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Original article

Oral administration of BDNF and/or GDNF normalizes serum BDNF level in the olfactory bulbectomized rats: A proof of concept study

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ABSTRACT

Background: Neurotrophins, especially brain-derived neurotrophic factor (BDNF) have gained significant therapeutic interest particularly in neurologic and psychiatric disorders and they have been found in human breast milk of mothers who suffered from adverse outcomes in pregnancy. This study tested the hypothesis that oral administration of BDNF/GDNF (glial cell line-derived neurotrophic factor) can exert a biological effect in a rat model of severe neuropathology induced by olfactory bulbectomy (OBX), which exhibits dysregulation of BDNF signaling and impaired blood-brain barrier.
Methods: Adult male albino Sprague-Dawley rats underwent the OBX surgery and separate groups of OBX and sham-operated controls received one oral dose of vehicle, BDNF (0.005 mg/kg), GDNF (0.03 mg/kg) or their combination. One week after neurotrophin dosing the rats were sacrificed and BDNF level was assessed by ELISA in the blood serum and cerebrospinal fluid.
Results: A significant decrease of serum BDNF level was found in the OBX model. This alteration was normalized by all types of treatment BDNF, GDNF, or their combination. No influence of sham surgery or treatment was observed in the control rats. BDNF levels in cerebrospinal fluid were below detection limit.
Conclusion: This study indicates that oral administration of neurotrophins is able to exert a biological effect in the OBX model. There is a number of potential mechanisms, which remain to be elucidated.

Introduction

Neurotrophins are closely related neuropeptides of the nerve growth factor family that control many aspects of maintenance of the neurons as well as other cell types in central nervous system (CNS) as well as peripheral nervous system (PNS) and affect strongly their functions such as synapse formation and synaptic plasticity [1,2]. Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4) are derived from a common ancestral gene and there is an extensive amount of literature published on their effects in PNS as well as CNS [3]. Apart from the very potent link of BDNF with the structure and function of neurons [4,5], the neurotrophins are involved in glial maintenance. The main player in the glial functioning is glial cell line-derived neurotrophic factor (GDNF) [6].

Considering the important protective and regenerative properties of neurotrophins, especially BDNF have gained significant therapeutic interest particularly in neurologic and psychiatric disorders [7,8]. However, BDNF has specific limitation to its therapeutic utility due to inherent issues with its structure and pharmacokinetic profile. The ability of neurotrophins to cross the blood-brain barrier (BBB) is unclear. Indeed, transport of BDNF across the brain capillary endothelial wall, which forms the BBB *in vivo*, is negligible for some authors [9]. However, other studies reported that BDNF is able to cross the BBB [10] and intravenously administered BDNF was shown to enter the rat brain [11]. In addition to its unresolved ability to cross the BBB, the half-life of BDNF in the bloodstream is also a limiting factor [9].

Recently, it has been reported that neurotrophins are present in human breast milk [12] and their levels seem to be elevated in the milk of mothers who suffered from adverse outcomes in pregnancy, such as preeclampsia [13]. This suggests potential

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existence of some trophic mechanisms of neurotrophins in the gastrointestinal tract of the new-born, however, very little is known about them. Currently, there are no studies available on the oral administration of BDNF in rat nor on potential antagonism/synergy with other factors, although synergistic or additive effect of BDNF combined with GDNF was proposed in several different models [14,15].

Therefore, this study aimed to prove the hypothesis that oral administration of BDNF is able to exert a biological effect in a rat model of severe neuropathology induced by olfactory bulbectomy (OBX), which features dysregulation of BDNF signaling [16] and impaired BBB [17]. Furthermore, we tested a synergistic effect of BDNF administered together with GDNF and we included sham-operated control animals where we presumed no effect of any neurotrophin administration.

Material and methods

Animals

Fifty male albino Sprague-Dawley rats (8 weeks old, with a weight range of 225–250 g at the beginning of the experiment) were purchased from Charles River (Germany). The rats were housed in standard rodent polycarbonate cages in sections of four. The final number of animals was n = 16 in the sham-operated group and n = 24 in the OBX group. The reasons for exclusion of the animals in the OBX groups were death during/after surgery. The success of the OBX lesion was verified after finishing the study by a dissection.

Environmental conditions during the whole study were constant: relative humidity 50–60%, temperature 23 °C ± 1 °C, inverted 12-hour light-dark cycle (6 a.m. to 6 p.m. darkness). Food and water were available *ad libitum*. All procedures were performed in accordance with EU Directive no. 2010/63/EU and approved by the Animal Care Committee of the Faculty of Medicine, Masaryk University, Czech Republic and the Czech Governmental Animal Care Committee, in compliance with Czech Animal Protection Act No. 246/1992.

Drugs and treatments

The outline of the study protocol is depicted in Fig. 1. Human recombinant BDNF and GDNF were purchased from PeproTech (New Jersey, USA) and R&D Systems (Minneapolis, MN, USA), respectively. Fourteen days after OBX surgery the animals were divided into treatment groups and neurotrophins were administered orally dissolved in full-fat cow milk (approx. 3.5% of fats). Due to the particularly challenging task of obtaining a sufficient amount of human or rat milk, cow milk was chosen as a widely available pathogen-free liquid with similar properties. This also ensures better data reproducibility, given the strictly monitored cow milk quality standards. BDNF was administered at a dose of 0.005 mg/kg in 2 ml of milk, GDNF was used at a dose of 0.03 mg/kg in 2 ml of milk. Cow milk from the same package was used as a vehicle. The doses of BDNF and GDNF were calculated in order to approach concentrations in human milk that were available from our previous studies. Treatment groups were as follows: SHAM-VEH (n = 6), SHAM-BDNF (n = 6), SHAM-BDNF + GDNF (n = 4), OBX-VEH (n = 6), OBX-BDNF (n = 5), OBX-GDNF (n = 6), OBX-BDNF + GDNF (n = 7). Due to the loss of several animals during the surgery, we were forced to decrease the number of sham-operated rats too. Therefore, the SHAM-GDNF group was excluded consistently with our hypothesis.

OBX surgery

Rats were randomly divided into two groups and the bilateral ablation of the olfactory bulbs was performed in accordance with a standard procedure routinely used in our laboratory [18–21]. In brief, animals were anesthetized with ketamine 50 mg/kg (Narkamon® 100 mg/ml, Bioveta a.s., Czech Republic) and xylazine 8 mg/kg (Rometar® 20 mg/ml, Bioveta a.s., Czech Republic) administered intraperitoneally. The top of the skull was shaved, swabbed with an antiseptic solution, after which a midline frontal incision was made through the skin on the skull. After exposure of the skull, 2 burr holes were drilled at the points 7 mm anterior to the bregma and 2 mm lateral to bregma suture. Olfactory bulbs were aspirated and the dead space was filled with a haemostatic sponge. The skin was sutured and the antibacterial neomycin and bacitracin powder were applied. Sham-operated animals underwent the identical procedures as OBX animals, but their bulbs were left intact. A period of 14 days was allowed for the recovery from the surgical procedure. During this period, animals were regularly handled to eliminate potential aggressiveness [22,23]. At the end of the study, rats were sacrificed by decapitation and the brains were dissected for confirmation of the successful removal of the olfactory bulbs.

Sacrifice of animals and sample harvest

Seven days after oral administration of neurotrophins animals were sacrificed by decapitation under injection anesthesia *ip* administration of 50 mg/kg ketamine plus 8 mg/kg xylazine). The selected time point of seven days after administration was chosen in order to safely eliminate the possibility of detecting the *per os* administered substance of interest. Animals were fixed in a stereotaxic frame; a skin incision was made on the top of the head and the caudal part of the skull was exposed in order to collect the cerebrospinal fluid from cisterna magna by aspiration with a disposable 1 ml syringe. Later, the trunk blood was collected by decapitation and centrifuged to obtain serum. All samples were shock frozen in dry ice and kept in the −70 °C freezer until analysis.

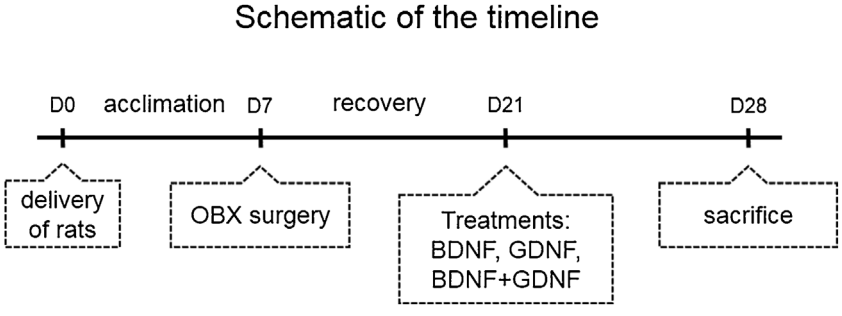

Fig. 1. Experimental design. The figure depicts a timeline of experimental procedures used in this study. Acronym D indicates day of the study.

ELISA analysis

BDNF levels were measured with a commercially available sandwich ELISA kits (MyBioSource, CA, USA) according to the manufacturer's instructions. Repeatability (expressed as the coefficient of variation CV) was less than 10%.

Statistical analysis

Standard descriptive analysis of the data was performed as the first step of the analysis. The data was divided into 7 treatment groups as defined in the previous section. Shapiro-Wilk normality test [24,25] was used to assess the normality in each group. Due to non-normal nature of BDNF in serum, a natural log transformation of the data was used in all further steps of the analysis. The boxplot [26] was selected as an appropriate visualization tool for transformed data.

Student's two-sample *t*-test was performed to assess the difference between groups SHAM-VEH and OBX-VEH. The effect of treatment on SHAM and OBX conditions was determined by one-way ANOVA [27]. The post-hoc differences between individual groups were computed using Tukey's Honest Significant Differences [28]. The normality assumption for the ANOVA was tested by the Shapiro-Wilk test and the homogeneity of variances assumption by Bartlett Test [29]. Minor violation of the normality assumption was allowed due to the robustness of the ANOVA method for non-normal data [30].

The statistical analysis was performed in the R software version 3.5.0 [31]. The significance level of 0.05 was used throughout the analysis.

Results

We observed a significant decrease of serum BDNF level in the OBX model (*t*-test, t = 2.318, $p = 0.043$), as indicated in the Fig. 2. As expected, the analysis in sham-operated rats revealed no changes of BDNF serum level after either BDNF or BDNF + GDNF oral administration (one-way ANOVA, $F_{(2,13)} = 0.201$, $p = 0.820$), as shown in the Fig. 3. On the other hand, there was a significant effect of treatment in the OBX rats (one-way ANOVA, $F_{(3,19)} = 4.979$, $p = 0.010$). The post-hoc analysis revealed a significant influence of all types of neurotrophin treatment: BDNF ($p = 0.031$), GDNF ($p = 0.024$), BDNF+GDNF ($p = 0.023$). The results are depicted in the Fig. 4. BDNF concentrations in cerebrospinal fluid (CSF) were below the detection limit and therefore are not given in a figure.

Discussion

Breast milk provides an optimal nutritional source for new-borns. It contains a myriad of constituents including the proteins that serve as a source of amino acids for rapidly growing infants. In addition, breast milk facilitates optimal new-born development *via* the action of trophic factors [32]. Several lines of evidence suggest that proteins such as BDNF and GDNF may be contributing to neurotrophic properties of milk that are essential for successful development of the nervous system [33–35]. This is of particular interest in infants with an increased risk of neurodevelopmental disorders due to adverse outcome of pregnancy. As suggested by the studies where the alterations in breast milk BDNF levels were present in mothers with preeclampsia, composition of breast milk might help ameliorate negative impact of such condition [13,36]. Importantly, since neurotrophins might cross the BBB and activate neuronal downstream signalling directly in the brain [10,11], the importance of oral route of BDNF delivery shall be further in-depth examined. Our aim was to show that BDNF/GDNF given orally in milk are able to exert a systemic effect, despite the common notion of being inactivated by digestion. To achieve this, we employed a rat OBX model that is associated with BBB impairment and BDNF dysregulation [16,17].

The main finding of this study is normalization of serum BDNF level in the OBX rats after oral administration of BDNF, GDNF or their combination. Both BDNF and GDNF alone restored the serum level of BDNF in OBX rats. We have not detected the synergistic effect of the neurotrophin combination. Interestingly, this phenomenon was present one week after acute dosing of the neurotrophins, which excludes the possibility of detecting orally delivered BDNF in the serum, as the BDNF is known to have a very short half-life [9]. No treatment effect was observed on the sham-operated control animals. Furthermore, our data indicate significantly decreased BDNF serum level in the OBX rats.

The change of BDNF levels is a commonly reported OBX-induced alteration reported both in blood and in several brain regions. Although there exists some controversy about BDNF levels in the rat and mouse OBX models and in different rat strains, the majority of the studies show a decline of BDNF levels in various part of the brain or in the system [37–43]. Hence, our study is in

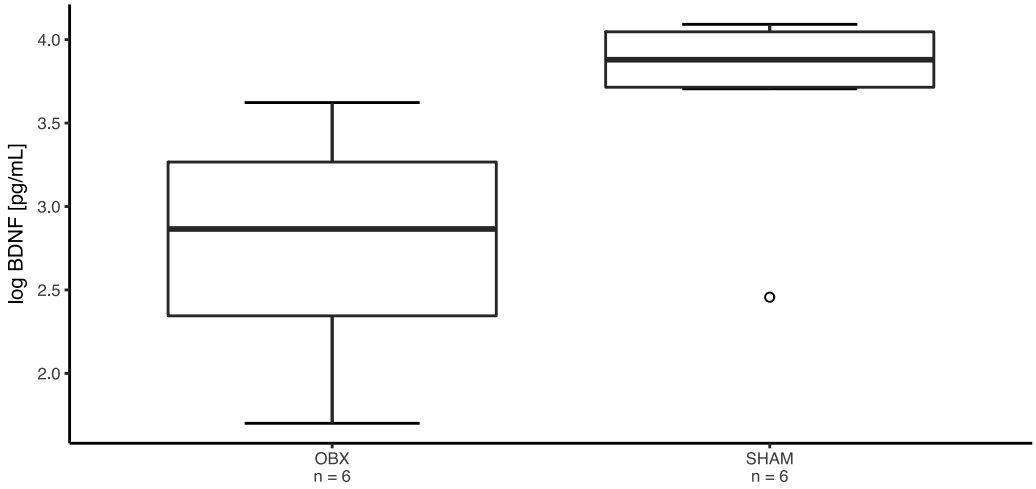

Fig. 2. Serum BDNF level in the OBX model. The boxplot indicates log transformed data of the serum BDNF level in the OBX rats and sham-operated control animals. OBX rats showed significantly lower values, *t*-test, $p = 0.043$.

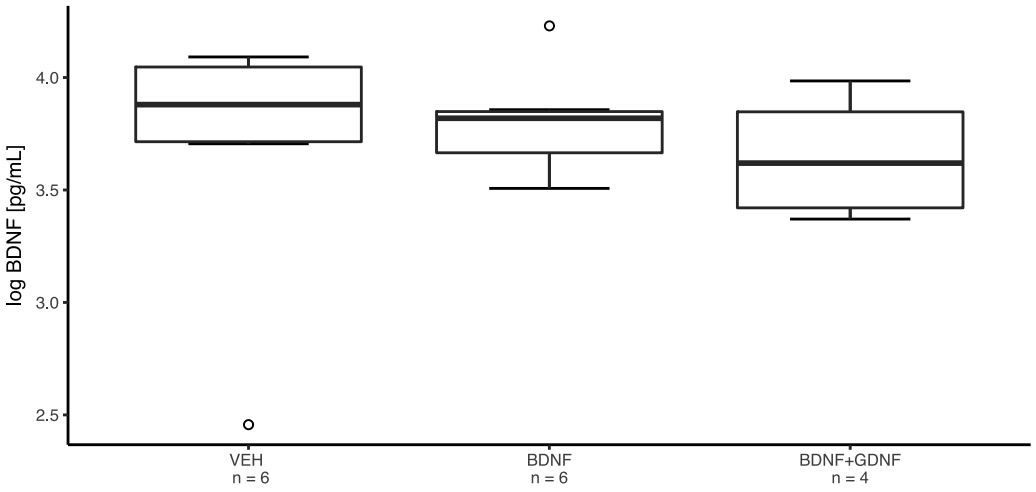

Fig. 3. Treatment effect on the serum BDNF level in the sham-operated rats. The graph indicates log transformed data of the serum BDNF level in the control rats treated with BDNF, and combination BDNF + GDNF or vehicle. One-way ANOVA did not detect any significant treatment effect.

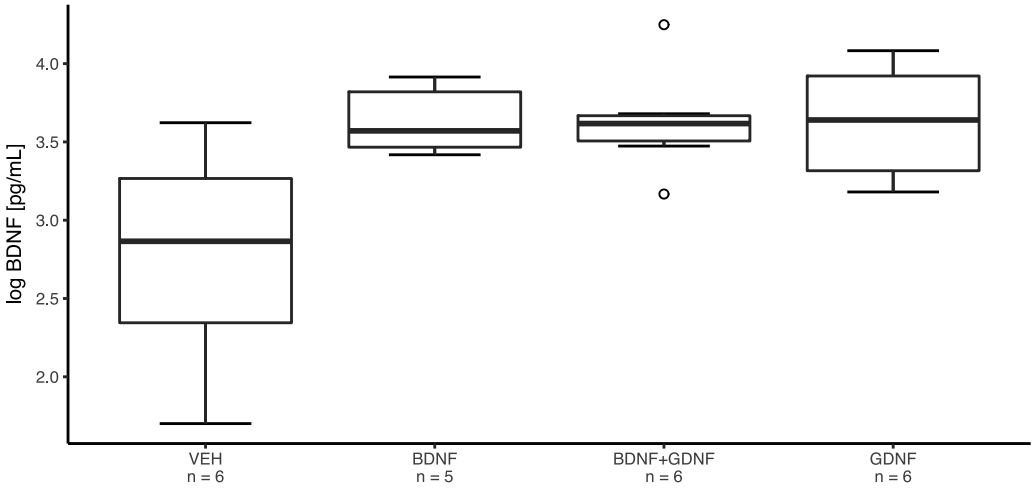

Fig. 4. Treatment effect on the serum BDNF level in the OBX operated rats. The graph indicates log transformed data of the serum BDNF level in the OBX rats treated with BDNF, GDNF, their combination or vehicle. One-way ANOVA with post-hoc test revealed a significant effect of all types of neurotrophin treatment: BDNF ($p = 0.031$), GDNF ($p = 0.024$), BDNF+GDNF ($p = 0.023$) compared to vehicle-treated group.

accordance with the published reports showing lower BDNF levels following the OBX. Although the origin of BDNF detected in the periphery is still a matter of debate, a growing body of evidence suggests that the brain is the main contributor. Importantly, several studies have reported that BDNF detected in peripheral blood reflects BDNF levels in the brain. Positive correlation has been found between serum and brain-tissue BDNF levels in young and adult Sprague-Dawley rats [44]. Klein et al. also reported a significant correlation between BDNF in rat blood and in hippocampal brain tissue. Interestingly, this study also revealed a correlation between plasma BDNF and hippocampal BDNF levels in pigs, suggesting that peripheral BDNF might reflect brain-tissue BDNF across the mammalian species [45]. These highly consistent findings are in line with our study showing significantly reduced serum BDNF levels 28 days after OBX lesion. The levels of BDNF in CSF were below the detection limit, which is not an uncommon phenomenon in human and rodent studies employing ELISA-based methodology [46–48]. This drawback can be overcome in future studies by the recently described impedimetric BDNF sensor that combines the advantages of in vivo detection and higher sensitivity and represents a promising alternative to the conventional methods [49].

Despite that nervous system is generally recognized as a principal source of BDNF in organism, many peripheral tissues are now documented to express BDNF in relatively high levels, including liver, spleen, skeletal muscle, cardiorespiratory system, urogenital systems and all of the gastrointestinal regions [50–52]. Given the oral administration of neurotrophins, we suppose that gastrointestinal interface is of supreme importance in our model. Gastrointestinal tract (GIT) is designed to absorb nutrients, water, and electrolytes. Simultaneously, it provides a tight biological and physical barrier that is protecting the organism against toxins, allergens, bacteria, and other potentially harmful substances. Following the consumption, the majority of proteins are hydrolyzed by the action of proteolytic digestive enzymes. This should inevitably lead to the loss of their biological functions during this step. However, multiple lines of evidence exist showing that peptides, partially digested proteins, and even intact proteins, although in a limited amount, can cross the intestinal barrier and enter the bloodstream [53]. Interestingly, proteolytic fragments of

nerve growth factors were shown to be more biologically active than structurally intact protein [54]. Given the inherent impairment of BBB in the rat OBX model [17] that can further facilitate transport of neurotrophins into the brain, we cannot exclude that BDNF, GDNF or at least biologically active peptides recognizable by receptors can act directly in the brain regions of OBX rats in an autoregulatory positive-loop fashion. Such control of BDNF gene expression by activated tyrosine kinase B (TrkB), the high-affinity BDNF receptor, have been recently described in a rat model [55].

Intriguingly, it was reported that inflammation of the viscera is associated with BDNF upregulation and that BDNF is expressed by inflammatory cells [56–58]. Therefore, partially digested proteins might also stimulate immunocompetent cells in GIT and further modulate intestinal permeability as well as endogenous neurotrophin production by activated cells of the immune system. Notably, even though milk itself contains leukocytes that were shown to express BDNF and GDNF [12,59], we didn't observe any effect in the vehicle-treated group. This could be explained either due to rather strictly monitored content of leukocytes in cow milk that was used as a vehicle or because of the effect of pasteurization. Unfortunately, to our knowledge, there is no study quantifying BDNF content in commercially processed cow milk. However, based on the several studies focusing on neurotrophin S100B we may assume that procedures routinely employed in the industrial preparation of milk cause substantial heat-induced modifications that reduce the biological activity of proteins or compromise the accuracy of the quantification [60,61].

The intestinal barrier consists of the mucus layer, the epithelial layer, and the lamina propria [62]. Epithelial cells are connected with tight junction proteins, including members of the claudin family, occludin, and junctional adhesion molecules that represent a critical regulator of intestine permeability [63]. Recently, BDNF was reported to be involved in the modulation of colonic epithelium ultrastructure and expression of tight junction proteins in mice GIT [64] which may represent another potential mechanism of its biological effect after oral administration.

Unfortunately, little is known about the intestinal transport of BDNF or GDNF. In general, transport of the proteins across the intestinal tract depends mainly on size, polarity, and structure of the protein [65]. Several authors reported that closely related nerve growth factor administered orally in milk or intraluminally is transported across the absorptive epithelium of young suckling rats (postnatal day 0–12) and readily enters the bloodstream and various organs including the brain [66,67]. With respect to the differences between GIT and BBB of adult and suckling rats, these results suggest the possibility that biologically active peptides are able to access the bloodstream *via* the digestive tract. Negligible changes of serum BDNF levels in sham-operated animals in this study point out that one of the critical factors for continuous endogenous neurotrophin production is the integrity of BBB known to be impaired in the OBX model [17].

The gut is innervated by several pathways comprising vagal afferents and efferents, sympathetic efferents, dorsal root afferents, and enteric neurons. Many of these pathways are reported to express BDNF and both receptors for BDNF; high-affinity receptor TrkB and low-affinity neurotrophin receptor p75 [68]. Following the binding of BDNF to its receptors at the axon terminal, signalling cascades involving the phospholipase C-gamma, mitogen-activated protein kinase/extracellular signal-regulated protein kinase (MAPK/ERK), and phosphatidylinositol-3-kinase (PI3K) are activated. The proteins of these signalling pathways are then packaged onto endocytic vesicular structures termed signalling endosome. BDNF/TrkB signalling endosome is then retrogradely transported through the neurons in dynein-dependent manners [69,70]. Notably, retrograde transport of GDNF has been reported as well, although the molecular mechanisms that underlie the trafficking remain elusive [70]. Signal transduction of GDNF is mediated through the multicomponent receptor complex that consists of the Ret receptor tyrosine kinase and a glycosylphosphatidylinositol-linked co-receptor named GDNF receptor α (GFRα). Binding of GDNF to GFRα induces Ret phosphorylation and activates several intracellular pathways, including MAPK/ERK and the PI3K [71]. Overlapping intracellular pathways responsible for the BDNF and GDNF signal transduction might explain the lack of synergistic effect observed in animals treated simultaneously with BDNF and GDNF. This is also in line with evidence that neuroprotective properties of GDNF might be partially mediated in BDNF-dependent manners, further supporting the concept of considerable BDNF and GDNF crosstalk described in different models [72,73]. Hence, changes elicited by orally delivered BDNF or GDNF could alter the signaling pathways and consequently lead to continuous BDNF production.

Conclusion

Our results indicate that the rat OBX model is associated with reduced BDNF levels in serum. Orally administered neurotrophins BDNF and GDNF are able to normalize decreased serum BDNF levels in the OBX model but do not exert any effect in sham-operated control animals. There is a number of potential mechanisms, which may contribute to the observed effect.

Conflict of interest disclosure

All authors declare no conflict of interest.

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