Effects of BDNF deficiency and endoplasmic reticulum stress on the GABAergic system

Purpose: Protective pathways against endoplasmic reticulum stress in neurons are activated via brain-derived neurotrophic factor. However, it is not known how the inhibitory intermediate neuron types expressing the different Ca\(^{2+}\)-binding proteins of GABAergic system will be affected with changes in Ca\(^{2+}\) homeostasis, in conditions of chronic reduction of brain-derived neurotrophic factor and endoplasmic reticulum stress. The study was planned to reveal the interaction of these factors.

Materials and methods: 6-8 months old (30-40 g), wild-type (WT) and brain-derived neurotrophic factor heterozygous (BDNF\(^{(+/-)}\)) male mice were used and 4 groups were formed. Groups 3 and 4 were treated with a single dose of tunicamycin to induce endoplasmic reticulum stress. On the 3rd day of tunicamycin injection, animals were sacrificed and blood and brain tissues were taken. In serum samples BDNF, in tissue homogenates GRP78, CHOP, Caspase-12, parvalbumin, calretinin, calbindin, GAD65 and GAD67 levels were investigated by ELISA method. One-way ANOVA and Tukey post-hoc tests were used for statistical evaluation.

Results: Serum BDNF levels were significantly lower in BDNF\(^{(+/-)}\) and tunicamycin-treated BDNF\(^{(+/-)}\) groups. Caspase-12 and CHOP levels significantly increased with tunicamycin injection. Calbindin level decreased significantly with endoplasmic reticulum stress. GAD65 and GAD67 levels were similar in WT and BDNF\(^{(+/-)}\) groups. However, GAD65 level was significantly decreased during endoplasmic reticulum stress in WT and BDNF\(^{(+/-)}\) groups.

Conclusion: Endoplasmic reticulum stress caused a significant decrease in glutamic acid decarboxylase GAD65 isoform and calbindin levels. This result indicates that the sensitivity of varied intermediate neurons in GABAergic system to endoplasmic reticulum stress may be different.

Key words: BDNF, endoplasmic reticulum stress, calcium homeostasis, GABA.
Sonuç: Endoplazmik retikulum stresi glutamik asit dekarboksilazın GAD65 izoformunda ve kalbindin düzeylerinde anlamlı bir düşüşe sebep olmuştur. Bu sonuç, GABAerjik sistemdeki çeşitli ara nöronların endoplazmik retikulum stresine duyarlıklarının farklı olabileceğini göstermektedir.

Anahtar kelimeler: BDNF, endoplazmik retikulum stresi, kalsiyum homeostazı, GABA.

Introduction

The major neurotransmitter of the inhibitory system in the brain is gamma-aminobutyric acid (GABA) and is secreted by the inhibitor intermediate neurons mainly present in cortical structures [1]. GABA plays an essential role in regulating the stimulating activity of pyramidal cells and thus specifying the function of cortical networks. Cortical GABA is synthesized at the terminals with 67- and 65-kDa glutamic acid decarboxylase (GAD) isoforms, which are products of separate genes and are subjected to different post-translational changes. It is thought that under steady-state conditions GAD65 is largely ineffective and provides the optional GABA pool, whereas GAD67 is active and provides the basal pool. Constituting the majority of GABA synthesis the half-life of GAD67 is short (~2 hours), within the cortex this enzyme is dispersed across intermediate neurons and its activity is mainly regulated by transcription. In contrast, GAD65 has a long half-life (>24 hours) and is efficiently transported to the axon terminals; cofactor-dependent activity is highly regulated in response to GABA concentration. Both enzymes have been shown to be expressed depending on neuronal activity, however, only post-translational regulation of GAD65 is thought to be associated with rapid functional adaptation of GABA release [2, 3].

In the inhibitory system, non-adaptive, fast-spiking chandelier-type neurons that synthesize parvalbumin (PVALB), a Ca\(^{2+}\) binding protein, usually determine cortical excitability and synchronization of pyramidal cells via perisomatic synapses [4,5]. Inhibitory neurons that express other Ca\(^{2+}\) binding proteins -calbindin (CB) or calretinin (CR), but do not express PVALB, preferably control the dendritic integration of synaptic inputs into pyramidal neurons or the activity of other intermediate neurons. These neurons with low frequency are adaptable cells and do not have fast spiking properties. PVALB positive neurons with fast spiking feature express GAD67, but CB and CR positive neurons preferentially express GAD65 [4-6].

In addition to its classic role on neuronal growth and differentiation, brain-derived neurotrophic factor (BDNF) is a rapid regulator of excitability, synaptic conduction and inhibition, and activity-dependent synaptic plasticity [7, 8]. Synaptic inhibition plays an important role in the regular operating of brain cortex functions. During the performance of sensory, motor, memory and high cognitive functions, the stability and proper running of all cortical tasks can be regulated by the correct working of these inhibitory mechanisms [9]. BDNF deficiency, which is specific to different regions of the brain, causes an irregularity in the expression of various neuropeptides and Ca\(^{2+}\)-binding proteins.

As part of the cellular reticular network, endoplasmic reticulum (ER) plays an important role in many processes such as Ca\(^{2+}\) balance, lipid and protein biosynthesis, translational modification and regulation of gene expression [10]. Disorders of cell Ca\(^{2+}\) and redox balance, hypoxia or glucose deprivation affect post-translational modifications in ER and cause abnormal protein folding. Accumulation of misfolded or unfolded proteins in ER initiates a process defined as “ER stress”, and prolongation of this process triggers apoptotic mechanisms leading to cell death [10-12]. Cellular defense mechanisms activated during ER stress are associated with survival and coping with stress.

Ca\(^{2+}\) homeostasis in cells is provided by the integrated and coordinated function of Ca\(^{2+}\)-transport molecules, Ca\(^{2+}\)-tampons and Ca\(^{2+}\)-binding proteins. For normal cellular functions, the flow of Ca\(^{2+}\) to cells and organelles should be regulated continuously in equilibrium. In this process, the majority of ER-associated proteins are involved in maintaining Ca\(^{2+}\) homeostasis. In ER stress deterioration of Ca\(^{2+}\) balance and
changes in expression of different Ca\(^{2+}\)-binding proteins were observed [13, 14].

Studies have shown that protective pathways against ER stress in neurons are activated via BDNF [15-17]. It has been reported that under conditions where ER stress is induced, BDNF specifically blocks the CCAAT/enhancer-binding protein homologous protein (CHOP) pathway, preventing apoptosis and preventing nerve cell damage and death [17]. Nevertheless, under conditions where BDNF is chronically reduced and with changes in Ca\(^{2+}\) homeostasis in ER stress, it is not known how the types of inhibitory intermediate neurons of the GABAergic system expressing different Ca\(^{2+}\)-binding proteins will be affected. Current study was designed to demonstrate the interaction between these factors.

**Materials and methods**

*Experimental animals and grouping:* This study was approved by the Local Institutional Animal Care and Use Committee of the Faculty of Medicine, Karadeniz Technical University. 6-8 months old, wild type (WT) and BDNF heterozygous (BDNF\(^{(+/-)}\)) male adult mice were used and 4 groups were formed: Group 1: Control-Wild-type mice WT (n=7); Group 2: Control-BDNF heterozygous mice BDNF\(^{(+/-)}\) (n=8); Group 3: Wild type-Tunicamycin (Tm) mice WT+Tm (n=8); Group 4: BDNF heterozygous-Tunicamycin mice BDNF\(^{(+/-)}\)+Tm (n=8). A BDNF heterozygous (knockdown) mouse model has been developed to better understand the functions of BDNF and to characterize the consequences of chronic BDNF deficiency under physiological conditions [18]. In this model, one of the alleles in the coding region of the BDNF gene was replaced with the neomycin-resistant gene. As a result, completely healthy and fertile animals were obtained. Polymerase chain reaction (PCR) analysis was performed to determine the type of animal from the tail tissues of mice [19].

**ER stress generation and application of Tunicamycin:** Tm is an agent that induces ER stress by inhibiting N-glycosylation in ER [20, 21]. In our study, ER stress was induced by intraperitoneal administration of 0.5 mg/kg Tm intraperitoneally to groups 3 and 4. In the control groups, the same volume of saline was administered intraperitoneally. Mice under general anesthesia (ketamine, 50 mg/kg, intramuscular) were sacrificed on the 3rd day of injection, then blood and tissue samples were taken. Brain tissues were placed in -80°C freezer.

**Biochemical parameters:** Serum BDNF levels were determined by ELISA. This parameter was used as an indicator of BDNF deficiency in BDNF heterozygous groups. 78-kDa glucose-regulated protein (GRP78), CHOP and cleaved-Caspase-12 levels in tissue homogenates were studied by ELISA method. These parameters, which are seen as evidence of ER stress [22, 23], showed the difference between wild and heterozygous groups in both basal and stress induced conditions in the brain. In addition to Ca\(^{2+}\)-binding proteins (PVALB, CB and CR) synthesized by inhibitory intermediate neurons, GAD65 and GAD67 enzymes were also examined by ELISA method in order to see how GABA synthesis has changed.

**Statistical evaluation:** Results are given as mean±standard deviation. Differences were analyzed via parametric one way analysis of variance (ANOVA) followed by Tukey's Post Hoc Test for normally distributed variables using GraphPad Prism 4.0 software. \(p<0.05\) was considered statistically significant.

**Results**

The circulating BDNF level in BDNF\(^{(+/-)}\) and BDNF\(^{(+/-)}\)+Tm groups showed a statistically significant decrease compared to WT and WT+Tm groups \((p<0.05)\) (Table 1).

The concentration of GRP78 as an ER stress marker was measured with ELISA in mice treated with saline solution or Tm. GRP78 level increased slightly in BDNF\(^{(+/-)}\) group compared to WT group, but it was not statistically significant (0.77±0.22 and 0.57±0.06 ng/mg prot, respectively). Tm injection did not cause a statistically significant change in the level of GRP78 (Figure 1).

CHOP level was similar in WT and BDNF\(^{(+/-)}\) groups (3.44±0.20 and 3.67±0.63 ng/mg prot, respectively). ER stress produced a significant increase in CHOP levels in both WT+Tm and BDNF\(^{(+/-)}\)+Tm groups (4.60±0.78 and 6.26±0.78 ng/mg prot, respectively). However, CHOP increase was more prominent in BDNF\(^{(+/-)}\)+Tm group, compared to WT+Tm group \((p<0.01)\) (Figure 2).
Table 1. Serum BDNF levels

|                | WT (n=7) | BDNF (+/-) (n=8) | WT+Tm (n=8) | BDNF (+/-)+Tm (n=8) |
|----------------|----------|------------------|-------------|---------------------|
| Serum BDNF (ng/ml) | 0.21±0.02 | 0.11±0.03*       | 0.24±0.02   | 0.14±0.03^          |

*p<0.05 (difference from WT), *p<0.05 (difference from WT+Tm)
BDNF: brain-derived neurotrophic factor; WT: wild type;
BDNF (+/-): BDNF heterozygous; Tm: tunicamycin

Figure 1. Brain GRP78 levels

GRP78: 78-kDa glucose-regulated protein; BDNF: brain-derived neurotrophic factor;
WT: wild type; BDNF (+/-): BDNF heterozygous; Tm: tunicamycin

Caspase-12 level was 0.68±0.08 in the WT group and 0.8±0.23 ng/mg prot in the BDNF (+/-) group. Tm injection significantly increased Caspase-12 levels, this increase was more evident in BDNF (+/-)+Tm group, but did not show statistical significance compared to WT+Tm group (1.41±0.51 and 1.15±0.05 ng/mg prot, respectively). Again, Tm injection caused a significant increment in Caspase-12 level in brain tissue of BDNF heterozygous animals, compared to saline applied BDNF (+/-) group (p<0.01) (Figure 3). Under basal conditions, levels of PVALB (0.68±0.14 and 0.85±0.38 µg/mg prot, for WT and BDNF (+/-) groups, respectively), CR
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Figure 3. Brain Caspase-12 levels
*p<0.05, **p<0.001 (difference from WT), #p<0.01 (difference from BDNF(+/-))
BDNF: brain-derived neurotrophic factor; WT: wild type; BDNF(+/-): BDNF heterozygous; Tm: tunicamycin

(0.86±0.21 and 1.17±0.51 ng/mg prot, for WT and BDNF(+/-), respectively) and CB (3.36±0.67 and 3.96±1.64 µg/mg prot, for WT and BDNF(+/-) groups, respectively) were higher in the BDNF(+/-) group, although this difference was not statistically significant (Figure 4). Changes induced by Tm application in PVALB and CR values were not statistically important. However, CB level decreased significantly with ER stress (2.37±0.87 and 2.26±0.31 µg/mg prot, for WT+Tm and BDNF(+/-)+Tm groups, respectively (Figure 4).

GAD65 (0.23±0.06 and 0.27±0.12 ng/mg prot, respectively) and GAD67 (0.21±0.04 and 0.26±0.12 ng/mg prot, respectively) levels were close to each other in WT and BDNF(+/-) groups. Reduction was observed in both groups after Tm injection (Figures 5 and 6). GAD65 level in BDNF(+/-) group decreased from 0.27±0.12 ng/mg prot to 0.16±0.05 ng/mg prot in BDNF(+/-)+Tm group (p<0.05) (Figure 5).

Discussion

Important findings of the current study are: (i) under basal conditions, endogenous BDNF deficiency did not affect the parameters associated with ER stress in the brain, as well as different types of inhibitory intermediate neurons expressing different Ca²⁺-binding proteins of the GABAergic system, and two different enzyme isoforms that synthesize GABA neurotransmitter; ii) a significant decrease in the levels of CB and GAD65 isofom of glutamic acid decarboxylase during ER stress showed that the sensitivity of different intermediate neurons in the GABAergic system to ER stress may be different; iii) endogenous BDNF deficiency did not alter the sensitivity of these intermediate neurons to ER stress.

Transgenic heterozygous mice show reduction in the expression of BDNF to nearly 50% in the brain cortex, but exhibit similar phenotypic characteristics with normal wild type mice [24]. In this study, chronic BDNF deficiency was also confirmed by BDNF measurements in serum of BDNF(+/-) groups. Previously, it was observed that decreased BDNF level induces hyperphagia, obesity, hyperglycemia, insulin resistance and behavioral abnormalities, including increased aggression and hyperactivity in these transgenic mice [25, 26]. Consistent with these findings, an increase in food intake, body weight, fat mass and aggressive behavior pattern was observed during the experiments in non-stressed heterozygous mice (data not shown).

ER coordinates synthesis, folding and post-translational modification of proteins, cytoplasmic and mitochondrial metabolism, Ca²⁺ storage and cell death. The protein folding capacity of ER is impaired under different physiological and pathological conditions that induce ER stress. During ER stress, the unfolded proteins deposited in the ER lumen trigger an adaptive cell response called unfolded protein response (UPR) to maintain cell homeostasis. Tm, an agent that inhibits N-glycosylation in ER, was used in this study to induce ER stress. Three days after the injection of Tm, known to cross the blood-brain barrier
Figure 4. Brain Ca\textsuperscript{2+}-binding proteins levels

\#p<0.05 (difference from BDNF\textsuperscript{(+/-)})

PVALB: parvalbumin; CR: calretinin; CB: calbindin;
BDNF: brain-derived neurotrophic factor; WT: wild type;
BDNF\textsuperscript{(+/-)}: BDNF heterozygous; Tm: tunicamycin
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Figure 5. Brain GAD65 levels

\#p<0.05, ##p<0.01 (difference from BDNF(+/-))

GAD65: glutamic acid decarboxylase 65-kDa isoform; BDNF: brain-derived neurotrophic factor; WT: wild type; BDNF(+/-): BDNF heterozygous; Tm: tunicamycin

Figure 6. Brain GAD67 levels

There was no statistically significant difference among the groups

GAD67: glutamic acid decarboxylase 67-kDa isoform; BDNF: brain-derived neurotrophic factor; WT: wild type; BDNF(+/-): BDNF heterozygous; Tm: tunicamycin

[27], the presence of ER stress in the brain was assessed by GRP78, CHOP and Caspase-12 markers. As an ER resident chaperone, GRP78 recognizes unfolded proteins and plays an important role in the regulation of UPR through activation of three pathways: PKR-like ER kinase (PERK), activated transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1) [12, 28, 29]. Activation of the UPR pathways first leads to a general weakening of protein synthesis and prevents further accumulation of unfolded proteins. Two other major adaptive responses are the increase of chaperones such as GRP78 and GRP94 that improve ER folding capacity and the rearrangement of the ER-associated degradation system that activates the breakdown of misfolded proteins. Despite these defense mechanisms, ER stress can trigger apoptosis depending on the severity and duration of stress [30]. Transcriptional activation of the CHOP, also known as growth arrest, activation of the cJUN NH2-terminal kinase (JNK) pathway and activation of ER-associated Caspase-12 are involved in ER stress mediated apoptosis [30, 31]. In a previous study, it was found that the amount of GRP78 reached the highest value in the twenty-fourth hour after ER stress was induced in the mouse breast gland and decreased rapidly after the forty-eighth hour; however, the level of caspase (Caspase-7), one of the apoptotic markers, increased gradually after the twenty-fourth hour and was determined by Western and immunohistochemical analyzes [11]. In our study, the level of GRP78 did not
differ significantly between the groups on the third day of ER stress, possibly due to the major neuronal loss caused by stress, while the amount of Caspase-12 increased significantly in the brain tissue of both control and heterozygous animals exposed to Tm.

Our BDNF analysis results showed that Tm-induced ER stress did not alter serum BDNF levels. Since BDNF is a secretory protein, a decrease in BDNF levels during ER dysfunction can be expected. Wei et al. [32] demonstrated that homocysteine-induced ER stress modeling was associated with a reduction in BDNF expression with induction of ER stress in the hippocampus after seven days of intracerebroventricular homocysteine administration. Although there are no studies in the literature that can be interpreted with our results, we can suggest that the short test period (3 days) may not be sufficient for such an effect. It is well known that BDNF, a neurotrophic factor affecting the central nervous system, prevents neuronal cell death induced by various stimulants. Molecular mechanisms involved in the protective effects of BDNF against ER stress have been scrutinized. Shimoke et al. [16] showed that BDNF limits ER stress-induced apoptosis by suppressing the activation of Caspase-12 via a phosphatidylinositol 3-kinase (PI3-K) dependent mechanism; Chen et al. [17] suggested that suppression of CHOP activation contributes to BDNF-mediated neuroprotection during ER stress. The current study showed that Tm administration significantly increased Caspase-12 and CHOP levels in both wild-type and BDNF heterozygous mice. Although the Tm-induced increase of Caspase-12 levels were similar in both groups, the increase in CHOP was greater in the BDNF heterozygous mice than the wild-type ones. This result suggests that endogenous BDNF may protect the brain against apoptosis due to ER stress by reducing CHOP elevation.

Inhibitor intermediate neurons make up approximately 20-30% of cortical neurons. Based on the expression of one of the three Ca²⁺-binding proteins (PVALB, CB or CR) most GABAergic neurons within the prefrontal cortex can be divided into non-overlapping subtypes [33]. CB, CR and PVALB neurons play different roles in the local cortical network. For instance, PVALB and CB neurons offer different strategies for regulating feedforward or feedback inhibition within and between cortical layers, as well as neuronal input-output transformations in cortical circuits. Thus, the amount and type of GABA inhibition that each cortical layer receives directly affects the integration of inputs into that layer (inhibition provided by CB and CR neurons) or the functional output of neurons present in the layer (inhibition provided by PVALB neurons).

In differentiated neurons, BDNF directly regulates synaptic transmission. In hippocampal cultures, BDNF induces expansion of soma of GABAergic neurons, increases GABA, receptor subunits and GAD expression, and facilitates high K⁺ mediated GABA release. Data to date have been controversial, but BDNF has many roles, particularly for the development and maintenance of interneurons. In general, however, BDNF deficiency does not seem to be of great importance for the development and maturation of presynaptic terminals [34]. Our study revealed that endogenous BDNF deficiency did not affect two different enzyme isoforms that synthesize inhibitory intermediate neuron types and GABA neurotransmitters in basal conditions. The significant decrease in levels of CB and GAD65 isoform during ER stress indicated that the sensitivity of different intermediate neurons in the GABAergic system to ER stress may be different, but endogenous BDNF deficiency did not increase the sensitivity of these intermediate neurons to stress.

According to our results, less loss was observed in CR and PVALB expressing neurons with ER stress, than in neurons expressing CB. This finding may suggest that neurons expressing CB are more sensitive and vulnerable to ER stress. In previous reports it was observed that Ca²⁺-binding proteins protect GABAergic neurons from an increase of Ca²⁺ overload in various pathological states such as hypoxia and ischemia [35], amyotrophic lateral sclerosis [36] and glutamate toxicity [37], thus maintain Ca²⁺ homeostasis. In different stress models, different subtypes of Ca²⁺-binding proteins stand out as protective of neurons. For instance, in ischemic conditions neuroprotective efficacy was demonstrated in a descending order like: CB≥CR>PVALB [35]. Therefore, in our study, we can speculate that especially PVALB and CR proteins, rather than CB, make GABAergic neurons more resistant to ER stress.
Dysfunction of inhibitor GABAergic interneurons have been implicated in various brain diseases with neurodevelopmental origin. It has been suggested that altered GABA neurotransmission in the subgroups of intermediate neurons in the prefrontal cortex contributes to the pathophysiology of psychiatric disorders including schizophrenia [38], mental retardation [39], and epilepsy [40], as well as to the pathobiology of major depression [41] and autism [42]. Inhibitor GABAergic interneuron abnormalities cause excitation-inhibition imbalance in neural circuits. Among these subtypes, PVALB-positive GABAergic intermediate neurons in the neocortex, were strongly associated with brain disorders. Therefore, further molecular, cellular and experimental animal model studies should be performed to increase our understanding of the pathophysiology of various neurodevelopmental and neuropsychiatric disorders associated with inhibitory intermediate neurons.

The limitation of the study is that the biochemical parameters were studied in brain tissue homogenates. If these parameters were supported by immunohistochemical analysis, the localization of GABAergic intermediate neurons could be demonstrated exactly.

Conflict of interest: No conflict of interest was declared by the authors.

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**Contributions of the authors to the article**

G.H.D. built the main idea, hypothesis and scientific infrastructure of the study. S.C. developed the idea. S.A. and İ.A. developed the method part of the study. G.H.D., S.C. and H.Y. conducted the experiments. G.H.D. and S.C. made the evaluation of the data in the results section. The discussion part of the article was written by G.H.D., then S.C., S.A. and İ.A. reviewed and made the necessary corrections. In addition, all authors discussed the entire study and approved its final version.