Inhibiting BDNF/TrkB receptor improves resiniferatoxin-induced postherpetic neuralgia through decreasing ASIC3 signaling in dorsal root ganglia

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Research

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Abstract

Background Postherpetic neuralgia (PHN) is a devastating complication after varicella-zoster virus infection. Brain-derived neurotrophic factor (BDNF) has been shown to participate in the pathogenesis of PHN. Tropomyosin receptor kinase B (TrkB), an endogenous high-affinity receptor of BDNF is abundantly expressed in primary sensory neurons. Acid-sensitive ion channel 3 (ASIC3) is involved in chronic neuropathic pain, but its relation with BDNF/TrkB during PHN is unclear. This study aimed to investigate whether BDNF/TrkB contributes to PHN through regulating ASIC3 signaling in dorsal root ganglia (DRGs).

Methods Resiniferatoxin (RTX) was used to induce rat PHN models. Mechanical allodynia was assessed by measuring the paw withdrawal thresholds (PWTs). Thermal hyperalgesia was determined by measuring the paw withdrawal latencies (PWLs). We evaluated the effects of TrkB/ASIC3 signaling inhibition on the behavioral test, neuronal excitability and inflammatory response in rat models of PHN. ASIC3 short hairpin RNA (shRNA) transfection was used to investigate the effect of exogenous BDNF on inflammatory response in cultured PC-12 cells.

Results RTX resulted in significant mechanical allodynia, upregulated the protein expression of BDNF, TrkB, ASIC3, TRAF6, nNOS and c-Fos, and increased neuronal excitability in DRGs. Inhibition of ASIC3 reversed the above-mentioned effects of RTX, except for BDNF and TrkB protein expression. In addition, inhibition of TrkB blocked RTX-induced mechanical allodynia, activation of ASIC3 signaling and hyperexcitability of neurons. RTX-induced activation of BDNF was found in both neurons and satellite glia cells in DRGs. Furthermore, exogenous BDNF activated ASIC3 signaling, increased NO level and enhanced IL-6, IL-1β, TNF-α level in PC-12 cells, which was blocked by shRNA-ASIC3 transfection.

Conclusion These findings demonstrate that inhibiting BDNF/TrkB reduced inflammation, decreased neuronal hyperexcitability and improved mechanical allodynia through regulating ASIC3 signaling pathway in DRGs, which may provide a novel therapeutic target for patients with PHN.

Introduction

Postherpetic neuralgia (PHN) is a chronic neuropathic pain syndrome induced by the reactivation of latent varicella-zoster virus infection. Its typical symptoms include continuous aching, burning and allodynia[1]. PHN causes physical disability, emotional distress and many other complications, which seriously debilitate the quality of life [2]. Current treatments for PHN include medical (systemic tricyclic antidepressants, topical lidocaine and capsaicin, anticonvulsants and opioids) and interventional therapies (subcutaneous botulinum toxin injections, nerve blocks and neurostimulation)[3]. However, these is still lack of effective clinical therapy and up to 50% of patients with PHN are refractory to management[4]. Thus, it is meaningful to develop novel efficacious therapeutic strategies for preventing PHN.

Brain-derived neurotrophic factor (BDNF), an important neurotrophin and neurotransmitter, plays a crucial role in neural maintenance and repair. Besides that, BDNF also contributes to sensitization of pain-related pathways in primary sensory neurons, making it a potentially novel therapeutic target [5–7]. Emerging evidence indicated that the synthesis and release of BDNF in dorsal root ganglia (DRGs) were significantly increased in the process of inflammatory pain, cancer pain and neuropathic pain [8–10]. In addition, BDNF heterozygous mutant mice

showed an obvious suppression of neuropathic pain[11]. Tropomyosin receptor kinase B (TrkB) has been identified as an endogenous high-affinity receptor of BDNF, and is abundantly expressed in the primary sensory neurons [12, 13]. By acting through this receptor, BDNF participates in nociceptive pain-related signaling of primary sensory neurons[6, 12]. Nevertheless, the detailed molecular mechanism that elicit signaling downstream of BDNF/TrkB in the development of PHN remain elusive.

Acid-sensitive ion channels (ASICs) are cation-selective channels that are widely expressed in peripheral and central nervous systems in mammals [14]. Some studies demonstrated that the ASIC1, ASIC2 and ASIC3 channel subtypes are mainly expressed in neurons using *in situ* hybridization experiments [15–17]. Among these subtypes, ASIC3 is the most abundant one in neurons of DRGs, as well as the most sensitive to extracellular acidification[18]. Sluka *et al.* [19] found ASIC3 knockout mice failed to develop chronic muscle pain in a mouse fibromyalgia model induced by repeated acid injections. Pro-inflammatory mediators, specifically nerve growth factor, interleukin-1, serotonin, and bradykinin, reportedly led to hyperexcitability of primary cultured DRG neurons through increasing ASIC3 expression[20]. Besides, BDNF from extracellular significantly increased excitability of cultured primary hippocampal neurons[21]. However, the exact regulation mechanism between BDNF/trkB and ASIC3 channels in PHN needs to be explored.

Based on the strong feasibility of resiniferatoxin (RTX) and its possible mimic of the PHN-like symptoms [22, 23], we used RTX to induce PHN in the present study. We hypothesize that RTX injection increased the synthesis and secretion of BDNF, and then the up-regulated BDNF activated ASIC3 signaling through TrkB receptor. Our present study helps to demonstrate the mechanism underlying the role of BDNF/TrkB-ASIC3 signaling which may be used as a potential therapeutic target for the prevention and treatment of PHN.

Materials And Methods

Animals

Adult healthy male Sprague-Dawley rats weighing 200 ± 20 g were provided by the experimental animal center of Soochow University (Animal license No. SYXK Jiang-su2017-0043). Animals were kept under controlled temperature of 24˚C-26˚C, relative humidity 40% − 60% and 12 h light-dark cycle with food and water available ad libitum. Animal care and handling were approved by the Institutional Animal Care and Use Committee of Soochow University (Suzhou, Jiangsu, China). All experiments were performed in accordance with the Guide for the Care of Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85 − 23, revised in 1996).

Establishment of a resiniferatoxin (RTX)-induced PHN model

Rats in the RTX group received single intraperitoneal injection of RTX (200 μg/kg, LC Laboratories, Woburn, MA, USA) with isoflurane inhalational (2% isoflurane in a mixture of oxygen and air) anesthesia as previously described[23]. RTX was dissolved in a mixture of 10% Tween 80 (Sigma, St. Louis, MO, USA) and 10% ethanol in normal saline, and rats in vehicle group received the same volume of the mixture solution.

Intrathecal injection

For intrathecal injection, the intrathecal catheter (PE-10 tube, Smiths Medical, Minneapolis, MN, USA) was administered 5 days before establishment of RTX-induced PHN model according to a previously described

method[24]. After rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), the polyethylene catheter was inserted into the subarachnoid space of the spinal cord between L4 and L5 spinous process. The correct position was confirmed by the tail flick response immediately after inserting the catheter.

Cell culture and BDNF treatment

Rat pheochromocytoma PC-12 cells were purchased from Stem Cell Bank, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Invitrogen, Carlsbad, CA, USA) containing 10% (v/v) heat-inactivated fetal bovine serum (Gibco, Life Technologies, Carlsbad, CA, USA), penicillin (100U/ mL) and streptomycin (100 μg/mL) in an incubator (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C with 5% CO2. Cells were treated with BDNF (2, 20, and 200 ng/mL, PeproTech, Cranbury, NJ, USA) for 20 min.

ASIC3-shRNAs transfection

Part I: For ASIC3 knockdown in PC-12 cells, cells were transfected with the short hairpin RNAs (shRNAs, Genechem, Shanghai, China) targeting ASIC3 (NM_173135). And cells were randomly divided into 4 groups: NC group (negative control transfection for 24 h), shRNA1 group, shRNA2 group and shRNA3 group (shRNAs-ASIC3 transfection for 24 h, respectively).

Part II: To investigate the effects of ASIC3 knock-down by shRNA on PC-12 cells during treatment with BDNF, cells were randomly divided into 4 groups: Control group, BDNF group (cells were incubated with 20 ng/ml BDNF for 20 min), BDNF + shRNA-ASIC3 group (cells were transfected with shRNA for 24 h prior to BDNF treatment), and BDNF + NC (negative control) group. Transfection was performed by using the jetOPTIMUS® transfection regent (Polyplus-transfection®SA, Strasbourg, Bas-Rhin, France). The shRNAs oligo sequences are shown in Table 1.

Table 1
Sequences of shRNAs oligonucleotides targeting ASIC3

Name	Direction	oligonucleotides sequences 5′- 3′
shRNA1	Sense	CCGGCAGCTGTGACTCTGTGTAATACTCGAGTATTACACAGAGTCACAGCTGTTTTTG
	Antisense	AATTCAAAAACAGCTGTGACTCTGTGTAATACTCGAGTATTACACAGAGTCACAGCTG
shRNA2	Sense	CCGGTGGCAACGGACTGGAGATTATCTCGAGATAATCTCCAGTCCGTTGCCATTTTTG
	Antisense	AATTCAAAAATGGCAACGGACTGGAGATTATCTCGAGATAATCTCCAGTCCGTTGCCA
shRNA3	Sense	CCGGTGGCTGTCGAATGATGCATATCTCGAGATATGCATCATTCGACAGCCATTTTTG
	Antisense	AATTCAAAAATGGCTGTCGAATGATGCATATCTCGAGATATGCATCATTCGACAGCCA

Behavioral tests

Mechanical allodynia was assessed using von Frey filaments (Stoelting Company, Wood Dale, IL, USA) as previously described[24]. Rat hindpaw was stimulated with a series of von Frey fiber (1, 1.4, 2, 4, 6, 8, 10, and 15 g). The paw withdrawal threshold (PWT) was defined as the lowest force of the filaments that produced at least three withdrawal responses in five tests. Thermal hyperalgesia was assessed by measuring the paw

withdrawal latencies (PWLs) in response to a radiant heat source (Ugo Basile, Gemonio, Italy). The investigators were blinded to group allocation during data collection and analysis.

Immunofluorescence

Animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and transcardially perfused with normal saline followed by 4% paraformaldehyde. The L4-L6 segments of DRGs were removed and post-fixed at 4 °C for 12 h, and then were dehydrated at 4 °C for overnight with 30% sucrose solution in phosphate-buffered saline (PBS). DRG slices of 10 μm thick were obtained by using a cryostat. For double immunofluorescence, the slices were incubated with primary antibodies: rabbit anti-TrkB (1:200; Abcam, ab18987, Cambridge, MA, USA), mouse anti-ASIC3 (1:100, Abcam, ab190638), rabbit anti-BDNF (1:200; Abcam, ab108319), mouse anti-neuronal nuclear protein (NeuN, 1:200, Abcam, ab104224), and mouse anti- Glial Fibrillary Acidic Protein (GFAP, 1:500, #3670,Cell Signaling Technology, USA). Images were captured under a fluorescence micro-scope (Nikon Corporation; Tokyo, Japan), and analyzed with Image Pro Plus software (Media Cybernetics, Silver Spring, MD, USA).

Western blotting

The L4-L6 segments of DRG were rapidly dissected out and total protein was extracted by using the RIPA reagents (Beyotime, Shanghai, China). Then, the protein concentration was determined with a bicinchoninic acid protein assay kit (Beyotime, Shanghai, China). The tissue protein was separated by sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gel electrophoresis, and transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore Corp., Bedford, MA). The membrane was incubated with the primary antibodies for overnight at 4 °C, then incubated with the horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 h at room temperature. Finally, the densities of protein were normalized to β-actin as control. The primary antibodies were used as the following: rabbit anti-ASIC3 (1:500, Abcam, ab190638), rabbit anti-TrkB(1:1000, Abcam, ab18987), rabbit anti-tumor necrosis factor receptor-associated factor 6 (TRAF6, 1:1000, Abcam, ab33915), rabbit anti-neuronal nitric oxide synthase (nNOS, 1:1000, C12H1, Cell Signaling Technology, Beverly, MA, USA), mouse anti-c-Fos (1:500, SC-52, Santa Cruz Technology, USA) and rabbit anti-β-actin(1:1000, 2148, Cell Signaling Technology, Beverly, MA, USA).

Quantitative- real time PCR (RT-qPCR)

Total RNA from L4-L6 DRG segments or PC-12 cells were extracted by using the Trizol reagent (Invitrogen; Thermo Fisher Scientific, Inc. Waltham, MA, USA). Absorbances at 260 and 280 nm were measured for RNA quantification and purity control. According to the manufacturer's instructions, the reverse transcription was performed using the cDNA Synthesis Kit (Applied Biological Materials, Richmond, BC, Canada). RNA samples were added to 5 × All-in-one and DEPC water to form a 20 μl reaction system for reverse transcription to cDNA. Quantitative PCR was conducted with EvaGreen qPCR MasterMix (Applied Biological Materials, Richmond, BC, Canada) in 10 μl reaction volumes on Roche Light Cycler R480 System (Roche, Bedford, MA, USA). And 10 μl reaction volume contained 1.5 μl of cDNA, 5 μl Eva Green, 1 μl of each pair of primers and 1.5 μl DEPC water. The cycle parameters were as following: pre-denaturation at 95 °C for 10 s, denaturation at 58 °C for 15 s, and annealing at 75 °C for 20 s for 40 cycles. The value obtained for the target gene expression was normalized to β-tubulin and analyzed by the relative gene expression $2^{-\Delta\Delta CT}$ method. And all the experiments were repeated three times. The primers were provided by Shanghai Shenggong Co., Ltd., and the sequences were:

ASIC3: Forward primer: 5'-CTGGCAACGGACTGGAGATTA-3', Reverse primer: 5'-TGTAGTAGCGCACGGGTTGG-3';

TrkB: Forward primer: 5'-AGGGATGGCGTGACTTTC-3', Reverse primer: 5'-GTTGGCTTCAGGCTTATGC-3';

β-actin: Forward primer: 5'-TCTATCCTGGCCTCACTGTC-3', Reverse primer: 5'-AACGCAGCTCAGTAACAGTCC-3';

Interleukin (IL)-1β: Forward primer: 5'-ATCTCACAGCAGCATCTCGACAAG-3', Reverse primer: 5'-CACACTAGCAGGTCGTCATCATCC – 3';

IL-6: Forward primer: 5'-CCAGTTGCCTTCTTGGGACT-3', Reverse primer: 5'-GGTCTGTTGTGGGTGGTATCC-3';

Tumor necrosis factor (TNF) -α: Forward primer: 5'-GCATGATCCGAGATGTGGAACTGG-3', Reverse primer: 5'-CGCCACGAGCAGGAATGAGAAG-3'.

β-actin: Forward primer: 5'-TCTATCCTGGCCTCACTGTC-3', Reverse primer: 5'-AACGCAGCTCAGTAACAGTCC-3'.

Acute isolation of DRG neurons and whole-cell patch clamp recording

After animals were anesthetized, the L4-6 DRGs of rats was quickly removed and transferred to an ice-cold oxygenated fresh dissection solution. DRGs were incubated with the dissection solution containing collagenase D (1.8-2.0 mg/ml, Roche; Indianapolis, IN, USA) and 0.25% trypsin (Sigma; St. Louis, MO, USA) for 1.5 h at 37 °C. At the end of digestion, the cells were allowed to adhere for 45 min on a glass cover slip, and then the adherent DRG cells were placed in a Petri dish and attached to a table of a reverse microscope. Small- and medium-sized DRG neurons were harvested and used in our study. The excitability of DRG neurons was measured by the whole-cell patch-clamp recording technique with an EPC10 patch-clamp amplifier (HEKA Electronics, Lambrecht, Germany). The data were obtained and analyzed by Fit Master from HEKA Electronics.

Determination of Nitric oxide (NO) levels

Nitric oxide release was quantified from the concentration of nitrite, a stable metabolite of NO. PC12 cells at the logarithmic growth phase were seeded in 6-well plates a density of approximately 1×10^6 cells/well overnight. Following the different interventions, the NO level was assessed using the Nitric Oxide Assay kit (Abcam, Cambridge, MA, USA), with triplicates for each sample. The final absorbance values were measured at 540 nm wavelength using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The NO level of each sample was calculated by a standard curve generated according to the manufacturer's guidance.

Statistical analysis

All data were expressed as mean ± standard error of the mean (SEM) and analyzed using the GraphPad Prism software (version 7.0, GraphPad, San Diego, CA, USA). Statistical significance was determined by using Student's *t*-test, one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test, or two-way repeated measures ANOVA followed by Bonferroni's post-hoc test, as appropriate. $P < 0.05$ was considered statistically significant.

Results

RTX injection increased ASIC3 and TrkB expression in DRGs

The effect of RTX administration on mechanical withdrawal threshold was examined using von Frey filaments. RTX significantly reduced the paw withdrawal thresholds (Baseline) 3 days after injection (Figure 1A). Meanwhile, the paw withdrawal latency to heat stimulus was obviously enhanced from 5 days after RTX

treatment (Figure 1B). In contrast, vehicle injection did not affect the paw withdrawal threshold or withdrawal latency to heat stimulus in rats. These results indicate the establishment of postherpetic neuralgia in rats [23, 25] . Then, DRGs were dissected out 14 days after RTX or Vehicle injection. RTX injection dramatically increased ASIC3 and TrkB mRNA expression (Figure 1C) in DRGs. Next, western blot results showed that RTX injection also significantly enhanced the protein expression of ASIC3 and TrkB (Figure 1D-1F). Immunostaining was performed 14 days after RTX injection. As shown in Figure 1G, TrkB positive cells (red) and ASIC3 positive cells (green) colocalized in the DRG neurons. Moreover, RTX injection significantly increased the number of positive neurons of TrkB and ASIC3 (Figure 1H).

Inhibition of ASIC3 attenuated mechanical allodynia and decreased TRAF6 signaling

To determine whether ASIC3 is involved in RTX-induced postherpetic neuralgia, Amiloride (Ami), a potent ASIC3 inhibitor, was intrathecally administrated at 14 days after RTX injection. Treatment with 50, 100 or 200 μg/kg Amiloride improved the paw withdrawal threshold in RTX-injected rats. The mechanism threshold was increased at 0.5h and lasted for 8h after Amiloride treatment in a dose dependent manner (Figure 2A). The use of 50 μg/kg Amiloride was less effective, and the effect of 200 μg/kg Amiloride was comparable to that of 100 μg/kg. Meanwhile, 100 μg/kg Amiloride had no significant effects on the mechanism threshold in healthy control rats (Figure 2B). Based on these results, 100 μg/kg Amiloride was administrated intrathecally from 7 days after RTX injection, once per day for consecutive 7 days. The ASIC3 inhibitor effectively blocked the decrease in paw withdrawal threshold of RTX-injected rats (Figure 2C). RTX injection also increased the protein expression of TRAF6 and nNOS, which was effectively reversed by consecutively Amiloride administration. c-Fos, serving as a marker of neuronal activity, was detected. As shown in Figure 2D, consecutively Amiloride administration led to lower c-Fos protein level in DRGs compared to RTX injection (Figure 2E-2G). However, Amiloride did not reversed the protein expression of BDNF and TrkB in DRGs induced by RTX injection (Figure 2H and 2I).

Inhibition of ASIC3 decreased hyperexcitability in DRGs neurons

We next determined the intrinsic membrane properties including resting membrane potentials (RP), current threshold (rheobase), and pattern of firings in response to depolarizing current stimulation of DRG neurons. RTX injection significantly increased RP (Figure 3A) and decreased rheobase (Figure 3B), which was partly blocked by consecutively Amiloride administration. Besides, RTX injection obviously increased the number of action potentials (APs) in response to 2× rheobase current stimulation, as well as 3× rheobase current stimulation (Figure 3C and 3D), while treatment with Amiloride reversed the increased response. In addition, the numbers of APs evoked by 100, 300 and 500 pA ramp current stimulation were determined. Amiloride significantly reduced the number of APs evoked by ramp current stimulation in DRG neurons of RTX-injected rats (Figure 3E-3G). Several additional membrane properties were also determined. Cell membrane capacitance (Cm), input resistance (Rin), AP threshold, AP duration and overshoot were not significantly altered in DRG neurons from rats after Vehicle, RTX or Amiloride injection (data not shown).

Inhibition of TrkB attenuated mechanical allodynia and decreased ASIC3 signaling

ANA-12, a specific TrkB inhibitor, was intrathecally administrated from day 7 after RTX injection, once per day for consecutive 7 days. The ANA-12 effectively blocked the decreased paw withdrawal threshold induced by RTX injection (Figure 4A). Besides, ANA-12 had no significant effects on the mechanism threshold in healthy

control rats (Figure 4B). ANA-12 also significantly inhibited the elevated protein expression of ASIC3, TRAF6, nNOS and c-Fos induced by RTX injection (Figure 4C-4G).

Inhibition of TrkB reduced hyperexcitability in DRGs neurons

Next, ANA-12 significantly blocked the increased RP (Figure 5A) and decreased rheobase (Figure 5B) in DRG neurons induced by RTX injection. Besides, ANA-12 also decreased the number of APs in response to 2× rheobase current stimulation, as well as 3× rheobase current stimulation (Figure 5C and 5D), induced by RTX injection. In addition, ANA-12 significantly decreased the number of APs evoked by 100, 300 and 500 pA ramp current stimulation in DRG neurons of RTX-injected rats (Figure 5E-5G).

TrkB receptor contributed to RTX-PHN through ASIC3 signaling

Next, 7,8-dihydroxyflavone (7,8-DHF), a potent small molecular TrkB agonist, was intraperitoneal administrated in healthy rats. 7,8-DHF (1, 3 or 6 mg/kg) significantly decreased the paw withdrawal threshold in healthy rats, and threshold was decreased at 0.5h and lasted for 4h after 7,8-DHF treatment in a dose dependent manner (Figure 6A). Besides, 1mg/kg 7,8-DHF was less effective, and the effect of 3 mg/kg was comparable to 6mg/kg. Based on these results, 3 mg/kg 7,8-DHF and 100 μg/kg Amiloride were administrated from day 7 after RTX injection, once per day for consecutive 7 days. The paw withdrawal threshold was significantly increased in RTX-PHN+DHF+Ami group compared to RTX-PHN group (Figure 6B). 3 mg/kg DHF and 100 μg/kg Amiloride consecutively administration also significantly inhibited the elevated protein expression of TRAF6, nNOS and c-Fos induced by RTX injection (Figure 6C-6F).

RTX injection increased BDNF expression in DRGs

DRGs were dissected out 14 days after RTX or Vehicle injection. RTX injection dramatically increased BDNF mRNA expression (Figure 7A) and enhanced BDNF protein expression in DRGs (Figure 7B and 7C). Next, double labeling studies were performed 14 days after RTX injection. As shown in Figure 7D, NeuN positive cells (green) and BDNF positive cells (red) colocalized in the DRG neurons (yellow), and the percentage of BDNF-positive cells in neurons were increased after RTX injection (Figure 7E). Besides, we found that GFAP positive cells (green) and BDNF positive cells (red) also colocalized in the satellite glia cells, the percentage of BDNF-positive cells in satellite glia cells were increased after RTX injection (Figure 7F and 7G).

Exogenous BDNF enhanced TrkB-ASIC3 signaling in PC-12 cells

To investigate whether the TrkB-ASIC3 signaling activation in DRGs was induced by BDNF secretion from glia or neurons, the cultured PC-12 cells were incubated with exogenous BDNF for 20 min. Treatment with BDNF (2, 20 or 200 ng/ml) significantly increased TrkB protein expression. Meanwhile, both 20 and 200 ng/ml BDNF significantly increased ASIC3 protein expression, but 2 ng/ml BDNF did not. In addition, the use of 20 ng/ml BDNF was more effective than 200 ng/ml BDNF (Figure 8A and 8B). Based on these results, 20 ng/ml BDNF was used in the following experiments. Besides, exogenous BDNF up-regulated the protein expressions of ASIC3, TRAF6 and nNOS in PC-12 cells, while these above changes were reversed by ANA-12 treatment (Figure 8C and 8D).

Knockdown of ASCl3 abolished TRAF6 signaling activation induced by exogenous BDNF treatment in PC-12 cells

Then, PC-12 cells were transfected with three different ASIC3 shRNAs (shRNA1, shRNA2, and shRNA3) for 24h. Transfection with shRNAs significantly reduced ASIC3 protein levels compared to the NC groups, and western blot results showed that shRNA3 was the most efficient one to inhibit the protein expression of ASIC3 (Figure 9A and 9B), while the ASIC3 protein levels in NC group was not changed compared to the control groups (Figure 9C and 9D). The shRNA3 was used in the following experiments, and we found that shRNA transfection significantly inhibited the increased protein expressions of ASIC3, TRAF6 and nNOS induced by exogenous BDNF (Figure 9E and 9F). Next, NO concentration was assessed using Nitric Oxide Assay kit. Exogenous BDNF also significantly increased NO level, which was reversed by shRNA transfection (Figure 9G). Besides, exogenous BDNF obviously upregulated mRNA expressions of IL-6, IL-1β and TNF-α, which was reversed by knockdown of ASIC3(Figure 9H-9J).

Discussion

The BDNF-TrkB signaling was implicated in the pathological development and functional amelioration of various neuronal disorders. Moreover, BDNF overexpression worsens pathological conditions [26]. One study found that BDNF activity was augmented in cultured neurons incubated with sera from patients with PHN [27]. In addition, ASIC3 is an important protein in the pathogenesis of chronic pain diseases, and this making it a good candidate for a pain sensor[28, 29]. Based on the current findings, this study investigated the exact regulation mechanism between BDNF/TrkB and ASIC3 signaling in a rat RTX-PHN model. To the best of our knowledge, this is the first study to demonstrate that inhibiting BDNF/TrkB reduced inflammation factor and NO levels, reversed hyperexcitability of DRGs neurons, and improved mechanical allodynia through regulating ASIC3 signaling (Figure 10).

Mamet *et al*. [20] found that chronic inflammatory pain elevates ASIC3 expression in the DRGs neurons, and ASIC3 blocker produced beneficial effects on the suppression of hyperalgesia in mice [30], rats [31] and acid-induced pain in human subjects [32]. In addition, one study demonstrated that hyperalgesia only occurs in wild type mice but not in ASIC3-knockout ones with mice model of joint inflammation[33]. These results were consistent with our current findings that ASIC3 expression was significantly increased in DRGs neuron of RTX-induced PHN rat model, and hyperalgesia was improved with Amiloride administration, implicating the importance of ASIC3 in RTX-induced pain sensation. Recent studies have shown that TRAF6 is closely related to neuropathic pain caused by the primary or secondary damage or dysfunction of peripheral of nervous system [34-36]. In consistent with the known response of TRAF6 activation in the immune system, our findings indicated that TNF- α, IL-1β, IL-6 and NO level were significantly upregulated in PC-12 cells in response to exogenous BDNF, and further revealed these increased changes was effectively blocked by knockdown of ASIC3. Our results also indicated that RTX enhanced neuronal excitability and preventing ASIC3 activation reversed the enhanced neural hyperexcitability of DRG neurons. The possible explanation for these findings is that ASIC3 depolarizes neuronal membrane and leads to an excitation of the neuron through regulating TRAF6-nNOS signaling.

TrkB is a high-affinity BDNF receptor, which is primarily present on large- and medium-sized DRG neurons [37]. Studies showed that TrkB receptor was increased in the primary sensory neurons of DRG during inflammatory

pain [38] or neuropathic pain [39]. We detected BDNF and TrkB protein levels were enhanced in the DRGs after RTX injection. Besides, one study found the increased ASIC1a activity through enhancing its surface expression and activity was required for BDNF-mediated hypersensitivity of spinal dorsal horn nociceptive neurons and central mechanical hyperalgesia[40]. Another recent study suggested that ASIC2 and ASIC3 transcripts in DRGs were upregulated in spinal cord injury mice, and the increased expression levels were reduced by anti-BDNF treatment[41]. Based on our current results, TrkB inhibitor ANA-12 attenuated mechanical allodynia, decreased ASIC3 signaling and reduced hyperexcitability in DRGs neurons of RTX-injected rats, which further indicates that BDNF/TrkB pathway contributes to RTX-induced PHN through regulating ASIC3 in the primary sensory neurons. The previous study demonstrated that elevating the extracellular concentration of BDNF significantly increased neuronal excitability of cultured primary hippocampal neurons [21]. Thus, BDNF/TrkB-regulated ASIC3 delivery may be an important mechanism for channel reactivation during neuropathic pain conditions, and increased amount of ASIC3 channels may further increase neuronal excitability[42].

BDNF is normally expressed and synthesized in neurons, and it could release from DRGs to provide neuronal development or participate in pain perception and sensitization[43]. A previous study showed that BDNF released from sensory neurons did not significantly contribute to acute pain, but was necessary for the transition from acute to chronic inflammatory pain and some neuropathic pain states[7]. Our results found that BDNF expression was upregulated in the neurons of DRGs at 14 days after RTX injection, and we speculated that BDNF mainly contributed to the chronic pain processing in rat PHN models. Interestingly, we also found that BDNF expression was also increased in the activated satellite glial cells, suggesting more studies are still needed to verify the pronociceptive role of BDNF in neuropathic pain processes in the peripheral nervous system with using the conditional knockout mice.

This study has several limitations. First, as this study aimed to explore whether targeting TrkB could affect RTX-induced postherpetic neuralgia through regulating ASIC3 signaling, the interaction between ASIC3 and TRAF6 was not evaluated. Second, the current results suggest that ASIC3 contributed to RTX-induced postherpetic neuralgia through enhancing the protein level. However, we did not detect whether the channel function of ASIC3 was involved in the postherpetic neuralgia through increasing ASIC3 current. Last, BDNF is synthesized by a subpopulation of unmyelinated primary afferents located in the DRGs, and some studies reported that anterograde transport of BDNF was released to the central terminals in the spinal cord dorsal horn, then interacted with receptors in spinal neurons to facilitate excitatory neurotransmission, or modulate inhibitory signaling[44]. Thus, the role of BDNF signaling in the spinal dorsal horn also needs to be investigated in further studies.

Conclusion

This present study revealed that inhibiting BDNF/TrkB reduced the RTX-induced mechanical allodynia, decreased inflammation factor levels and reversed hyperexcitability of DRGs neurons through regulating ASIC3 signaling, which may provide a potential novel therapeutic target for the prevention and treatment of patients with PHN.

List Of Abbreviations

PHN: Postherpetic neuralgia

BDNF: Brain-derived neurotrophic factor

TrkB: Tropomyosin receptor kinase B

ASIC: Acid-sensitive ion channel

DRGs: Dorsal root ganglia

RTX: Resiniferatoxin

PWTs: Paw withdrawal thresholds

PWLs: Paw withdrawal latencies

shRNA: Short hairpin RNA

RPMI: Roswell Park Memorial Institute

NeuN: Neuronal nuclear protein

GFAP: Glial Fibrillary Acidic Protein

SDS-PAGE: Sodium dodecyl sulphate-polyacrylamide

HRP: Horseradish peroxidase

nNOS: Neuronal nitric oxide synthase

NO: Nitric oxide

TRAF6: TNF receptor associated factor 6

RP: Resting membrane potentials

Aps: Action potentials

Cm: Cell membrane capacitance

Rin: Input resistance

7,8-DHF: 7,8-dihydroxyflavone

Ami: Amiloride

i.p.: Intraperitoneal

NC: Negative control

PBS: Phosphate-buffered saline

IL: Interleukin

TNF: Tumor necrosis factor

Declarations

Ethics approval and consent to participate: Animal care and handling were approved by the Institutional Animal Care and Use Committee of Soochow University (Suzhou, Jiangsu, China). All experiments were performed in accordance with the Guide for the Care of Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised in 1996).

Consent for publication: Not applicable

Availability of data and materials: The data used to support the findings of this study are available from the corresponding authors upon request.

Competing interests: There is no conflict of interest regarding the publication of this paper.

Authors' contributions: Study concept and design: JPY and XWM. Acquisition of data: XW, XWM and JH. Analysis and interpretation of data: XW, LNW, KP, XHJ, BZ and FHJ. Drafting of the manuscript: XWM and XW. Statistical analysis: FHJ and KP. Critical revision of the manuscript for important intellectual content: LNW and JPY.

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Figures

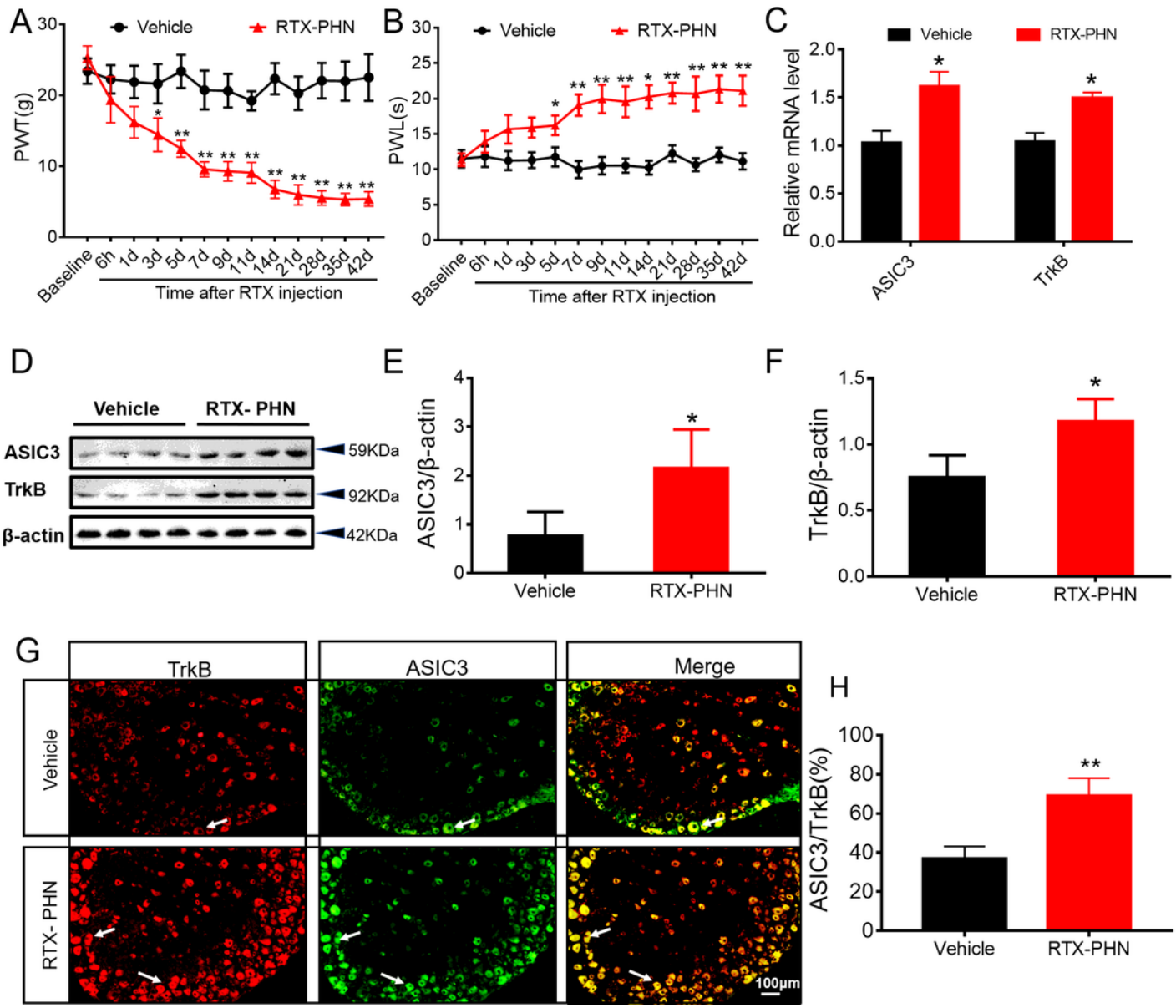
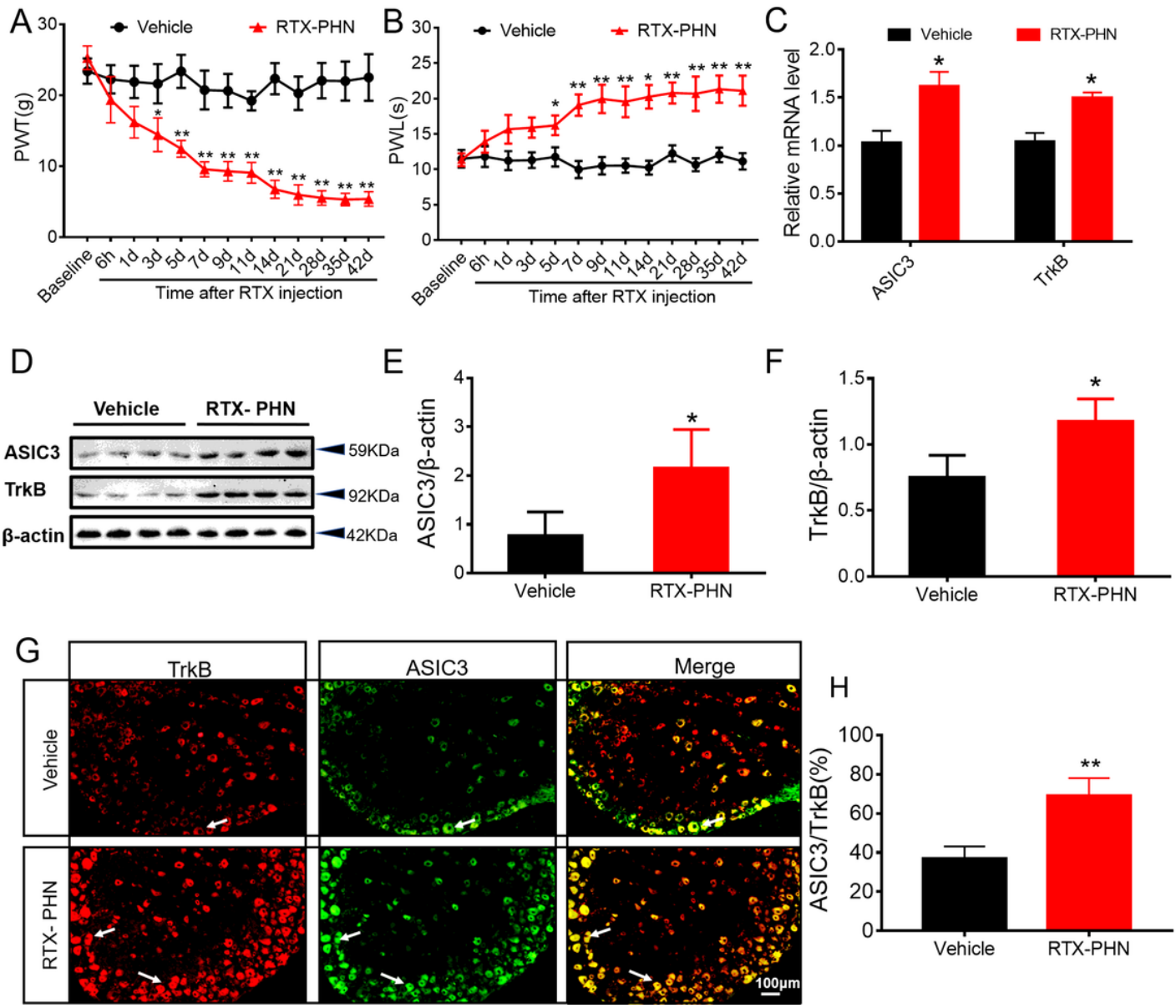


Figure 1

RTX injection increased ASIC3 and TrkB expression in DRGs. (A) Paw withdrawal thresholds (n=10, *P < 0.05, **P < 0.01, two-way repeated measures ANOVA followed by Bonferroni's post-hoc test). (B) Paw withdrawal latency (n=10, *P < 0.05, **P < 0.01, two-way repeated measures ANOVA followed by Bonferroni's post-hoc test). (C) Increased ASIC3 and TrkB mRNA expression after RTX injection (n=4, *p < 0.05 vs. Vehicle, Student's t-test). (D-F) ASIC3 and TrkB protein expression in DRGs (n=4, *p < 0.05 vs. Vehicle, Student's t-test). (G) Merge of double labeling of TrkB and ASIC3 (Scale bar = 100 μm). (H) The percentage of ASIC3 labeled in TrkB positive cells (n=5, *p < 0.05 vs. Vehicle, Student's t-test).



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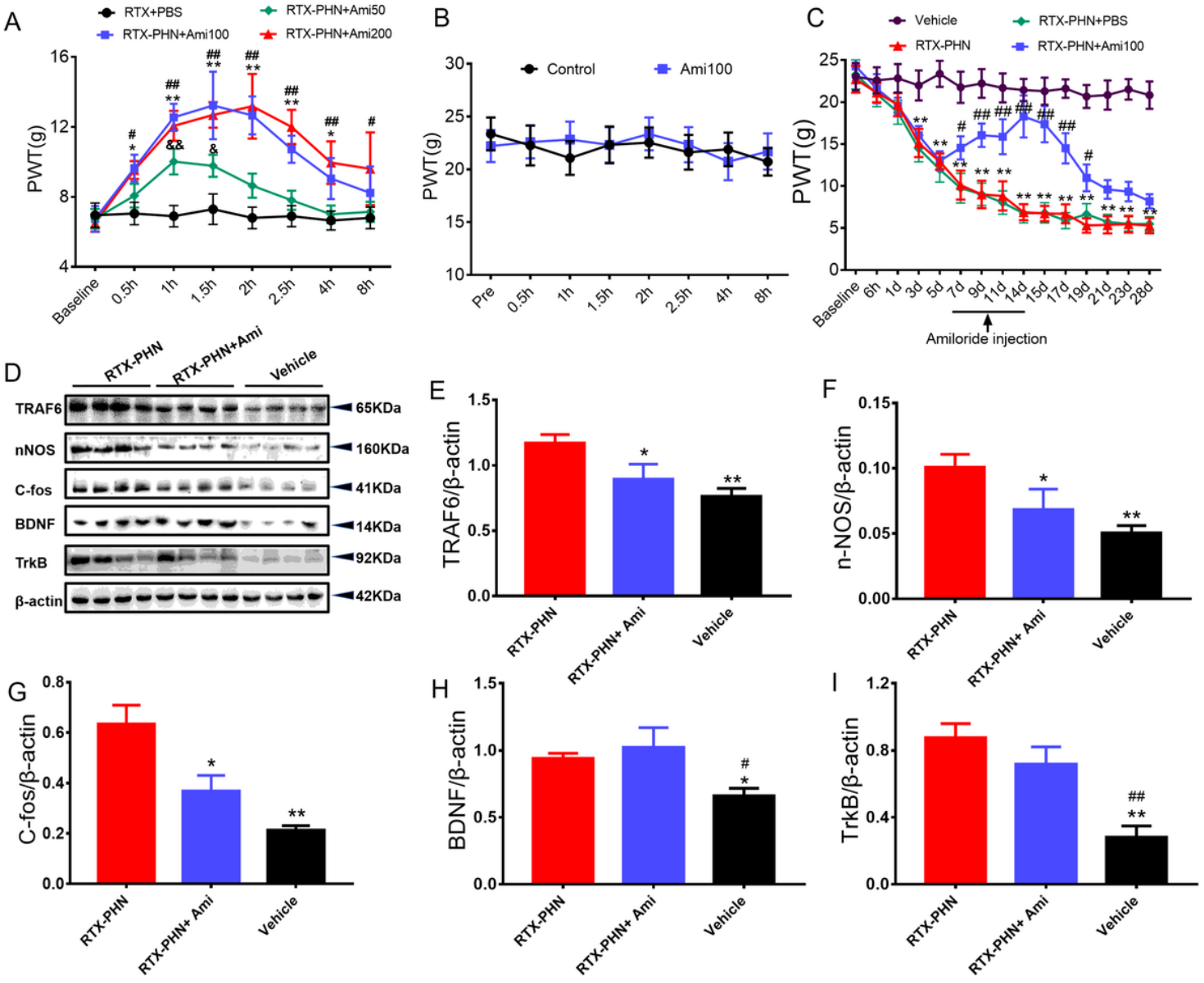
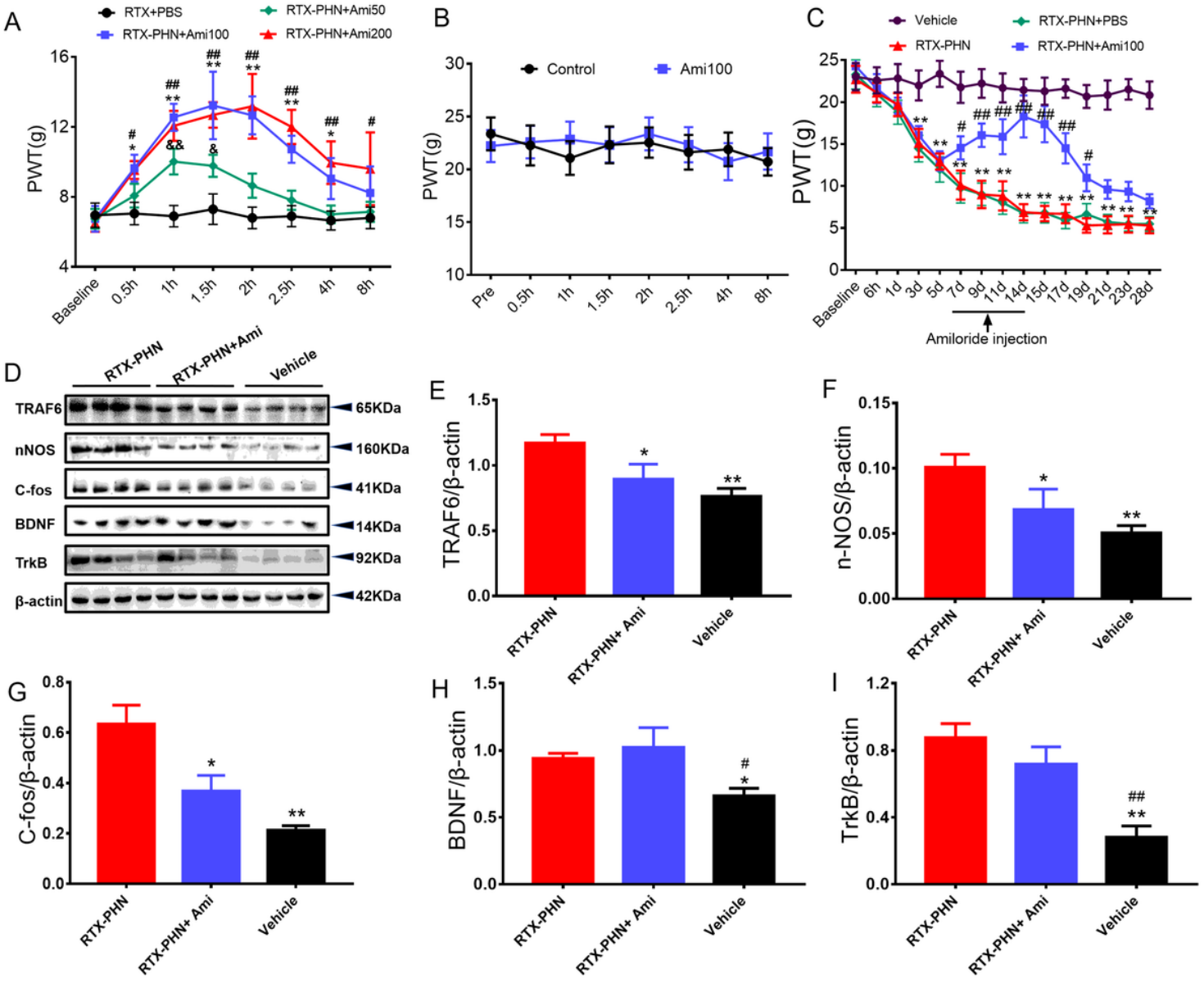


Figure 2

Inhibition of ASIC3 attenuated mechanical allodynia and decreased TRAF6 signaling. (A) Amiloride improved the paw withdrawal threshold (n=10, &p < 0.05, &&p < 0.01 vs. RTX-PHN+PBS; *p < 0.05, **p < 0.01 vs. RTX-PHN+PBS; #p <0.05, ##p <0.01 vs. RTX-PHN+PBS; two-way repeated measures ANOVA followed by Bonferroni's post-hoc test). (B) Amiloride at 100μg/kg had no significant effects on the mechanism threshold in healthy control rats(n=10). (C) Amiloride(100μg/kg) administration with once per day for consecutive 7 days effectively blocked the decreased in paw withdrawal threshold (n=10; **p < 0.01 vs. Vehicle; #p <0.05, ##p <0.01 vs. RTX-PHN; two-way repeated measures ANOVA followed by Bonferroni's post-hoc test). (D, E) protein expression of TRAF6, nNOS, c-Fos, BDNF and TrkB in DRG (n=4, *p < 0.05, **p < 0.01 vs. RTX-PHN; #p < 0.05, ##p < 0.01, vs. RTX-PHN+Ami; one-way ANOVA followed by Tukey's post-hoc test).



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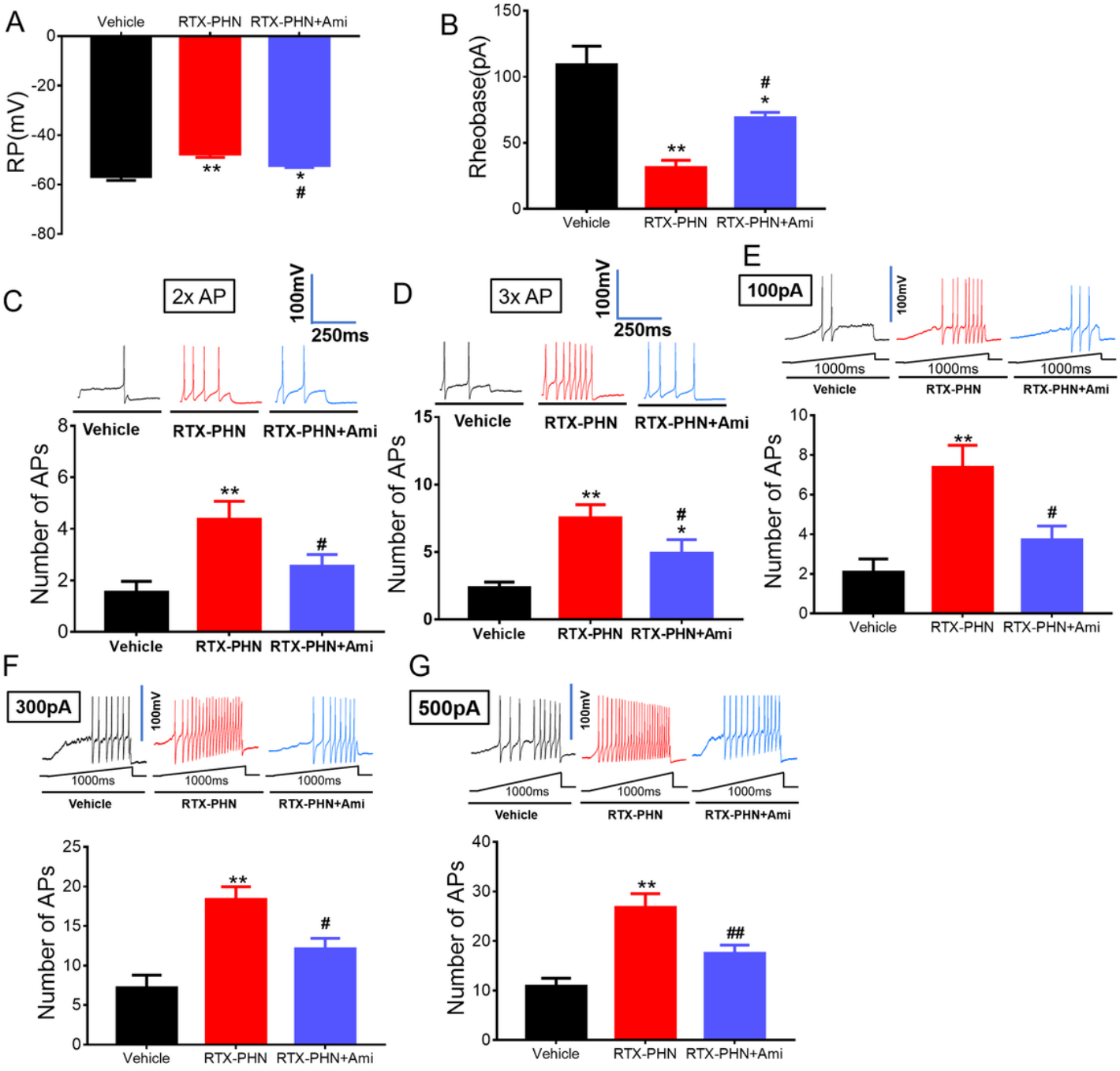
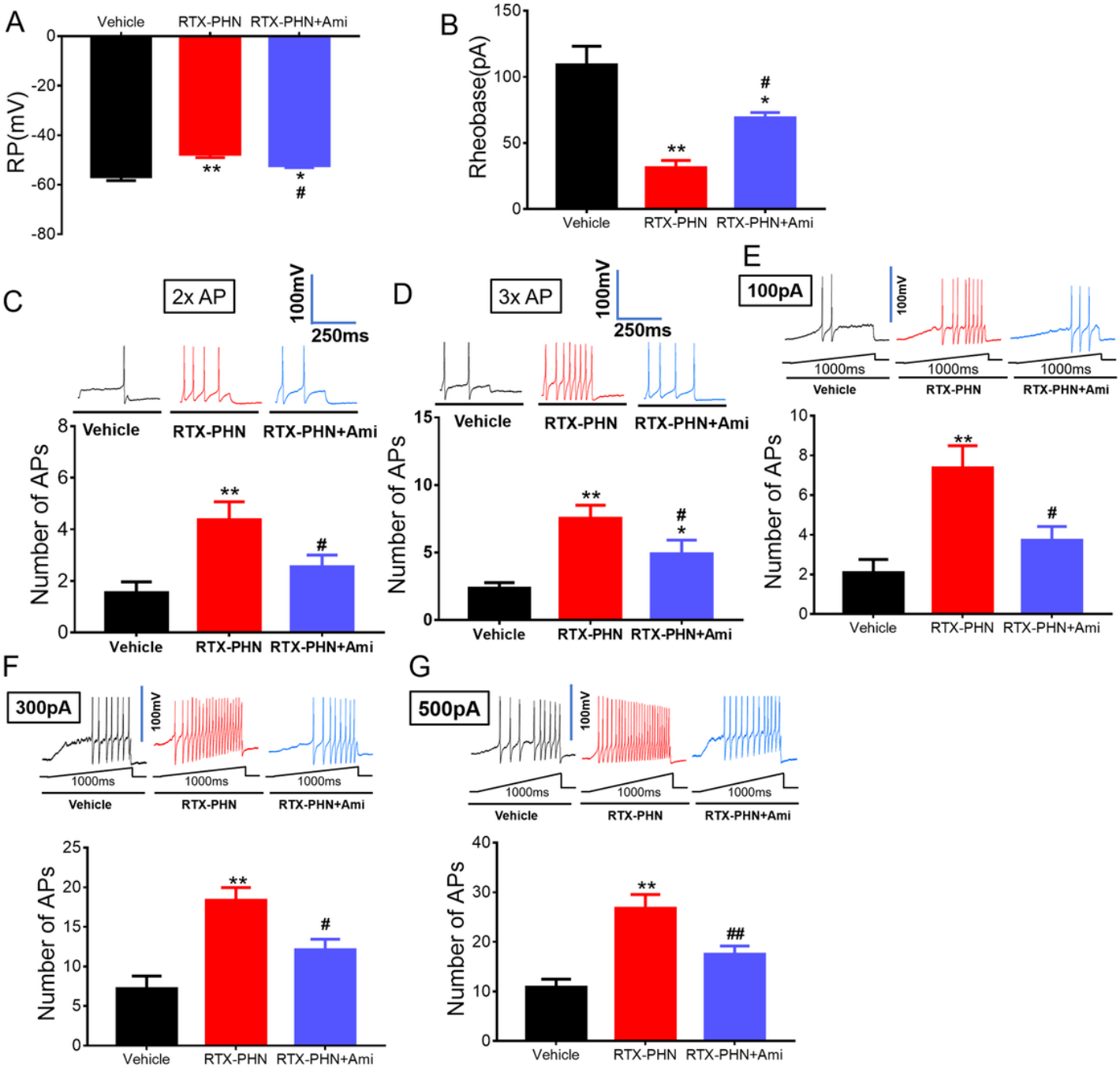


Figure 3

Inhibition of ASIC3 decreased hyperexcitability in DRGs neurons. (A) Amiloride reduced RP of DRG neurons. (B) Amiloride increased rheobase of DRG neurons. (C, D) Amiloride reversed numbers of APs in response to 2× and 3× rheobase current stimulation. (E-G) Amiloride reduced numbers of APs evoked by 100, 300 and 500pA ramp current stimulation. n=11, *p < 0.05, **p < 0.01, vs. Vehicle group; #p<0.05, ##p<0.01 vs. RTX-PHN; one-way ANOVA followed by Tukey's post-hoc test).



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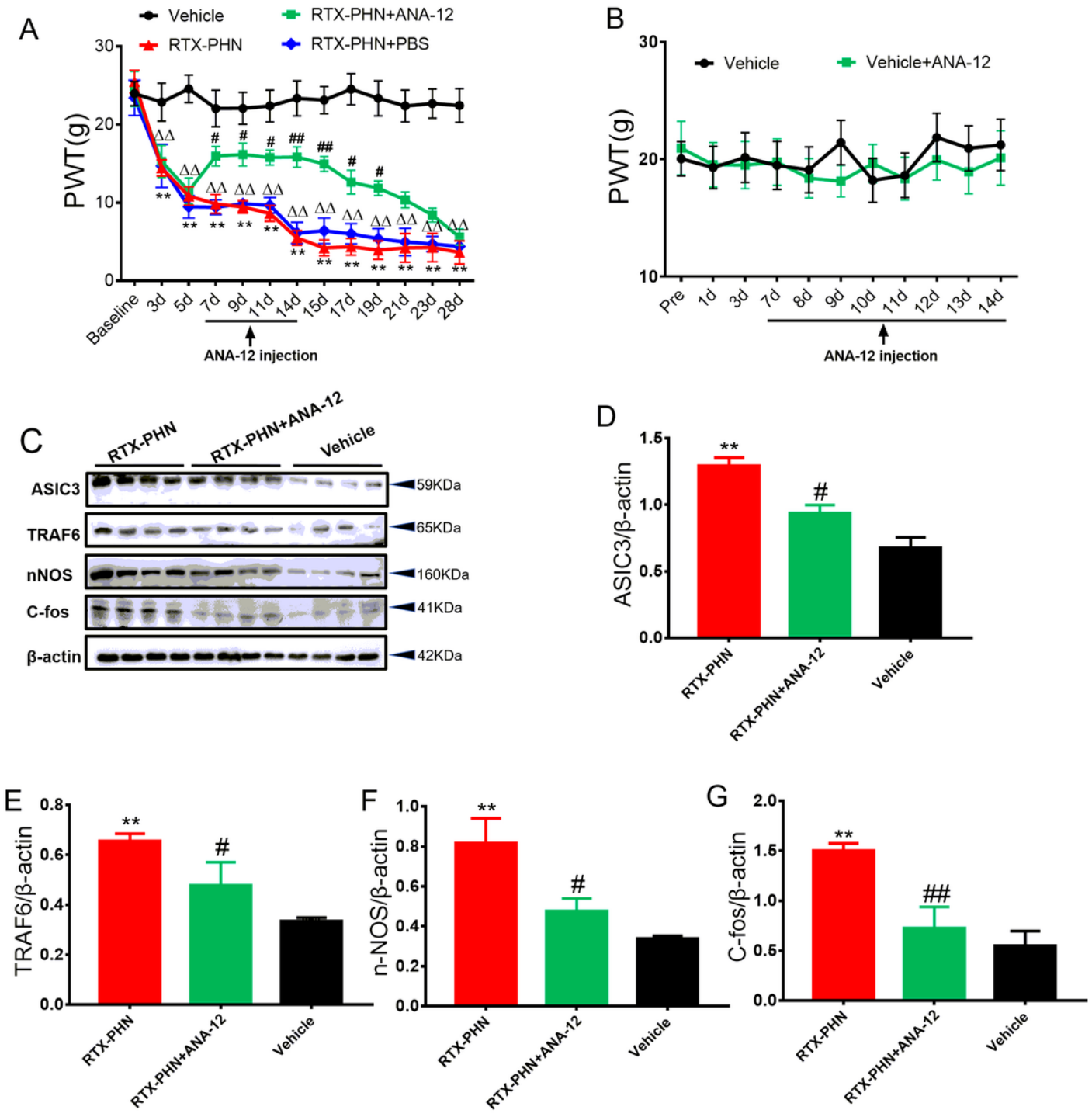
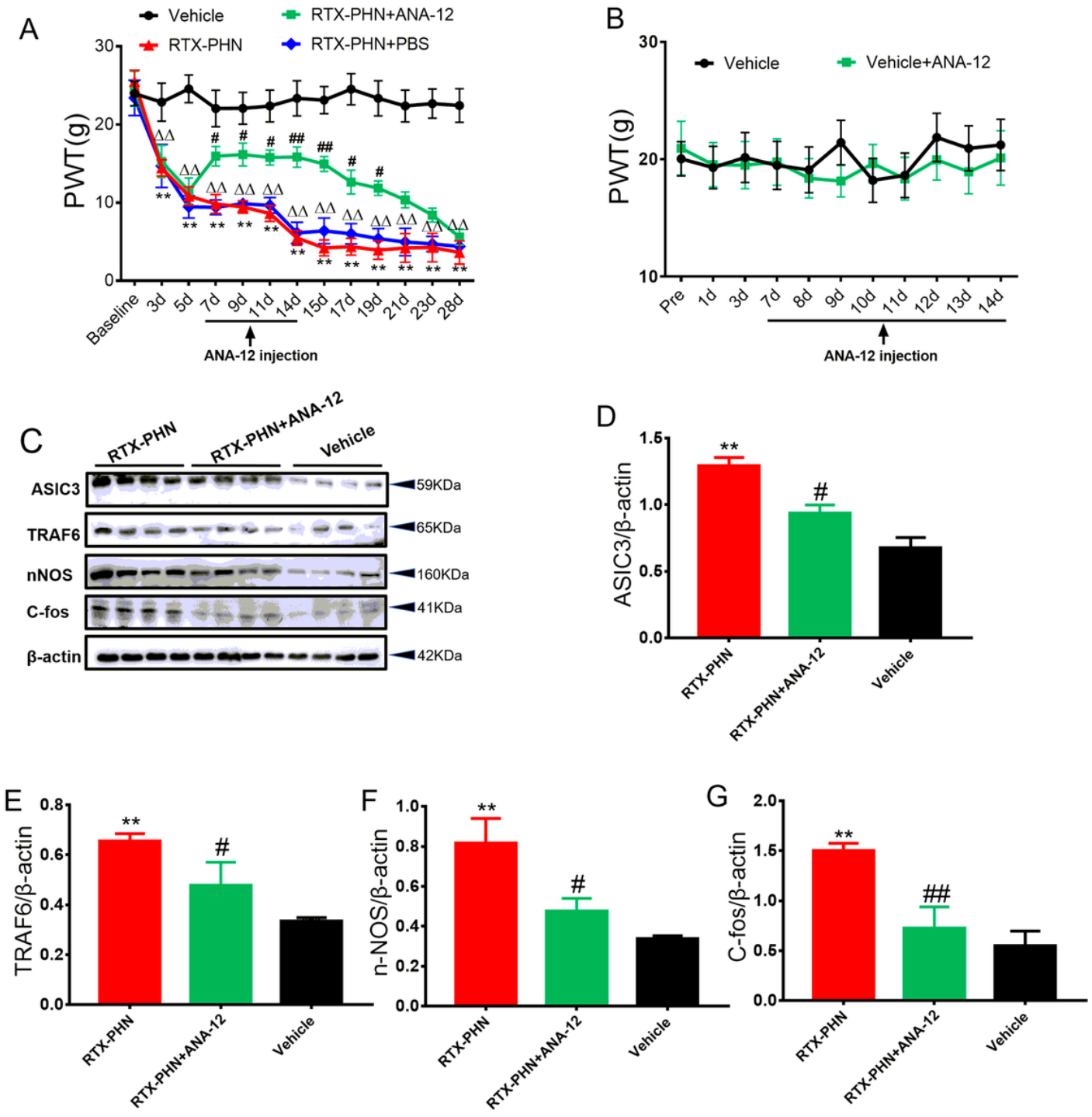


Figure 4

Inhibition of TrkB attenuated mechanical allodynia and decreased ASIC3 signaling. (A) ANA-12 increased paw withdrawal threshold (n=10; **p < 0.01, ΔΔp < 0.01 vs. Vehicle; #p <0.05, ##p <0.01 vs. RTX-PHN; two-way repeated measures ANOVA followed by Bonferroni's post-hoc test). (B) ANA-12 had no significant effects on the mechanism threshold in healthy control rats (n=10). (C-G) Protein expression of ASIC3, TRAF6, nNOS and c-Fos in DRGs (n = 4, *p < 0.05, **p < 0.01 vs. Vehicle, #p <0.05, ##p <0.01 vs. RTX-PHN; one-way ANOVA followed by Tukey's post-hoc test).



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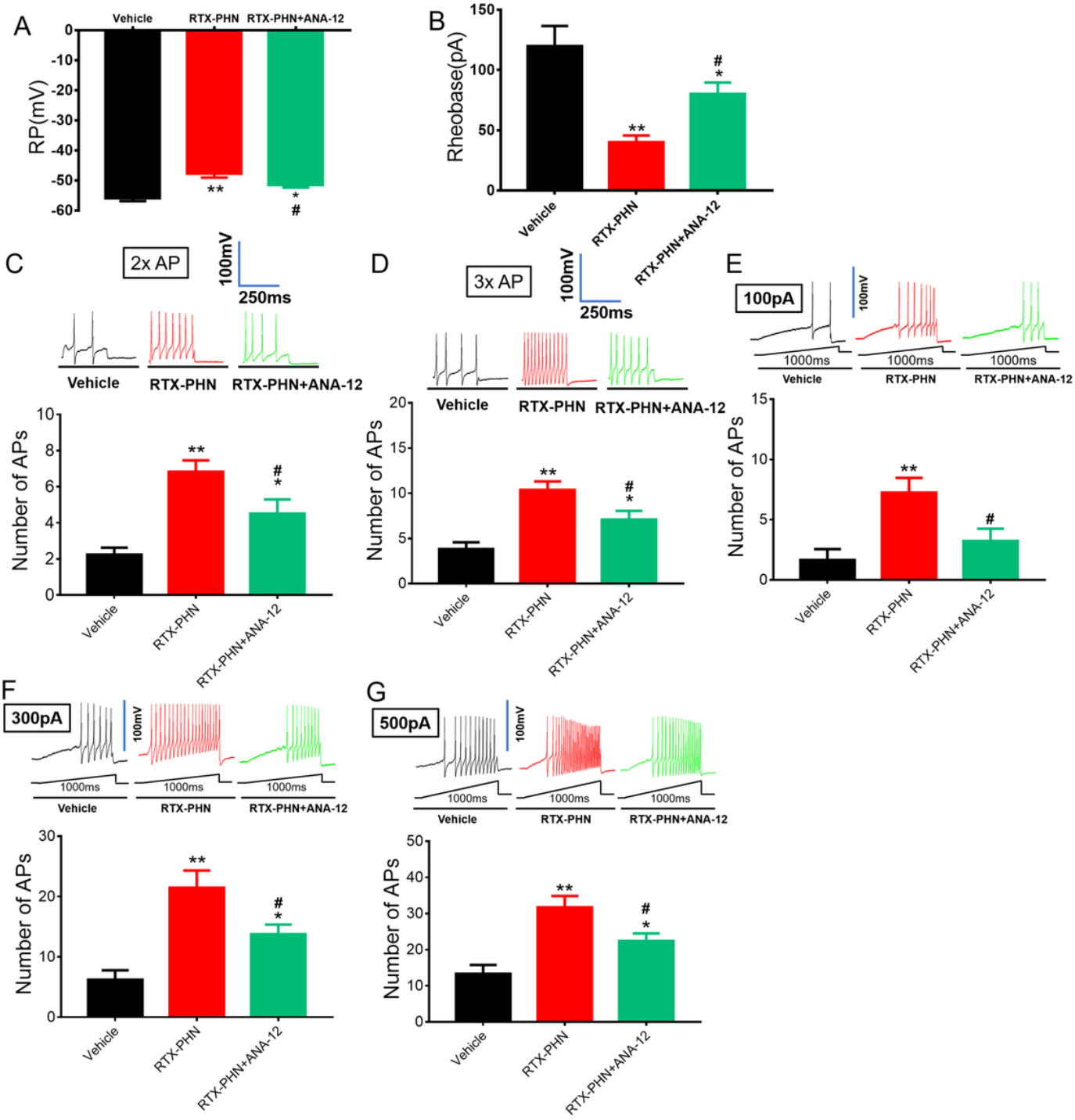

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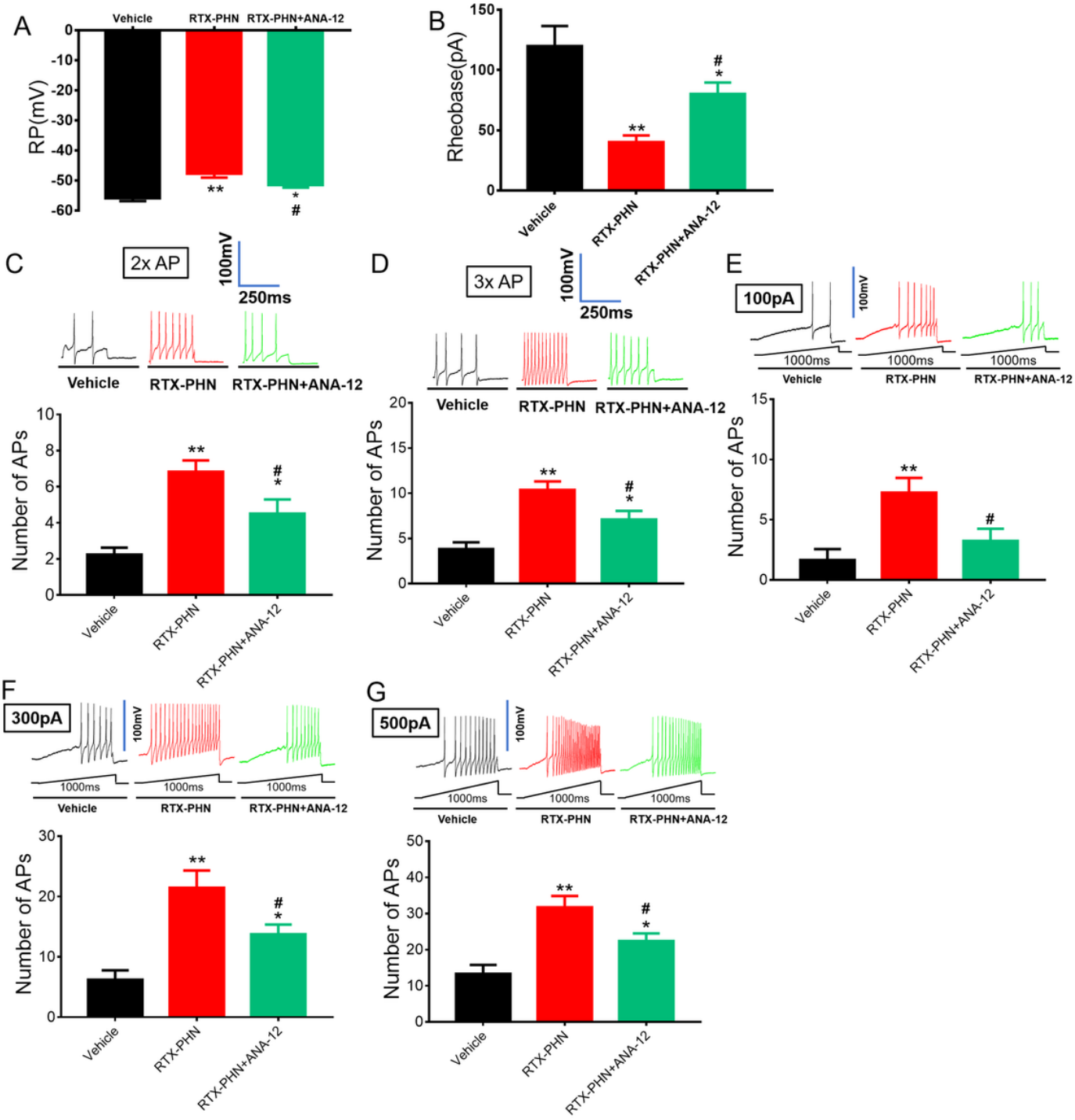

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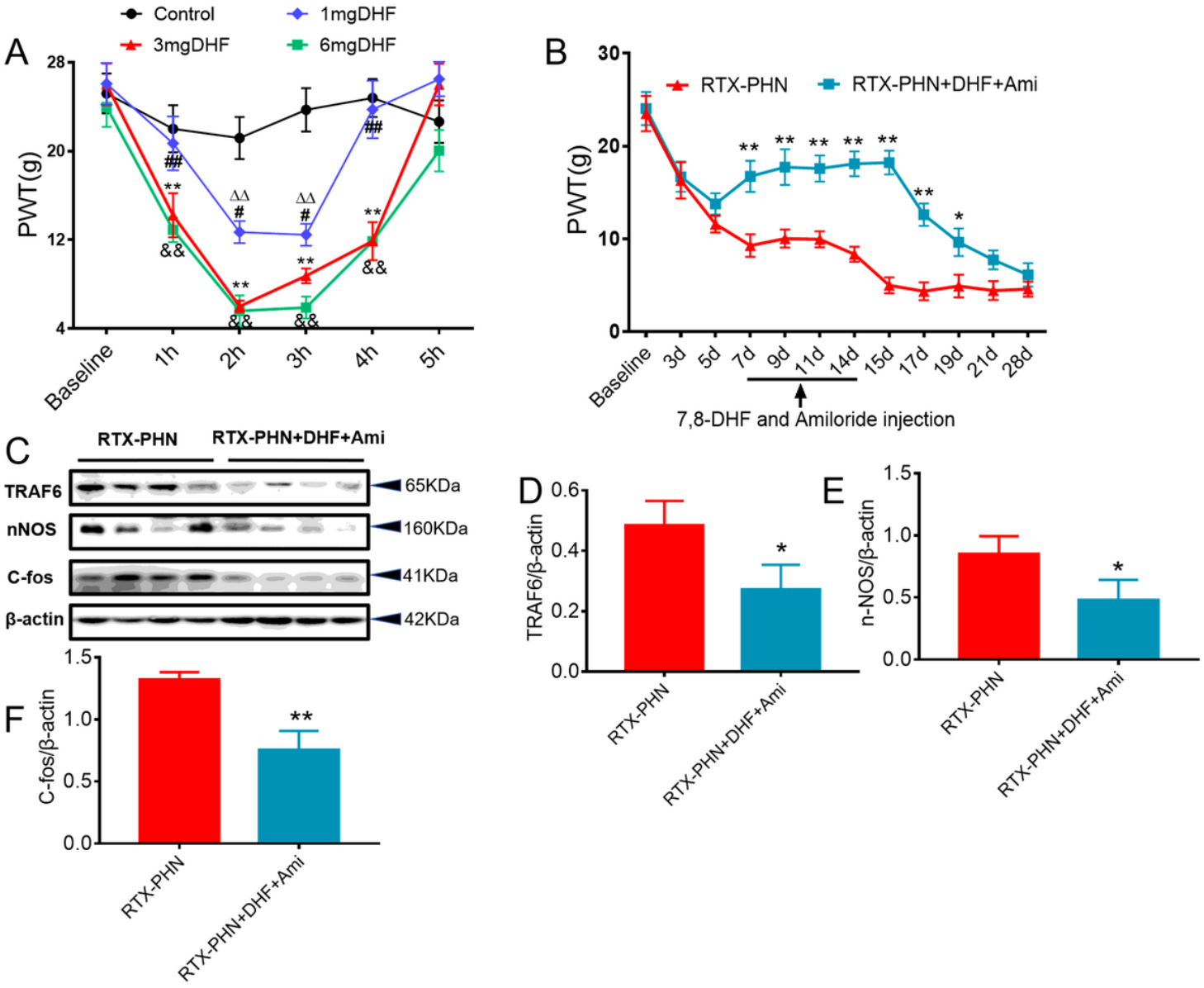
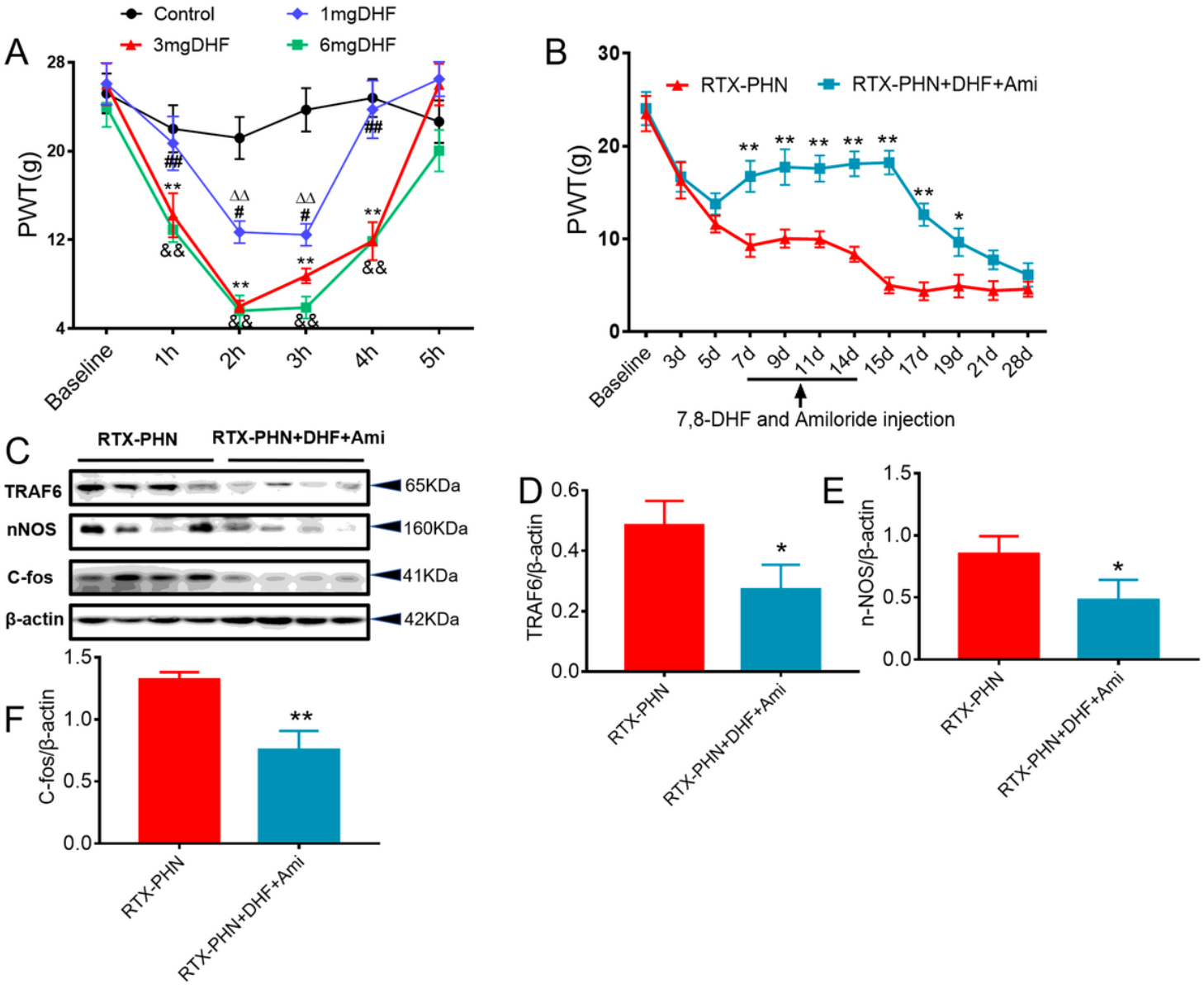


Figure 6

TrkB receptor contributed to RTX-PHN through ASIC3 signaling. (A) 7,8-DHF decreased the paw withdrawal threshold of healthy rats (n = 10; ΔΔp < 0.01, Control vs. 1mg DHF; **p < 0.01, Control vs. 3mg DHF; &&p <0.01, Control vs. 6mg DHF; #p <0.05, ##p <0.01, 1mg DHF vs. 3mg DHF; two-way repeated measures ANOVA followed by Bonferroni's post-hoc test). (B) 7,8-DHF and Amiloride treatment reversed the decreased paw withdrawal threshold (n=10; *p < 0.05, **p < 0.01 vs. RTX-PHN; two-way repeated measures ANOVA followed by Bonferroni's post-hoc test). (C-F) 7,8-DHF and Amiloride treatment inhibited protein expression of TRAF6, nNOS and c-Fos (n = 4, *p < 0.05, **p < 0.01 vs. RTX-PHN; Student's t-test).



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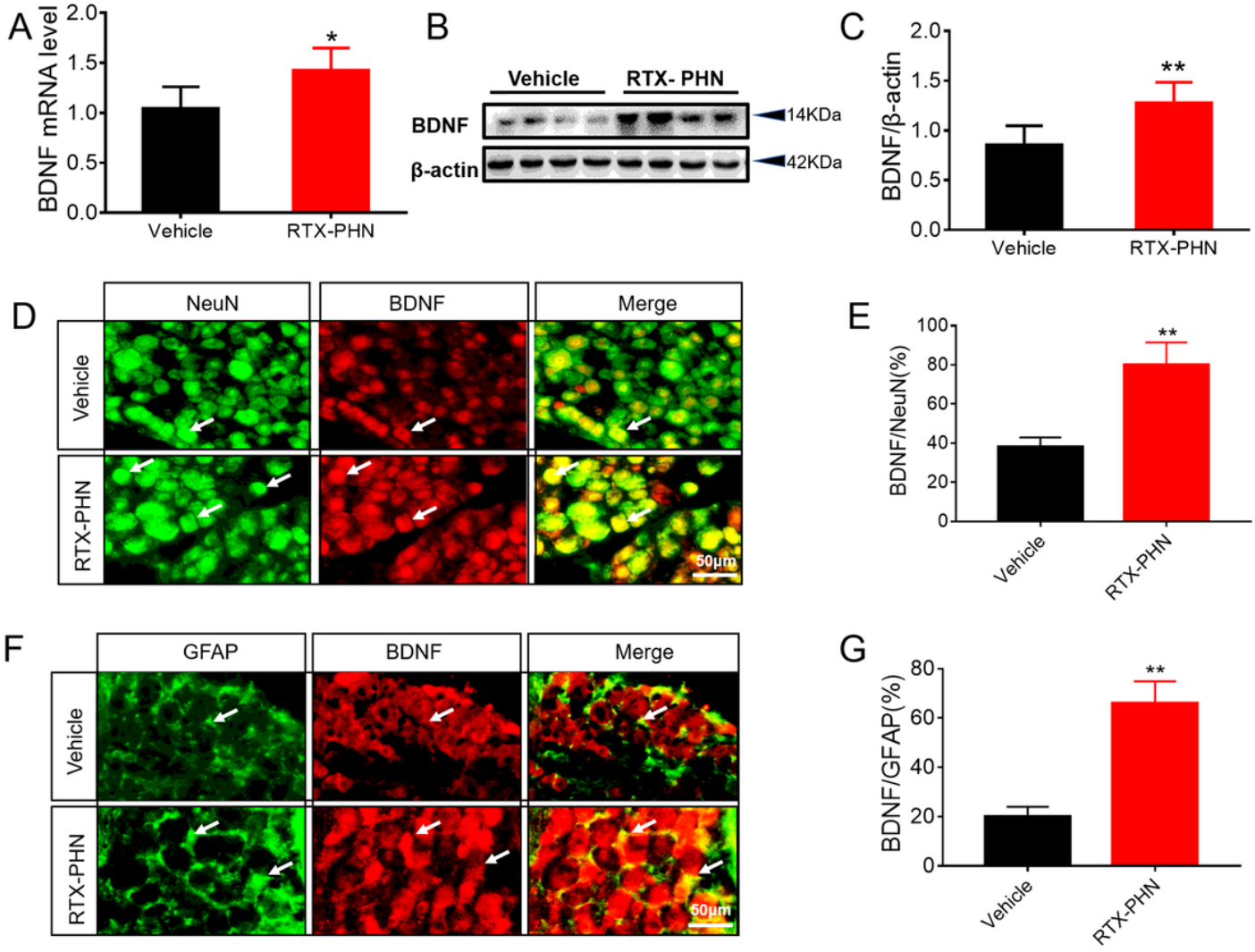
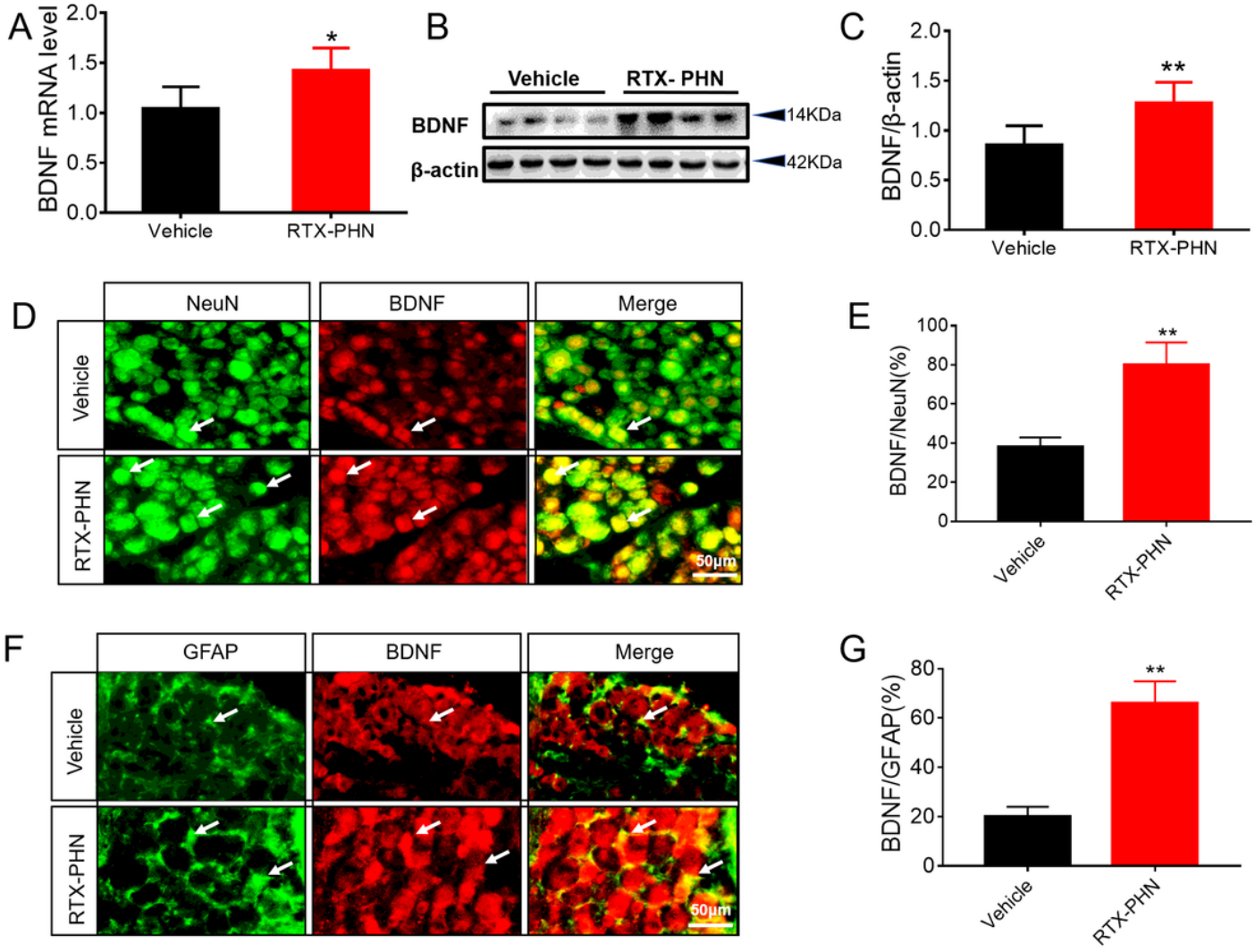


Figure 7

RTX injection increased BDNF expression in DRGs. (A) Increased BDNF mRNA expression after RTX injection(n=4). (B, C) Increased BDNF protein expression in DRGs after RTX injection(n=4). (D, E) Upregulated NeuN and BDNF protein co-expression after RTX injection. (F, G) Enhanced GFAP and BDNF protein co-expression after RTX injection (n=5). *p < 0.05, **p < 0.01 vs. Vehicle group, Student's t-test. Scale bar = 50μm.



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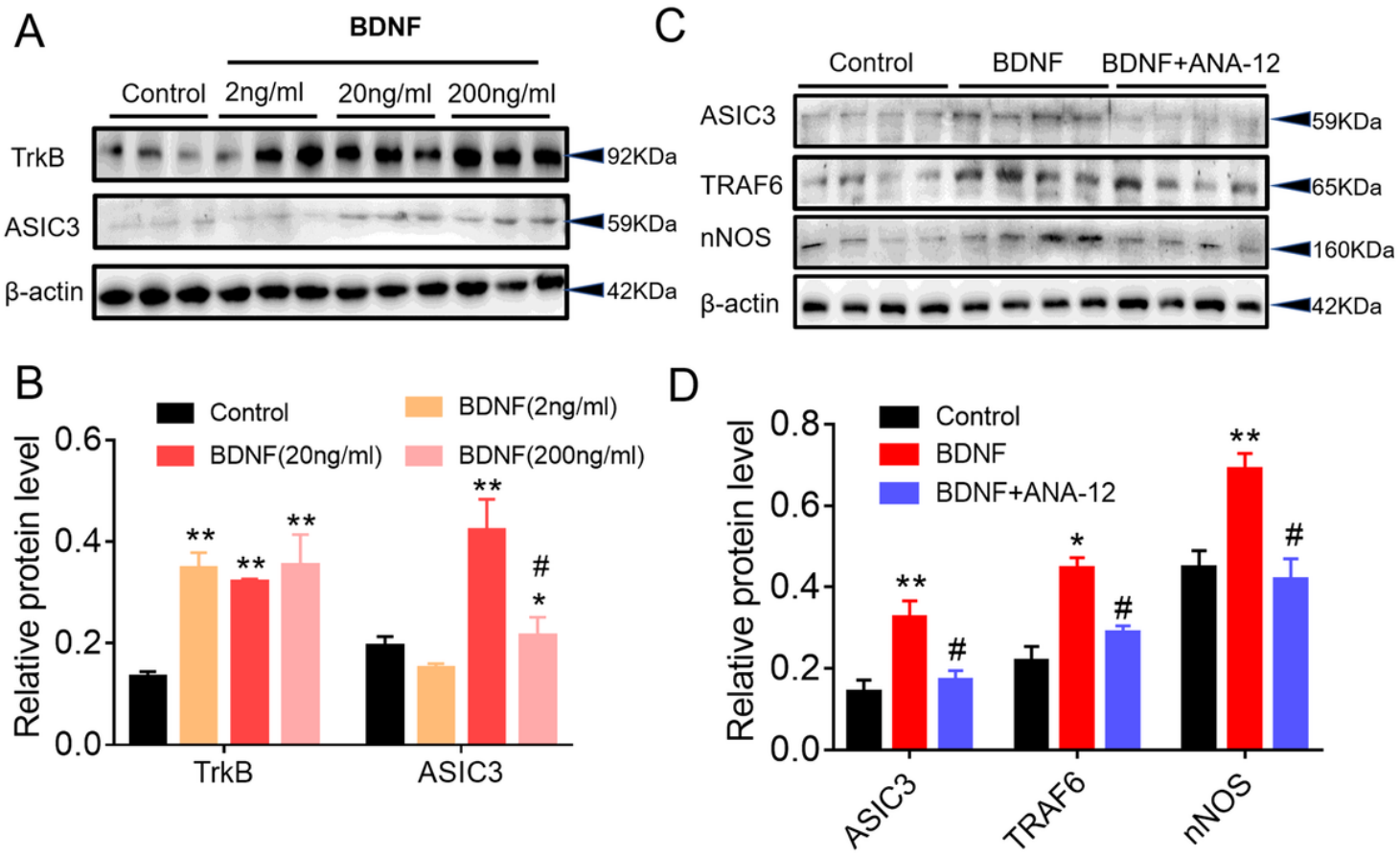
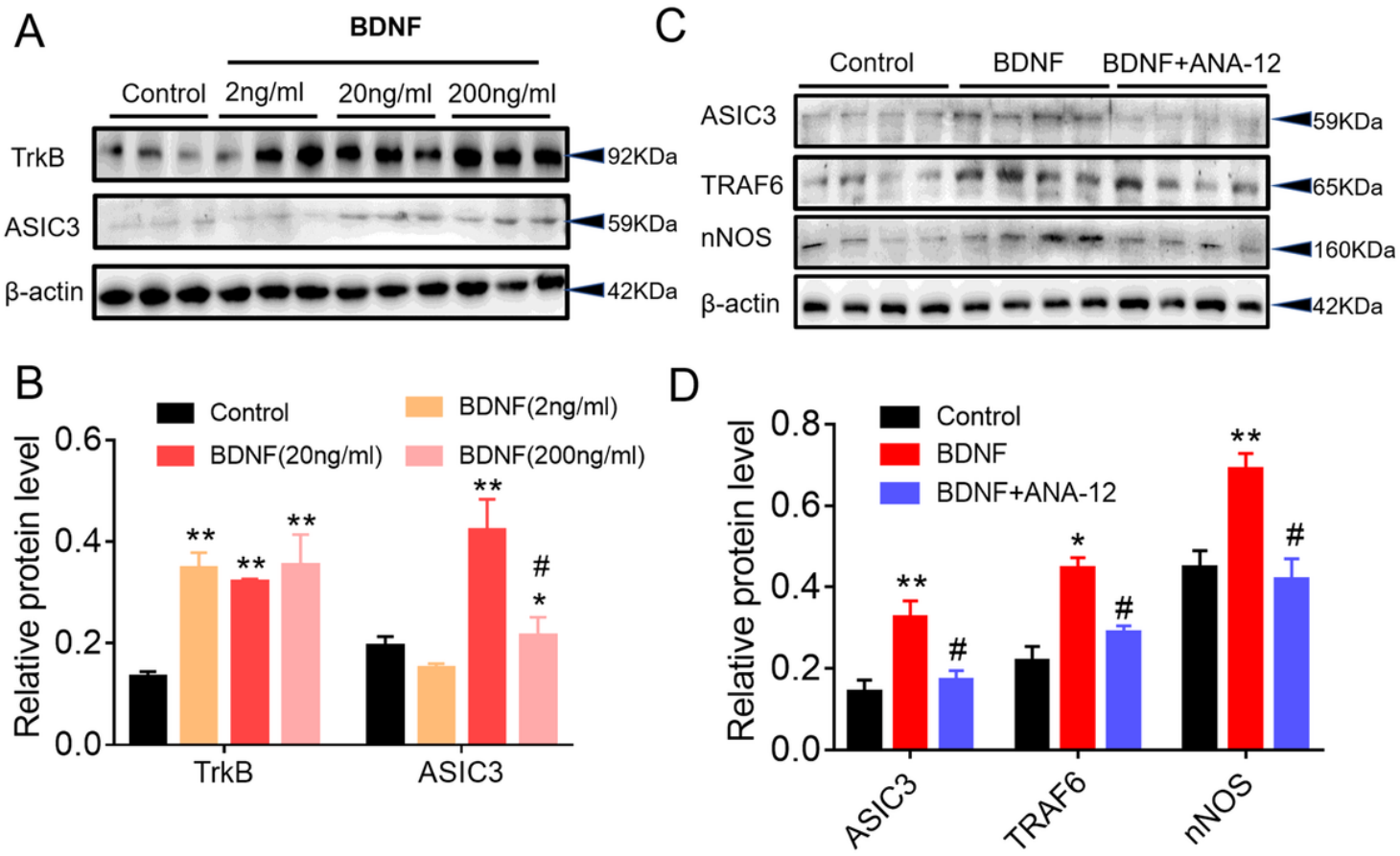


Figure 8

Exogenous BDNF enhanced TrkB-ASIC3 signaling in PC-12 cells. (A, B) Exogenous BDNF increased TrkB and ASIC3 protein expression in PC-12 cells (n=3, *p < 0.05, **p < 0.01 vs. Control group; #p<0.05 vs. BDNF (20ng/ml) group; one-way ANOVA followed by Tukey's post-hoc test). (C, D) ANA-12 decreased protein expressions of ASIC3, TRAF6 and nNOS (n=4, *p < 0.05, **p < 0.01 vs. Control group; #p<0.05 vs. BDNF group; one-way ANOVA followed by Tukey's post-hoc test).



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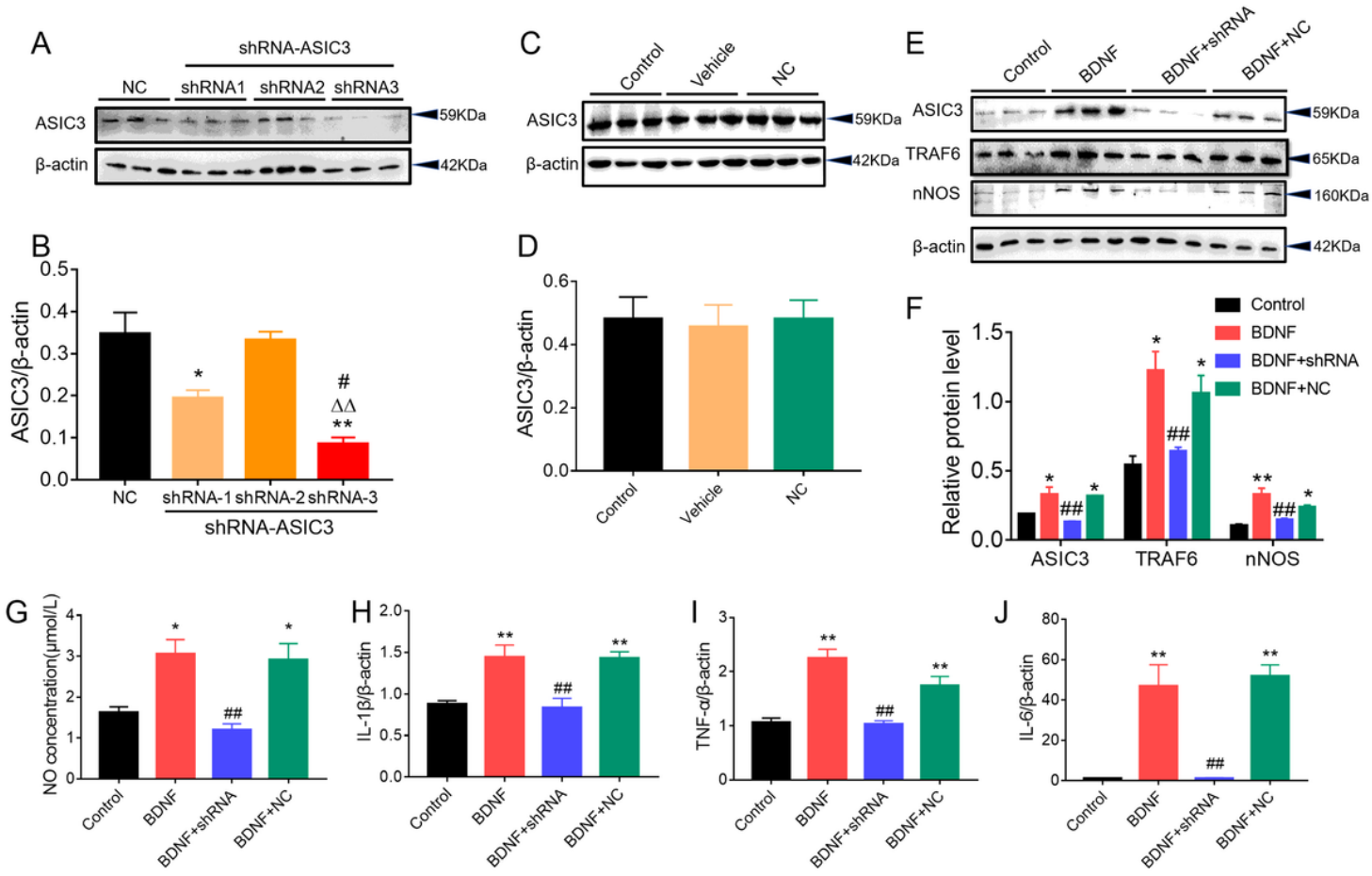
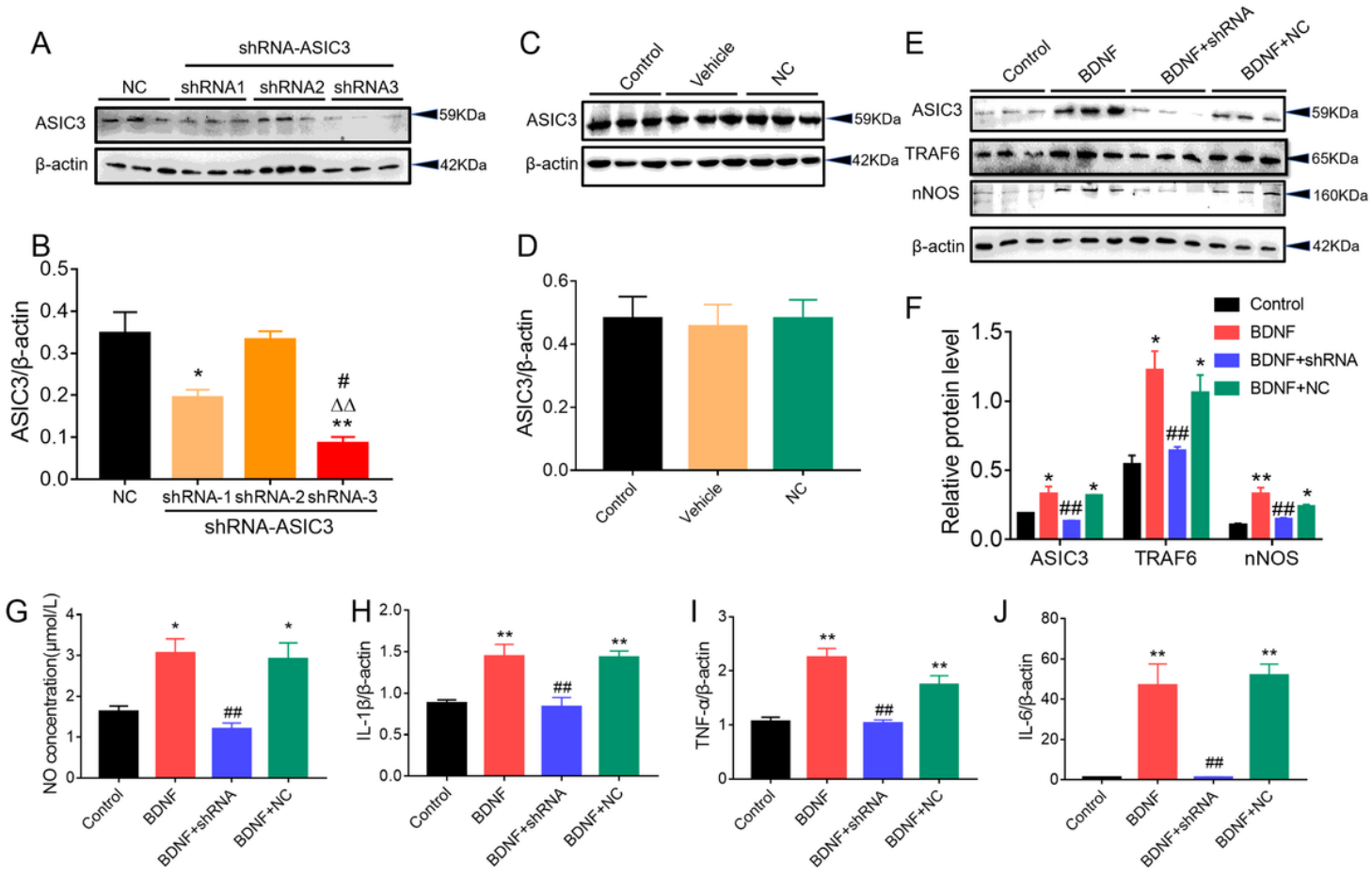


Figure 9

Knockdown of ASCI3 abolished TRAF6 signaling activation induced by exogenous BDNF treatment in PC-12 cells. (A, B) Transfection with shRNAs reduced ASIC3 protein levels (n=3, *p < 0.05, **p < 0.01 vs. NC group; ΔΔ p < 0.01 vs. shRNA2 group; #p<0.05 vs. shRNA1 group; one-way ANOVA followed by Tukey's post-hoc test) (C, D) ASIC3 protein levels in NC group was not changed compared to the control group (n=3). (E, F) shRNA transfection inhibited the protein expressions of ASIC3, TRAF6 and nNOS induced by exogenous BDNF(n=3). (G) Exogenous BDNF increased NO level, which was reversed by shRNA transfection(n=5). (H-J) Exogenous BDNF upregulated mRNA expressions of IL-6, IL-1β and TNF-α, which was reversed by shRNA transfection(n=5). *p < 0.05, **p < 0.01 vs. Control group; #p<0.05, ##p<0.01 vs. BDNF group; one-way ANOVA followed by Tukey's post-hoc test.



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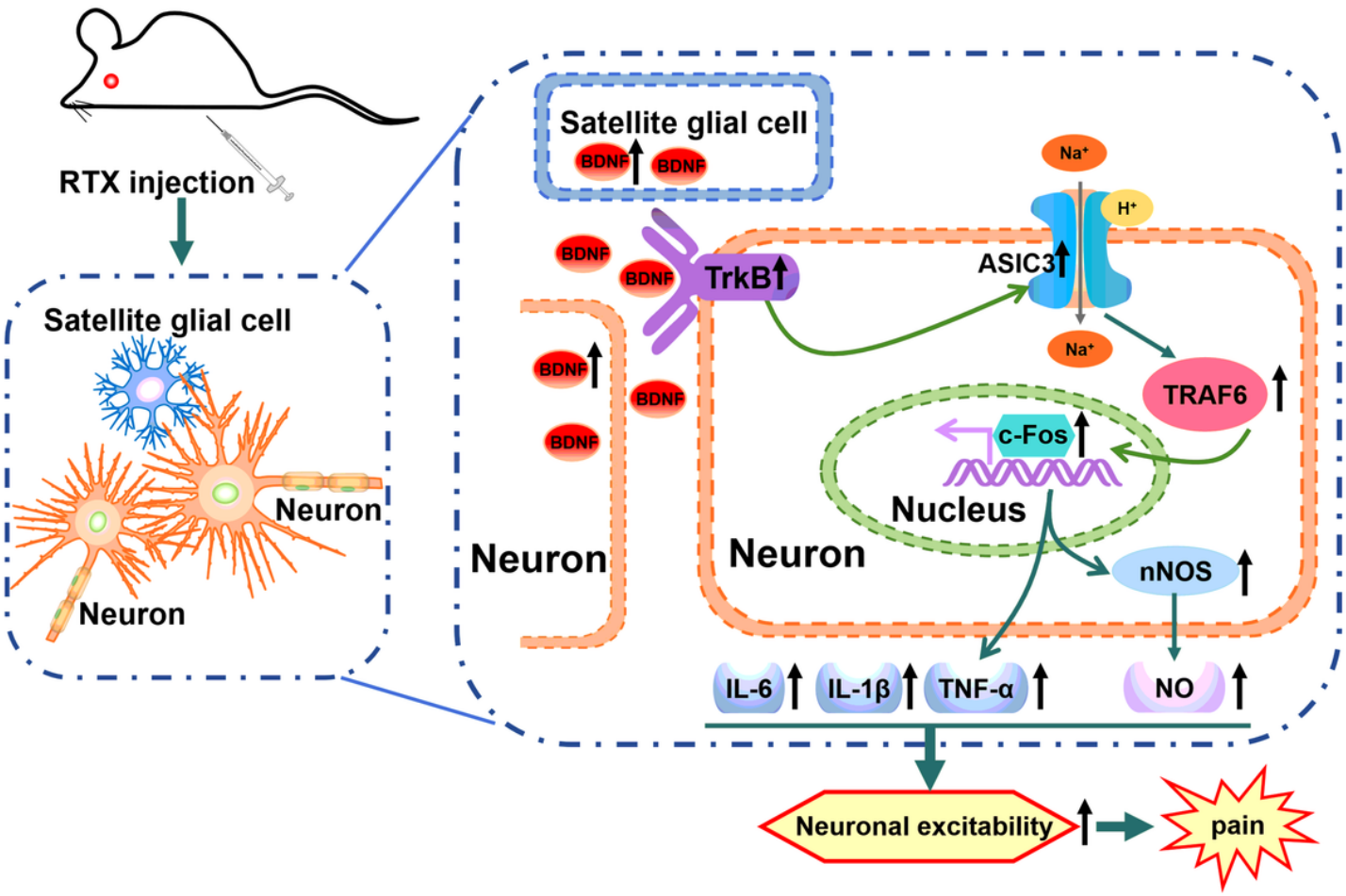
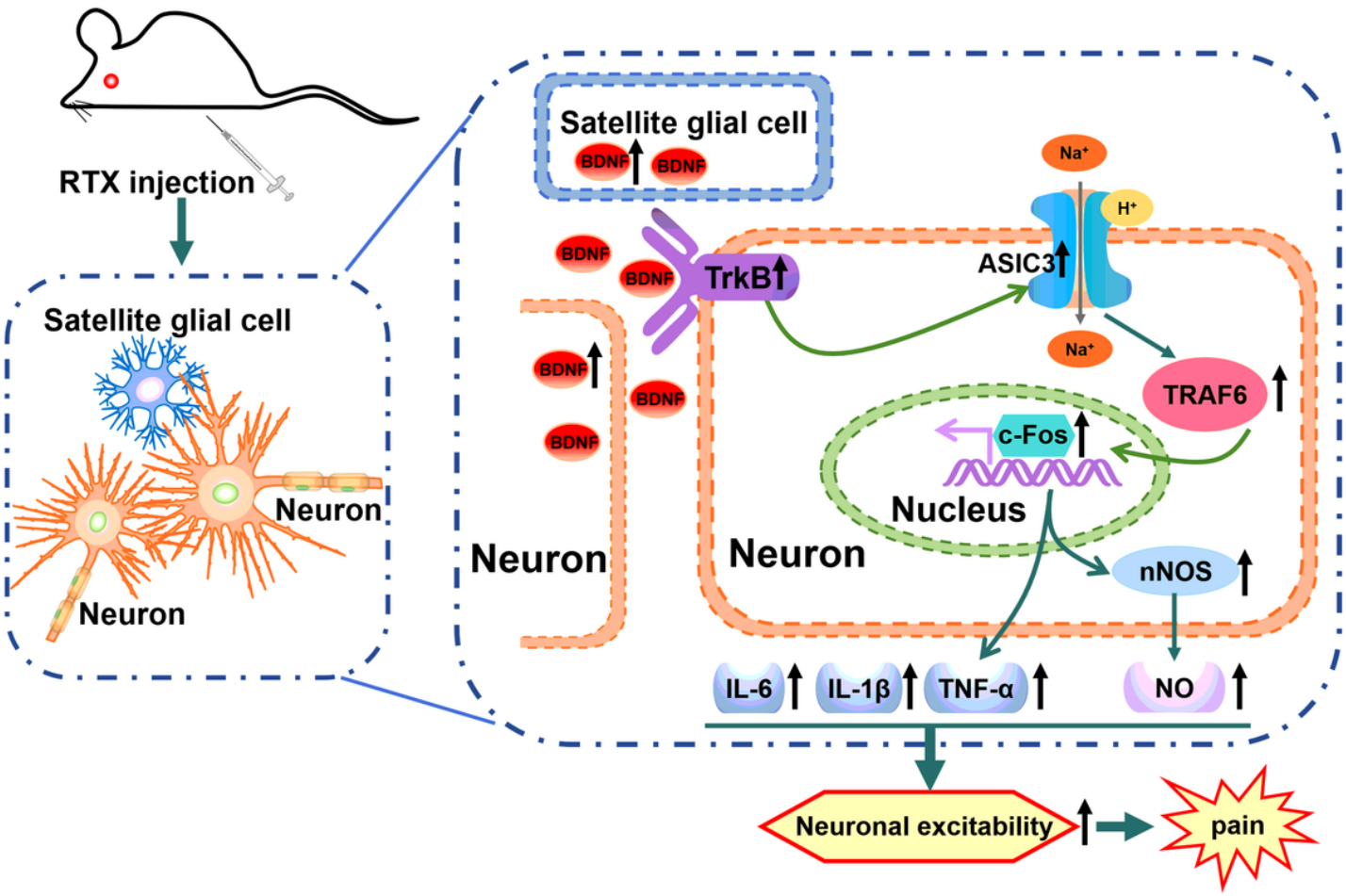


Figure 10

Schematic showing BDNF/TrkB contributes to RTX-induced postherpetic neuralgia through regulating ASIC3 signaling. RTX injection increased the synthesis and secretion of BDNF from activated satellite glial cell and neurons in DRGs. Then, BDNF activated ASIC3 signaling through increasing TrkB receptor in neurons, which led to elevated expression of c-FOS, TRAF6 and nNOS, as well as enhanced transcription of IL-6, IL-1β, TNF-α. Finally, the inflammatory response and NO production induced the neuronal excitability and promoted the development and maintenance of pain.



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