Clinical and Experimental Health Sciences

Effect of Reboxetine Treatment on BDNF, Synaptophysin, and PSD-95 Levels in the Spinal Dorsal Horn of Rats with Diabetic Neuropathy

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ABSTRACT

Objective: It is known that neuropathic pain is accompanied by alterations in the levels of neurotrophic factors and synaptic proteins in the microenvironment of the spinal dorsal horn. Such changes contribute to hyperalgesia and allodynia processes; thus, analgesic drugs can exert their pharmacological effects by affecting the expressions, levels, or functions of these endogenous substances. In this study, based on the knowledge that reboxetine (a selective noradrenaline reuptake inhibitor) has the potential for antihyperalgesic efficacy in diabetic neuropathy, we aimed to examine the probable effects of this drug on diabetes-induced changes in brain-derived neurotrophic factor (BDNF), synaptophysin (the pre-synaptic marker of synaptic integration), and postsynaptic density-95 (PSD-95) (the postsynaptic marker of synaptic integration) levels in the superficial laminae of the dorsal horn.

Methods: Experimental diabetes was induced by a single-dose injection of streptozotocin (STZ) (50 mg/kg) in rats. After four week-long induction period of painful diabetic neuropathy, rats were treated orally with 8 mg/kg reboxetine for two weeks. Hyperalgesia responses were evaluated by using the Randall–Selitto and Hargreave's tests. Following the pain tests, immunohistochemical studies were performed.

Results: Two weeks of reboxetine administration increased the reduced paw withdrawal thresholds and shortened the paw withdrawal latencies of diabetic rats in neuropathic pain tests, indicating the antihyperalgesic efficacy of this drug. Moreover, augmented BDNF and synaptophysin levels in diabetic rats reversed by reboxetine treatment. However, there was no alteration in the densities of PSD-95, in both STZ-diabetic and reboxetine-treated STZ-diabetic rats.

Conclusion: The obtained results suggested that inhibition of central sensitization and modulation of spinal plasticity seem to be pharmacological mechanisms underlying reboxetine's antihyperalgesic effects on diabetic rats. However, further studies are still needed to clarify the exact mechanism of action.

Keywords: Brain-derived neurotrophic factor, diabetes mellitus, neuropathic pain, postsynaptic density-95, synaptophysin.

1. INTRODUCTION

Neuropathic pain is a highly complex pain condition that usually results from nerve injury or dysfunction of the nervous system. Amputations, disc herniation, traumas, tumors compressing nerves, some chemotherapeutic agents, some viral infections, and some diseases are among the main reasons causing neuropathic pain (1). *Diabetes mellitus* is one of the major causes of neuropathy. Chronic exposure to hyperglycemia leads to detrimental changes in the nervous system's sensory and motor (somatic and autonomic) components (2,3,4). This metabolic disease gives rise to abnormal sensory symptoms such as hyperalgesia (augmented pain sensitivity), and allodynia (painful sensation against innocuous stimuli) (5). Patients with diabetic neuropathy may experience burning, tingling, shooting, sharp, lancinating, or sometimes electric shock-like sensations (6).

Polyol pathway hyperactivity, oxidative and nitrosative stress, microvascular changes, channels sprouting, microglial activation, central sensitization, and alterations in synaptic plasticity are the main mechanisms underlying the physiopathology of neuropathic pain in diabetes (6).

Neurotrophins, which regulate the growth, maintenance, and apoptosis of neurons in the developing nervous system as well

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as injured neurons, have been reported for their substantial roles in the development and transmission of neuropathic pain (1,7). These endogenous molecules have been shown to contribute to the pathogenesis of neuropathic pain as they have critical roles in the complex mechanisms that underpin peripheral and central sensitization (8,9,10). Moreover, results of numerous studies demonstrated that the changes in synaptic morphology and synaptic protein levels in spinal dorsal horn neurons are also associated with the development of chronic pain (11,12,13). The enhanced synaptic plasticity of nociceptive interneurons in this region has been suggested as the basis of central sensitization in neuropathic pain (14). Especially synaptophysin (a presynaptic vesicle glycoprotein that is an indicator of synaptic connections' efficiency) and PSD-95 (a scaffold protein on the postsynaptic membrane playing a critical role in the modulation of the size and shape of dendritic spines) are important synaptic proteins mediating enhancement of synaptic plasticity and playing critical roles in the development of neuropathic pain (13). In this context, reducing neuropathy-induced alterations in neurotrophic factors and synaptic protein levels in the dorsal horn may be an important strategy to alleviate chronic pain.

Reboxetine is a potent and selective noradrenaline reuptake inhibitory antidepressant (15). It has been reported that this drug has analgesic activity in acute and chronic pain (16,17,18). The catecholaminergic and opioidergic systems mediated beneficial effects of reboxetine against diabetes-induced neuropathic pain have also been shown previously (19). However, the potential efficacy of this drug on diabetes-induced changes in spinal neurotrophic factors or synaptic proteins has not been clarified, yet. Therefore, in this study, we aimed to investigate the effect of reboxetine on the brain-derived neurotrophic factor (BDNF), synaptophysin, and postsynaptic density-95 (PSD-95) densities in the spinal dorsal horn of rats with diabetic neuropathy, in order to clarify molecular mechanisms underlying the antihyperalgesic effects of this drug.

2. METHODS

2.1. Animals

Inbred male Sprague Dawley rats weighing 300–350 g at the same age were obtained from the Anadolu University Research Unit for Experimental Animals, Eskişehir, Turkey. Rats were housed under controlled temperature at 24 °C, 12/12-h light/dark cycle, and 50% humidity in well-ventilated rooms. The rats had access to food and water provided ad-lib. The Local Ethics Committee on Animal Experimentation confirmed the experimental protocol (Protocol code 2022-15 and date of approval 11.05.2022).

2.2. Establishment of Streptozotocin-Induced Experimental Diabetes

Streptozotocin (STZ) was used for the experimental diabetes induction (20). The rats were denied access to food overnight before the STZ injection. STZ was dissolved in citrate buffer (pH=4.5; 0.1 M). A single dose of STZ (50 mg/kg) through the tail vein was administered to the rats of the diabetic groups. An equal volume of citrate buffer was injected into the tail veins of the healthy controls. Plasma glucose levels were quantified after 72 hours of the STZ administration. The rats were considered diabetic with over 300 mg/dL plasma glucose levels.

2.3. Drug Administrations

4 weeks after inducing the experimental diabetes model, reboxetine was administered to the diabetic rats at doses of 8 mg/kg (*p.o.*) for 2 weeks. This is the effective antihyperalgesic dose of reboxetine in rats with diabetic neuropathy (19). Rats in the healthy and diabetic control groups were administered a physiological saline solution (0.9% sodium chloride), that was used to dissolve reboxetine.

2.4. Assessment of Neuropathic Pain

Mechanical hyperalgesia was evaluated by using the Randall–Selitto analgesiometer (Ugo-basile, Varese, Italy), as described previously (20). The device is used to apply an increasing pressure stimulus to the dorsal parts of the hind paws of rats and the force (grams) with which the rat withdraw its paw was accepted as the mechanical nociceptive threshold. The maximum force to be applied was determined as 250 grams in order to prevent tissue damage (19,20).

Thermal hyperalgesia was evaluated with the Hargreave's test device (Ugo-basile, 37370, Verase, Italy). This test is based on measuring the duration of the "paw withdrawal" reaction of rats against radiant heat focused on their hind paws. At the beginning of the test, the rats were placed in the plexiglass compartments of the device and waited for 30 minutes to acclimate to the environment. Then, paw withdrawal latency (time between activating the heat source and withdrawal of the back paw), was recorded with an accuracy of 0.1 seconds by the automatic timer of the device. Response times were calculated by taking the average of three measurements made with a 5-minute interval. Heat was not applied for more than 20 seconds in order to avoid a possible paw damage (19,20).

2.5. Spinal Cord Dissection

After completion of neuropathic pain experiments, rats were anesthetized with halothane and perfused transcardially with 0.1 M pH 7.4 phosphate-buffered saline (PBS) and then 4% paraformaldehyde in PBS (20). Midlumbar L4-L5 segments of the spinal cord were dissected and stored at 4 °C overnight in the fixative solution.

2.6. Immunohistochemistry of Spinal Dorsal Horn

After dehydration through an ascending concentration series of ethanol, spinal tissues were cleared in xylene and embedded in paraffin blocks (20). Transverse sections of the L5-level spinal cord (21) were collected by a microtome and sections (3 µm thick) were mounted on positively charged slides. To prevent antigen masking, the sections were treated with 1:10 EDTA Buffer (AP-9004-999 Thermo Scientific) under high pressure and cooled down to room temperature. Endogenous peroxidase activity was blocked to prevent non-specific background staining with 3% hydrogen peroxide solution (TA-125-HP ThermoScientific). Thermo Scientific UltraVision protein block solution (TA-125-PBQ Thermo Scientific, USA) was applied to slides for 10 minutes after washing with PBS.

Mouse monoclonal anti-synaptophysin antibody [SY38] (ab8049) 1:10 was used to investigate presynaptic protein expression; rabbit polyclonal anti-PSD-95 antibody-synaptic marker (ab18258) 1:250 was used to investigate postsynaptic protein expression and recombinant anti-BDNF antibody [EPR1292] (ab108319) 1:250 was used to label BDNF immunopositive somas and terminals in the spinal sections, respectively. After incubation with primary antibodies (Abcam PLC, Cambridge, UK) for 2 hours at room temperature, the sections were incubated with Primary Antibody Amplifier Quanto (TL-125-QPB), HRP Polymer Quanto, and Ultra V Block (TA-125-UB) for 30 minutes at room temperature, respectively. Each preparation was washed with PBS for all steps of UltraVision™ Quanto Detection System HRP (Thermo Scientific, USA). Sections were washed for 10 minutes three times in PBS. After that, sections were exposed to 2 µg/ml 3,3' – diaminobenzidine for 2-3 minutes. After the reaction product was revealed, sections were washed, dehydrated, placed in xylene for 5 minutes and coverslipped.

2.7. Light Microscopy, Immunohistochemical Analysis

Photomicrographs were obtained through the light microscope with Olympus CX31RTSF (Olympus GmbH, Hamburg, Germany) with an integrated camera with a 4x objective lens and LCmicro (Olympus GmbH, Hamburg, Germany) imaging software. Digital photomicrographs were taken under a 40x lens from all prepared sections and then evaluated by using the ImageJ 1.50i (U.S. NIH, Bethesda, MD, USA) image analysis program.

For calculating immunoreactivity, the rectangular fields (200 x 100 µm), including the superficial layer (laminae I and II), were taken from four consecutive sections at 200 µm intervals per L4-L5 spinal segments as presented in Figure 1. The auto thresholding tool of the ImageJ program was used to evaluate the percentage of immunoreactive area in the superficial laminae of the dorsal horn. All individual values were averaged to procure a mean value for each animal (21).

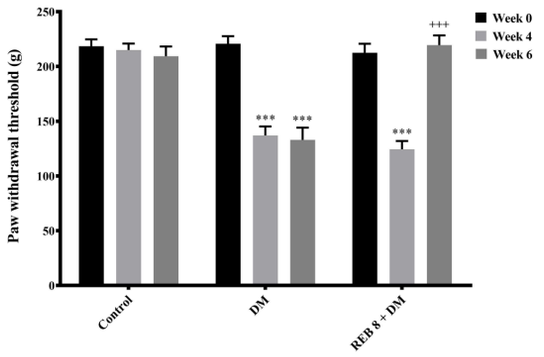

Figure 1. *Change in the paw-withdrawal thresholds of control (normoglycemic) and diabetic rats and the effects of reboxetine (8 mg/kg) treatment on these alterations, in the Randall–Selitto test. Significant difference compared to week 0 group $^{***}p < 0.001$; significant difference compared to week 4 group $^{+++}p < 0.001$. Two-way repeated-measures analysis of variance, followed by the Bonferroni test, n =8 (REB: Reboxetine, DM: Diabetes mellitus).*

2.8. Statistical Analysis

Statistical analysis of the data was carried out with GraphPad Prism (ver. 8.3.4.). The data obtained from Randall-Selitto and Hargreaves tests were evaluated using two-way repeated measures ANOVA followed by Bonferroni multiple comparison test. The data obtained from immunohistochemical studies were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD multiple comparison test. $p<0.05$ was considered significant.

3. RESULTS

3.1. Results of Neuropathic Pain Tests

Figure 2 demonstrates the changes of paw-withdrawal thresholds in the control and diabetic rats and the effect of reboxetine treatments on these alterations, in the Randall–Selitto test.

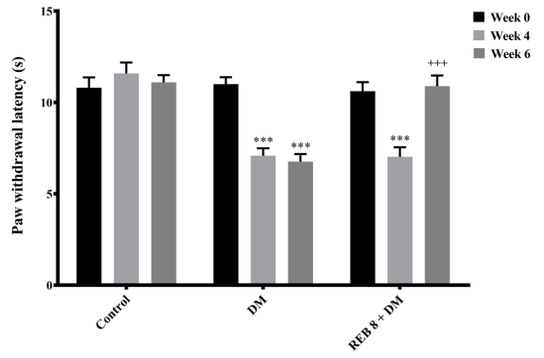

Figure 2. *Change in the paw-withdrawal latencies of control (normoglycemic) and diabetic rats and the effects of reboxetine (8 mg/kg) treatment on these alterations, in the Hargreave's test. Significant difference compared to week 0 group $^{***}p < 0.001$; significant difference compared to week 4 group $^{+++}p < 0.001$. Two-way repeated-measures analysis of variance, followed by the Bonferroni test, n =8 (REB: Reboxetine, DM: Diabetes mellitus).*

Two-way repeated-measures ANOVA results indicated that animals' withdrawal threshold in this test were affected not only by the treatment factor [F (2, 21) = 28.77, P < 0.001], but also by the time factor [F (2, 42) = 39.12, P < 0.001]. In addition, a significant interaction was found between treatment and time [F (4, 42) = 20.78, P < 0.001].

The Bonferroni test showed that no changes were observed in the paw-withdrawal thresholds of normoglycemic rats, throughout the experiments. However, paw-withdrawal thresholds of diabetic rats, measured at fourth (p<0.001) and sixth (p<0.001) weeks, were significantly lower than the values measured at the beginning of the experiments. Moreover, subacute administration of reboxetine at 8 mg/kg daily dose for two weeks produced marked increases (p<0.001) in the paw-withdrawal thresholds of diabetic rats (Figure 2).

Figure 3 demonstrates the changes of paw-withdrawal latency in the control and diabetic rats and the effect of reboxetine treatments on these alterations, in the Hargreave's test. Two-way repeated measures ANOVA results indicated that animals' paw withdrawal latency in this test were affected by both the treatment factor [F (2, 21) = 22.94, P < 0.001] and the time factor [F (2, 42) = 16.71, P < 0.001]. In addition, a significant interaction was found between treatment and time [F (4, 42) = 14.57, P < 0.001].

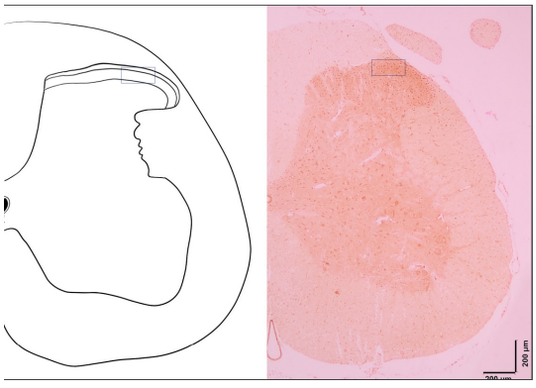

Figure 3. *Representative drawing and image of the spinal cord for the region of interest for immunohistochemical analysis.*

The Bonferroni test revealed that, similar to Randall–Selitto test, no changes were detected in the paw-withdrawal latencies of normoglycemic rats, during the experiments. On the other hand, paw-withdrawal latencies of diabetic rats, measured at fourth (p<0.001) and sixth (p<0.001) weeks, were significantly lower than the values measured at the beginning of the experiments. Furthermore, subacute administration of reboxetine at 8 mg/kg daily dose for two weeks induced notable increases (p<0.001) in the paw-withdrawal latencies of diabetic rats (Figure 3).

3.2. BDNF Immunoreactivity in the Spinal Dorsal Horn

Representative images of BDNF immunoreactivities in the superficial layers of the spinal dorsal horn were presented in Figure 4. In these photomicrographs, the brown-stained regions represent BDNF-IR neurons and synaptic terminals of the first-order sensory neurons in the spinal sections. BDNF immunostainings revealed that 6-week STZ-induced diabetes increases the immunoreactive areas compared to non-diabetic healthy rats (Figures 4A and 4B) and 2-week reboxetine treatment has ameliorated the BDNF overexpression (Figure 4C).

Alterations of the BDNF-IR % area in the spinal dorsal horn [F (2, 15) = 11.35; *p<0.01*] were presented in Figure 4D. Tukey's multiple comparison tests showed that rats with experimental diabetes significantly elevated BDNF-IR % area in the marginal layer and *substantia gelatinosa* of the spinal dorsal horn compared to healthy rats. On the other hand, 2-week reboxetine treatment administered to diabetic rats significantly reduced the percentage of BDNF-IR areas compared to non-treated diabetic rats.

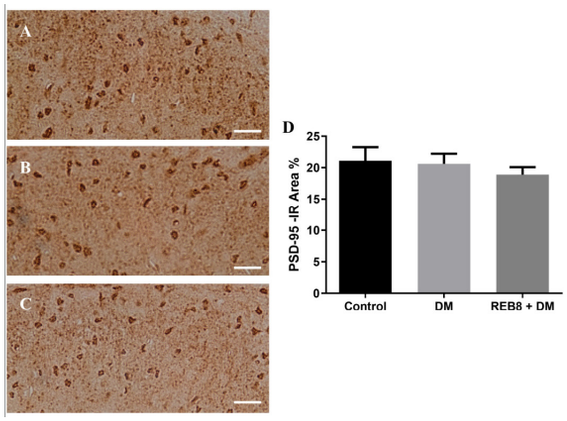

Figure 4. *Representative images of BDNF immunopositive transverse sections of L5 level spinal dorsal horn* ***(A)*** *physiological saline-administered healthy rats (Control) and* ***(B)*** *physiological saline-administered diabetic rats (DM),* ***(C)*** *8 mg/kg reboxetine-treated diabetic rats (REB 8 + DM), Scale bar: 20µm.* ***(D)*** *Percentage of BDNF-IR area in the superficial dorsal horn of healthy rats (Control), physiological saline-administered diabetic rats (DM), or 8 mg/kg reboxetine-treated diabetic rats (REB 8 + DM). The significant difference compared to the control group* ****p<0.001; Significant difference compared to the DM group* $^{+}$*p<0.05; One-way analysis of variance, followed by Tukey's HSD multiple comparison test, n=6 (REB: Reboxetine, DM: Diabetes mellitus).*

3.3. Immunoreactivity of Synaptic Proteins in the Spinal Dorsal Horn

Representative images of synaptophysin immunoreactivities in the superficial layers of the spinal dorsal horn were presented in Figure 5. Brown-stained regions represent synaptophysin-IR synaptic terminals of somatosensory neurons. It can be observed that the synaptophysin immunoreactivities were increased in diabetic rats compared

to normoglycemic animals (Figures 5A and 5B) and reboxetine treatment for 14 days (Figure 5C) significantly reversed this augmentation in the superficial layers of the spinal dorsal horn.

Alterations of the synaptophysin-IR % area in the superficial dorsal horn [$F(2, 15) = 6.129$; $p<0.05$] were presented in Figure 5D. Obtained results from the posthoc test indicated that the percentage of synaptophysin-IR areas was significantly elevated in rats with experimental diabetes than in healthy rats. On the other hand, 2-week reboxetine treatment in diabetic rats normalizes the increased synaptophysin levels.

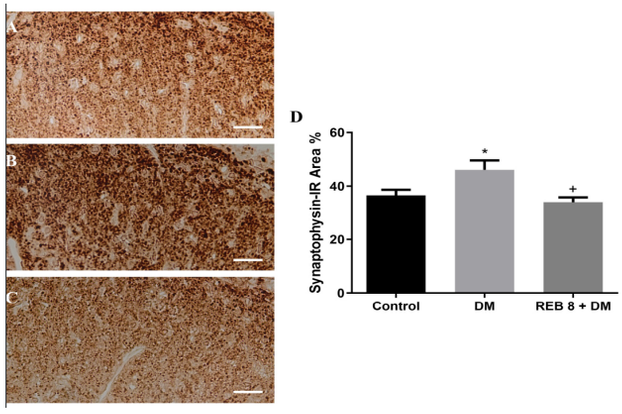

Figure 5. *Representative images of synaptophysin immunopositive transverse sections of L5 level spinal dorsal horn* ***(A)*** *physiological saline-administered healthy rats (Control) and* ***(B)*** *physiological saline-administered diabetic rats (DM),* ***(C)*** *8 mg/kg reboxetine-treated diabetic rats (REB 8 + DM), Scale bar: 20µm.* ***(D)*** *Percentage of the synaptophysin-IR area % in the superficial dorsal horn of healthy rats (Control), physiological saline-administered diabetic rats (DM), or 8 mg/kg reboxetine-treated diabetic rats (REB 8 + DM). The significant difference compared to the control group* $^{*}p<0.05$*; Significant difference compared to the DM group* $^{+}p<0.05$*; One-way analysis of variance, followed by Tukey's HSD multiple comparison test, n=6 (REB: Reboxetine, DM: Diabetes mellitus).*

Representative images of PSD-95 immunoreactivities in the superficial layers of the spinal dorsal horn were presented in Figure 6. Brown-stained regions represent PSD-95 immunoreactive postsynaptic terminals and cell bodies of dorsal horn neurons. It can be seen that the PSD-95 immunoreactivities did not alter between the experimental groups (Figures 6A-6C).

Alterations of the PSD-95 immunoreactive area % in the superficial dorsal horn [$F(2, 15) = 0.459$; $p>0.05$] were presented in Figure 6D. Tukey's HSD multiple comparisons test for PSD-95 immunoreactivities has revealed that the healthy and diabetic groups did not show any differences. Although reboxetine treatments slightly tend to reduce PSD-95 immunoreactive area % compared to untreated diabetics, the difference is not significant.

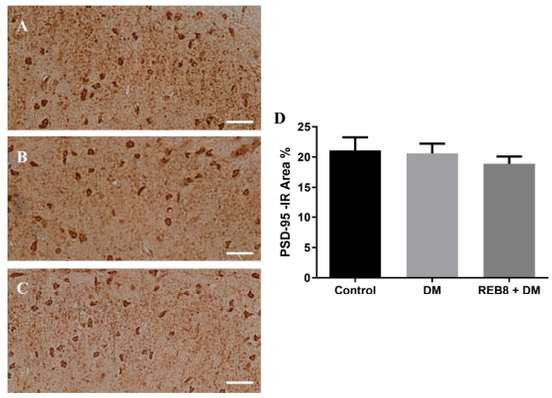

Figure 6. *Representative images of PSD-95 immunopositive transverse sections of L5 level spinal dorsal horn* ***(A)*** *physiological saline-administered healthy rats (Control) and* ***(B)*** *physiological saline-administered diabetic rats (DM),* ***(C)*** *8 mg/kg reboxetine-treated diabetic rats (REB 8 + DM), Scale bar: 20µm.* ***(D)*** *Percentage of PSD-95 – IR area in the superficial dorsal horn of healthy rats (Control), physiological saline-administered diabetic rats (DM), or 8 mg/kg reboxetine-treated diabetic rats (REB 8 + DM). One-way analysis of variance, followed by Tukey's HSD multiple comparison test, n=6 (REB: Reboxetine, DM: Diabetes mellitus).*

4. DISCUSSION

In the first step of the study, diabetes was induced with STZ. After four weeks, Randall–Selitto and Hargreave's tests were performed to evaluate whether hyperalgesia developed in rats (19,20). The results of these tests revealed that four-week diabetic rats had reduced paw withdrawal thresholds and shortened paw withdrawal latencies indicating that diabetic animals developed hyperalgesia against both mechanical and thermal nociceptive stimuli. On the other hand, two weeks of reboxetine treatment significantly increased the reduced paw withdrawal thresholds and shortened paw withdrawal latencies of diabetic rats. These findings confirm our previous results reporting the antihyperalgesic efficacy of reboxetine against diabetes-induced hyperalgesia (19).

In the second step of this study, based on the antihyperalgesic activity of reboxetine on diabetes-induced neuropathic pain, we aimed to investigate the potential effect of this drug on diabetes-induced alterations in spinal neurotrophin and synaptic protein levels.

BDNF is a member of neurotrophins, essential for neuronal differentiation, maturation, survival, and synaptic plasticity (22,23). It is also a critical protein in the microglia-neuron signaling pathway. In the spinal dorsal horn, an increment of BDNF release by neurons and microglia would lead to the disinhibition of nociceptive neurons and enhancement of excitatory synaptic transmission (24,25). The primary sensory neurons are known to synthesize BDNF, which can be transported anterogradely to the axon terminals in the spinal dorsal horn (26,27). BDNF binds to tyrosine kinase receptor B (TrkB) on the second-order sensory neurons. This triggers intracellular signaling cascades through phosphorylation

and alters synaptic transmission (28,29). Increased BDNF expression caused by a peripheral tissue or nerve injury diminishes the presynaptic and postsynaptic GABAergic inhibition, which leads to thermal and static mechanical hypersensitivity, respectively (30). In addition, BDNF-induced activation of the dorsal horn NR2B-containing N-methyl-D-aspartate (NMDA) receptors (NMDA-2B) has been associated with developing and maintaining neuropathic pain (31). Another mechanism suggested for the role of BDNF is related to its regulatory role on the expression and activity of the TRPV1, which plays roles in the transmission of mechanical, chemical, and thermal nociceptive stimuli (32).

As a modulator of central and peripheral nociception, BDNF has been studied widely in experimental animal models, and several studies point out the importance of this protein in the pathophysiology of neuropathic pain (10,24,33,34). It has been reported that targeting this neurotrophin might be a beneficial therapeutic strategy for chronic and persistent pain treatment (35). Therefore, we investigated the potential efficacy of reboxetine on the BDNF immunoreactivity in the dorsal horn, in painful diabetic neuropathy (PDN).

Obtained results of this study demonstrated that six-week STZ-induced diabetes increased the BDNF immunoreactivity in the superficial laminae of the dorsal horn. This data is aligned with the previous papers reporting the overexpression of BDNF in several neuropathic pain models (1,31,36,37). Moreover, our experimental data supported some recent findings about the alterations of BDNF levels in diabetes-induced neuropathic pain conditions. For example, in a recent study, twenty-six-day-long STZ-induced diabetes was reported to increase BDNF expression significantly in the ipsilateral spinal cord in Western-Blot and immunohistochemical measurements compared to the control group (28). Similarly, in a study evaluating BDNF / TrkB expressions in comorbid depression with chronic unpredictable mild stress model in STZ-induced painful diabetic neuropathic rats, BDNF / TrkB expressions were found to be significantly increased in both the spinal dorsal horn and DRG (38). Based on the role of BDNF in diabetes-induced neuropathic pain, we investigated whether an antihyperalgesic drug reboxetine alters the expression of this neurotrophin in dorsal horns. Our results indicated that a two-week treatment of diabetic rats with reboxetine significantly decreased the BDNF overexpression in the superficial laminae of the dorsal horn. This finding suggested that the beneficial effect of this drug on diabetic neuropathic pain might be related to the inhibition of central sensitization induced by enhanced BDNF levels.

In addition to neurotrophic factors, changes in synaptic plasticity induced by synapse-associated proteins also play a critical role in the development of neuropathic pain (13,14,39,40). Increased synaptic plasticity of nociceptive interneurons in the spinal dorsal horn has been reported as the source of central sensitization in neuropathic pain (14). Long-term potentiation of synaptic plasticity of nociceptive neurons in the spinal dorsal horn requires the participation of both pre-and postsynaptic structures (41). Active zones are the sites specialized for the exocytosis of synaptic vesicles at the pre-synaptic axon terminals (42). The size of the active zone is an important morphological parameter of synapses that effectively reflects the area of neurotransmitter release. Increased synaptic vesicle proteins in the pre-synaptic active zones may lead to synaptogenesis, thus resulting in neuropathic pain (43).

Synaptophysin is the first cloned marker of synaptic vesicles and the second most abundant synaptic vesicle glycoprotein (14,44). This protein is the main component of the small vesicles of neuroendocrine cells and neurons and modulates the synaptic vesicle cycle (45). The level of synaptophysin is an agreeable indicator of the synapse number and synaptic connections' efficiency. In addition, synaptophysin expressions in DRG and the spinal dorsal horn have been associated with the severity of neuropathic pain (14). Therefore, we examined the alterations of synaptophysin levels in the superficial dorsal horn of diabetic rats. Our findings indicated that six-week diabetes increased the levels of synaptophysin in the axon terminals of the primary sensory neurons forming synapses in the spinal dorsal horn. These results correlate with some previous findings indicating the diabetes-induced increase in synaptophysin immunoreactivity and numerical enhancement of synapses in the dorsal horn (13,14,40). It can be suggested that this augmentation in the presynaptic region increases the release of neurotransmitters and neuromodulators, provides faster and more intense transmission of the nociceptive signal, and reduces the pain threshold. Our study examining the potential effect of reboxetine on this increased synaptophysin immunoreactivity in the spinal dorsal horn demonstrated that administration of this drug for 2 weeks significantly reversed the diabetes-induced alterations. These findings suggest that the analgesic effect of reboxetine against diabetic neuropathic pain may be related to its inhibitory effect on synaptophysin overexpression in the dorsal horn, as well as BDNF.

Forming and developing the postsynaptic terminals in excitatory synapses is as crucial as pre-synaptic partners for synaptic plasticity in neuropathic pain (46,47). PSD-95 also known as SAP-90 encoded by the DLG4 gene is a main postsynaptic scaffolding protein in excitatory glutamatergic neurons. This protein is a member of the membrane-associated guanylate kinase (MAGUK) superfamily. Structurally, it has three PDZ domains in the membrane that anchor receptor proteins to cytoskeletal components and an SH3-GUK (Src homology 3-guanylate kinase) domain (48,49). PSD-95, located at the postsynaptic compartment of excitatory synapses called "postsynaptic density", is a regulator of synaptic plasticity and synaptogenesis (49). This postsynaptic marker has been reported to lie behind central sensitization in neuropathic pain via interacting with glutamatergic NMDA and AMPA receptors (14). In this study, in order to clarify diabetes-induced alterations in the postsynaptic terminals, we investigated the immunoreactivity of PSD-95 in the superficial dorsal horn of the spinal cord. Obtained findings showed that levels of

PSD-95, the postsynaptic marker of synaptic integration, have not changed for diabetic rats. Considering the findings of this study pointing to synaptophysin overexpression in diabetic conditions, it can be suggested that the unchanged expression of PSD 95 may be a compensatory mechanism following excessive neuromediator release from presynaptic neurons. Indeed, in a recent study, Calabrese et al. also reported that one-month-long diabetes in rats induced a significant increase in presynaptic proteins (synapsin-1 and syntaxin-1) but they did not detect an alteration in diabetes on the expressions of PSD proteins (PSD-95, GluN1, GluN2A, and GluN2B) in the spinal dorsal horn (50). The findings of this study are consistent with our data. On the other hand, some other studies suggested that diabetic animals showed an enhancement in the levels of both synaptophysin and PSD-95 (13,14). The contradictory findings may be related to the differences between the experimental conditions. Actually, more detailed and time-dependent experiments are needed to clarify changes in synaptic protein expressions during diabetes progression.

In this study, our further experiments about the effect of 14-day-reboxetine-treatment on PSD-95 immunoreactivity in the superficial dorsal horn of diabetic rats revealed that PSD-95 has not been changed following the drug administrations, which suggests that alteration in PSD-95 expression did not mediate the efficacy of reboxetine on diabetic neuropathic pain.

A limitation of this study is the lack of data on the effects of reboxetine on normoglycemic rats, as reboxetine treatment may also cause significant changes independent of diabetic conditions. Indeed, previous studies reporting a direct effect of reboxetine on brain BDNF levels in healthy (51,52) and depressed (53,54,55) subjects may point to the potential of this drug to alter spinal neurotrophin levels. Similarly, reboxetine may also have direct effects on synaptophysin levels. Therefore, it is clear that new studies investigating the effects of reboxetine treatment on neurotrophin and other synaptic protein levels are needed.

5. CONCLUSION

In conclusion, the results of the present study revealed that subacute reboxetine administration in diabetic rats suppresses the over-expressions of both BDNF and synaptophysin in the spinal dorsal horn. Therefore, it can be suggested that reboxetine may reveal its antihyperalgesic effect on PDN by preventing the establishment of central sensitization and weakening the presynaptic neurotransmitter release.

To our knowledge, this is the first study associated with the antihyperalgesic effect of reboxetine with some molecular mechanisms in the superficial dorsal horn of diabetic rats. On the other hand, assessing the effects of reboxetine on diabetes-induced changes in the structural synaptic plasticity parameters such as, the number of dendritic branches, the width of the synaptic cleft, and the thickness of postsynaptic matter, etc. may provide further understanding of the exact activity mechanism of this drug against neuropathic pain.

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