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Brain-derived neurotrophic factor and Glial cell line-derived neurotrophic factor expressions in the trigeminal root entry zone and trigeminal ganglion neurons of a trigeminal neuralgia rat model

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Abstract

Microvascular compression on the trigeminal root entry zone (TREZ) is the main etiology of trigeminal neuralgia (TN) patients. To investigate brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) in the trigeminal ganglion (TG) and TREZ, immunofluorescence staining and Western blot were used in a rat TN model. Both BDNF and GDNF were observed in the TG neurons and TREZ. The expression of the BDNF dimer in the TG was increased in the TN group, while GDNF expression was decreased after compression injury. The BDNF dimer/pro-BDNF ratio in the TREZ of the TN group was higher than that in the sham group, but the GDNF expression in the TREZ was significantly lower than that in the sham group. These results suggested that compression injury in the TREZ of rats induced dynamic changes in BDNF and GDNF in both the TG and TREZ, and these changes are involved in the nociceptive transmission of the TN animal model.

KEYWORDS

brain-derived neurotrophic factor, glial cell line-derived neurotrophic factor, trigeminal neuralgia, animal model, trigeminal root entry zone, trigeminal ganglion, chronic compression

1 | INTRODUCTION

Trigeminal neuralgia (TN) is one of the most severe orofacial neuropathic pain conditions affecting patients. While accumulating evidence has indicated that aberrant neurovascular compression on the trigeminal root entry zone (TREZ) is the main etiology of TN (Cruccu et al., 2016), its pathogenesis remains unknown. The trigeminal ganglion (TG) is the peripheral ganglion of the trigeminal sensory pathway, which contains different sensory neurons and satellite glial cells (SGCs). Neurons in the TG respond to innocuous and noxious stimuli from the orofacial area. Interestingly, neurons in the TG have no dendrites, but several SGCs wrap each neuron to form a completed glial sheath, which is a unique morphological and, most likely, functional unit (Hanani, 2005).

Brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) are two important

Abbreviations: BDNF, brain-derived neurotrophic factor; CCT, chronic compression of the trigeminal root; CNS, central nervous system; GDNF, glial cell line-derived neurotrophic factor; POD, postoperation day; PNS, peripheral nervous system; SGCs, satellite glial cells; TG, trigeminal ganglion; TN, trigeminal neuralgia; TREZ, trigeminal root entry zone.

Daoshu Luo and Lili Luo contributed equally to this study.

growth factors synthesized in TG neurons in rodents and humans (Merighi, 2016; Merighi et al., 2008; Quartu, Geic, & Del Fiacco, 1997; Salio & Ferrini, 2016) and play important roles in mediating nociceptive neurotransmission and neuropathic pain (Khan & Smith, 2015; Merighi, 2016; Shinoda, Fukuoka, Takeda, Iwata, & Noguchi, 2019; Smith, 2014). Evidence has indicated that BDNF can modulate central nociceptive pain in the trigeminal nerve and that GDNF can modulate peripheral pain (Constandil et al., 2012; Taylor & Ribeiro-da-Silva, 2011). However, there are few reports on the expression of BDNF and GDNF in the TG and TREZ of the peripheral nervous system (PNS) in the TN animal model under the condition of TREZ compression injury.

In this study, we used immunohistochemical staining and Western blot to investigate the expression of BDNF and GDNF in the TG and TREZ of trigeminal primary afferent pathways in a TN animal model induced by mechanical compression to better understand and interpret the special functions of BDNF and GDNF in the pathogenesis of TN.

2 | MATERIALS AND METHODS

2.1 | Animals and Surgery

Adult male Sprague–Dawley rats weighing 150 ± 20 g were used in this study. All animal experiments were performed in accordance with the National Institutes of Health laboratory animal use guidelines and approved by the Fujian Medical University Institutional Animal Care and Use Committee. Rats were housed in a temperature- and humidity-controlled room on a 12 hr-12 hr light/dark cycle and provided water and food *ad libitum*. The rats were randomly divided into two groups: a (1) TN group ($n = 32$) and a (2) sham group ($n = 32$).

Rats in the TN group were subjected to chronic compression of the trigeminal root (CCT) by retrograde insertion of a plastic filament from the right inferior orbital fissure to compress the TREZ as previously described (Lin et al., 2018; Luo et al., 2012, 2019). Rats in the sham group underwent the same procedure to expose the right infraorbital nerve without inserting the filament to compress the trigeminal nerve root.

The investigators who performed the immunohistochemistry and Western blot analyses were blinded to the experimental groups. Substantial effort was made to minimize the number of animals used and their suffering in this study.

2.2 | Tissue Preparation

Rats were deeply anesthetized with sodium pentobarbital (200 mg/kg) and transcardially perfused through the left ventricle with saline followed by 4% paraformaldehyde phosphate buffer (pH 7.4). The trigeminal nerve root along the brainstem junction and the TG was dissected and then cryoprotected in 0.1 M phosphate buffer containing 30% (w/v) sucrose overnight at 4°C. Vertical sections of the trigeminal nerve root attached to the TG were sectioned at 10 μm thickness with a cryostat microtome (Leica CM1950, Germany).

2.3 | Immunofluorescence Staining

For immunohistochemical staining, sections were preincubated in 0.01 M phosphate buffered saline containing 3% bovine serum albumin for 30 min, incubated with appropriate mixtures of two primary antibodies overnight at room temperature, and then continuously incubated at 4°C for 48 hr. The following primary antibody mixtures were used in this study: rabbit anti-BDNF polyclonal antibody (1:200, Abcam, MA)/mouse anti-GFAP monoclonal antibody (1:1,000, Proteintech, IL) and rabbit anti-GDNF monoclonal antibody (1:200, Abcam, MA)/mouse anti-GFAP monoclonal antibody (1:1,000, Proteintech, IL). The sections were then incubated with the following secondary antibodies overnight at 4°C: goat anti-rabbit 488 (1:500, Invitrogen, CA) and biotinylated donkey anti-mouse IgG (1:200; Vector, CA). Finally, the sections were incubated with Cy3-avidin (1:1,500, Jackson ImmunoResearch, PA) for 4 hr at room temperature, and nuclei were stained with DAPI (1:1,000, Invitrogen, CA).

2.4 | Western Blot Analysis

Rats in the TN and sham groups were sacrificed on postoperation days (PODs) 7, 14, 21, and 28 under deep anesthesia with sodium pentobarbital (200 mg/kg), and segments of the trigeminal nerve root and TG were quickly removed and frozen in liquid nitrogen. Tissues were homogenized in an extraction buffer (100 mM Tris, pH 7.4) with 2 mM phenylmethanesulfonylfluoride and 10 mg/mL aprotinin and centrifuged at 12,000×*g* for 10 min at 4°C. The protein concentrations were measured using the Pierce BCA Protein Assay Kit. Equal amounts of protein were separated by electrophoresis on 10% polyacrylamide SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes (Millipore, MA). The membranes were blocked with 5% nonfat milk for 1 hr at room temperature and then incubated overnight at 4°C with the following primary antibodies: rabbit anti-BDNF polyclonal antibody (Abcam, 1:1,000), mouse anti-GDNF monoclonal

antibody (Abcam, 1:500) and mouse anti-β-actin (1:1,000, TransGen, Beijing, China). Then, the membranes were incubated with a goat anti-rabbit IgG (H + L)-HRP (1:5,000, Bioworld, OH) or goat anti-mouse IgG-HRP (1:100,000, EarthOX, CA) secondary antibody at room temperature for 2 hr. Finally, bands were detected using the Immobilon Western Chemiluminescent reagent (Merck Millipore P90720).

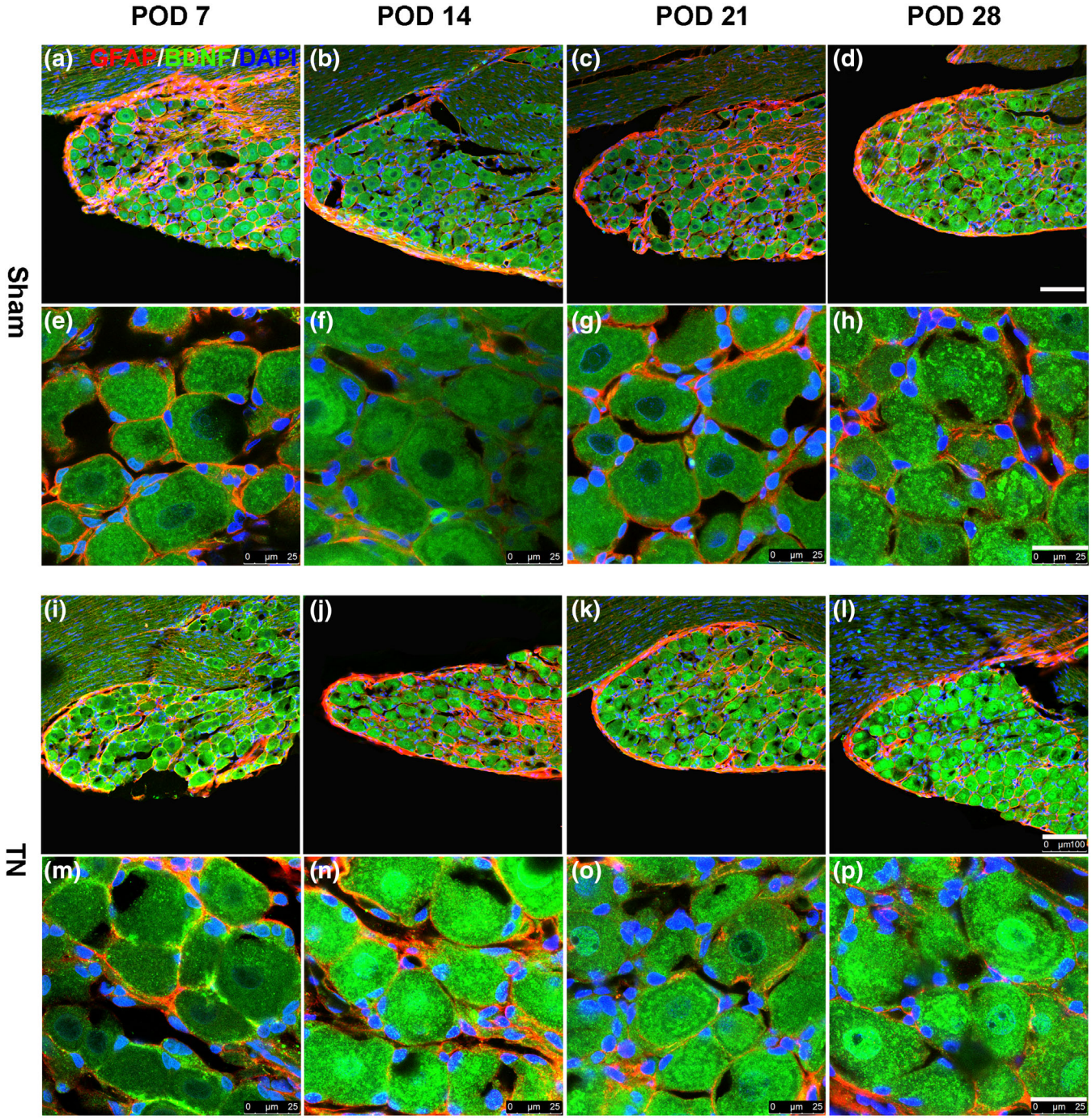

FIGURE 1 Distribution of BDNF and GFAP in the TG. BDNF (green) was expressed in TG neurons, and GFAP-immunoreactive satellite cells (red) were distributed around the TG neurons to form a thin glial sheath. Representative images of the distribution of BDNF and GFAP in the sham group (a–d, bar = 100 μm) and TN group (i–l, bar = 100 μm) in the TG; enlarged images of the sham group (e–h, bar = 25 μm) and TN group (m–p, bar = 25 μm) on postoperation days (PODs) 7–28 are also shown. BDNF, brain-derived neurotrophic factor; TG, trigeminal ganglion; TN, trigeminal neuralgia

2.5 | Statistical Analysis

The band intensities on Western blots were quantified with ImageJ software (v1.8.0, National Institutes of Health). Data are presented as the mean ± SEM and were analyzed by two-way ANOVA followed by Sidak's multiple comparisons test. $p < .05$ was considered statistically significant.

3 | RESULTS

3.1 | Morphological Distribution of BDNF in the TG and TREZ

In the TG, some BDNF immunoreactivity was observed in the TG neurons, and most of the GFAP-immunoreactive satellite cells formed a thin glial sheath to envelope the

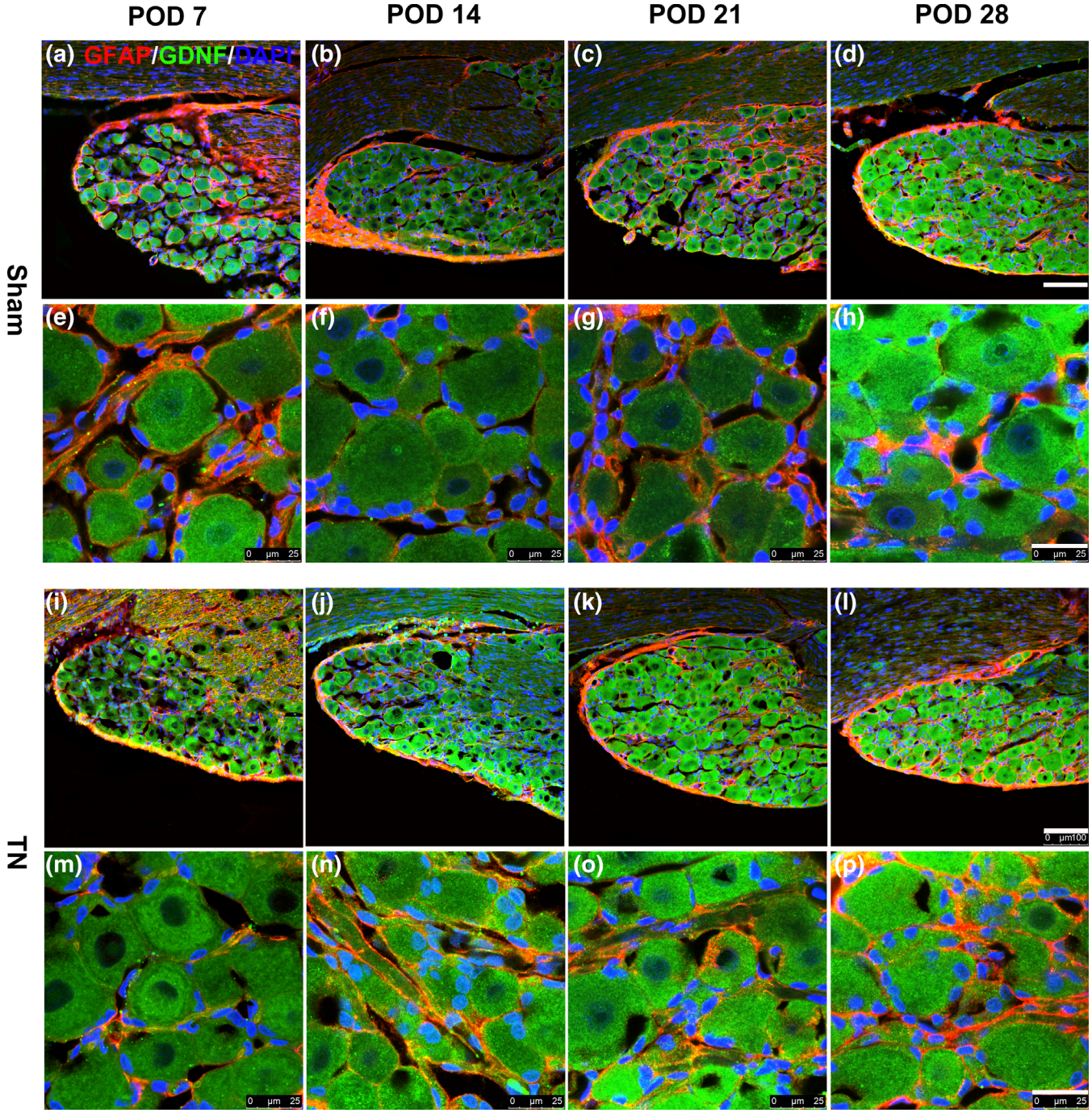

FIGURE 2 Distribution of GDNF and GFAP in the TG. GDNF (green) was expressed in TG neurons, and GFAP-immunoreactive satellite cells (red) wrapped around the TG neurons. Representative images of the distribution of GDNF and GFAP in the sham group (a–d, bar = 100 μm) and TN group (i–l, bar = 100 μm) in the TG; enlarged images of the sham group (e–h, bar = 25 μm) and TN group (m–p, bar = 25 μm) on PODs 7–28 are also shown. GDNF, glial cell line-derived neurotrophic factor; PODs, postoperation days; TG, trigeminal ganglion; TN, trigeminal neuralgia

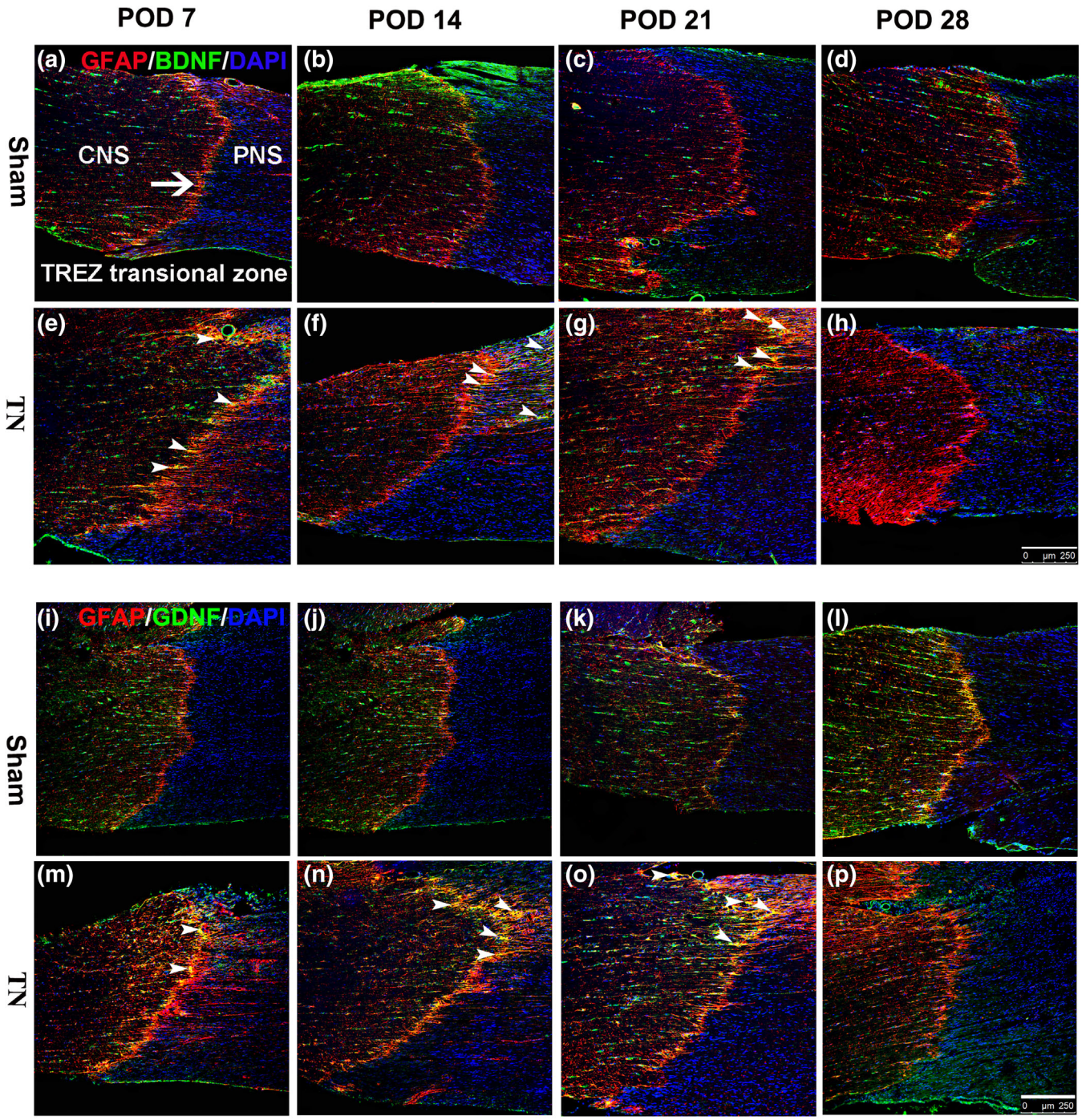

FIGURE 3 Distribution of BDNF/GFAP and GDNF/GFAP in the TREZ. The representative glial fringe of the CNS–PNS transition zone of the TREZ (white arrow) is distinguished by GFAP-positive astrocytes (red) and DAPI (blue) as determined by immunofluorescence staining (a). A small number of BDNF-immunoreactive fibers (green, a–h) and GDNF-immunoreactive fibers (green, i–p) were mainly distributed longitudinally on the central side of the TREZ, while the GFAP-positive fibers (red, e–h, m–p) obviously extended distally to the peripheral side of the CNS–PNS transition zone in the TN group from PODs 7–28 after compression injury. A few double-labeled BDNF/GFAP or GDNF/GFAP immunoreactive signals (yellow, e-g and m-o, representative white arrowhead) were observed on the astrocyte interface, which also extended to the peripheral side of the TREZ. Bar = 250 μm. BDNF, brain-derived neurotrophic factor; CNS, central nervous system; GDNF, glial cell line-derived neurotrophic factor; PNS, peripheral nervous system; POD, postoperation day; TN, trigeminal neuralgia; TREZ, trigeminal root entry zone

neurons (Figure 1). Most of the neuron-SGC complexes were stained with GFAP- and BDNF-immunoreactive antibodies. GFAP expression in the TN group was significantly increased in the TG after compression injury.

As determined by the double labelling of GFAP and GDNF in the TG, GDNF-positive neurons wrapped around GFAP-immunoreactive satellite cells in the sham and TN groups, but the expression of GDNF in the TN group on PODs 14–28 was more obvious than that in the sham group (Figure 2).

In the TREZ, GFAP-immunoreactive astrocytes formed a distinguished dome-shaped glial interface in the central nervous system (CNS)-PNS transition zone in the sham group (Figure 3a, white arrow shows the boundary profile), and a few BDNF-immunoreactive cells were mainly distributed longitudinally on the central side of the TREZ (Figure 3a–h). Little BDNF immunoreactivity was observed in the peripheral part of the CNS–PNS transition zone in the sham group (Figure 3a–d). However, the GFAP-positive astrocyte glial interface obviously extended distally to the peripheral side of the CNS–PNS transition zone in the TN group from PODs 7 to 28 after compression injury (Figure 3e–h), and a few double-labeled BDNF- and GFAP-immunoreactive cells were also observed on the astrocyte interface in the TN group (white arrowhead). Furthermore, some BDNF-immunoreactive cells also extended to the peripheral side of the TREZ, accompanied by sprouting GFAP-positive astrocytes.

3.2 | Morphological Distribution of GDNF in the TG and TREZ

Similarly, GDNF-immunoreactive fibers were mainly distributed in the central part of the CNS–PNS transition

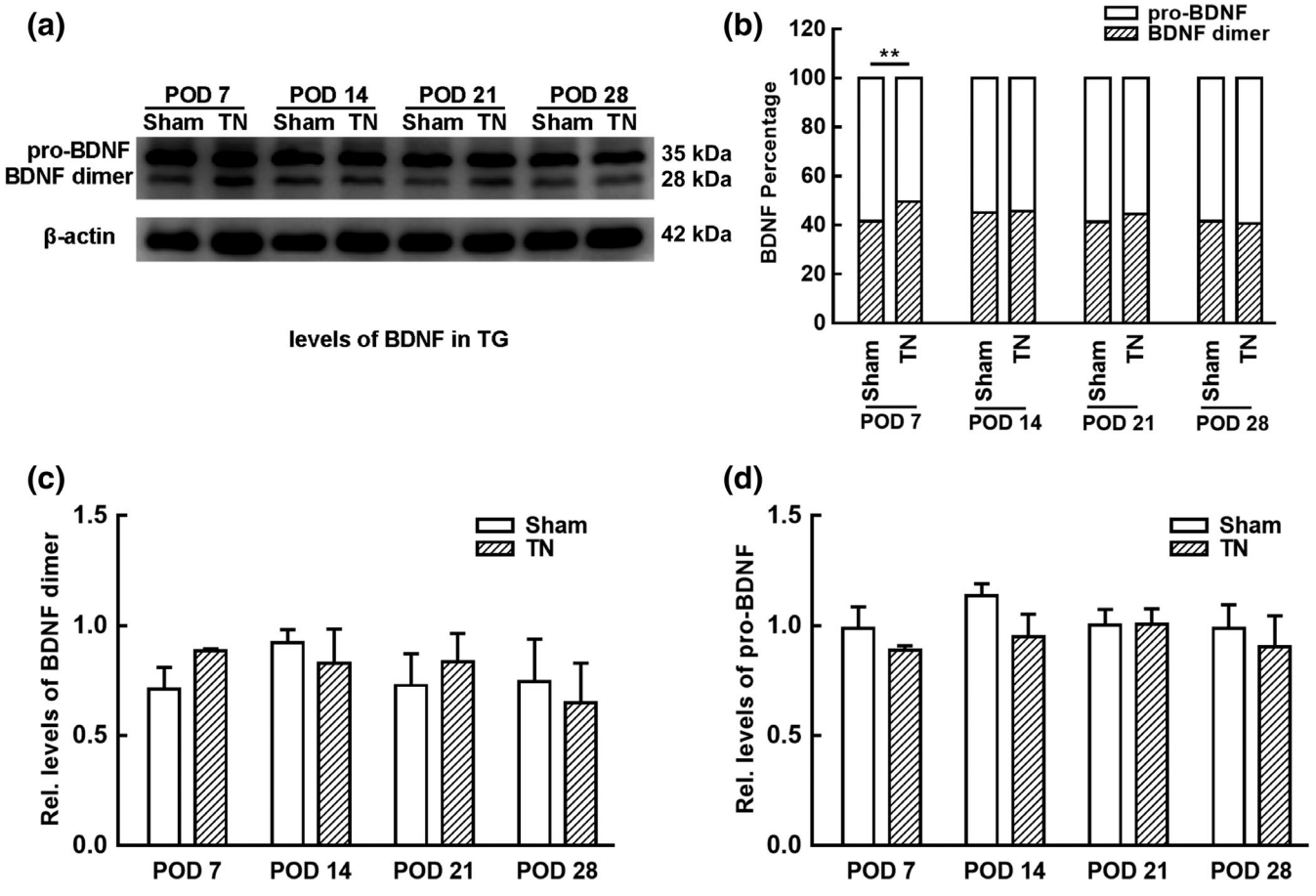

FIGURE 4 Western blot analysis of BDNF in the TG. Both 35 kDa pro-BDNF and a 28 kDa BDNF dimer were detected in the TG (a). The BDNF dimer/pro-BDNF ratio in the TN group was higher than that in the sham group on POD 7 (b, $p < .01$). There were no significant differences in the expression levels of pro-BDNF and the BDNF dimer in the sham and TN groups (c and d). The expression levels of BDNF were detected by Western blot analysis using β-actin as an internal control. BDNF, brain-derived neurotrophic factor; POD, postoperation day; TG, trigeminal ganglion; TN, trigeminal neuralgia

zone of the TREZ in the sham group (Figure 3i–l), and some of the GDNF-immunoreactive fibers also extended into the peripheral part of the CNS–PNS transition zone of the TREZ, accompanied by GFAP-positive sprouting astrocytes in the TN group from PODs 7 to 28 (Figure 3m–p).

3.3 | Western Blot Analysis of BDNF and GDNF Expression

Western blot analysis identified both 35 kDa pro-BDNF and a 28 kD BDNF dimer in the TG (Figure 4a). The BDNF dimer/pro-BDNF ratio in the TN group was higher than that in the sham group on POD 7 (Figure 4b) ($p < .01$), but there were no significant differences in the expression levels of pro-BDNF and the BDNF dimer in the sham and TN groups (Figure 4c and d). The TREZ protein expression levels of the 35 kDa pro-BDNF and the 28 kDa BDNF dimer were also detected by Western blot (Figure 5a). The BDNF dimer/pro-BDNF ratio in the TN group was obviously higher than that in the sham group on PODs 7 and 14 ($p < .001$) and slightly higher than that in the same group on POD 21 (Figure 5b) ($p < .05$). There were no significant differences in the expression levels of pro-BDNF in the sham and TN groups (Figure 5d) ($p > .05$), but the levels of the BDNF dimer in the TN group on PODs 7 and 14 were higher than those in the sham group (Figure 5c) ($p < .01$).

Western blot analysis showed that the protein levels of 23 kDa GDNF in the TG of the TN group were lower than those in the sham group on PODs 21 ($p < .05$) and

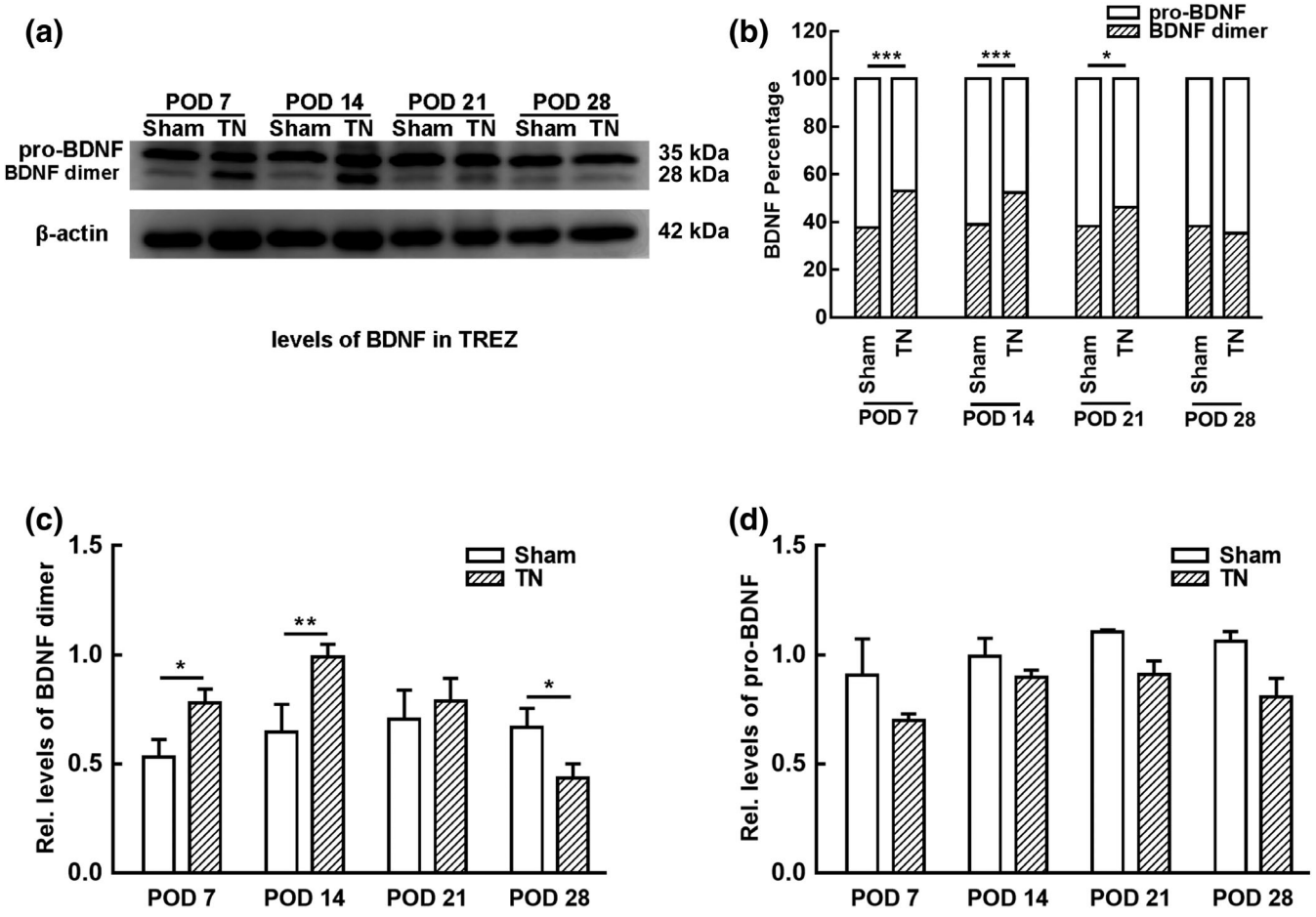

FIGURE 5 Western blot analysis of BDNF in the TREZ. The TREZ protein expression of 35 kDa pro-BDNF and the 28 kDa BDNF dimer was also detected by Western blot (a). The BDNF dimer/pro-BDNF ratio in the TN group was obviously higher than that in the sham group on PODs 7 and 14 ($p < .001$) and slightly higher than that in the sham group on POD 21 ($p < .05$) (b). The levels of the BDNF dimer in the TN group on PODs 7 and 14 were higher than those in the sham group ($p < .01$) (c). There were no significant differences in the expression levels of pro-BDNF in the sham and TN groups (d). The expression levels of BDNF were detected by Western blot analysis using β-actin as an internal control. BDNF, brain-derived neurotrophic factor; POD, postoperation day; TN, trigeminal neuralgia; TREZ, trigeminal root entry zone

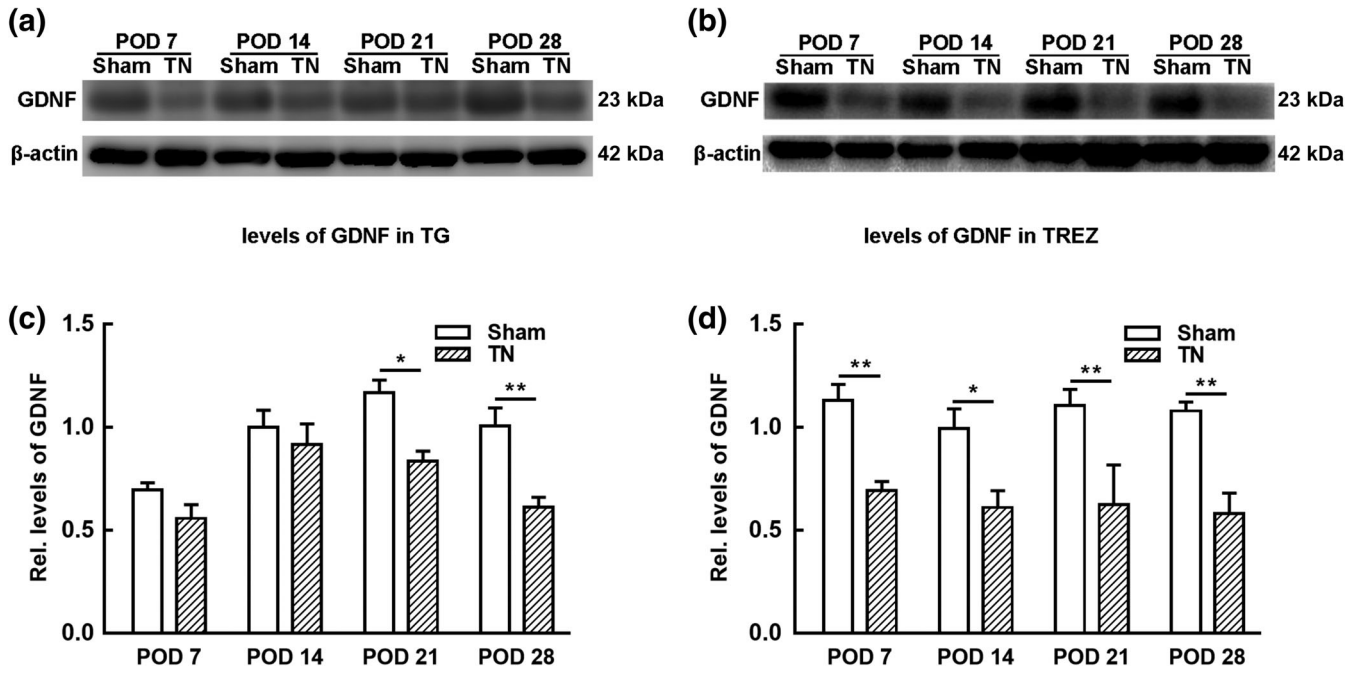

FIGURE 6 Western blot analysis of GDNF in the TG and TREZ. The protein expression of 23 kDa GDNF was detected in both the TG and the TREZ (a and b). The protein levels of GDNF in the TG of the TN group were lower than those in the sham group on PODs 21 ($p < .05$) and 28 ($p < .01$) (c). The level of GDNF in the TREZ of the TN group was significantly lower than that in the sham group on POD 7 ($p < .01$), POD ($p < .05$) and POD 21 to 28 ($p < .01$) (d). GDNF expression levels were detected by Western blot analysis using β-actin as an internal control. GDNF, glial cell line-derived neurotrophic factor; POD, postoperation day; TG, trigeminal ganglion; TN, trigeminal neuralgia; TREZ, trigeminal root entry zone

28 (Figure 6a,c) ($p < .01$), but the level of GDNF in the TREZ of the TN group was significantly lower than that in the sham group on POD 7($p<.01$), POD 14 ($p<.05$) and POD 21 to 28 ($p<.01$) (Figure 6b,d).

4 | DISCUSSION

BDNF and GDNF, two important neurotrophic factors that are simultaneously required for the development and survival of primary sensory neurons, promote myelination and axonal regeneration (Erickson, Brosenitsch, & Katz, 2001; Hoke et al., 2003; Santos, Gonzalez-Perez, Navarro, & Del Valle, 2016; Terenghi, 1999) and modulate the excitability of nociceptive TG neurons under inflammatory and neuropathic pain conditions (Boucher & McMahon, 2001; Takeda, Takahashi, Hara, & Matsumoto, 2013). BDNF- and GDNF-like immunoreactivities in the human TG at ages ranging from gestation to adulthood were studied by immunohistochemistry. Most of them were observed to be localized in small- and medium-sized TG neurons and spinal trigeminal nuclei, which suggested that both BDNF and GDNF may play a functional role in human trigeminal primary sensory neurones and be involved in the regulation of pain-related neural pathways in the human trigeminal sensory system (Quartu et al., 1997, 1999). However, the morphological changes and expression evaluation of BDNF and GDNF over time have not previously been characterized in a TN animal model induced by TREZ compression injury. We therefore evaluated the time course of these neurotrophic factor changes to further understand the process of TN development.

Determining the BDNF and GDNF expression patterns in the TREZ and TG under TREZ compression injury is important preliminary information and will contribute to further studying their functional roles in the pathogenesis of TN. As previously described, we established a TN rat CCT animal model induced by mechanical compression on the TREZ, and the animals developed hypersensitivity in the late postoperation period; this model is comparatively better than other models and more closely matches the clinical TN etiology (Lin et al., 2018; Luo et al., 2012). We also studied the histone acetylation of glial cells and glial plasticity changes in the TREZ in the CCT animal model (Lin et al., 2018; Luo et al., 2019).

The morphological localizations of BDNF and GDNF in the present study are consistent with the histological

observations in the human TG (Quartu et al., 1997, 1999), and the expression alterations of GDNF are similar to those in the trigeminal neuropathic pain rat model induced by the infraorbital nerve chronic constriction injury (Costa et al., 2016). GDNF was shown to protect against neuropathic pain (Shi et al., 2011), and exogenous BDNF application in the rat TG was shown to increase the hyperexcitability of neurons, as determined by an electrophysiology technique, suggesting that BDNF may increase pain sensitivity (Wang et al., 2019). However, no studies on the time course of BDNF expression alterations have been reported in the TN animal model.

In the present study, the expression levels of the 28 kDa BDNF dimer and 35 kDa pro-BDNF in the TG and TREZ were not significantly different in the sham and TN groups. Interestingly, the BDNF dimer/pro-BDNF ratio in the TREZ of the TN group was higher than that in the sham group from PODs 7 to 21, which suggested that trigeminal root compression injury may increase the transformation of different BDNF forms from pro-BDNF to the BDNF dimer in the TREZ. Furthermore, the BDNF dimer/pro-BDNF ratio in the TG of the TN group increased only slightly on POD 7 and soon recovered to levels roughly equal to those in the sham group from PODs 14 to 28. However, the mature BDNF/pro-BDNF ratio in the TREZ of the TN group increased from PODs 7 to 21 and recovered on POD 28. The expression of GDNF in the TG and TREZ of the TN group decreased significantly after trigeminal nerve compression injury.

We considered that although the expression of the 28 kDa BDNF dimer may have been slightly increased in the early phase of TREZ compression injury, as the TG neurons were the source of and repository for BDNF in the peripheral trigeminal nerve, the expression of the 28 kDa BDNF dimer quickly recovered. The TREZ served as the CNS–PNS tissue junction, and BDNF was transported from the brain stem or TG, which was more susceptible to changes in the external environment, such as those induced by mechanical compression injury. The decreased GDNF expression in both the TG and TREZ of the TN group was also more susceptible to compression injury.

In addition, other studies have demonstrated SGC proliferation and activation in the TG following chronic constriction injury of the infraorbital nerve (Donegan, Kernisant, Cua, Jasmin, & Ohara, 2013; Iwasa et al., 2019). Our study also observed GFAP-positive SGCs wrapped with primary sensory neurons in the TG. GFAP-immunoreactive astrocytes in the TREZ showed a distinct central-peripheral glial boundary and changed dynamically after compression injury. Neuron–glia crosstalk in the TG may also play an important role in the development of TN (Hossain, Unno, Ando, Masuda, & Kitagawa, 2017).

There are also limitations to our study. First, although our study was based on the TN animal model, which thus far most closely resembles the clinical etiology of TN patients, finding a vascular substitute to mimic the complex pathophysiology of the compression microvasculature in the TREZ remains difficult. Second, microvascular compression on the TREZ in TN patients is almost congenital and continues from birth to old age for decades; thus, matching the duration of TN in animal models with that in TN patients is difficult, similar to problems with other studies. Third, we studied the expression of BDNF and GDNF in the primary afferent of the trigeminal sensory pathway to provide a preliminary research basis. Further studies should be performed to understand the roles of BDNF and GDNF in the pathogenesis of TN by altering their expression levels under compression injury.

In summary, these results suggest that BDNF and GDNF may be involved in the nociceptive neuronal excitability of TG neurons in the CCT animal model of TN and affect the transmission of peripheral orofacial nociceptive stimuli from afferent fibers through the TREZ to the brain stem and higher level brain regions.

CONFLICT OF INTEREST

All authors have no conflicts of interest to declare.

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