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Reduction of TRPV1 expression in the trigeminal system by botulinum neurotoxin type-A

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ABSTRACT

Botulinum neurotoxin type-A (BoNT-A) is clinically used for patients with pain disorders and dystonia. The precise mechanism whereby BoNT-A controls pain remains elusive. Here, we studied how BoNT-A affects the expression of the transient receptor potential vanilloid subfamily member 1 (TRPV1), a cation channel critically implicated in nociception, in the trigeminal system. Histological studies revealed that subcutaneous BoNT-A injection (0.25, 0.5, or 5 ng/kg) into the face targeted the ophthalmic division of trigeminal ganglion (TG) neurons and decreased TRPV1-immunoreactive neurons in the TG and TRPV1-immunoreactive fibers in rat trigeminal terminals. Of note, TG neurons that received projections from the dura mater, a principal site of headache generation, had reduced TRPV1 expression. BoNT-A-induced cleavage of SNAP25 (synaptosomal-associated protein of 25-kDa) in the TG became obvious 2 days after BoNT-A administration and persisted for at least 14 days. Quantitative real-time RT-PCR (reverse transcription-polymerase chain reaction) data indicated that the TRPV1-decreasing effects of BoNT-A were not mediated by transcriptional downregulation. By employing a surface protein biotin-labeling assay, we demonstrated that BoNT-A inhibited TRPV1 trafficking to the plasma membrane in primary TG neurons. Moreover, Y200F-mutated TRPV1, which is incapable of trafficking to the plasma membrane, was expressed in PC12 cells by transfection, and pharmacological studies revealed that TRPV1 in the cytoplasm was more predisposed to proteasome-mediated proteolysis than plasma membrane-located TRPV1. We conclude that the mechanism by which BoNT-A reduces TRPV1 expression involves the inhibition of TRPV1 plasma membrane trafficking and proteasome-mediated degradation in the cytoplasm. This paradigm seems to explain how BoNT-A alleviates TRPV1-mediated pain. Our data reveal a likely molecular mechanism whereby BoNT-A treatment reduces TRPV1 expression in the trigeminal system and provide important clues to novel therapeutic measures for ameliorating craniofacial pain.

Introduction

Botulinum neurotoxin type-A (BoNT-A) cleaves the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) synaptosomal-associated protein of 25-kDa (SNAP25) and inhibits regulated exocytosis (Dolly et al., 2009; Meng et al., 2007; Südhof and Rothman, 2009). BoNT-A potently inhibits exocytosis at presynaptic terminals, causing flaccid muscle paralysis and is regarded as one of the most lethal toxins known to man. However, its pharmacological properties can be strategically utilized to modulate the exocytosis-mediated release of neurotransmitters, particularly acetylcholine, at the neuromuscular junction. Accordingly, BoNT-A and botulinum neurotoxin type-B (BoNT-B) are now used in clinical practice for a variety of muscular problems, including dystonia and spasticity (Lim and Seet, 2010). In addition, there is good evidence that BoNT-A is effective in treating pain disorders. After an extensive review of the literature, the Therapeutics and Technology Assessment Subcommittee of the American Academy of Neurology (TTAS/AAN) concluded that botulinum neurotoxin (BoNT) may be an effective treatment for lower back pain and should be considered for this condition (Naumann et al., 2008). BoNT-A has been shown to also be effective for craniofacial pain. The utility of BoNT-A in the treatment of chronic migraine has been demonstrated in randomized, placebo-controlled parallel studies performed in the United States and Europe (Aurora et al., 2010; Diener et al., 2010). As a consequence, the agent is now approved for patients with chronic migraine. Gazerani et al. (2009) examined the effect of subcutaneous BoNT-A treatment on trigeminal pain and vasomotor reactions caused by the local injection of capsaicin, the pungent ingredient in chili pepper, into the foreheads of 14 human volunteers (Gazerani et al., 2009). Strikingly, BoNT-A decreased capsaicin-induced trigeminal pain intensity, pain area, secondary hyperalgesia, flare area and vasomotor reactions. The receptor that binds capsaicin has been identified as a non-selective cation channel termed the transient receptor potential

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vanilloid subfamily member 1 (TRPV1), which is abundantly expressed in nociceptive cells. TRPV1 is also responsive to protons and noxious heat (>42 °C) and plays a role in transducing chemical and thermal nociceptive stimuli into neural signals that are eventually perceived as pain (Szallasi et al., 2006). TRPV1 mobilization from intracellular stores to the plasma membrane involves regulated exocytosis (Dolly et al., 2009; Morenilla-Palao et al., 2004). Hence, BoNT-A is likely to inhibit the trafficking of TRPV1 to the plasma membrane. There is histological evidence that local BoNT-A administration decreases TRPV1 expression levels in the human bladder (Apostolidis et al., 2005). At present, the molecular mechanism by which BoNT-A-mediated exocytosis inhibition reduces TRPV1 expression remains elusive. Unraveling this mechanism should contribute to the design of novel therapeutic strategies against pain disorders, especially inflammatory pain. TRPV1 functions are enhanced by inflammatory mediators, which cause TRPV1 phosphorylation by activating various protein kinases (Bhave et al., 2003; Bonnington and McNaughton, 2003; Gunthorpe and Chizh, 2009; Morenilla-Palao et al., 2004; Stein et al., 2006; Zhang et al., 2005; Zhu and Oxford, 2007). TRPV1 function is facilitated by enhanced channel activity and/or recruitment of TRPV1 from intracellular stores and their subsequent insertion into the plasma membrane, which leads to inflammatory hyperalgesia (Bonnington and McNaughton, 2003; Morenilla-Palao et al., 2004; Stein et al., 2006; Van Buren et al., 2005; Zhang et al., 2005). Therefore, inhibiting exocytosis and subsequent attenuation of TRPV1 expression appears to be useful in preventing the development of inflammatory hyperalgesia. We recently demonstrated the existence of TRPV1-immunoreactive nerve fibers in the dura mater that originate in the trigeminal ganglion (TG) (Shimizu et al., 2007). The dura mater is densely innervated by trigeminal nociceptors, and inflammatory processes often involve this exquisitely pain-sensitive intracranial structure. Clinically, meningitis causes excruciating pain that is often refractory to existing pain medications.

Here, we provide in vivo and in vitro evidence that BoNT-A hinders the recruitment of TRPV1 to the plasma membrane, and we show that the failure of TRPV1 plasma membrane trafficking leads to intracellular TRPV1 degradation by the proteasome. We further use behavioral analysis to demonstrate that the antinociceptive action of BoNT-A is selective for TRPV1-mediated pain. We propose that therapeutic measures that interfere with TRPV1 plasma membrane trafficking could be effective, especially against inflammatory hyperalgesic conditions.

Material and methods

Animals

A total of 71 male Sprague Dawley (SD) rats weighting 200–250 g were used for the study (32 for immunohistochemistry, 21 for Western blotting, 12 for RT-PCR, and 35 for behavior analysis). In addition, a total of 73 neonatal SD rats were used for TG primary cultures. The experimental procedures were approved by the Animal Welfare Committee of Keio University (No. 08076). All the procedures were undertaken with utmost caution to minimize the suffering of the animals.

True blue injection to the skin region innervated by V1 of the trigeminal nerve

To confirm the distribution pattern of TG neurons innervating the skin, three SD rats were injected with 1% retrograde axonal tracer (true blue, 5-benzofurancarboximidamide, 2,2′-[1,2-ethenediyl] bis-, dihydrochloride [Invitrogen, Carlsbad, CA] solution, 30 μl) into the left dorso-lateral side of the nose between the upper whisker pad and the ipsilateral eye, and the left TG was dissected out after perfusion fixation at 14 days.

BoNT-A and true blue injection to the skin region innervated by V1 of the trigeminal nerve

Fifteen SD rats were divided into 3 groups (control, BoNT-A 0.5 ng/kg, BoNT-A 5 ng/kg [Wako, Osaka, Japan]). The animals were deeply anesthetized with an intraperitoneal injection of pentobarbital (30 mg/kg body weight). Control animals were injected with 30 μl saline containing 1% true blue in the left dorso-lateral nose between the upper whisker pad and the ipsilateral eye.

In the other two groups, BoNT-A 0.5 ng/kg or BoNT-A 5 ng/kg containing 1% true blue solution (30 μl) were injected into the left side of the face. After 14 days, each group of animals was perfused, and the left TG was dissected out.

BoNT-A injection to the facial skin

Five SD rats were injected with saline as control, and the other 5 animals were injected with 0.5 ng/kg BoNT-A into the left side of the face, and the left facial dermis around the injection site was dissected out after perfusion fixation at 14 days.

True blue application to the dura mater and BoNT-A injection to the skin

Ten SD rats were anesthetized with an intraperitoneal injection of pentobarbital (30 mg/kg body weight). The heads of the animals were fixed in a stereotaxic frame, and a small burr hole was made in the left parietal bone around the middle meningeal artery. The dura mater was kept intact. A bank was built around the burr hole using dental cement (Ionosit, DMG) to prevent the spreading of what was to be locally administered to the dura. True blue was applied to the dura mater, and the application site was covered with the skull bone and dental cement. Seven days after tracer application, 0.5 ng/kg BoNT-A was injected into the left side of the face in 5 animals, and the 5 control animals were injected with saline into the left side of the face. After 7 days, the animals were sacrificed, and the left TGs were dissected out.

Immunohistochemical procedures

All animals were perfused with 200 ml of 0.01 M phosphate-buffered saline (PBS, pH 7.2), followed by a mixture of 2% formaldehyde and 0.2% picric acid in 0.1 M phosphate buffer, pH 7.0. Immediately after the perfusion-fixation, the TG was dissected out and immersed overnight in phosphate buffer containing 20% sucrose at 4 °C and then processed into 10-μm sections with a cryostat (Reichert-Jung Cryocut 1800; Leica Instruments).

Sections were preincubated with 10% normal donkey serum for 30 min followed by incubation with primary antibodies for 48–72 h at room temperature. The primary antibodies were then washed off with 0.01 M PBS, and the tissue specimens were incubated with species-specific secondary antibodies for 2 h at room temperature. Specimens were mounted in buffered glycerol (pH 8.6).

Slides were labeled with anti-TRPV1 receptor antibody corresponding to residues 824–838 (EDAEVFKDSMVPGEK) of rat TRPV1 (raised in rabbit; code KM 018; Trans Genic, Kumamoto, Japan). The specificity of the anti-TRPV1 receptor antibody was previously demonstrated.

Slides of TG sections were labeled with anti-TRPV1 receptor antibody (raised in rabbit; Trans Genic). Slides of the facial dermis were double-labeled with anti-TRPV1 receptor antibody and anti-CGRP antibody (raised in guinea pig; code B-GP 640-1; Euro-Diagnostica). Immunoreactivities with the primary antibodies were visualized with species-specific secondary antibodies raised in donkey and conjugated to Cy-3 or fluorescein isothiocyanate (FITC) that was obtained from Jackson Immunoresearch Laboratories (West Grove, PA).

Microscopy and quantitative analyses

Immunolabeled specimens were examined under an Olympus BX 50 microscope (Olympus, Tokyo, Japan) fitted with highly discriminating filters. Images were captured via a Sony CCD video camera (model XCD-SX 900; Sony, Tokyo, Japan) connected to an EPSON computer. The images were stored, and the area of the cell bodies that contained nuclei was measured using image analysis software (CHORI Imaging Corporation, Yokohama, Japan).

For quantitative analysis of TG tracer experiments, we estimated the number of the tracer-positive neurons in every third section that contained nuclei. We also counted the number of TRPV1-IR neurons with true blue accumulation. Cell counting was performed by a researcher blinded to the experimental conditions.

For quantification of TRPV1-IR in the nerve terminal of the dermis, we chose 5 dermal sections near the BoNT-A injected side in each animal. Section images were captured and binarized into black and white pixels using Adobe Photoshop (version 7.0; Adobe Systems, Mountain View, CA), and the areas of individual nerve bundles and the areas of TRPV1 containing nerve fibers within each nerve bundle were measured using the Image J analysis software (National Institutes of Health, Bethesda, MD). We calculated the proportions of the area of TRPV1-positive nerve fibers within the nerve bundle containing them.

Western blot analysis for tissue samples of TG and facial dermis

Twenty-one SD rats were divided into 7 groups (3 animals in each group). BoNT-A (0.25 ng/kg or 0.5 ng/kg) was injected into the face bilaterally. The animals were sacrificed on Day 2, 7 or 14 after injection. Control animals were injected with saline and were sacrificed on Day 7. After excision, TG tissues were homogenized in ice-cold lysis buffer (20 mM Tris [pH 7.4], 150 mM NaCl, 1 mM EDTA, 0.1% SDS and Complete Protease Inhibitor Cocktail [Roche, Mannheim, Germany]). A portion of the dermis around the BoNT-A injection site was removed, finely chopped and homogenized. The homogenates were centrifuged, and the supernatants were used for Western blot analysis. The primary antibodies used were rabbit anti-SNAP25 (Covance, Berkeley, CA), rabbit anti-TRPV1 antibody (Trans Genic), mouse anti-α-tubulin (Sigma, St. Louis, MO) and rabbit anti-neurofilament (Chemicon, Temecula, CA). Immunoreactive bands were developed with enhanced chemiluminescence reagent (ECL-Plus; Perkin-Elmer, Waltham, MA) and detected with a LAS-4000 lumino-image analyzer (Fuji Film, Tokyo, Japan).

RT-PCR

Twelve SD rats were divided into 3 groups (4 animals in each group). BoNT-A (0.5 ng/kg) was injected into the face bilaterally, and the rats were sacrificed at 2 or 7 days after BoNT-A injection. Untreated rats were used as controls. Total RNA was prepared from TG tissue with TRIzol reagent (Invitrogen). cDNA synthesis was performed using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen). Target amplification for *TRPV1* and *GAPDH* cDNAs was carried out with a TaqMan® Gene Expression Assay system (Applied Biosystems, Carlsbad, CA). We used a TaqMan® gene expression assay [20×] for *TRPV1* cDNA [Rn01460299_m1, ION11000001109900000] or GAPDH assay mix: rat GAPD endogenous control kit for *GAPDH* cDNA. Real-time quantitative PCR was performed by using a StepOnePlus™ instrument (Applied Biosystems). The quantification of TRPV1 transcripts was performed according to the comparative calculated threshold cycle method.

Pain-related behavior analysis

SD rats were anesthetized with halothane, and their faces were shaved. Capsaicin (3.1 mg, Wako Co. LTD., Japan) was dissolved in a solution of 100% ethanol (62.5 μl) and Tween-80 (65.6 μl) in saline (871.8 μl) to produce a 10 mM capsaicin solution. Then, an 8×8 mm cotton patch was soaked in the 10 mM capsaicin solution and placed on the surface of the lateral facial skin of each animal for 30 s without anesthesia in the capsaicin-treated group (Honda et al., 2008). BoNT-A (0.5 ng/kg) was injected into the left side of the face 7 days before capsaicin administration. The TRPV1 antagonist capsazepine was dissolved in dimethylsulfoxide (DMSO) at a final concentration of 10 mM. The capsazepine solution was subcutaneously administered into the lateral facial skin (infraorbital region) using a 29 G×1/2 needle under light halothane anesthesia (AstraZeneca) 30 min prior to capsaicin patch application. We measured the frequency of wipes over the face using the forelimb as an index of nociception. Only unilateral wipes with the forelimb that were not part of grooming behavior were counted (Shimada and LaMotte, 2008). These wipes usually consisted of a gentle, single caudal to rostral stroke of the lateral face. We never observed back and forth stroking suggestive of scratching. The $P2X_3$ agonist, α,β-methyleneATP, was dissolved in saline to a concentration of 20 mM and subcutaneously injected into the lateral facial skin using a 29 G×1/2 needle under light halothane anesthesia. Pain-related behaviors were induced by mechanical stimulation with home-made von Frey hairs (VFHs, diameter: 0.5 mm; bending forces: 92.2 mN). Rats were allowed to acclimate to their surroundings for 60 min before testing. During the mechanical sensitivity assessment, the animals were treated gently and restrained at the trunk with a towel. Each VFH was applied ten times (once every 2–3 s) to the lateral facial skin, and the number of forelimb or withdrawal responses was counted (Shinoda et al., 2007). The frequency of nocifensive behaviors (head withdrawal or wiping) was measured in 10 trials. Stimulation of the skin with the 92.2-mN VFHs elicits a sensation of painful pricking. A significant increase in the frequency of face withdrawal and wiping in response to these mechanical stimuli was interpreted as punctate hyperalgesia. Behavioral tests were carried out at four time points: before and 5, 20 and 30 min after α,β-methyleneATP administration.

Primary TG neuronal cultures and immunocytochemistry

Primary cultures of TG neurons were prepared from neonatal SD rats (1–3 days old). Briefly, the brains were dissected, and TG tissue samples were minced in ice-cold HBSS (Hank's balanced saline solution; Invitrogen). After centrifugation, the pellet was resuspended in Leibovitz L15 (Invitrogen) containing 250 U/ml collagenase (Worthington, Lakewood, NJ), 1 U/ml elastase (Worthington), and 5 U/ml papain (Worthington), and the suspension was incubated for 1.5 h at 37 °C. After quenching the proteases, the suspension was centrifuged, and cells were dissociated by trituration. Dissociated cells were grown in Dulbecco's modified Eagle's medium (DMEM)/F12 (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 50 ng/ml NGF (Sigma), and 1% penicillin/streptomycin (Invitrogen). For immunocytochemical analysis, cells were fixed with 4% paraformaldehyde/PBS, permeabilized with 0.1% TritonX-100/PBS, and blocked with 1% normal goat serum/PBS. They were subsequently incubated with the following primary antibodies: rabbit anti-TRPV1 (Trans Genic) and guinea pig anti-CGRP (EuroDiagnostica, Malmö, Sweden). Immunoreactivity (IR) was visualized with appropriate FITC- and Cy3-conjugated secondary antibodies (Jackson ImmunoResearch). The nuclei were stained with DAPI, and confocal images were captured using a Leica TCS-SP5 confocal microscope.

Cell surface biotinylation of TG primary neurons

The biotinylation and isolation of cell surface proteins in TG primary neurons were performed using a Pierce® Cell Surface Protein Isolation Kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. Twenty-four hours after treatment with either BoNT-A (1 nM) or vehicle (water), the TG primary neurons were incubated

with sulfo-NHS-SS-biotin to biotin-label cell surface proteins before they were collected and disrupted in lysis buffer. The solutions were centrifuged, and a small aliquot of supernatant was preserved as whole cell lysate. The remaining supernatants were incubated with NeutrAvidin agarose, and biotinylated proteins attached to the NeutrAvidin agarose were eluted with Laemmli sample buffer. Biotinylated fractions of TRPV1 and the α1 subunit of Na/K ATPase were analyzed by Western blotting using antibodies for anti-TRPV1 (Trans Genic), anti-$P2X_3$ (Chemicon), and anti-Na/K-ATPase α1 subunit (Abcam, Cambridge, MA). For normalization, the results were compared with Western blot data from whole cell lysate samples, which were obtained with rabbit anti-TRPV1 (Trans Genic), rabbit anti-SNAP25 (Chemicon) and rabbit anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive band densitometric analysis was performed with Multigauge software version 3.3 (Fuji Film, Tokyo, Japan). For assessing the plasma cell membrane translocation of TRPV1, we calculated the ratio of TRPV1 level in the biotinylated fraction sample to that in the corresponding whole cell lysate sample.

Transfection of TRPV1 expression vectors into PC12 cells

Using the total RNA sample from TG tissue, full-length rat *TRPV1* cDNA was amplified with the following set of oligonucleotide primers: forward: 5′-ggaattctggaacaacgggctagcttagac-3′; reverse: 5′-ggggtaccttatttctcccctgggaccat-3′. The PCR product was subcloned into pEGFP-C3 (Clontech, Mountain View, CA). To create a point mutation (Tyr200Phe [Y200F]), we used a QuikChange® site-directed mutagenesis kit (Stratagene, Santa Clara, CA). PC12 cells were grown in DMEM/F12 supplemented with 15% horse serum, 2.5% FBS, and 1% penicillin–streptomycin solution. The plasmids for wild-type and Y200F TRPV1 were transfected using Lipofectamine 2000 (Invitrogen). The expression of the EGFP–TRPV1 fusion proteins was confirmed by capturing the florescence images using a Leica confocal TCS-SP5 microscope. Cell lysates were subjected to Western blot analyses using rabbit anti-GFP (MBL, Nagoya, Japan) and mouse anti-α-tubulin (Sigma). Immunoreactive band densitometric analysis was carried out employing the Multigauge software version 3.3 (Fuji Film, Tokyo, Japan).

Statistical analysis

Unless otherwise stated, all the numerical data are expressed as the mean ± SD. SPSS for Windows (SPSS Inc., Chicago, IL), version 19, was used for all statistical analyses. Differences of quantitative data between the two groups were assessed using Student's *t*-tests. The densitometric data for EGFP–TRPV1 fusion protein expression levels, TG cell numbers and behavioral parameters were compared using one-way analysis of variance (ANOVA), followed by Scheffé's post hoc test. Statistical significance was set at $p<0.05$.

Results

We injected true blue tracer into the left dorso-lateral portion of the nose between the upper whisker pad and the ipsilateral eye to identify the TG neurons innervating the injected region. As shown in Fig. 1, tracer accumulation was observed in the ophthalmic division of the TG. In other sections, a small number of true blue-accumulating neurons were present in the maxillary division (data not shown).

In Fig. 2, neurons retrogradely labeled with true blue showing TRPV1-IR were observed in the TG of each group. When we compared the proportion of TRPV1-IR-containing neurons in true blue-containing neurons among the three groups, the ratio was significantly different between the control and BoNT-A-injected groups (Figs. 2B, C, E, F, H, and I). In the control group, the proportion of TRPV1-IR-containing neurons in true blue-accumulated neurons was 28% (n = 1432 neurons from 5 animals). However, in animals injected with 0.5 ng/kg or 5 ng/kg BoNT-A, the proportion of TRPV1-IR-containing neurons in true blue-accumulated neurons was 11% (n = 961 neurons from 5 animals) and 9% (n = 727 neurons from 5 animals), respectively. These decreases were significant compared to the ratio observed in the control group (one-way ANOVA, followed by Scheffé's post hoc test analyses, $p<0.05$; Fig. 2J). We also observed TRPV1-IR in the skin to determine whether BoNT-A affects TRPV1 expression in the nerve terminals. In control animals, TRPV1-IR nerve fibers with varicosities were observed in the dermis near the injection site (Fig. 3A). Several nerve fibers colocalized with CGRP-IR (Figs. 3B, C). After BoNT-A injection, the number of TRPV1-IR nerve fibers was decreased (Fig. 3D). However, the CGRP-IR-containing nerve fibers remained intact following BoNT-A injection (Figs. 3E and F). The proportion of the area of TRPV1-positive nerve fibers within the nerve bundle containing them in BoNT-A treated animals was 3.8 ± 2.1%, which was significantly lower than that in the control group (22.4 ± 9.1%; Student's *t*-test, $p<0.0001$). When we unilaterally applied a tracer to the dura mater, tracer-accumulated neurons were observed in the TG on the ipsilateral side. As shown in Figs. 4A–C, neurons retrogradely labeled with true blue showing TRPV1-IR were observed in the control group. The proportion of TRPV1-IR-containing neurons that were also true blue-positive was 27% (Fig. 4G; n = 572 neurons from 5 animals). In BoNT-A treated animals, tracer-accumulation and TRPV1-IR were also observed (Figs. 4D and E). However, the number of TRPV1-IR neurons retrogradely labeled with true blue was reduced (Fig. 4F). The proportion of TRPV1-IR-containing, true blue-positive neurons in BoNT-A treated animals was 11% (n = 697 neurons from 5 animals), and this was significantly decreased

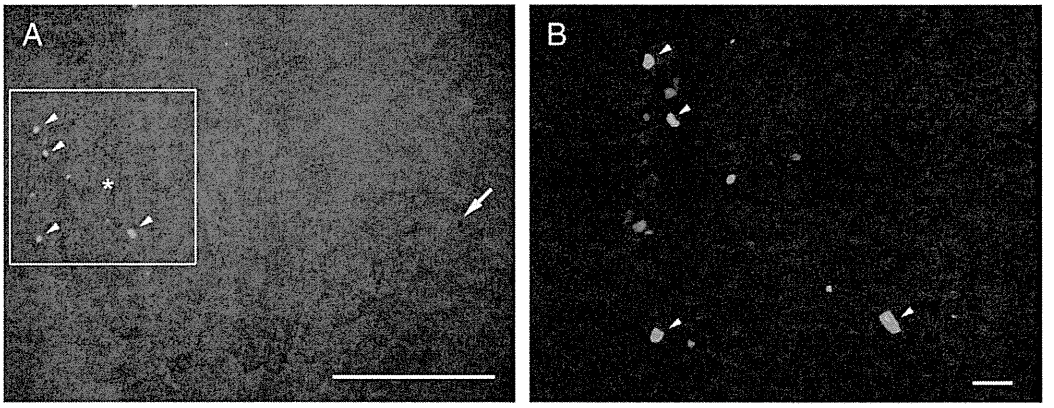

Fig. 1. The distribution pattern of TG neurons retrogradely labeled with true blue. (A) Sections of TG neurons retrogradely labeled with true blue. This picture was produced by merging fluorescence and transillumination images in the same field. The asterisk indicates the ophthalmic division, and the arrow points to the mandibular division of the TG. The arrowheads show neurons labeled with true blue, most of which are localized in the ophthalmic division of the TG. (B) Enlarged fluorescence image of (A). Scale bars: 1 mm (A) and 100 μm (B).

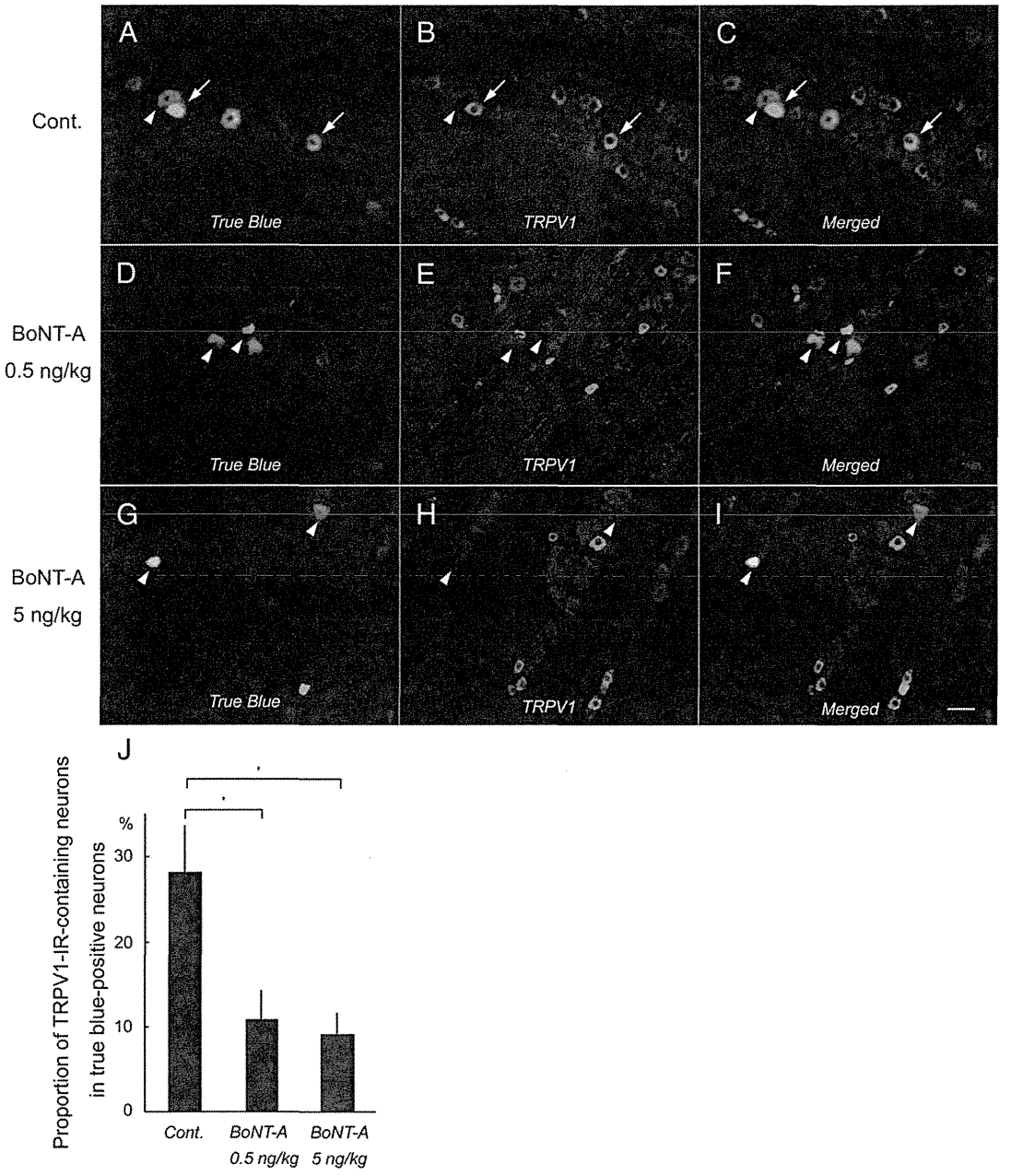

Fig. 2. Decreased number of TRPV1-IR neurons after TG BoNT-A treatment. TG sections containing neurons retrogradely labeled with true blue (A, D, G) and TRPV1-IR (B, E, H). (C) Merged image of (A) and (B). (F) Merged image of (D) and (E). (I) Merged image of (G) and (H). In control animals (A–C), neurons retrogradely labeled with true blue (A) and colocalized with TRPV1-IR (B) are indicated by arrows, and true blue-containing neurons without TRPV1-IR are indicated by arrowheads. In BoNT-A-treated animals (D–F, injected with 0.5 ng/kg BoNT-A containing 1% true blue solution and G–I, injected with 5 ng/kg containing 1% true blue solution), most of the neurons retrogradely labeled with true blue did not colocalize with TRPV1-IR (arrows). Scale bar: 50 μm for all images. (J) Histogram summarizing the quantitative data on the proportion of TRPV1-IR-containing neurons in true blue-positive neurons. Differences between means were considered statistically significant at $p<0.05$ (one-way ANOVA followed by Scheffé's post hoc test).

compared to the ratio observed in the control group (Student's *t*-test, $p<0.0001$; Fig. 4G).

Western blot data confirmed decreased TRPV1 expression in the TG and facial dermis 7 days after BoNT-A administration at a dose of 0.25 ng/kg (Fig. 5A). As for BoNT-A-induced cleavage of SNAP25 in the TG, SNAP25 was cleaved in a dose-dependent manner beginning 2 days after BoNT-A administration (Fig. 5B). SNAP25 cleavage was evident for up to 14 days.

We next considered whether reduced TRPV1 expression was due to inhibited *TRPV1* gene transcription. Quantitative real-time RT-PCR using TaqMan® probes showed that the ratios of *TRPV1* gene transcription to the basal level *TRPV1* gene transcription were 0.942 ± 0.272 (mean ± SD) and 1.151 ± 0.584 at 2 and 7 days following BoNT-A administration, respectively (Fig. 5C). The trend toward an increased mRNA level at Day 7 might reflect a compensatory change that occurred in response to decreased protein expression. These data suggest that the decrease in TRPV1 expression was due to a post-transcriptional mechanism(s) rather than transcriptional downregulation.

We subsequently carried out pain-related behavior assays to confirm the functional significance of the BoNT-A-induced reduction in TRPV1 expression. The number of wipes on the left face was counted for 45 min after applying a cotton patch soaked with 10 mM capsaicin to the left face for 30 s. These induced nocifensive behaviors in the capsaicin-treated group (caps), such as wipes with the forelimb over

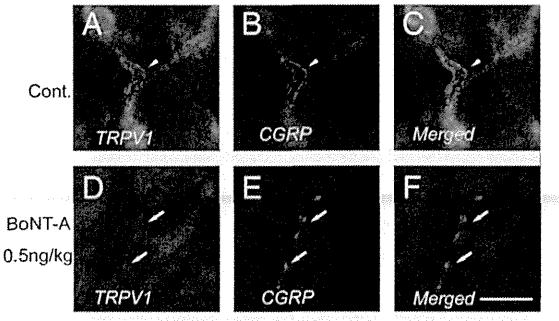

Fig. 3. TRPV1-IR nerve fibers were reduced by BoNT-A treatment of the facial dermis. Double labeling for TRPV1 (green; A, D) and CGRP (red; B, E) in the facial dermis of control (A–C) and 0.5 ng/kg BoNT-A (D–F) groups. (C) Merged image of (A) and (B). (F) Merged image of (D) and (E). As indicated by the arrowheads, the CGRP-IR nerve fibers (B) colocalized with TRPV1-IR (A). As indicated by the arrows, the CGRP-IR nerve fibers (E) did not colocalize with TRPV1-IR (D). Scale bar: 50 μm for all images. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the face skin (82 ± 46 times; n = 11; Fig. 6A open bar). However, animals that received an injection of 0.5 ng/kg BoNT-A into the left side of the face 7 days earlier (Fig. 6A, solid bar) had a significantly lower number of wipes (29 ± 26 times; n = 10) compared to caps (*$p < 0.001$, one-way ANOVA followed by Scheffé's post hoc test). Likewise, animals pretreated with 10 mM capsazepine (Fig. 6A, gray bar) had a significantly decreased number of wipes (26 ± 14 times; n = 9) compared to caps (*$p < 0.001$). There was no significant difference in the number of wipes between the capsazepine-pretreated and BoNT-A-injected animals.

Next, we assessed the selectivity of the BoNT-A action by examining the effect of BoNT-A pretreatment on $P2X_3$-mediated nociception. The injection of a well-established $P2X_3$ agonist, α,β-methyleneATP, to the lateral facial skin did not elicit any nocifensive behaviors. However, the threshold for the induction of nocifensive behavior (head withdrawal and wipes with the forelimb over the skin) with von Frey hair (VFH) stimulation was lowered, which was interpreted as the development of mechanical allodynia. We estimated the number of nocifensive responses to 10 times VFH stimulation before and after injection of

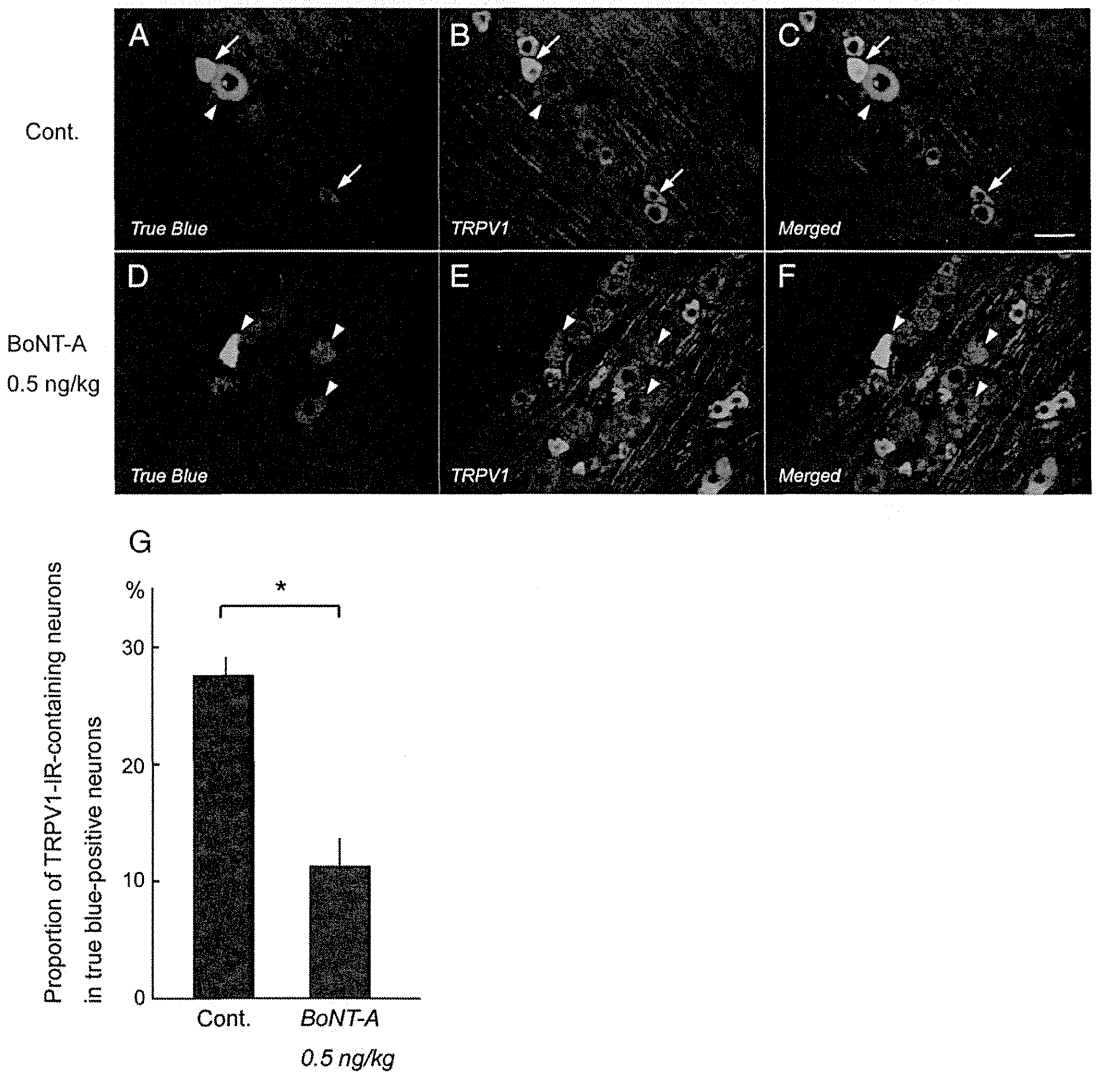

Fig. 4. Reduced number of TRPV1-IR neurons innervating the dura mater after BoNT-A treatment in the TG. Sections of the TG containing neurons retrogradely labeled with true blue (A, D) and TRPV1-IR (B, E). (C) Merged image of (A) and (B). (F) Merged image of (D) and (E). In control (A–C), neurons retrogradely labeled with true blue (A) colocalized with TRPV1-IR (B) are indicated by arrows, and true blue-containing neurons without TRPV1-IR are indicated by arrowheads. In BoNT-A-treated animals (D–F; injected with 0.5 ng/kg BoNT-A containing 1% true blue solution), most of the neurons retrogradely labeled with true blue did not colocalize with TRPV1-IR (arrowheads). Scale bar: 50 μm for all images. (G) Histogram summarizing the quantitative data on the proportion of TRPV1-IR-containing neurons in true blue-positive neurons. Differences between means were considered statistically significant at $p < 0.0001$ (Student's *t*-test).

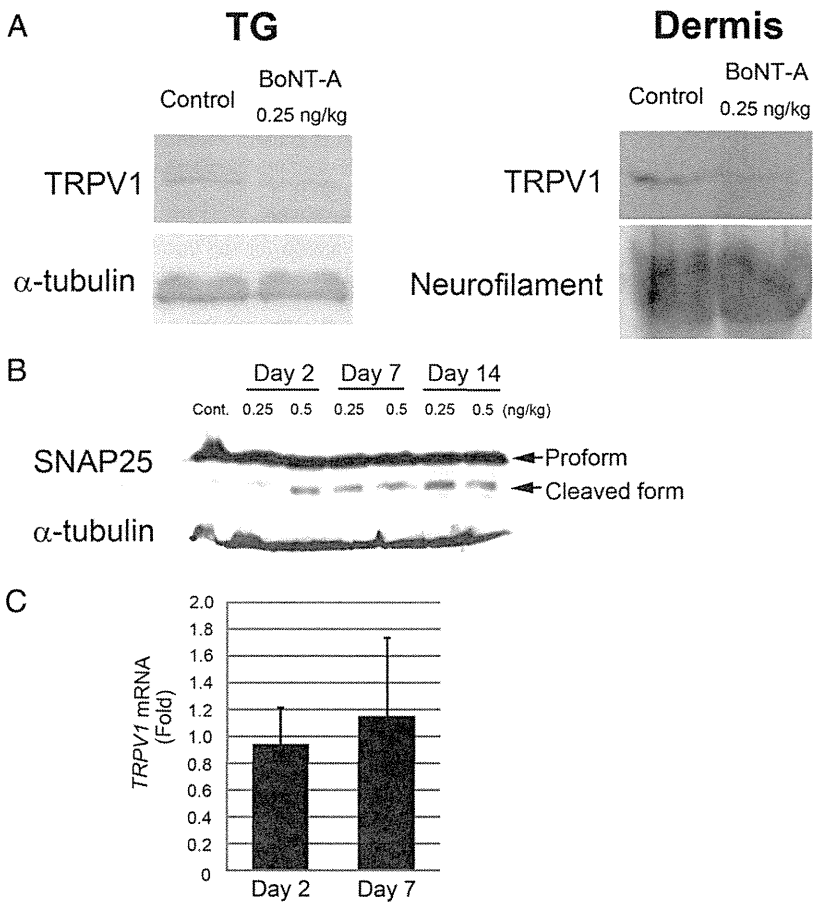

Fig. 5. The BoNT-A-induced reduction in TRPV1 protein expression was not mediated by transcriptional downregulation. (A) BoNT-A administration (0.25 ng/kg) reduced TRPV1 protein expression levels in the TG and facial dermis that contained trigeminal terminals on Day 7. Neurofilament was used to assess the equality of trigeminal nerve-derived protein loading for the dermal samples. (B) Western blot analysis indicated that BoNT-A-mediated cleavage of SNAP25 occurred on Day 2 in a dose-dependent manner. At later time points (Days 7 and 14), SNAP25 cleavage was clearly observed in animals that had received either dose (0.25 or 0.5 ng/kg) of BoNT-A. (C) Quantitative RT-PCR analysis using TaqMan® probes did not reveal significant changes in *TRPV1* transcript levels at Days 2 or 7 after BoNT-A administration (0.5 ng/kg). Statistical significance was set at $p<0.05$ (one-way ANOVA followed by Scheffé's post hoc test).

α,β-methyleneATP into the left lateral face in control animals (Fig. 6B, solid circles; $n=5$) and animals pretreated with 0.5 ng/kg BoNT-A (Fig. 6B, open circles; $n=5$). In control animals, the number of nocifensive responses was 2.0 ± 1.8 times before α,β-methyleneATP injection (before), 4.6 ± 2.3 times at 5 min, 5.4 ± 4.5 times at 20 min and 5.4 ± 4.4 times at 30 min. The number of nocifensive responses after drug administration was significantly increased compared with the baseline value (*$p<0.001$, one-way ANOVA followed by Scheffé's post hoc test). In BoNT-A-pretreated animals, the number of nociceptive response was 1.4 ± 1.5 times before α,β-methyleneATP injection, 6.4 ± 2.1 times at 5 min, 5.4 ± 3.5 times at 20 min and 5.4 ± 3.7 times at 30 min. The number of nocifensive responses was significantly increased after the drug administration compared with the value before α,β-methyleneATP injection (*$p<0.001$, ANOVA followed by Scheffé's post hoc test). However, two-way ANOVA did not detect significant differences in the number of nocifensive responses between the control and BoNT-A-pretreated groups, and BoNT-A pretreatment failed to affect $P2X_3$-mediated threshold alterations.

Next, we performed a surface protein biotin-labeling assay in TG primary neurons. Approximately 90% of TRPV1-positive cells were also CGRP positive (Fig. 7A). In our cultures, BoNT-A was capable of cleaving SNAP25 in a dose-dependent fashion in the range of 0.1–10 nM (Fig. 7B). As BoNT-A has been recognized as a neurotoxin, we first examined the effect of BoNT-A on cell viability. TG neurons did not exhibit any morphological change suggestive of cell death (Fig. 7C) or caspase-3 activation (data not shown) when subjected to 1 nM BoNT-A for 24 h. We treated TG primary cultures with either 1 nM BoNT-A or vehicle for 24 h before labeling cell surface proteins with biotin. We then examined TRPV1 and $P2X_3$ levels in whole cell lysates and eluates from NeutrAvidin agarose beads (biotinylated fractions). We compared the ratios of TRPV1 in the biotinylated fraction to that in the corresponding whole cell lysate and found a reduction to $20.6\pm30.9\%$ in the BoNT-A-treated sample compared to the control (vehicle-treated) sample (Figs. 7D and E). The biotin-labeled $P2X_3$ level was not altered by BoNT-A treatment (Fig. 7E). Such differential actions of BoNT-A were consistent with the behavioral analysis results. To verify the efficiency of biotinylation in each sample, we also looked at biotin-labeled Na/K-ATPase, a representative membrane-bound protein. The results excluded the possibility that the decrease in biotinylated TRPV1 in the BoNT-A-treated fraction was due to inefficient cell surface biotin labeling. We also observed that the BoNT-A treatment caused decreased total TRPV1 expression in TG primary neurons (Fig. 7F).

We then addressed whether impaired trafficking of TRPV1 to the plasma membrane explained the decline in TRPV1 expression. Phosphorylation at tyrosine residue 200 (Y200) is critical for plasma membrane trafficking of TRPV1 (Zhang et al., 2005). We synthesized expression plasmids encoding enhanced GFP (EGFP)-tagged wild-type TRPV1 and mutant TRPV1 (tyrosine 200 to phenylalanine, Y200F), and these plasmid vectors were transfected into PC12 cells. We found that EGFP-tagged Y200F TRPV1 mutant was poorly translocated to the plasma membrane, whereas the EGFP-tagged wild-type TRPV1 was appropriately

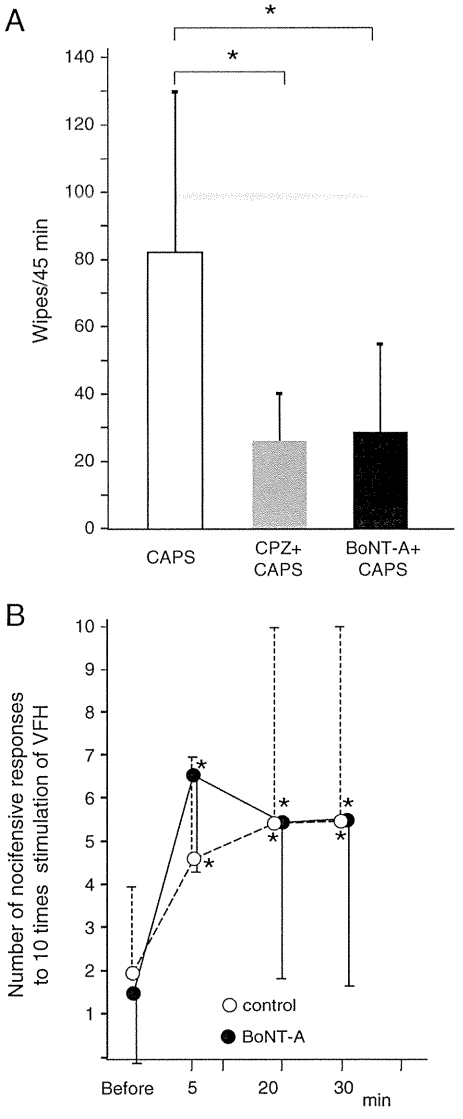

Fig. 6. Effects of BoNT-A on capsaicin-induced nocifensive behaviors. A. The number of wipes of the left face was counted over 45 min after application of a cotton patch soaked with 10 mM capsaicin to the left side of the face for 30 s. B. The number of nocifensive responses to 10 times stimulation using VFH before and after injection of the $P2X_3$ agonist α,β-methyleneATP into the left lateral face in control animals (solid circles) and animals pretreated with 0.5 ng/kg BoNT-A 7 days previously (open circles). In both groups, there was a significant increase of the number of nocifensive responses after drug administration compared with the baseline value (*$p<0.001$, one-way ANOVA followed by Scheffé's post hoc test). Two-way ANOVA did not detect significant differences in the number of nocifensive responses between the control and BoNT-A-pretreated groups.

localized in the plasma membrane (Fig. 8A). Western blot analysis demonstrated that Y200F TRPV1 expression was much lower than that of the wild-type TRPV1 (Fig. 8B). The results implied that impaired plasma membrane trafficking of TRPV1 led to reduced expression levels. However, at this point, a causal link between the two phenomena was unclear. We hypothesized that a certain degradation mechanism might affect cytoplasmic TRPV1. We investigated whether the ubiquitin-proteasome or lysosome-mediated protein degradation system might be involved in cytoplasmic TRPV1 proteolysis. First, we treated PC12 cells expressing either the wild-type or Y200F-mutant TRPV1 with MG-132, a well-established proteasome inhibitor (Goldberg, 2003), for 24 h. This treatment increased the expression levels of both types of TRPV1 in a dose-dependent manner (wild-type: 181.3 ± 37.2% at 1 μM, 291.4 ± 59.3% at 5 μM; Y200F: 271.6 ± 60.4% at 1 μM, 838.7 ± 189.2% at 5 μM), which indicated that proteasomal degradation was implicated in TRPV1 catabolism (Figs. 8C, E). The immunoreactivity in the high molecular range appeared to represent poly-ubiquitinated EGFP–TRPV1 proteins (Fig. 8C). In addition, MG-132-induced potentiation of protein expression was more robust in the cells expressing Y200F-mutant TRPV1 than in cells expressing wild-type TRPV1. This indicated that cytoplasmic TRPV1 was more vulnerable to proteasomal degradation than plasma membrane-bound TRPV1, which provided a good reason for the instability of TRPV1 that failed to translocate to the plasma membrane. We also assessed the effects of lysosomal protease inhibition with a cocktail of E64d (30 μM), pepstatin A (15 μM), and leupeptin (20 μM) (Mizushima et al., 2010). This intervention did not alter wild-type or Y200F-mutant TRPV1 expression levels (wild-type, 104.6 ± 17.6%; Y200F, 104.3 ± 12.5%), indicating that lysosomal proteases did not play a major role in TRPV1 catabolism (Figs. 8D, E).

Discussion

This study demonstrated that subcutaneous BoNT-A administration to the region of the face innervated by the ophthalmic division of the trigeminal nerve causes SNAP25 cleavage in TG neurons and decreases TRPV1 protein expression in both trigeminal terminals and neurons independently of transcriptional downregulation. Notably, TG neurons that exhibited reduced TRPV1 expression after BoNT-A treatment contained those receiving projections from the dura mater. Pain behavior analysis showed that BoNT-A treatment attenuated TRPV1-mediated nocifensive behaviors. The antinociceptive action of BoNT-A appeared selective because it did not affect $P2X_3$-mediated pain-induced behaviors. In our primary TG neuronal cultures, BoNT-A treatment concomitantly induced SNAP25 cleavage and reduced TRPV1 protein expression. More importantly it inhibited TRPV1 trafficking to the plasma membrane, implying that the exocytosis-mediated TRPV1 delivery system is an important therapeutic target of BoNT-A. Moreover, the pharmacological assay using Y200F-mutant TRPV1 revealed that TRPV1 is prone to ubiquitination and subsequent proteasomal degradation, especially when its plasma membrane trafficking is blocked, which accounted for its decreased expression levels following BoNT-A treatment (Fig. 9).

Our Western blot data clearly demonstrated cleaved SNAP25 in the TG after local BoNT-A administration in the vicinity of trigeminal terminals, which implies that BoNT-A effects are not localized; rather, BoNT-A can affect neuronal somata that project axons to injection sites. Antonucci et al. (2008) histologically demonstrated that BoNT-A can be retrogradely transported, and even transcytosed, to afferent synapses (Antonucci et al., 2008). Moreover, Matak et al. (2011) confirmed the appearance of BoNT-A-truncated SNAP-25 in the trigeminal nucleus caudalis (TNC) neurons after BoNT-A administration to the whisker pad, which was abolished by pretreatment with colchicine (Matak et al., 2011). Their observation provided clear evidence for transcytosis of peripherally-administered BoNT-A up to the brainstem. Our observation that TG neurons innervating the dura mater also had decreased TRPV1 expression strongly suggests that the effect of BoNT-A is not restricted to neurons directly treated with the toxin. In this regard, Kitamura et al. (2009) demonstrated that intradermal administration of BoNT-A to the whisker pad induced a potent inhibition of the vesicular neurotransmitter release from the acutely dissociated TG neurons, which was indicative of the occurrence of transcytosis within TG (Kitamura et al., 2009). Taken together, our data lend support to accumulating evidence for active transcytosis of BoNT-A within the sensory nervous system.

A TRPV1-null mouse study confirmed that TRPV1 plays a pivotal role in the development of inflammatory thermal hyperalgesia (Caterina et al., 2000). In addition, it is known that TRPV1 function can be enhanced by increased plasma membrane expression levels and/or upregulation of TRPV1 cation channel activity, and TRPV1 phosphorylation is important in these phenomena (Bhave et al., 2003; Bonnington and McNaughton,

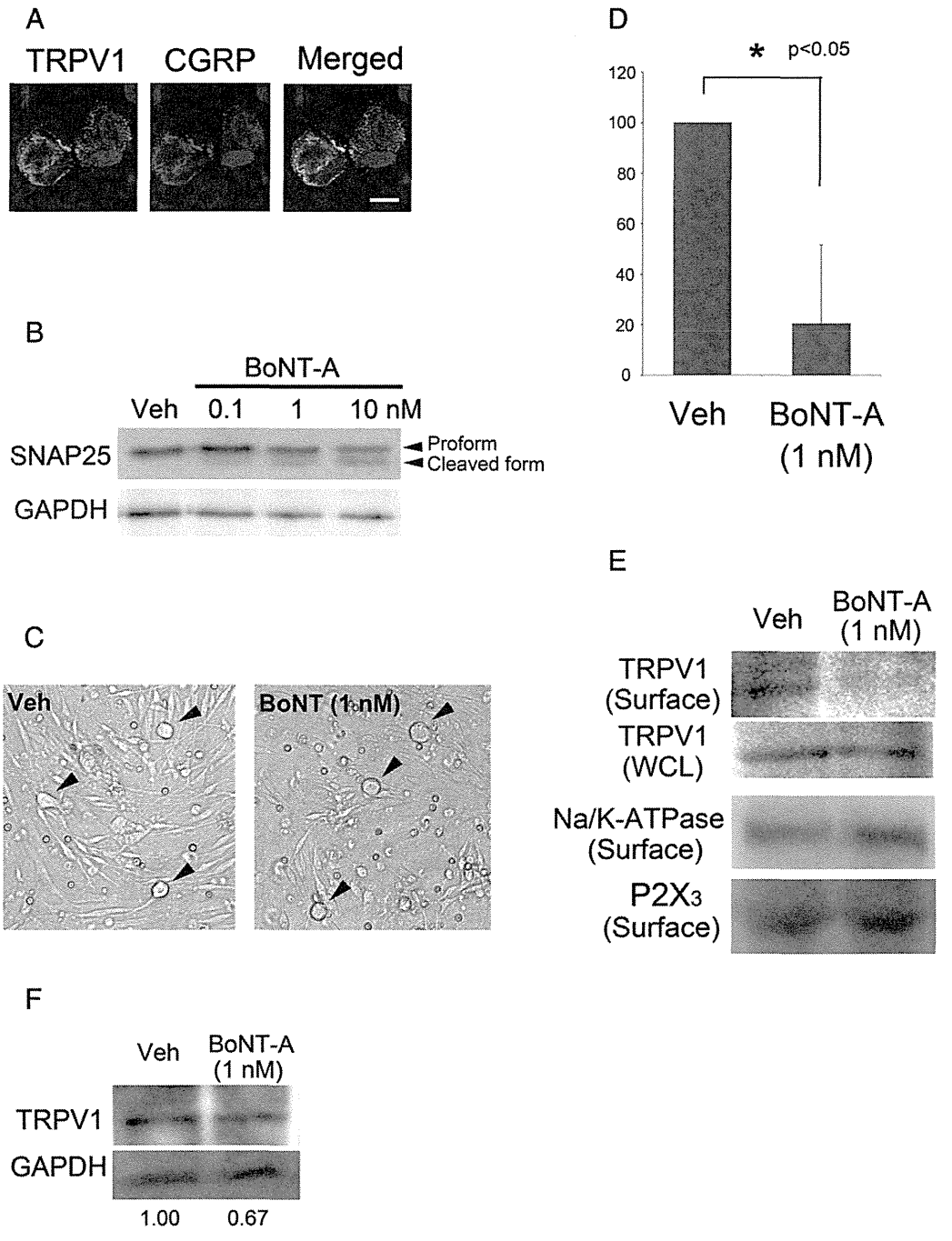

Fig. 7. BoNT-A inhibited TRPV1 trafficking to the plasma membrane in TG neurons. (A) TRPV1 immunostaining showed that TRPV1 was expressed in CGRP-positive small-diameter (~20 μm) neurons. Approximately 90% of TRPV1-positive cells were immunoreactive for CGRP. Green: TRPV1; red: CGRP; blue: nuclei; scale bar: 10 μm. (B) BoNT-A cleaved SNAP25 in a dose-dependent manner in the range of 0.1–10 nM. The molecular weights of the pro and cleaved forms were 25 and 23 kDa, respectively. The blot was probed for GAPDH to confirm equal sample loading. (C) BoNT-A (1 nM) treatment for 24 h did not result in any morphological changes suggestive of cell death. Arrowheads indicate TG neurons. (D) The ratio of TRPV1 in the plasma cell (biotinylated) fraction to that in the whole cell lysate was significantly reduced to 20.6±30.1% ($p<0.05$, Student's *t*-test) in the BoNT-A-treated cell culture compared to the vehicle-treated cell culture. The bar represents SD. (E) Cell surface biotin labeling indicated that TRPV1 plasma membrane expression in TG neurons decreased 24 h after BoNT-A (1 nM) treatment. The decrease in TRPV1 band intensity in the biotinylated fraction sample was obvious, even after the whole cell lysate TRPV1 level in the BoNT-A-treated sample was matched with that in the vehicle-treated sample. BoNT-A treatment did not affect $P2X_3$ cell surface expression. The identical whole cell lysate and biotinylated protein samples were examined for Na/K-ATPase α subunit, a representative plasma membrane protein, which confirmed that the decreased biotinylated TRPV1 level induced by BoNT-A treatment was not due to inefficient biotin labeling. (F) BoNT-A reduced TRPV1 expression in the whole cell lysate of the TG culture. As indicated by the numbers below the blots, quantification revealed that BoNT-A (1 nM) treatment for 24 h reduced TRPV1 level, normalized by the GAPDH level by 33%. The blots were representative of three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2003; Gunthorpe and Chizh, 2009; Morenilla-Palao et al., 2004; Stein et al., 2006; Zhang et al., 2005; Zhu and Oxford, 2007). In particular, Y200 phosphorylation of TRPV1 was found to be crucial in plasma membrane translocation evoked by NGF, an algogenic inflammatory mediator that acts on the tyrosine kinase receptor, TrkA (Zhang et al., 2005). As our PC12 cells were grown in NGF-free medium, the basal trafficking of TRPV1 to the plasma membrane in our cultures was largely dependent on Y200 phosphorylation. NGF-induced Y200 phosphorylation of

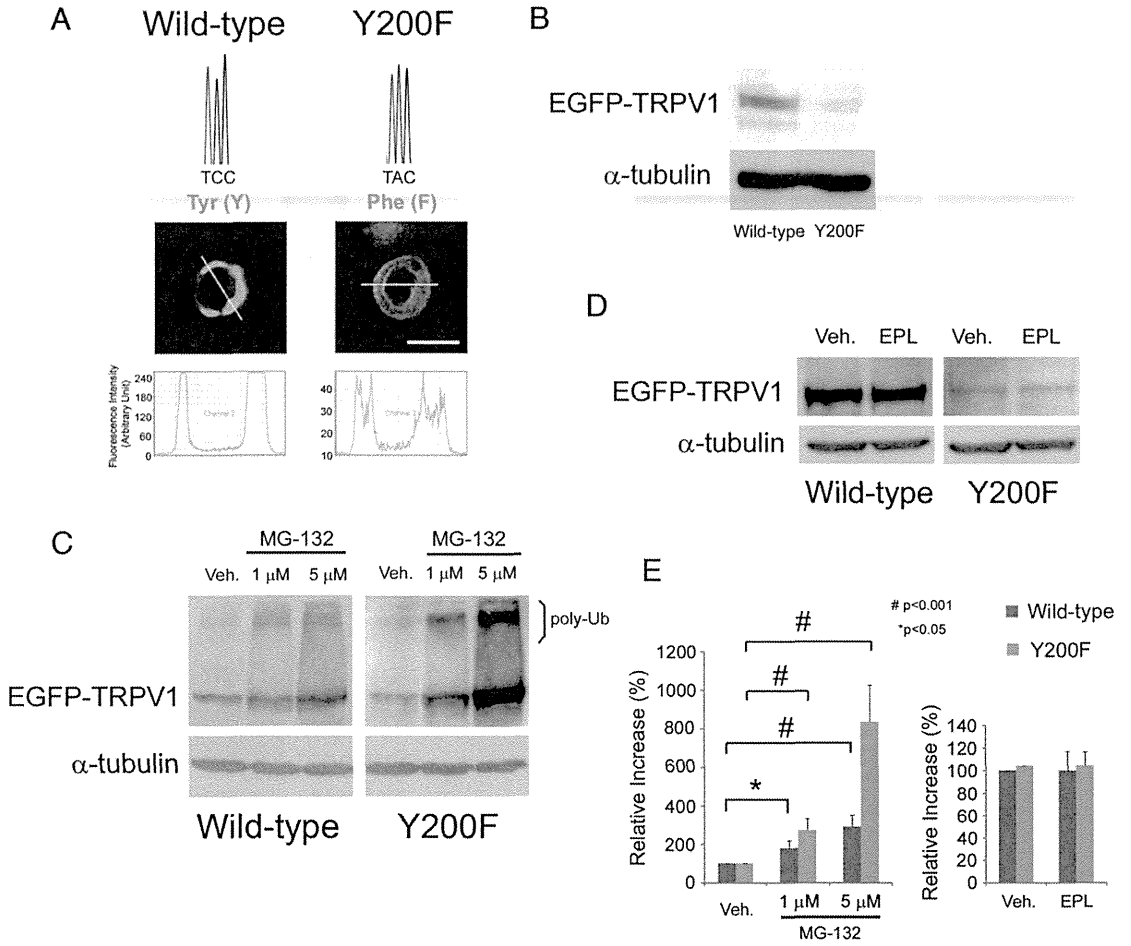

Fig. 8. Failure of TRPV1 to translocate to the plasma membrane led to proteasomal degradation. (A) EGFP-tagged wild-type and Y200F-mutant TRPV1 were transfected into PC12 cells. EGFP-tagged wild-type TRPV1 was primarily localized in the plasma membrane. The Y200F mutation, confirmed by DNA sequencing, resulted in altered localization; the EGFP-tagged Y200F TRPV1 mutant was diffusely present in the cytoplasm. As the GFP signal emitted by the Y200F-mutant was weak, a longer exposure condition was adopted when the image of this Y200F-expressing cell was acquired. Scale bar: 10 μm. GFP signal quantification along the thin white lines revealed that the GFP signal in the plasma membrane (~250 arbitrary units [AU]) was more intense than that in the cytoplasm (~50 AU). The analysis was carried out using Leica LAS AF software. (B) Western blot analysis showed that the steady-state expression level of Y200F-mutant TRPV1 was lower than that of wild-type TRPV1. (C) Proteasomal inhibition using MG-132 (1 and 5 μM; Sigma) led to increased expression of both wild-type and Y200F-mutated EGFP–TRPV1 in a dose-dependent manner. The magnitude of the elevation in MG-132-induced expression was greater in PC12 cells expressing Y200F-mutant TRPV1, indicating that cytoplasm-localized TRPV1 was more vulnerable to proteasomal degradation than was membrane-localized TRPV1. (D) Lysosomal protease inhibition with a combination of E64d (30 μM; Peptide Institute, Osaka, Japan), pepstatin A (15 μM; Peptide Institute), and leupeptin (20 μM; Peptide Institute) did not alter the expression of wild-type or Y200F-mutant TRPV1. EPL: E64d + pepstatin A + leupeptin. (E) Quantification of wild-type and Y200F-mutant EGFP–TRPV1 protein level alterations following proteasome and lysosomal protease inhibition.

TRPV1 is mediated by the sequential activation of phosphatidylinositol 3-kinase (PI3-K) and Src kinase (Stein et al., 2006; Zhang et al., 2005; Zhu and Oxford, 2007). Protein kinase C (PKC) also plays an important role in NGF-driven, exocytosis-mediated TRPV1 surface membrane expression, and the PKCδ-specific inhibitor rottlerin has been shown to inhibit NGF-dependent phosphorylation and functional potentiation of TRPV1, implying that PKC, particularly PKCδ, acts upstream of the Src kinase (Bhave et al., 2003; Bonnington and McNaughton, 2003; Morenilla-Palao et al., 2004; Zhang et al., 2005). This is supported by the finding that angiotensin-induced Src phosphorylation was abrogated by rottlerin in rat liver endothelial cells. Collectively, these results suggest that the role of PKCδ in TRPV1 Y200 phosphorylation appears likely to be mediated by Src kinase activation (Shah and Catt, 2002). Regulated exocytosis is accomplished when vesicles fuse with the plasma membrane due to the coordinated actions of SNARE proteins (Dolly et al., 2009; Südhof and Rothman, 2009). Recently, Camprubí-Robles et al. (2009) demonstrated that a lipopeptide that specifically inhibits SNAP25 activity attenuated NGF-induced TRPV1 trafficking to the plasma membrane (Camprubí-Robles et al., 2009), suggesting that Y200 phosphorylation is closely associated with TRPV1 mobilization by BoNT-A-sensitive exocytosis. Furthermore, a yeast two-hybrid study revealed that the N-terminal domain of TRPV1, including the Y200 residue, can interact with Snapin and synaptotagmin IX (Syt IX), which are both vesicular proteins related to exocytosis. Therefore, we reasoned that our results from Y200F-mutant TRPV1-expressing PC12 cells are pertinent to our in vivo findings regarding BoNT-A action.

The present study demonstrated the differential effect of BoNT-A on the trafficking of TRPV1 over $P2X_3$ in the TG neurons. Contrary to our finding, Apostolidis et al. (2005) reported concomitant decreases of TRPV1 and $P2X_3$ in the suburothelial nerve fibers after intradetrusor injections of BoNT-A in humans (Apostolidis et al., 2005). This discrepancy may reflect differences in tissues and/or species examined. Along with TRPV1, $P2X_3$ also plays an important role in the nociception of the trigeminal system (Shinoda et al., 2007, 2008; Staikopoulos et al., 2007). Hsieh et al. reported that the potent TRPV1 agonist, resiniferatoxin (RTX), induced degeneration of TRPV1-positive neuron in the dorsal root ganglia (DRG), followed by an increase in $P2X_3$ expression in the remaining DRG neurons. The number of $P2X_3$-positive neurons correlated with the magnitude of sensitization that developed after the RTX treatment. Their finding implies that $P2X_3$ expression

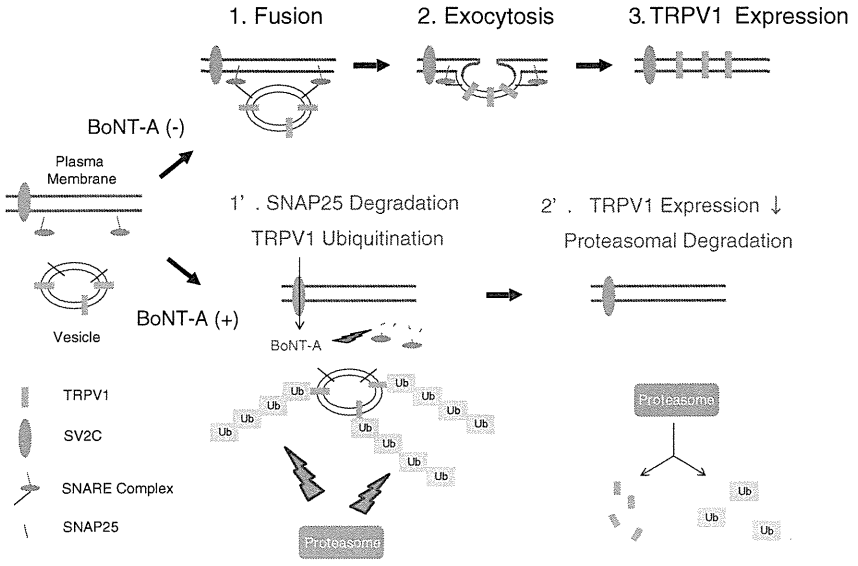

Fig. 9. Schematic model of intracellular events associated with the mechanism whereby BoNT-A reduces TRPV1 expression. TRPV1 plasma membrane expression is accomplished by regulated exocytosis. Under appropriate circumstances, TRPV1-carrying vesicles fuse with the plasma membrane (1). The ensuing exocytosis leads to plasma membrane surface expression of TRPV1, which is dependent on the coordinated actions of the SNARE complex (2, 3). In the presence of BoNT-A, which enters the cell through its acceptor protein, SV2C, SNAP25 is cleaved by the protease activity of BoNT-A (1′) (Dong et al., 2006), which inhibits exocytosis. The failure of TRPV1 to translocate to the plasma membrane renders TRPV1 vulnerable to ubiquitination and subsequent proteasomal degradation, thus resulting in decreased TRPV1 levels (2′).

increased in primary sensory neurons to compensate for the decreased TRPV1 expression. In this sense, in addition to BoNT-A administration, a therapeutic measure that controls $P2X_3$ expression level may be required to alleviate pain disorders.

The present study provides novel evidence that TRPV1 is susceptible to proteasomal degradation in the cytoplasm, unless it translocates to the plasma membrane. Proper cell surface receptor regulation is very important for various physiological functions. As wild-type TRPV1 was also shown to be sensitive to 24-hour-long MG132 treatment (Fig. 6C), proteasome-regulated TRPV1 turnover appears to be dynamic and is probably related to the fine-tuning of nociception. In this regard, the balance between neuronal receptor synthesis and degradation is expected to be tightly regulated. The ubiquitin-proteasome system is known to be involved in receptor catabolism. For example, NR1, an NMDA receptor subunit, is ubiquitinated by the F-box protein, Fbx2, a component of E3 ligase, and is then degraded by a proteasome (Kato et al., 2005). Details regarding the TRPV1 ubiquitination mechanism, including the identification of the responsible E3 ligase, remain to be elucidated. Nonetheless, it is a significant discovery of this study that blocking TRPV1 trafficking to the plasma membrane markedly decreases total TRPV1 expression due to proteasomal degradation. This finding provides important insight into therapeutic measures against pathological conditions in which total TRPV1 expression and recruitment to the plasma membrane are increased, as seen in inflammation-induced hyperalgesic states. Our results also revealed that there is no discernible contribution of lysosomal proteases to TRPV1 degradation.

This study provides a novel molecular basis for the efficacy of BoNT-A in the treatment of pain disorders. Previously, Gazerani et al. (2009) demonstrated that subcutaneous BoNT-A administered to the forehead reduced capsaicin-induced trigeminal pain in humans, thus providing strong evidence for a link between BoNT-A and TRPV1 in the human trigeminal system (Gazerani et al., 2009). The results of the rat pain behavior assay supported these findings and revealed the selectivity of the antinociceptive action of BoNT-A against TRPV1. In addition, the failure of BoNT-A pretreatment to affect $P2X_3$-mediated nocifensive behaviors made it unlikely that the molecular functions implicated in pain signal conduction and transmission, such as ion channels and neuronal receptors, were influenced by BoNT-A administration. Any detailed analysis of BoNT-A-induced molecular events has been impossible using only in vivo studies; therefore, the mechanism by which BoNT-A attenuated TRPV1 functions has remained unclear. Here, we demonstrated that local administration of BoNT-A to the face can target TG neurons of the ophthalmic division, including those receiving projections from the dura mater, and these neurons are related to nociception associated with headaches. Thus, these findings have clinical implications for headache disorders. Infectious and granulomatous diseases often affect meninges, and resultant meningitis causes severe pain that is refractory to conventional therapy. TRPV1 channel blockers have been used in humans for the therapeutic purpose (Meents et al., 2010). Among them, AMG 517, a highly TRPV1 selective antagonist, was administered to alleviate pain after molar extraction, which caused long-lasting hyperthermia with maximal body temperature above 40 °C (Gavva et al., 2008). This is consistent with the body temperature-regulating function of TRPV1 (Romanovsky et al., 2009), and the unfavorable side-effect hampers the further clinical application of this agent. BoNT-A-mediated inhibition of intracellular TRPV1 trafficking seems to be superior to TRPV1 channel blockage with respect to the development of hyperthermia.

BoNT-A has been demonstrated to be effective for chronic migraine (Aurora et al., 2010; Diener et al., 2010). Although the precise pathophysiology of migraine is still largely unknown, some researchers postulate that neurogenic inflammation in the dura mater plays an important role in the generation of migraine pain (Moskowitz, 1984). Following this line, long-term inflammation likely causes TRPV1 upregulation in the trigeminal nociceptors and may be involved in chronic migraine pathophysiology; the efficaciousness of BoNT-A against chronic migraine seems consistent with this hypothesis. Ideally, oral drugs capable of regulating exocytosis-mediated TRPV1 plasma membrane trafficking should be developed. Such drugs could be expected to have a broad spectrum of clinical applications, including the treatment of a variety of non-neurological disorders associated with TRPV1 hyperactivity, such as respiratory tract hypersensitivity (Lee and Gu, 2009) and inflammatory bowel diseases (Akbar et al., 2010).

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Alterations in microglia and astrocytes in the trigeminal nucleus caudalis by repetitive TRPV1 stimulation on the trigeminal nociceptors

Toshiya Kuroi, Toshihiko Shimizu, Mamoru Shibata, Haruki Toriumi, Megumi Funakubo, Tatsuo Iwashita, Hitoshi Sato, Kenzo Koizumi and Norihiro Suzuki

TRPV1 is a nonselective cation channel in nociceptors. TRPV1 stimulation has been shown to lead to the activation of microglia and astrocytes in the dorsal horn of the spinal cord. However, information on the effect of TRPV1 stimulation on glial activation in the trigeminal nucleus caudalis (TNC) is lacking. Here, we stimulated TRPV1 in the trigeminal afferents by a repetitive injection of 10 mmol/l capsaicin into the whisker pad for 2 days (d2 group), 4 days (d4 group), or 6 days (d6 group). As a control (c group), the vehicle was injected for 2 days. Anti-Iba1 and anti-glial fibrillary acidic protein antibodies were used to immunostain microglia and astrocytes in the TNC, respectively. The ratio of the cross-sectional area immunoreactive for Iba1 to the entire area of the TNC was increased in the d2 group compared with the c group on the injected side. Microglia were recruited to the superficial layers of the TNC. The numbers of microglia were reduced in the d4 group and the d6 group compared with the d2 group. The ratio of the cross-sectional area immunoreactive for glial fibrillary acidic protein to the entire TNC showed a significant increase in d2 group and the d4 group compared with the c group on the injected side. Behavioral analysis indicated that mechanical allodynia began to develop after 2 days of capsaicin treatment and persisted for at least 6 days after the onset of the repetitive capsaicin injection. These data indicate that TRPV1 stimulation activates the microglia and astrocytes in temporally distinct ways and that the development of mechanical allodynia is independent of such glial activation. *NeuroReport* 23:560–565 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Keywords: astrocyte, mechanical allodynia, microglia, trigeminal nucleus caudalis, TRPV1

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Introduction

Glial cells are known to play a vital role in chronic pain. Several studies have shown the activation of the microglia and astrocytes in the spinal cord after inflammatory tissue damage [1,2] or a peripheral nerve injury [3,4]. The transient receptor potential, subfamily V, member 1 (TRPV1) receptor is a capsaicin-sensitive, proton-sensitive, and heat-sensitive nonselective cation channel and plays a role in nociception [5]. There are several inflammatory diseases involving the trigeminal nerve-innervated areas that cause severe and intractable pain, as exemplified by meningitis and pulpitis. As TRPV1 function is known to be upregulated by inflammatory mediators, the cation channel plays a pivotal role in the evolution of inflammatory pain. Although the precise relationship between nociceptive stimulation and glial activation in the central nervous system remains unclear, recent evidence has shown TRPV1-mediated activation of spinal cord glial cells [6]. An intraplantar capsaicin injection induced thermal hypersensitivity, which was accompanied by an increase in immunostaining for glial markers in the dorsal horn. However, information on the relationship between TRPV1 stimulation and glial activation in the brainstem trigeminal nucleus region appears to be lacking.

In this study, we aim to observe the alterations in the microglia and astrocytes in the trigeminal nucleus caudalis (TNC) by a repetitive TRPV1 stimulation using immunohistochemical techniques and to determine the correlation between the immunohistological data and the development of nocifensive behaviors.

Materials and methods

Animals

Experiments were performed on male Sprague–Dawley rats ($n = 27$; body weight, 250–300 g), 12 of which were used for immunohistochemistry, with the remaining 15 used for behavioral testing. These experimental procedures were approved by the Animal Welfare Committee of Keio University (No. 08075). Furthermore, all the procedures were undertaken with the utmost care to minimize the suffering of the animals.

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Drug administration

After deep anesthetization with halothane, either 100 µl of a 10 mmol/l 8-methyl-*N*-vanillyl-*trans*-6-nonenamide (capsaicin) solution (3.1 mg/ml in saline with 6% ethanol and 7% Tween-80; Sigma-Aldrich, St Louis, Missouri, USA) or a vehicle (saline with 6% ethanol and 7% Tween-80) was subcutaneously injected into the left whisker pad of each animal. In the immunohistochemical experiment, animals were divided into four groups ($n = 3$ in each group) as follows: rats treated with the vehicle for 2 days (c group), and with capsaicin for 2 days (d2 group), 4 days (d4 group), and 6 days (d6 group). In clinically important disease conditions, such as meningitis and pulpitis, nociceptive stimulation is present for a certain period. Therefore, we chose repetitive capsaicin treatment in this study to mimic clinical situations as in the inflammatory diseases.

Immunohistochemistry

On the day after the last injection in each group, the animals were deeply anesthetized and transcardially perfused with a mixture of 2% formaldehyde and 0.2% picric acid in 0.1 mol/l phosphate buffer (pH 7.0). Immediately after the perfusion fixation, the brainstem was dissected out and the portion harboring the TNC was processed into frozen serial sections of 12 µm thickness using a cryostat (Reichert-Jung Cryocut 1800; Leica Instruments, Buffalo Grove, Illinois, USA). The first ones of three consecutive sections were used for immunostaining. The frozen sections were preincubated with 10% normal donkey serum for 30 min. The sections were incubated with primary antibodies for 48 h at room temperature. The sections were then rinsed with 0.01 mol/l PBS and incubated with species-specific secondary antibodies for 2 h at room temperature. The specimens were mounted in buffered glycerol (pH 8.6). In this manner, the slides were double labeled with the anti-ionizing calcium-binding adapter molecule 1 (Iba1) antibody (raised in rabbits; code 019-19741; Wako Chemicals, Osaka, Japan; 1:200) and the anti-glial fibrillary acidic protein (GFAP) antibody (raised in mice; code G3893; Sigma-Aldrich; 1:500). Immunoreactivities with the primary antibodies were visualized with species-specific secondary antibodies raised in donkeys and conjugated to Cy3 or fluorescein isothiocyanate; all the antibodies were obtained from Jackson Immunoresearch Laboratories (West Grove, Pennsylvania, USA).

The immunolabeled specimens were examined under a Keyence BIOREVO BZ-9000 microscope (Keyence, Osaka, Japan) fitted with a highly discriminating filter.

Quantitative analysis of immunoreactivities for glial cells

We chose eight brainstem sections at the level of the TNC on the capsaicin-injected side in each animal (three animals per group). The sections were immunostained for Iba1 and GFAP to label the microglia and the astrocytes, respectively. Digital images were obtained using a high-quality CCD camera attached to a fluorescent microscope (BIOREVO BZ-9000; Keyence) and saved as Tiff files. The TNC area on the section images was determined according to the method of Paxinos and Watson [7]. Subsequently, we calculated the ratio of cross-sectional area immunoreactive for Iba1 or GFAP within the TNC area and estimated the number of Iba1-immunoreactivity (IR) cells per 1 mm^2 of the TNC area using an image analysis software (Keyence). In these quantitative analyses, we applied an identical fluorescence excitation condition to all of the sections examined. All enhancements were made uniformly across entire images. For capturing fluorescence signals representing immunoreactivities for Iba1 and GFAP, we used a distinct optimal condition for each marker in terms of segregation and threshold settings.

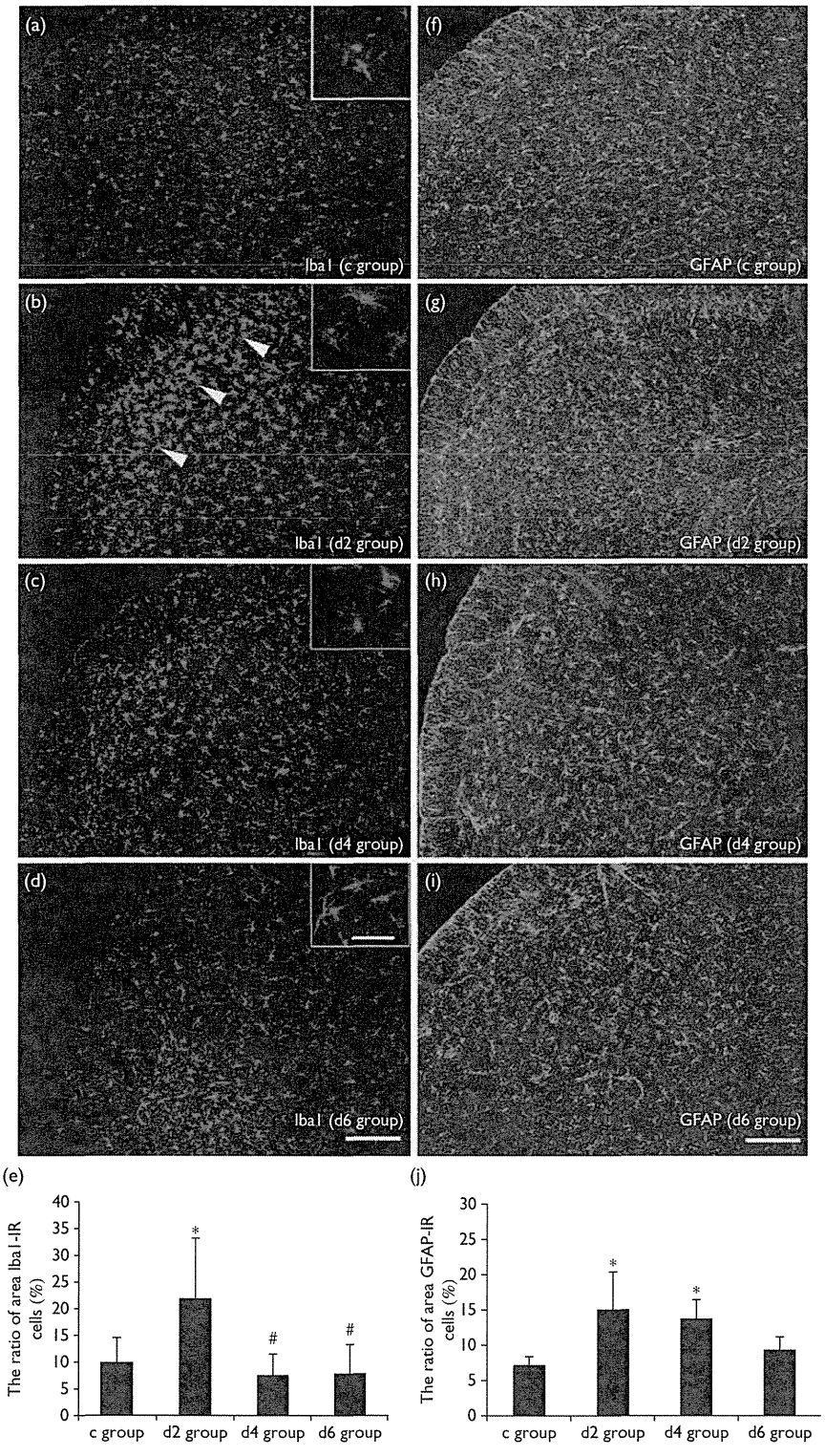
Data were presented as mean±SD. Differences among the groups were assessed by one-way analysis of variance, followed by individual post-hoc comparisons (Bonferroni's test), and P value less than 0.05 was considered significant (SPSS for Windows, version 19; SPSS Inc., Chicago, Illinois, USA).

Behavioral testing

Pain-related behaviors were induced by mechanical stimulation using home-made von Frey hairs (VFHs; diameter 0.5 mm; bending forces 34.3 mN). Rats were allowed to acclimate to their surroundings for 30 min before testing. During the procedure for assessment of sensitivity to mechanical stimuli, animals were restrained at the trunk with a towel to calm them and treated gently. Each VFH was applied ten times (once every 10 s) to the lateral facial skin, and the number of wiping instances by forelimb or withdrawal response was then counted [8]. The frequency of nocifensive behaviors (head withdrawal or wiping by the forelimb) was measured in 10 trials. Animals were subjected to the behavioral test on two occasions: before the injection [1 day before injection (pre 2)] and just before the first injection (pre 1). Subsequently, 10 mmol/l capsaicin (100 µl) was injected into the left whisker pad. The same injection procedure was repeated once a day for the designated period. Before each injection procedure, the behavioral test was carried out. In the control animals, mechanical sensitivity was assessed using the same protocol without the administration of capsaicin. All the behavioral tests were carried out by an examiner blinded to the identity of the animals.

Data were presented as mean±SD. Differences between groups were evaluated by one-way analysis of variance, followed by individual post-hoc comparisons (Bonferroni's test), and P value less than 0.05 was considered significant.

Fig. 1



Alterations in Iba1 (a–d) and glial fibrillary acidic protein (GFAP) (f–i) expressions in the ipsilateral trigeminal nucleus caudalis (TNC) by a repetitive capsaicin injection into the whisker pad. Vehicle injection for 2 days (a, f). Capsaicin injection for 2 days (b, g). Arrowheads indicate the superficial laminae of TNC. Capsaicin injection for 4 days (c, h). Capsaicin injection for 6 days (d, i). The scale bars = 200 μm. Enlargements in the insets show the alterations in the morphology of microglia. The scale bar in the inset = 50 μm. Quantitative analysis of Iba1-immunoreactivity (IR) cells and GFAP-IR cells (e, j). The ratio of area immunoreactive for Iba1 (e) and GFAP (j) to the total area of the ipsilateral TNC (a–i). The bars show the mean ± SD. *$P < 0.05$ versus c group. #$P < 0.05$ versus d2 group.

Results

Effect of trigeminal nociceptive stimulation on Iba1 and glial fibrillary acidic protein immunoreactivity in the trigeminal nucleus caudalis

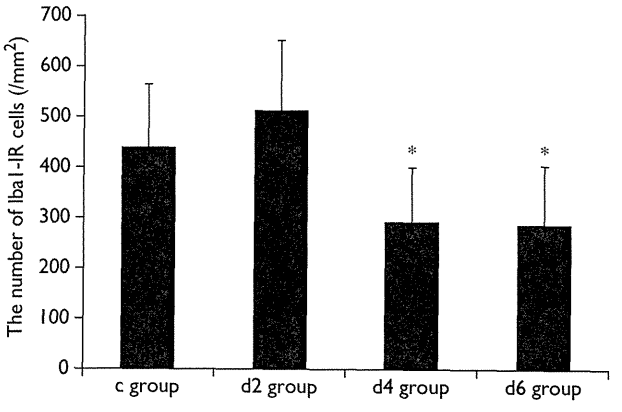
As shown in Fig. 1a, Iba1-IR was observed sparsely in the TNC in the c group. However, after trigeminal capsaicin stimulation, Iba1-IR was increased especially in the superficial laminae of TNC, where the central axons of trigeminal ganglion neurons make synaptic contact with the second-order trigeminal neurons, in the d2 group (Fig. 1b, arrowheads). In addition, marked hypertrophy of Iba1-IR cells was observed in the d2 group (Fig. 1b, inset). Morphologically, the majority of the hypertrophic microglia appeared to be ramified microglia, and only a few ameboid microglia were identified. There were fewer Iba1-IR cells and the extent of hypertrophy was less conspicuous in the d4 group and the d6 group as compared with the d2 group (Fig. 1c and d). Quantitatively, the ratio of the cross-sectional area of Iba1-IR cells to the TNC area was 9.9±4.7% in the c group, 23.7±11.4% in the d2 group, 7.4±4.1% in the d4 group, and 7.8±5.6% in the d6 group (mean±SD; Fig. 1e). There was a significant difference between the d2 group and the c group ($P < 0.05$). Also, the estimated numbers of Iba1-IR cells within the TNC region were 438.0±124.9/mm^2 in the c group, 511.8±139.8/mm^2 in the d2 group, 292.4±109.0/mm^2 in the d4 group, and 286.2±117.3/mm^2 in the d6 group (mean±SD; Fig. 2). The estimated numbers in the d4 group and the d6 group were significantly decreased, compared with the d2 group ($P < 0.05$). The alterations in GFAP-IR in TNC after capsaicin injections showed a temporal profile distinct from those of Iba1-IR. As shown in Fig. 1f, GFAP-IR was observed in the TNC before capsaicin stimulation in the c group. The IR of GFAP cells was also increased in the capsaicin injection groups (Fig. 1g–i), which was accompanied by cytoplasmic hypertrophy. However, there was a trend toward reversion to the basal level in the d6 group (Fig. 1i).

The ratio of the cross-sectional area of GFAP-IR cells to the TNC area was 7.3±1.2% in the c group, 15.1±5.4% in the d2 group, 13.8±2.7% in the d4 group, and 9.3±1.9% in the d6 group (mean±SD; Fig. 1j). There was a significant increase in the d2 group and the d4 group compared with the c group ($P < 0.05$). The number of GFAP-IR cells was not calculated in this study because of technical difficulty in the precise counting of these cells.

Effects of trigeminal nociceptive stimulation on nocifensive behaviors

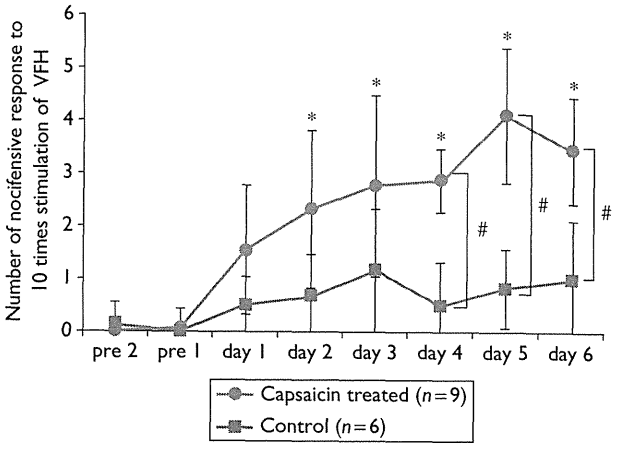
As shown in Fig. 3, the VFH stimulation procedure that we utilized did not induce a nocifensive response before trigeminal capsaicin stimulation (Fig. 3, pre 1 and pre 2). However, after the repetitive administration of capsaicin, the number of nocifensive responses increased gradually (day 1, 1.5±1.2 [mean±SD] times; day 2, 2.3±1.5 times; day 3, 2.8±1.7 times; day 4, 2.8±0.6 times; day 5, 4.1±1.3 times; day 6, 3.4±1.0 times) and the maximal number of nocifensive responses was observed on day 5. The number of nocifensive responses was significantly increased on days 2, 3, 4, 5, and 6 compared with pre 1 and pre 2. Meanwhile, the control animals did not show a significant change in the nocifensive responses during the observation period. Significant differences ($P < 0.05$) were found between the capsaicin-treated animals and the control animals at days 4, 5, and 6 (day 4, 0.5±0.8 times; day 5, 0.8±0.8 times; and day 6, 1.0±1.1 times).

Fig. 2



Quantitative analysis of Iba1-immunoreactivity (IR) cells per 1 mm^2 area of the ipsilateral trigeminal nucleus caudalis. The bars show the mean ± SD. *P<0.05 versus d2 group.

Fig. 3



Nocifensive responses to mechanical stimulation to the ipsilateral face using von Frey hair (VFH). The bars represent the mean ± SD. *P<0.05 versus pre 1 and 2. #P<0.05 capsaicin-treated versus control.

Discussion

The present study showed that repetitive TRPV1 stimulation at trigeminal nociceptors induced transient morphological alterations in the microglia and astrocytes in the TNC. Both microglia and astrocytes showed cytoplasmic hypertrophy and, consequently, the areas immunoreactive for their markers, Iba1 and GFAP, increased after TRPV1 stimulation. Such changes were the most conspicuous at day 2, after which immunoreactivities for both markers gradually became less marked. At day 2, microglia were recruited to superficial layers of the TNC, where the first-order trigeminal neurons make synaptic contact with the second-order neurons. The number of microglia was reduced after day 2. Furthermore, our data indicated a temporal discrepancy between the glial morphological alterations and the occurrence of mechanical allodynia. Several lines of evidence have indicated that glial cell activation is responsible for the induction of mechanical allodynia in neuropathic pain models [9,10]. In response to a nerve injury, microglia have been shown to be rapidly activated and involved in the initiation of chronic pain. Meanwhile, astrocytes are activated on a slower timeline, and are involved in the maintenance of chronic pain [11,12]. Hence, the present study has shown a novel pattern of glial activation, apparently irrelevant to the development of mechanical allodynia.

Chen *et al.* [6] have previously reported that a single intraplantar injection of capsaicin induced hypertrophic changes in the microglia and astrocytes in the dorsal horn of the mouse lumbar spinal cord at day 3, but they did not provide any quantitative data for a longer time period. Despite the differences in animal species and experimental settings, the rapidly induced morphological changes after capsaicin treatment appear to be a common feature of glial cells irrespective of their locations in neural tissue.

Whether the activation of microglia and astrocytes is related to the development of mechanical allodynia is controversial. As mentioned above, most studies using neuropathic pain models support the role of glial activation in the occurrence of allodynia [9,10]. However, in inflammatory pain caused by complete Freund's adjuvant injection, mechanical allodynia has been shown to be induced in a manner independent of glial activation [13,14]. It is inferred that the mode of action of glial cells differs in accordance with the experimental settings. Glial cells in the central nervous system serve a number of housekeeping functions required for healthy neuronal communication [9]. Particularly, microglia are known to play a role in the maintenance of synaptic integrity. Morphologically, resting microglia are ramified and they dynamically extend and retract their processes for surveillance of the microenvironment [15]. In response to nerve injury and brain ischemia, activated microglia migrate to synapses and secrete neurotrophic factors capable of repairing neurons [16,17]. In our study, we observed that Iba1-IR cells were recruited to the superficial laminae of the TNC, where the central axons of trigeminal ganglion neurons make synaptic contact with the second-order trigeminal neurons. We did not observe any upregulation of IL-1β, P_2X4, or phosphorylated p38, which have been shown to contribute to neuronal damage [18–20] (data not shown). Moreover, there were only a few microglia with thick and retracted processes (ameboid microglia), which are associated with their neurotoxic actions [21]. Hence, it is likely that the activation of microglia by repetitive peripheral TRPV1 stimulation in this study was neuroprotective. The number of microglia located in the TNC decreased at days 4 and 6. Microglia may have exited from the TNC. Alternatively, they may have died through an apoptotic process. Activation-induced microglial apoptotic death has been reported in inflammatory and ischemic conditions [22,23]. Moreover, the decrease in neuroprotective microglia may have contributed toward the development of mechanical allodynia. Interestingly, TRPV1 knockout mice have a higher density of microglia and astrocytes in the spinal dorsal horn as compared with wild-type mice [6], which raises the possibility that there is a certain TRPV1-mediated machinery that decreases glial numbers. Our observations that glial cell numbers declined after 2 days of capsaicin treatment may reflect such a modulatory role of TRPV1.

Our results have provided a novel insight into the roles of brainstem glial cells under the TRPV1-relevant pathological pain conditions, which include meningitis, pulpitis, and possibly migraine.

Conclusion

In summary, we have shown the novel dynamics of glial activation in a TRPV1-associated pain model. TRPV1 induced a transient activation of glial cells in temporal profiles discrepant from the development of mechanical allodynia, and activated microglia appeared to exert protective effects on trigeminal synaptic functions.

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Conflicts of interest

There are no conflicts of interest.

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Original Article

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Suppressive effect of chronic peroral topiramate on potassium-induced cortical spreading depression in rats

Miyuki Unekawa, Yutaka Tomita, Haruki Toriumi and Norihiro Suzuki

Abstract

Objective: To evaluate the chronic effect of topiramate (TPM) on cortical spreading depression (CSD), which is thought to be related to migraine aura.

Methods: Male rats (n = 30) were randomized to once-daily peroral treatment with TPM (50, 100, 200 or 600 mg/kg) or vehicle for 6 weeks. We evaluated the characteristics of CSD induced by topical application of KCl under isoflurane anesthesia and the changes in plasma level of TPM in each group. The effect of single administration of TPM on CSD was also evaluated.

Results: After the final administration of TPM, when the plasma level of TPM was high, KCl-induced CSD frequency and CSD propagation velocity were dose-dependently reduced and the interval between CSD episodes was elongated, compared with the vehicle control. However, before the final administration of TPM, when the plasma level was very low, the KCl-induced CSD profile was the same as that in the vehicle control. Single administration of TPM did not alter the CSD profile. Local cerebral blood flow was not significantly altered by chronic administration of TPM.

Conclusion: TPM suppressed the frequency and propagation of CSD along the cerebral cortex, and might be a candidate for relief of migraine.

Keywords

Topiramate, cortical spreading depression, chronic treatment, migraine, prophylaxis

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Introduction

Migraine is a common, chronic, incapacitating neurovascular disorder characterized by severe headache, autonomic nervous system dysfunction, and in some patients an aura involving neurologic symptoms (1). Recently developed selective serotonin receptor agonists, such as triptans, are useful to treat migraine, but strict compliance with the dosing schedule is essential for effective treatment. Furthermore, frequent migraine attacks may lead to excessive acute medication without proper supervision, potentially resulting in medication-overuse headache (2). Therefore, prophylaxis of migraine is an important goal.

The pathophysiology of migraine is very complex, but one of the main correlates is thought to be cortical spreading depression (CSD) (3,4). CSD is a transient neuronal depolarization that slowly propagates along the cerebral cortex, followed by long-lasting suppression of neuronal activity (5). It has been proposed that CSD is a neuronal mechanism underlying migraine aura (6) and is involved in vasodilation of the middle meningeal artery during headache, which is linked to changes of neurometabolic brain activity with transmission via the trigeminal nerve (7).

A β-adrenoceptor antagonist, propranolol, has been widely used in the prophylaxis of human migraine (8) and has been shown to suppress CSD in an animal model (9). However, the mechanism of its action remains unclear. Topiramate (TPM; 2,3:4,5-bis-*O*-(1-methylethylidene)-beta-D-fructopyranose sulfamate; $C_{12}H_{21}NO_8S$), which is used as an anticonvulsant

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