Comparison of the influence of two models of mild stress on hippocampal brain-derived neurotrophin factor (BDNF) immunoreactivity in old age rats

Ewa Badowska-Szalewska*, Beata Ludkiewicz, Rafał Krawczyk, Natalia Melka, and Janusz Moryś

Department of Anatomy and Neurobiology, Medical University of Gdańsk, Gdańsk, Poland,
* Email: ewabadowska@gumed.edu.pl

The way hippocampal neurons function during stress in old age (critical times of life) is dependent on brain derived neurotrophin factor (BDNF). This study examined the influence of acute and chronic forced swim (FS) or high-light open field (HL-OF) stimulation on the density of BDNF immunoreactive (ir) neurons in the hippocampal pyramidal layers of CA1, CA2, CA3 regions and the granular layer of dentate gyrus (DG) in old (postnatal day 720; P720) Wistar Han rats. Our data showed that in comparison with non-stressed rats, acute FS caused a significant increase in the density of BDNF-ir neurons in CA2 and CA3, while acute HL-OF led to an increase in this factor in all hippocampal subfields with the exception of DG. However, the density of BDNF-ir cells remained unchanged after exposure to chronic FS or HL-OF in the hippocampal regions in relation to the control rats. These results indicate that acute FS or HL-OF proved to be a stressor that induces an increase in the density of BDNF-ir pyramidal neurons, which was probably connected with up-regulation of HPA axis activity and short-time memory processing of the stressful situation. Moreover, as far as the influence on BDNF-ir cells in hippocampus is concerned, chronic FS or HL-OF was not an aggravating factor for rats in the ontogenetic periods studied.

Key words: brain-derived neurotrophin factor, immunohistochemistry, mild stress, hippocampus, old age

INTRODUCTION

Brain-derived neurotrophin factor (BDNF) is a member of the nerve growth factor family, which is necessary for neurite outgrowth, survival and maintenance of neuronal function (Russo-Neustadt 2003, Mattson et al. 2004, West 2008, Cirulli and Alleva 2009). These diverse effects on neuronal processes modulate the establishment of neuronal circuits to regulate behavior (Greenberg et al. 2009). Moreover, BDNF is a stress-responsive intercellular messenger involved in regulating hypothalamic-pituitary-adrenal (HPA) axis activity (Givalois et al. 2004, Schulte-Herbrüggen et al. 2006). Due to its capability of protecting neurons against dysfunction and damage, this factor has been implicated in neurobiological mechanisms underlying brain plasticity after stress (McAllister et al. 1999, Poo 2001, Greenberg et al. 2009, Li et al. 2009). One of the structures of the limbic system, in which functioning BDNF plays an important regulatory role throughout lifetime (including old age), both in healthy organisms and under stress, is the hippocampus (Smith et al. 1995, Alleva and Santucci 2001, Silhol et al. 2005, Tapia-Arançibia et al. 2008).

As is known, the hippocampus is involved in learning, memory and emotional processes (McEwen and Magarinos 2001, Bartolomucci et al. 2002, Richter-Levin 2004). Moreover, it is a key element in the neuroendocrine response to stress (Fuchs and Flügge 2003, Herman et al. 2005). This structure regulates the HPA axis through inhibiting its activity (Jacobson and Sapolsky 1991, see Herman et al. 2005, Jankord and Herman 2008): it causes the trans-synaptic inhibition of corticotrophin-releasing hormone (CRH) neurons of paraventricular nucleus (PVN), which is where the HPA axis activation starts. This occurs via glutamatergic hippocampal projections to GABAergic neurons in regions such as the bed nucleus of the stria terminalis, which in turn can inhibit PVN neurons (Herman et al. 2003, Ulrich-Lai and Herman 2009).

The longer the life and consequently, the longer the period of senescence, the more necessary it is to have knowledge about processes leading to problems caused in old age by physiological, motoric and cognitive deficiencies, especially in the context of being able to cope with environmental changes. Hippocampal neurons are very susceptible to stress and senescence...
Hippocampal BDNF-ir under stress in old rats

The ageing process reveals diminished cognitive capabilities and reduced hippocampal plasticity observed in old rats (Silhol et al. 2005). While hippocampal BDNF in response to stress in adult animals has been studied extensively (Smith et al. 1995, Marmigère et al. 2003, Murakami et al. 2005, Li et al. 2009), there are few reports on changes in this factor under stress (especially mild) in old rats (Smith and Cizza 1996, Shi et al. 2010). In particular, there are no data on the effect of mild stimulation on hippocampal BDNF-immunoreactive (-ir) neurons in old age. Mild stressors cause no damage to the body, nor are they painful and, what is particularly important, they frequently occur in the natural environment. Moreover, mild stress-induced changes in the BDNF-ir neurons may be particularly relevant to the cognitive changes that occur in ageing (Adlard et al. 2011).

Due to the significant contribution of BDNF to the hippocampal neuronal functioning in old age (predominantly a protective mechanism) and the involvement of protein in regulating the hippocampus stress response in the senescence (Smith and Cizza 1996, Silhol et al. 2005), the aim of the study was to examine the effect of two stressors i.e. forced swim (FS) or high-light open field (HL-OF) on the density of BDNF immunoreactive (-ir) neurons in the hippocampal pyramidal layers, located in the CA1, CA2 and CA3 subfields, and the granular layer, situated in the dentate gyrus (DG) (which constitute the principal neuronal cell types in the hippocampus) in 24-month-old rats. FS and HL-OF were selected because of their similarities and differences. Both may be regarded as aversive naturalistic stressors (Dal-Zotto et al. 2000, Bouwknecht et al. 2007) (i.e. such that are encountered but avoided by rats in their natural habitat), both contain the psychological component (no possibility of escaping, novelty – acute stress and unfriendly environment). Differences lie in the fact that the main stressor in HL-OF is the psychological component (strong white light in the open field area is particularly stressful, because rats are nocturnal animals and they tend to avoid brightly lit places) (Bouwknecht et al. 2007) and in FS, the physical component (exercise – swimming) (Dal-Zotto et al. 2000), which can be more stress-inducing and aggravating in old age.

METHODS

All experimental procedures involving animals, care and treatment were approved by the National Institute of Health, as well as by the Local Ethical Committee of the Medical University of Gdańsk (opinion date: 090106; opinion number: 3/6).

Animals

Our studies were carried out on old (P720; P – postnatal day) male Wistar Han rats. The rats were bred until they reached the appropriate age under standard conditions in the Tri-City Academic Laboratory Animal Centre – Research and Services Centre. Two weeks before the experiments started, the rats were transferred to the Department Animal Center and housed socially in polysulfone cages (T. IV, 56×36×20 cm+7 cm cage lid) containing dust-free sawdust on the floor (2–3 animals per cage). The animals were kept in air-conditioned rooms under a constant temperature (22±2°C), a humidity of 55±10% and a lighting regimen (light on from 7:00 a.m. to 7:00 p.m.) with free access to water and food pellets.

Test model

The P720 rats were divided into control and experimental groups. The control group consisted of non-stressed rats that were handled for a few minutes daily by the same operator and remained in their home cage (in the same conditions as the experimental groups) until being anaesthetised. The experimental groups were exposed to acute or chronic stressors in the forced swim (FS) test or in the high-light open-field (HL-OF) test. Each of the groups (control or experimental) consisted of 6 or 7 rats. Acute stimulation was performed once in a 15–20 minute session, whereas the chronic test was conducted once a day in 15–20 minute sessions for 21 consecutive days and at the same time – between 9:00 a.m. and 2:00 p.m. After the end of the experiments, the rats were returned to their respective home cages for 90 minutes before killing.

FS test

In the FS test, the rats were placed individually in a transparent cylindrical glass tank (50 cm high, 30 cm in internal diameter) which was placed on the floor in the experimental room and was filled with clear, fresh water (at 22°C) up to a height of 30 cm. After each test, the tank was washed and disinfected with 70% ethanol.

HL-OF test

The apparatus for the HL-OF test consisted of a square (100×100×40 cm) wooden box with white walls and floor, illuminated with a 500-watt halogen lamp throughout the box and was set in a dark room. In order to provoke a stress reaction, each animal was gently placed in the centre of the open field arena. After each test, the box was cleaned with water and 70% ethanol.
Tissue collection and preparation and immunohistochemical methods

The detailed procedure of tissue collection and preparation as well as the procedure for the immunohistochemical labelling for BDNF were described previously (Badowska-Szalewska et al. 2012). A primary polyclonal rabbit anti-BDNF antibody (Millipore-Chemicom International Inc, AB1534SP; dilution 1:300) was used and then an appropriate secondary antibody, i.e. the Cy3-conjugated goat anti-rabbit (Jackson ImmunoResearch Laboratories Inc., West Baltimore Pike, PA, USA, 711-165-152; dilution 1:600) was applied. The specificity of the BDNF antibody administered was verified by Western blotting and described earlier (Badowska-Szalewska et al. 2016a, supplementary material). Additionally, controls for the immunohistochemistry, negative for any reactivity, were obtained by subjecting some tissue sections to the same procedure with the omission of the primary or secondary antibodies and did not show any signals.

Quantitative analysis

The newCAST ver.4.4.4.0 (Visiopharm, Denmark) image analysis system based on an Olympus BX51 microscope equipped with a DP72 camera (Olympus, Japan) was used to assess the density of BDNF-immunostaining neurons in the hippocampal pyramidal layer of CA1, CA2 and CA3 and granular layer of DG. The areas investigated were selected on the basis of Paxinos and Watson’s rat brain atlas (2007), Bregma points from −2.40 to −3.60.

The regions being investigated were outlined at 4-times magnification. The number of BDNF-ir profiles of cells in every area was calculated separately with computer-aided estimation. Under a 40× objective lens, cell profiles were counted in a single layer of cells separately for each hippocampal subfield. 60% of the total area of the section of the subfield on a given slide was chosen at random and automatically by the program, and then recalculated proportionally into 100% of the field. The total number of cells was converted into a surface area of 1 mm². Four to six sections of the same subfield from each rat were evaluated and the data were averaged. The results were grouped and statistically analysed separately for each hippocampal subfield.

Statistical analysis

All statistical analyses were carried out using InStat (GraphPad Software Inc, 1998), version 3.0. Each time before making final statistical inference by ANOVA, their assumptions were checked by means of a normality test (Kolmogorov-Smirnoff), and the equality of variances test (Bartlett). Upon checking those assumptions, the differences for CA1, CA2, CA3 and DG between the groups studied (intact-control, acutely stressed and chronically stressed) were assessed using a parametric analysis of variance (ANOVA). For the individual group differences between experimental groups (intact-control, acutely stressed and chronically stressed), the multiple comparison Tukey test was used. The precise F-value of ANOVA (with degrees of freedom) and the q-value of the Tukey test, along with the corresponding approximate P-levels, were provided. The whole process of statistical inference was performed at a significance level of P<0.05. The results of the immunohistochemical study were expressed as a mean density (number of labelled BDNF-immunoreactive cells/mm²) ±standard deviation (SD).

RESULTS

Parametric one-way Analysis of Variance (ANOVA) revealed statistically significant differences between the groups studied (control, FS acute, FS chronic, HL-OF acute, HL-OF chronic) in the density of BDNF-ir neurons in the hippocampal pyramidal layer of CA1 (F(4,23)=6.291, P=0.001, P<0.001), CA2 (F(4,23)=8.090, P=0.0002, P<0.001), CA3 (F(4,23)=5.092, P=0.0034, P<0.001) and granular layer of DG (F(4,23)=2.808, P=0.0482, P<0.05) in old (P720) rats. BDNF immunoreactive staining was found to be localized in the cytoplasm of neurons sometimes extending into the apical fibres (data not shown).

The density of BDNF-ir neurons in the hippocampal CA1, CA2, CA3 and DG in old rats after FS stimulation

A post-hoc Tukey multiple comparisons test showed that acute FS caused a marked increase in the density of BDNF-ir neurons in CA2 (q=4.594, P<0.05) and CA3 (q=5.332, P<0.01) but not in CA1 (q=3.898, P<0.05) and DG (q=3.712, P>0.05) in relation to non-stressed aged rats (Figs 1A–1D, 2A–2B). However, after chronic FS no significant changes in the density of BDNF-ir neurons in all the hippocampal areas vs. control rats (CA1: q=1.163, P>0.05; CA2: q=0.104, P>0.05; CA3: q=2.983, P>0.05; DG: q=2.892, P>0.05) were observed (Figs 1A–1D, 2A, 2C). In contrast, in CA2, a statistically significant decrease in the density of BDNF-ir cells after chronic FS in comparison with acute FS (q=4.490, P<0.05) was noted (Figs 1B, 2B–2C).
The density of BDNF-ir neurons in the hippocampal CA1, CA2, CA3 and DG in old rats after HL-OF stimulation

Post-hoc Tukey test analyses demonstrated that with the exception of DG ($q=3.854, P>0.05$) (despite statistically significant differences in the ANOVA), acute HL-OF led to a significant increase in the density of BDNF-ir cells in the hippocampal CA1 ($q=6.540, P<0.001$), CA2 ($q=5.794, P<0.01$) and CA3 ($q=5.058, P<0.05$) as compared with the control group (Figs 1A–1D, 2A, 2D). Chronic HL-OF, however, did not affect significantly the changes in BDNF-ir neurons in any of the areas tested in relation to non-stressed rats (CA1: $q=3.182, P>0.05$; CA2: $q=0.074, P>0.05$; CA3: $q=1.596, P>0.05$; DG: $q=3.854, P>0.05$) (Figs 1A–1D, 2A, 2E). In contrast, as compared with acute, chronic HL-OF caused a significant decrease in BDNF-ir cells in CA2 ($q=5.720, P<0.01$) (Figs 1B, 2D–2E).

**DISCUSSION**

In old non-stressed rats, BDNF immunoreactive neurons were abundantly expressed in the hippocampal granular layer of DG as well as in the pyramidal layer of CA2, CA3 with somewhat less expression in the CA1. Strong
immunostaining for BDNF in the hippocampal neurons was found previously in both middle-aged (Li et al. 2009, Badowska-Szalewska et al. 2010) and aged (Katoh-Semba et al. 1998) intact rats. Thus, our recent observations are

Fig. 2. Distribution of BDNF-ir neurons in the hippocampal pyramidal layer of the CA1, CA2, CA3 and granular layer of the dentate gyrus (DG) in old (P720) rats from the control group (A) and from the groups exposed to acute (B) and chronic (C) forced swim (FS) or acute (D) and chronic (E) high-light open-field (HL-OF) stimulation. The figure shows the increase in BDNF-ir neurons after acute FS in CA2, CA3 and after acute HL-OF in CA1, CA2, CA3 vs. non-stressed rats but no changes in the density of BDNF-ir neurons after chronic FS or chronic HL-OF vs. control in CA1, CA2, CA3 were observed. The scale bar is 200 μm.
in accordance with other studies (Smith and Cizza 1996, Katoh-Semba et al. 1998, Silhol et al. 2005), which stated that BDNF is continuously expressed in the hippocampus in the senescence.

We suppose that the abundant presence of BDNF-ir neurons in the CA1–CA3 and DG which was observed in healthy non-stressed old rats could result from the requirement for BDNF, in order to maintain the electrophysiological function of the hippocampus (predominantly cognitive and memory processes – Marmigére et al. 2003, see Silhol et al. 2005). Added to that, the necessity of supporting the survival of these neurons in the stages of the rats’ lives (for example, through buffering of toxic calcium, regulation of glucose metabolism and glutamate or other neuropeptides, facilitate long-term potentiation – see Smith 1996) could be the cause of numerous occurrences of BDNF-ir neurons in the hippocampal areas of non-stressed old rats. However, according to Smith (1996), in old age there seems to be a reduction in the ability of neurons to induce the response to BDNF. Thus, in spite of the high density of basal BDNF-ir hippocampal neurons in our study, disturbances in BDNF signaling are possible (as a result of the reduction of the axonal transport caused by the senescence – Mattson et al. 2004).

Our study found that in rats subjected to acute FS there was a marked increase in the density of BDNF-ir neurons in the pyramidal layers of CA2 and CA3 but not in CA1. However, in those rats in which acute HL-OF was applied the density of BDNF-ir neurons increased significantly in all pyramidal areas. Dentate granular neurons were unaffected by these two stressors. Moreover, no significant changes in the density of BDNF-ir cells after both chronic FS and HL-OF in comparison with non-stressed rats in any of the hippocampal subfields were noted. In contrast, a significant decrease in the density of BDNF-ir neurons in the CA2 was found in rats exposed to chronic FS or HL-OF vs. acute FS or HL-OF. Generally, we may conclude that as far as the influence of FS or HL-OF on the density of BDNF-ir hippocampal neurons in old rats is concerned, the results were similar for both stressors.

Other research revealed that short-time (10 minutes) forced swim in 4°C cold water caused a rapid increase in BDNF after 15 minutes (as well as 30, 60 or 90 minutes) of stressing in the hippocampus of aged (22-month-old) Sprague-Dawley rats measured by Western blotting, but when the rats were chronically forced to swim (10 minutes for 21 days) in 25°C water, the protein expression decreased after 60 minutes and later from the last experiment in comparison with control group (Shi et al. 2010). Similarly, a different duration of chronic unpredictable mild stress (8, 21 and 28 days) in the same strain aged rats induced a decrease in BDNF mRNA in the hippocampus, as detected by RT-PCR (Shao et al. 2010). Smith and Cizza (1996), however, using acute (2 h) immobilization in 24-month-old Fischer 344/N rats which were decapitated immediately after the end of the experiment, showed a decrease in BDNF mRNA (detected by in situ hybridization) in DG, but in CA1 and CA3 there were no significant changes.

From the above data, we can conclude that the influence of stress on hippocampal BDNF in aged rats depends not only on the kind of emotional stressor applied, its duration and frequency, but also other factors such as the strain of rats, the time of sacrifice after stress, the methods used for BDNF detection and its detectable forms (BDNF or BDNF mRNA) could affect the research findings. We think that acute FS and HL-OF in our study was effective in evoking a neuronal response in hippocampal pyramidal neurons predominantly in CA2 and CA3 in old Wistar Han rats, which caused an increase in their BDNF-ir.

The activation of the HPA axis after acute FS or HL-OF causes an increased secretion of glucocorticoids, which in turn affect the activation of hippocampal pyramidal neurons (via their glucocorticoids receptors) (Herman 1993), including them in feedback regulation of the HPA axis. However, after chronic FS or HL-OF, the activity of the HPA axis decreased (indirectly, it is supported by decreasing the density of the c-Fos-ir cells after chronic FS or HL-OF in PVN; c-Fos is a marker of neuronal/trans-synaptic activations – Kovács 1998, Thrivikraman et al. 2000) (see Badowska-Szalewska et al. 2016b, supplementary data). Probably, repeated exposure to FS or HL-OF can result in the habituation of the HPA axis response, characterized by decreasing secretion of glucocorticoids (see Dhabhar et al. 1997), which in turn decreases the activation of hippocampal neurons.

It is obvious that BDNF expression is regulated by neuronal activity (see Tapia-Arancibia et al. 2004). BDNF is involved in neural plasticity processes (Theoen 1995), which are in turn regulated by synaptic activity (Poo 2001). The increase in the density of BDNF-ir neurons in hippocampal pyramidal layers of CA1–CA3 we observed and no changes in BDNF-ir in granular layer of DG in response to acute FS or HL-OF probably indicates that, first of all, glutamatergic projection neurons are sensitive to the action of these stressors, which in contrast to the granular neurons of DG, form the important efferent nerve tracts, including those which in consequence connect the hippocampus with the hypothalamus (see Amaral and Witter 1995). It has been reported that glucocorticoids rapidly increase excitatory amino acid release in the hippocampus (Venero and Borrell 1999), which could potentiate a glutamate stimulatory effect in the early stress response (Marmigère et al. 2003). In hippocampal neurons, BDNF enhances glutamatergic synaptic transmission (Lessmann et al. 1994). According to Marmigère and others (2003), a short-term increase in BDNF synthesis (and probably in the density of BDNF-ir
pyramidal neurons in our study) could help stimulate some hippocampal functions, which are beneficial in the alert reaction triggered by stress.

In our research, the density of BDNF-ir pyramidal neurons after chronic FS or HL-OF was comparable to BDNF-ir neurons in non-stress rats. In the opinion of Marmigére and others (2003), the decrease in BDNF expression after long-time stress application could help to stop the hyperactivity favored in the short-term response, because its prolongation may become harmful for cell survival. Although it is believed that old animals have less resistance to stressful stimuli (Li et al. 2009), taking into consideration the influence on the density of BDNF-ir neurons in the hippocampal pyramidal layers, our results may suggest that the applied chronic stressors were not an aggravating factor for these neurons in old rats.

It should be pointed out that the observed lack of density changes in BDNF-ir cells after chronic FS or HL-OF (vs. control) does not exclude the presence of certain immunohistochemically undetectable variations of protein level or mRNA in the marked neurons, as observed by other authors (Shao et al. 2010, Shi et al. 2010). The ageing process is connected with the reduction in the ability to cope with stress (Pedersen et al. 2001, Pardon 2007). On the other hand, according to Frolkis (1993), compensating mechanisms are formed in the process of ageing, for example, the activation of systems participating in the response to stress is observed even in the absence of stressors – the “state of readiness” is supported in order to adapt easily to the changing environmental conditions. The raised level of neurons activation (thus, as we may suppose, the increased level of BDNF, which is known to be activity-dependent) in the hippocampus of old rats not affected by stressors was observed by Meyza and others (2007). This may be one of the ways of preventing the visible reduction in BDNF level (and lack of changes of BDNF-ir neurons density) after chronic stress, even in view of age-related deficiencies of the central control of the stress axis.

In the light of our earlier (Badowska-Szalewska et al. 2010) and current research results, we may state that the influence of chronic FS on BDNF-ir hippocampal neurons depends on the stage of life of the rats. Prolonged FS resulted in a change (decrease) in the density of BDNF-ir cells in 28-day-old rats (the period of adolescence includes rapid behavioural changes connected with the intensive re-organization of the brain structures and the ultimate formation of their connections), but not in 1- or 2-year-old rats. It might indicate that this chronic FS is a factor which does not influence the number of BDNF-ir neurons in rats’ hippocampus with ageing.

Hippocampal pyramidal cells are particularly involved in creating and maintaining long-term potentiation (LTP) (memory formation) (see Kim and Diamond 2002, Rex et al. 2005), and in its induction BDNF takes part (Smith 1996, McAlister et al. 1999). Marmigére and others (2003) suggests that short-term stress might directly or indirectly result in an increase in neurotrophins, which may contribute to storing information about this short, not harmful event (facilitating the short-term effect of stress on memory through increased BDNF expression). Storing such information would serve to prepare a further response in case of a new stress stimulus (Marmigére et al. 2003). Thus, the increase in the density of BDNF-ir in pyramidal neurons after short-term FS or HL-OF in our study might have had a stimulating effect on the memory processes in old age.

CONCLUSIONS

As far as the influence on the density of BDNF-ir hippocampal neurons in old rats is concerned, the results of FS and HL-OF action were similar.

Exposure to acute FS or HL-OF was associated with an increase in the density of BDNF-ir, predominantly in hippocampal CA2 and CA3 pyramidal neurons. These changes were presumably connected with the need for the stress-dependent BDNF to increase in order to intensify the activity of pyramidal hippocampal neurons involved in regulating the HPA-axis (in the alert reaction triggered by stress) and might have helped to create the memory traces connected with the situation under stress.

Repeated exposure to FS or HL-OF did not determine changes in the density of BDNF-ir in the hippocampal pyramidal neurons in old rats. This was probably due to the decrease in HPA-axis activity, which in turn caused the decrease in the activity of hippocampal neurons and, finally, the decline for the demand for BDNF. These results may indicate that in old rats there are some adaptation mechanisms which still work.

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