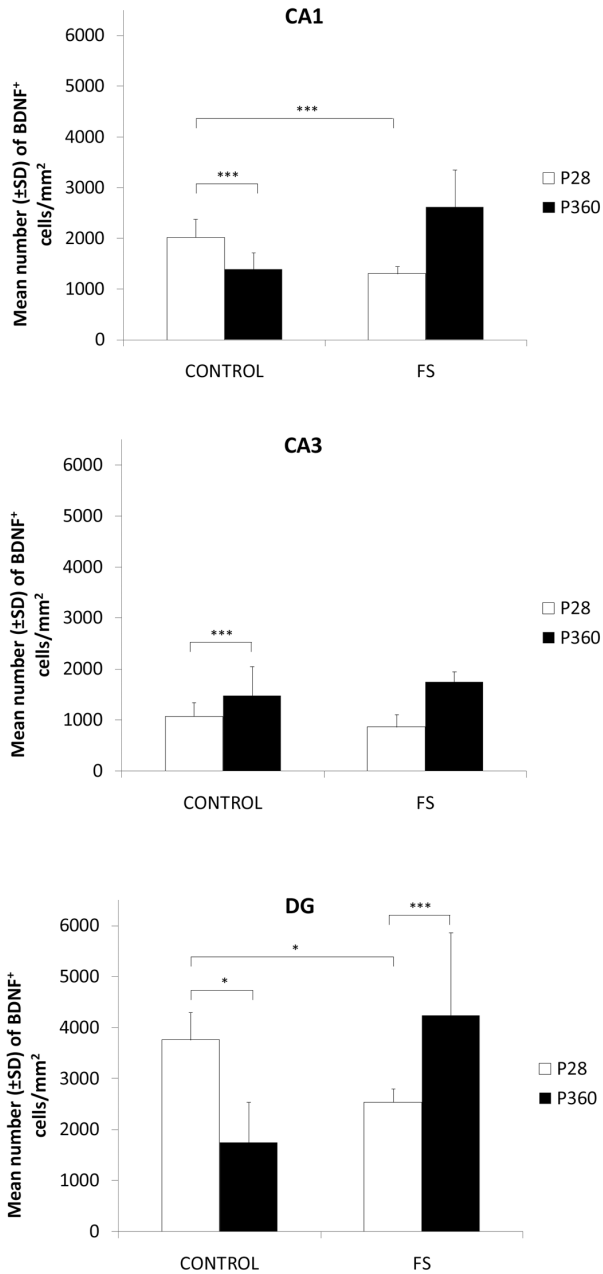

Fig.1. Effects of chronic forced swim stress (FS) on mean number (± SD) of BDNF-immunoreactive cells/mm² of juvenile (P28) and aged (P360) rats in the pyramidal cells of CA1 and CA3 hippocampal regions and in the granular cells of dentate gyrus (DG). Juxtaposed animals: experimental group with control group and juvenile (P28) rats with aged (P360) rats. *$p<0.05$, ***$p<0.001$

RESULTS

BDNF and TrkB immunoreactive cells in P28 and P360 control rats

In juvenile (P28) and aged (P360) non-stress control rats, BDNF and TrkB immunoreactive cells were expressed in the pyramidal layer of CA1 and CA3 areas as well as in the granular layer of DG (Fig. 1-4.). Both P28 and P360 groups displayed more TrkB-ir than BDNF-ir neurons in each studied section of the hippocampus. The density of BDNF-ir cells in CA1 and CA3 was comparable in juvenile and aged animals (CA1 - 2024.09±358.39, CA3 - 1064.5±278.9 and CA1 - 1386.43±139.3, CA3 - 1480.0±241.37, respectively), however, in DG it was significantly lower in P360 than in P28 rats (1748.41±252.61 and 3760.02±536.39, $p<0.05$) (Fig. 1.). Immunostaining for TrkB revealed a statistically smaller number/mm² of TrkB-ir cells in CA1 (2916.01±344.66, $p<0.005$), CA3 (2580.82±283.07, $p<0.05$) and DG (3610.63±586.6, $p<0.001$) in non-stress aged rats (P360) in comparison with control juvenile animals (P28) (CA1 - 3679.27±692.24, CA3 - 3060.97±343.69, DG - 5182.48±878.7) (Fig. 3.).

Influence of chronic exposure to the FS on the density of BDNF-immunoreactive neurons

Compared with control rats, the juvenile (P28) animals which underwent chronic FS test demonstrated a marked decrease in the density of BDNF-immunopositive cells in CA1(1306.02±334.02, $p<0.001$) and DG (2542.5±800.18, $p<0.001$) subfields, unlike in CA3, where no changes were noted. (Fig. 1., 2.). In P360 rats after chronic exposure to FS stress, we observed a trend towards an increase in the density of BDNF-ir cells in all hippocampal subfields in comparison to control groups, but the changes were statistically insignificant (Fig. 1., 2.). By contrast with juvenile animals, prolonged FS stimulation, however, led to a substantial increase in the density of BDNF-ir neurons in CA1(2618.21±730.82, $p<0.001$) and CA3 (1742.49±212.77, $p<0.001$) as well as in DG (4234.8±1634.72, $p<0.05$) in aged rats (P360) (Fig. 1.).

Effects of prolonged exposure to the FS on the density of TrkB-immunoreactive cells

A stress-induced elevation of TrkB-immunoreactive cells/mm² was recorded only in CA3 (3514.78±163.06,

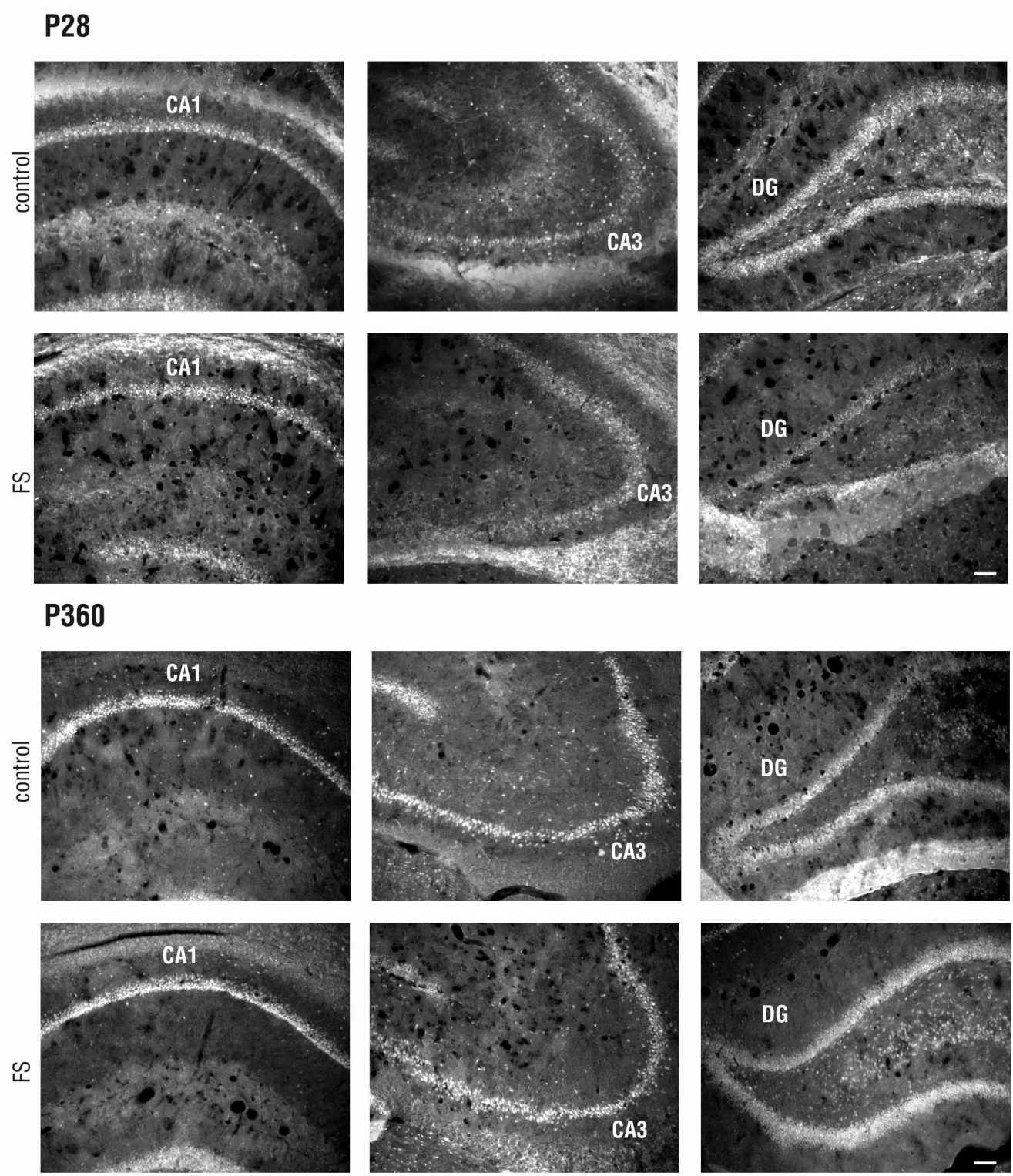

Fig. 2. BDNF-immunoreactive neurons in control and after forced swim test (FS) in hippocampal regions: CA1, CA3 and DG of juvenile (P28) and aged (P360) rats. Scale bar: 100 μm.

p<0.05) in P28 rats in comparison with control animals (Fig. 3., 4.), whereas in P360 rats chronic FS stress stimulation had no influence on the density of TrkB-positive neurons in all studied areas of the hippocampus (Fig. 3., 4.). Furthermore, as in control groups, after prolonged exposure to FS stress stimulus the number of TrkB-immunopositive cells/mm² was lower in CA1(2755.63±357.26, p<0.005), CA3 (2740.4±301.63, p<0.001) and DG (3703.29±446.21, p<0.001) subfields in aged animals (P360) than in juvenile (P28) rats (CA1 - 3555.4±264.79, CA3 - 3514.78±163.06, DG - 4834.98±753.7) (Fig. 3.).

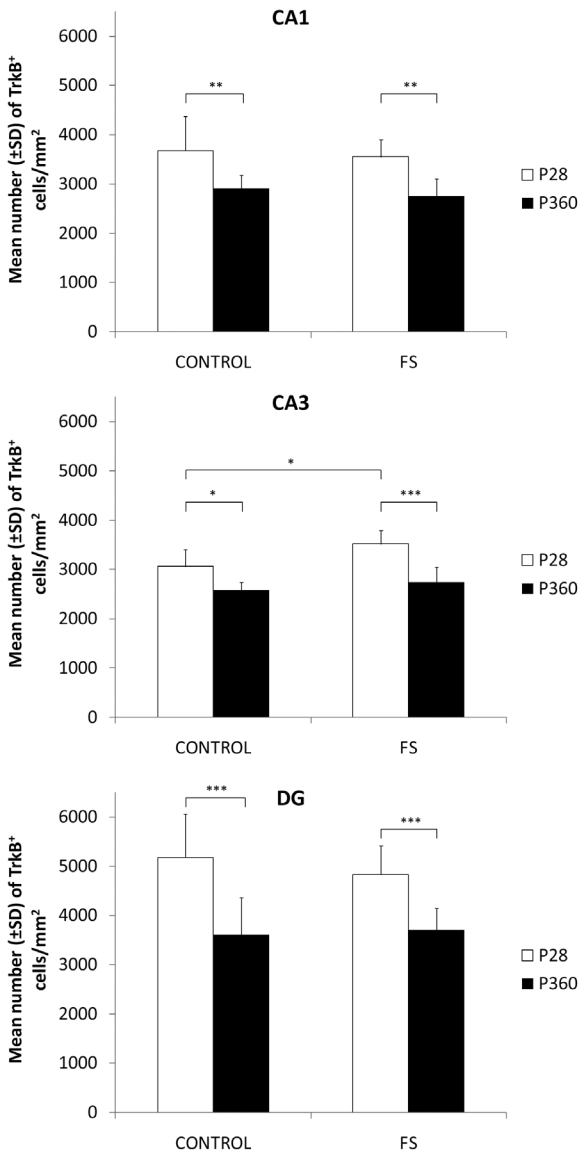

Fig. 3. Effects of chronic forced swim stress (FS) on mean number (± SD) of TrkB-immunoreactive cells/mm² in the pyramidal cells of CA1 and CA3 hippocampal regions, and the granular cells of dentate gyrus (DG) of juvenile (P28) and aged (P360) rats. Juxtaposed animals: experimental group with control group and juvenile (P28) rats with aged (P360) rats. *p<0.05, **p<0.005, ***p<0.001

DISCUSSION

The present study documents age-related changes in the density of BDNF-ir and TrkB-ir neurons in the studied subfields of the hippocampus as a consequence of chronic FS stress exposure. Prolonged FS has been found to affect the amount of BDNF-ir and TrkB-ir cells in the hippocampus of juvenile, not aged rats.

Both P28 and P360 control groups revealed BDNF and TrkB immunolabelled neurons in the granular cells of DG as well as in the pyramidal cells of CA1 and CA3. The number of cells that contains these proteins was higher in DG than in CA1 or CA3, which was probably related to numerous intrinsic and extrinsic connections of DG (Amaral and Witter 1989). Uneven distribution of BDNF and TrkB immunoreactive cells in the mentioned regions of the hippocampus has been recorded before (Katoh-Semba et al. 1997, Sato et al. 2001), but the reports mostly concerned brains of adult rats (Dugich-Djordjevic et al. 1995, Kawamoto et al. 1996, Drake et al. 1999). The high levels of BDNF and TrkB that persist in the hippocampus indicate that these molecules have important physiological functions in different stages of life (Ridder et al. 2005, Lipsky and Marini 2007, Tapia-Arancibia et al. 2008).

During adolescence, BDNF influences almost all aspects of development, including stimulation of growth, differentiation of neuronal stem cells into neurons and promotion of the survival of newly generated cells and synaptogenesis (Mattson et al. 2004, Tapia-Arancibia et al. 2004). On the other hand, during ageing BDNF may play a protective role by preventing neurodegeneration, stimulating sprouting and synaptic reorganization in the hippocampus (Smith 1996, Tapia-Arancibia et al. 2004), or by encouraging neuronal repair (Smith et al. 1995). As for TrkB, it exerts positive influence on dendritic branching and dendrtic integrity in the hippocampus (Sato et al. 2001), therefore a reduction in the level of this protein may underlie the synaptic changes that occur with age in the hippocampus (Geinisman et al. 1992).

The forced swim test (FS) correspondents to a psychophysical stressor (Dayas et al. 2001) and is deemed

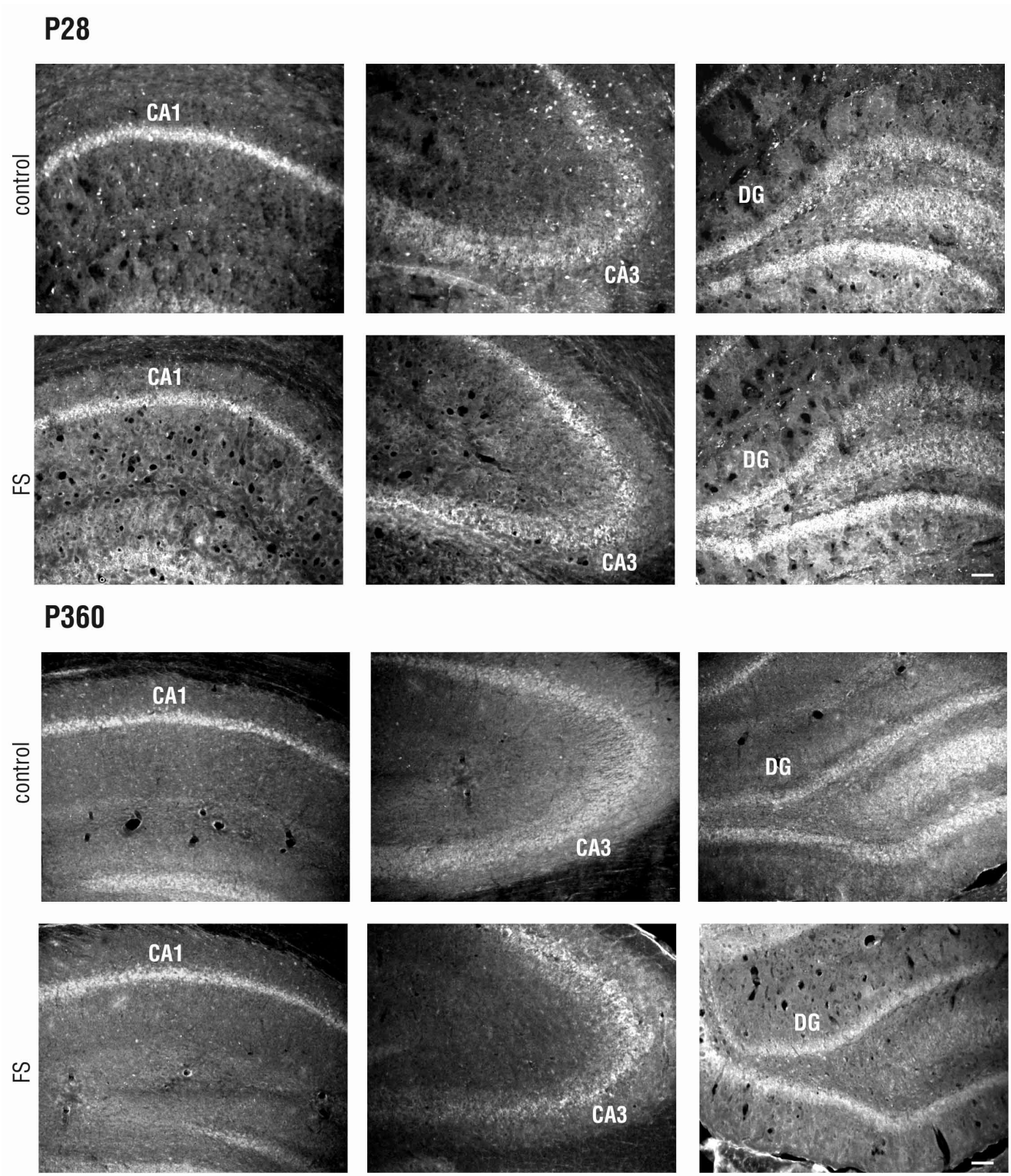

Fig. 4. TrkB-immunoreactive neurons in control and after forced swim test (FS) in the hippocampal regions: CA1, CA3 and DG of juvenile (P28) and aged (P360) rats. Scale bar: 100 μm.

to be an aversion stimulus, therefore it is used as an experimental model for an assessment of despair/depression-like behavior (Muigg et al. 2007, Stone et al. 2007).

In our research, it was observed that in juvenile rats under chronic FS, the density of BDNF-ir containing neurons significantly decreased in the studied areas of the hippocampus (in CA1 and DG), except for CA3, which displayed their unaltered amount, but the density of TrkB-ir cells increased in none but CA3 subregion of the hippocampus in comparison with control groups. According to Smith and coworkers (1995), BDNF support of CA3 neurons could be derived from target cell in CA1 pyramidal layer, where BDNF is normally expressed in low levels and further reduced by immobilization stress. The possibility of TrkB-ir up-regulation in CA3 cell layers was a compensatory response to a prolonged lack of changes in BDNF-ir cells in this region after chronic FS (Nibuya et al. 1999).

It is a well-known fact that adolescent rats, as opposed to adult specimens, tend to be more susceptible to the influence of aversive stimuli, which is manifested by extended time of HPA axis activation (Avital and Richter-Levin 2005, Lupien 2009), and consequently, prolonged time of glucocorticoid secretion and a growth of their level (Romeo et al. 2004, Cruz et al. 2008, McCormick et al. 2010). As Goldman and coauthors (1973) and Lupien (2009) claim, it ensues from the immaturity of the reversible system suppressing stress axis. During maturation many stressors (including chronic ones) change their mode of affecting hippocampal structure and its functioning (McEwen and Magarinos 2001, Sapolsky 2003, Fenoglio et al. 2006) by developing different means of remodeling neurons, including, for example, shortening of dendrites or loss of spine synapses. Stress-associated variations in the level of BDNF in the hippocampus may, in turn, contribute to chronic disinhibition of the HPA axis (Thoenen 1995, Rot et al. 2009). Furthermore, BDNF can act as a stress-responsive intercellular messenger modifying HPA axis activity (Schulte-Herbrüggen et al. 2006). Since hippocampal neurons display high concentrations of glucocorticoid receptors (McEwen 1999, Sapolsky 2001, Duman 2004), another explanation of our findings is that BDNF levels could be down-regulated by glucocorticoids (Rage et al. 2002, De Kloet et al. 2009). As evidenced by previous studies, juvenile rats exhibit a delayed rise and prolonged release of glucocorticoids after repeated exposure to stressors (Vazquez and Akil 1993, Romeo and McEwen 2006), which may cause dysregulation of HPA axis (Murakami et al. 2005, Girotti et al. 2006).

Our experiment shows that in aged (P360) rats exposed to chronic FS, the density of BDNF-ir and TrkB-ir cells is suppressed in none of the examined subregions of the hippocampus in relation to control group. There are contradictory findings regarding the aging effects of BDNF and/or TrkB expression in the hippocampus after chronic stress. Some researchers report a decrease in the amount of these proteins (Roceri et al. 2004), while others note lack of changes in this component of the brain (Schaaf et al. 2000). The aging process involves an impeded ability to manage with stress, being an outcome of the deficiency of physiological, motor and cognitive functions (Pedersen et al. 2001, Pardon 2007). Within this period, the regulatory activities of HPA axis in response to stress become dysregulated, which might be demonstrated by an increased or a decreased degree of its activation (McEwen 1999, Lucassen et al. 2001, De Kloet et al. 2007, McEwen and Gianaros 2010). Pardon (2007) maintains that age entails the need of providing a stronger stimulus in order to trigger stress reaction, but at the same time, the intensity of this reaction is also stronger. On the basis of these and other authors' reports (Pardon et al. 2005, Takeda et al. 2006) we can presume that the reason behind the absence of changes in the density of BDNF-ir and TrkB-ir cells in response to chronic exposure to FS is the fact that through its repetitiveness, prolonged application of this stress factor might have been too weak a stimulus for this group of animals to provoke neuronal response. On the other hand, owing to its capacity for preventing age-related neuronal loss in the hippocampus, long-lasting and repeatable physical activity in the FS test may have been an important, beneficial agent for the overall health and cognitive function of aging rats (Larsen et al. 2000, Tapia-Arancibia et al. 2004).

The older (P360) animals that underwent the FS test in our study were observed to have a significantly higher density of BDNF-ir neurons than juvenile (P28) experimental rats, but the density of TrkB-ir cells in P360 was markedly lower. Our results support the finding that the age of rats exposed to stress was the the main factor affecting the expression of BDNF (Li et al. 2009). Moreover, expression of BDNF and TrkB is con-

sidered to be regulated in opposite direction – the growth of BDNF content occurres along with a fall in the level of TrkB in the hippocampal cells of aged rats (Frank et al. 1997, Nibuya et al. 1999, Sommerfeld et al. 2000, Silhol M et al. 2007, Tapia-Arancibia et al. 2008). Sirianni and coworkers (Sirianni et al. 2010) noted that the rats which exhibited antidepressant-like behavior in the FS test had lower levels of TrkB than the animals that did not show this kind of behaviour. We therefore think that the higher density of BDNF-ir and lower density of TrkB-ir cells in P360 group of experimental rats may signify their protective effects against hippocampal damage of ageing animals in stress conditions.

CONCLUSION

This study has demonstrated that hippocampal subfields of juvenile and aged rats show different density of BDNF and TrkB immunostaining cells. Chronic FS stress influenced the density of BDNF-ir and TrkB-ir containing neurons merely in juvenile animals. The age of rats exposed to chronic FS stress occurred to be a decisive factor determining changes in the density of BDNF-ir and TrkB-ir in the studied hippocampal structures.

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