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Research Paper

Phosphorylation of TRPV1 by cyclin-dependent kinase 5 promotes TRPV1 surface localization, leading to inflammatory thermal hyperalgesia

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ABSTRACT

Cyclin-dependent kinase 5 (Cdk5) is an important serine/threonine kinase that plays critical roles in many physiological processes. Recently, Cdk5 has been reported to phosphorylate TRPV1 at threonine 407 (Thr-407) in humans (Thr-406 in rats), which enhances the function of TRPV1 channel and promotes thermal hyperalgesia in the complete Freund's adjuvant (CFA)-induced inflammatory pain rats. However, the underlying mechanisms are still unknown. Here, we demonstrate that Cdk5 phosphorylates TRPV1 at Threonine 406 and promotes the surface localization of TRPV1, leading to inflammatory thermal hyperalgesia. The mutation of Thr-406 of TRPV1 to alanine reduced the interaction of TRPV1 with the cytoskeletal elements and decreased the binding of TRPV1 with the motor protein KIF13B, which led to reduced surface distribution of TRPV1. Disrupting the phosphorylation of TRPV1 at Thr-406 dramatically reduced the surface level of TRPV1 in HEK 293 cells after transient expression and the channel function in cultured dorsal root ganglion (DRG) neurons. Notably, intrathecal administration of the interfering peptide against the phosphorylation of Thr-406 alleviated heat hyperalgesia and reduced the surface level of TRPV1 in inflammatory pain rats. Together, these results demonstrate that Cdk5-mediated phosphorylation of TRPV1 at Thr-406 increases the surface level and the function of TRPV1, while the TAT-T406 peptide can effectively attenuate thermal hyperalgesia. Our studies provide a potential therapy for inflammatory pain.

1. Introduction

Cyclin-dependent kinase 5 (Cdk5), a unique member of the cyclin-dependent kinase family, is a small proline-directed serine/threonine kinase that exists in almost all mammalian tissues but has the highest activity in the nervous system due to the restricted expression of its specific activators, p35, p25, p39 and p29, in neurons (Lew et al., 1994; Patzke and Tsai, 2002; Tang et al., 1995; Tsai et al., 1994; Tsai et al., 1993). Cdk5 is crucial for many nervous system events such as neuronal migration, neuronal differentiation, synapse development, synaptic plasticity and neuron survival (Gilmore et al., 1998; Meyer et al., 2014; Mishiba et al., 2014; Nikolic et al., 1996; Ohshima et al., 1999; Ohshima et al., 1996; Tanaka et al., 2001; Wang et al., 2006; Xie et al., 2009).

The expression and activity of Cdk5/p35 in the peripheral nervous system were first reported by Ino et al., without a demonstration of its specific molecular functions (Ino et al., 1994). Intrathecal administration of the Cdk5 inhibitor roscovitine effectively attenuates antinociceptive morphine tolerance (Wang et al., 2004) and the formalin-induced nociceptive response in rats (Wang et al., 2005), indicating that Cdk5 may be involved in nociception. Cdk5 and p35 are expressed in the primary afferent neurons of the dorsal root ganglion (DRG), trigeminal ganglion (TG) as well as in spinal cord (SC) and Cdk5 activity is increased during the peripheral inflammatory response (Pareek et al., 2006). More importantly, p35 knockout mice ($p35^{-/-}$), in which Cdk5 activity is significantly reduced, show delayed responses to painful thermal stimulation (Pareek et al., 2006). In addition, our previous studies revealed that Cdk5/p35 are expressed in dorsal horn neurons and that CFA-induced activation of Cdk5 contributes to inflammatory thermal hyperalgesia (increased sensitivity to noxious heat) but not to mechanical allodynia (Yang et al., 2007). Some inflammatory mediators released during the inflammatory response have been reported to be regulators of Cdk5 activity, including neurotrophins, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF), and several cytokines, such as interleukin-6 (IL-6), interleukin-18 (IL-18), interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α) and transforming growth factor-β1 (TGF-β1) (Bogen et al., 2008; Harada et al., 2001; Ojala et al., 2008;

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Quintanilla et al., 2004; Song et al., 2005; Tokuoka et al., 2000; Utreras et al., 2009b; Utreras et al., 2012). Cdk5 can also inhibit neurotransmitter release through phosphorylation and downregulation of the activity of P/Q-type voltage-gated calcium channels (VGCCs), which are involved in pain processing (Kim and Ryan, 2010; Tomizawa et al., 2002; Zamponi et al., 2009). The evidence described above indicates that Cdk5 and its activators play a critical role in inflammatory heat pain signaling. However, the regulatory mechanisms of Cdk5 in thermal sensitization are not clear yet.

The transient receptor potential vanilloid 1 (TRPV1) is a calcium-permeable, nonselective cation channel gated by heat, low pH or endogenous ligands termed "endovanilloids" and is highly expressed in primary sensory neurons, which can detect a variety of noxious stimuli that cause pain, inflammation and hyperalgesia (Wang, 2008). The function of TRPV1 is regulated by many types of protein kinases, such as MAPK, PKD, PKC, PKA, CaMKII, PI3K and Cdk5 (Bhave et al., 2002; Holland et al., 2011; Jeske et al., 2008; Ji et al., 2002; Stein et al., 2006; Yang et al., 2007; Zhang et al., 2007; Zhu et al., 2008; Zhu and Oxford, 2007). TRPV1 channel is a confirmed downstream target of Cdk5 in heat hyperalgesia, and human TRPV1 has been reported to be directly phosphorylated at Thr-407 (threonine −406 in rats) by Cdk5, increasing capsaicin-induced Ca^{2+} influx, whereas conditional deletion of Cdk5 in pain-sensing neurons causes abrogation of TRPV1 Thr-407 phosphorylation and induces hypoalgesia (Pareek et al., 2007). As a transmembrane receptor, TRPV1 channel function can be potentiated by increasing its membrane expression level (Lainez et al., 2010; Shimizu et al., 2012), which is a potential mechanism underlying proinflammatory mediator-induced heat hyperalgesia as has been reported for GluR1 and Nav1.8 (Choi et al., 2010; Swanwick et al., 2010). Our previous study indicates that Cdk5 can bind and phosphorylate Thr-506 of the motor protein KIF13B to regulate the interaction of TRPV1 and KIF13B, promoting the cell membrane translocation of TRPV1, which leads to thermal hyperalgesia (Xing et al., 2012). These findings provide further evidence of the mechanism of the functional regulation of TRPV1 by Cdk5 in pain sensation. All of these studies suggest that TRPV1 Thr-407 phosphorylation may affect cell-surface expression of TRPV1. Here, we used in vitro and in vivo approaches to investigate the role of Cdk5-mediated phosphorylation of TRPV1 at Thr-407 in inflammatory pain signaling.

2. Materials and methods

2.1. Plasmids, antibodies, and chemicals

cDNA fragments encoding the corresponding domains of KIF13B and TRPV1 were generated by PCR amplification from a rat brain cDNA pool and subcloned into pEGFP–C2 (Clontech, Palo Alto, California, USA) and the EGFP tag was at the C terminal of TRPV1 or KIF13B motor FHA (the motor and forkhead-associated domains of KIF13B, 1–557 amino acids) (Xing et al., 2012). A mutation (T406A) within TRPV1 was created with the QuikChange site-directed mutagenesis kit (Stratagene, Santa Clara, California, USA). All plasmids were verified by DNA sequencing.

All drugs and antibodies were purchased from Sigma Aldrich (St Louis, Missouri, USA) unless otherwise stated. The polyclonal anti-TRPV1 (P-19), anti-GFP (FL), anti-Cdk5 (C-8) and anti-p35 (C-19) antibodies were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). The polyclonal rabbit anti-TRPV1 (extracellular) antibody was purchased from Alomone Labs (Jerusalem, Israel). The monoclonal anti-β-actin (TA-09) antibodies and rabbit anti-goat secondary antibodies were purchased from Zhongshan Goldenbridge Biotechnology. The TAT fusion peptides were synthesized by GL Biochem, Shanghai, China.

2.2. Cell culture and treatment

HEK 293 cells were routinely cultured in DMEM supplemented with 10% FBS. F11 cells were routinely cultured in Ham's F-12 medium supplemented with 20% FBS and 1 × HAT (sodium hypoxanthine, aminopterin, and thymidine; Sigma-Aldrich, St. Louis, Missouri, USA). Cell transfections were performed with Lipofectamine 2000 (Invitrogen, Carlsbad, California, USA). Cells were harvested 48 h after transfection.

2.3. DRG culture

DRGs aseptically removed from neonatal rats (10–15 d) were digested with 0.25% trypsin (Sigma-Aldrich, St. Louis, Missouri, USA) for 35 min at 37 °C, followed by trituration with a flame-polished Pasteur pipette. Dissociated cells were collected and resuspended in plating medium (DMEM containing 10% FBS), and 2×10^5 DRG neurons were plated onto a 35 mm dish coated with poly-D-lysine (Sigma-Aldrich, St. Louis, Missouri, USA). After 15 min, the medium was replaced with Neurobasal medium supplemented with 2% B27, 2 mM GlutaMAX-I (Invitrogen, Carlsbad, California, USA), and 100 ng/ml mouse NGF (Promega, Madison, Wisconsin, USA). Five micromolar AraC (Sigma-Aldrich, St. Louis, Missouri, USA) was added to the culture 18–24 h after plating and maintained until the end of the experiments. The cultures were treated with the TAT fusion peptides (3 μM) on day 3.

2.4. Western blot

Cells were homogenized in ice-cold lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 10% glycerol, 1% Triton X-100, 5 mM EGTA, 0.5 μg/ml leupeptin, 1 mM PMSF, 1 mM Na_3VO_4, 10 mM NaF, and a proteinase inhibitor mixture) and rotated at 4 °C for 20 min before the supernatant was extracted by centrifugation at 12,000 × *g* at 4 °C for 5 min. Equal amounts of the protein extracts were denatured and subjected to SDS-PAGE. After separation, proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, California, USA). The membranes were blocked with 5% nonfat milk in TBST (25 mM Tris, pH 7.4, 137 mM NaCl, 2.7 mM KCl, and 0.05% Tween 20) for 1 h at room temperature and incubated with an anti-TRPV1 antibody (1:200 dilution), anti-β-actin (1:2000 dilution) or anti-GFP antibody (1:1000 dilution) overnight at 4 °C. After washing three times with TBST, the membranes were incubated with rabbit anti-goat (Zhongshan Goldenbridge Biotechnology, Beijing, China) (1:2000 dilution) or goat anti-mouse (Sigma-Aldrich, St. Louis, Missouri, USA) (1:5000 dilution) secondary antibody overnight at 4 °C, then washed again, and finally developed with ECL solution (Santa Cruz Biotechnology, Dallas, Texas, USA).

2.5. Cell-surface biotinylation assay

Surface biotinylation was performed on HEK 293 cells and acutely dissociated DRG neurons following established protocols (Xing et al., 2012; Zeng et al., 2013). Cells were biotinylated with 500 μg/ml EZ-link sulfo-NHS-LC-Biotin (Thermo, Scientific, Waltham, Massachusetts, USA) in PBS +/+ (PBS containing Ca^{2+} and Mg^{2+}) solution at 4 °C for 30 min. Unreacted biotin was quenched using PBS +/+ solution that contained 0.1 M glycine for 15 min. Then, cells were lysed in RIPA buffer (25 mM Tris, 137 mM NaCl, 2.7 mM KCl, 1% Triton X-100, 0.1% SDS, pH 7.4, and a proteinase inhibitor mixture). A 10% volume of the lysate was saved for the determination of total protein, and the remainder was incubated with NeutrAvidin plus Ultralink beads (Thermo, Scientific, Waltham, Massachusetts, USA) overnight at 4 °C. After three washes with RIPA buffer, bound proteins were eluted with 6 × SDS loading buffer by boiling for 5 min and were analyzed by Western blot with an anti-TRPV1 antibody.

2.6. Coimmunoprecipitation

For coimmunoprecipitation (Co-IP), protein samples (400–500 μg of protein) extracted from transfected HEK 293 cells were incubated at 4 °C for 3 h with 2 μg of anti-TRPV1 antibody before being incubated

with protein A-Sepharose CL-4B resin (GE Healthcare, Little Chalfont, Buckinghamshire, UK) overnight at 4 °C. The next day, the beads were washed three times with TBS containing 0.1% Triton X-100 and then eluted with 6 × SDS loading buffer by boiling for 5 min and immunoblotted with an anti-GFP antibody.

2.7. Immunofluorescence

Transfected HEK 293 cells that were grown on glass coverslips were blocked (3% FBS in PBS, 30 min at room temperature), and surface channels were labeled with a rabbit anti-TRPV1 (extracellular) antibody (1:800 dilution) (Alomone, Jerusalem, Israel) at 4 °C for 2 h. Unbound antibody was removed by washing three times with PBS. Cells were fixed for 15 min with ice-cold 4% paraformaldehyde (PFA) in PBS and then were labeled with Alexa Fluor-594-conjugated goat anti-rabbit secondary antibody (Invitrogen, Carlsbad, California, USA). After extensive washing with PBS, the coverslips were mounted with 70% glycerol in PBS.

2.8. Animals

Male Sprague–Dawley (SD) rats (180–220 g) were used. All experiments were performed according to the guidelines of the Animal Care and Use Committee of Peking University. The animals were housed in climate-controlled rooms on a 12/12-h light–dark cycle with free access to food and water. For the intrathecal delivery of drugs, a PE-10 polyethylene catheter was implanted into the intrathecal space, reaching the lumbar enlargement of the spinal cord (Yang et al., 2007). Surgeries were performed under 10% chloral hydrate anesthesia (0.3 g/kg, i.p.), and rats were allowed 4–5 d for recovery.

2.9. Behavioral assessment

Heat sensitivity was assessed by measuring the paw-withdrawal latency in response to a radiant heat stimulus applied to the core of the plantar surface of the hindpaw (Hargreaves et al., 1988; Zhu et al., 2008). Mechanical sensitivity was tested using von Frey filaments (Stoelting, Wood Dale, Illinois, USA). The paw-withdrawal threshold was calculated using the up–down method (Chaplan et al., 1994). The basal heat sensitivity and basal mechanical sensitivity of the rats that had recovered from the catheter implantation surgery were tested before drug administration. Ten microliters of TAT-T406 peptide (1 μg/μl) or TAT-T406A peptide (1 μg/μl) was intrathecally administered 30 min before the injection of 100 μl of 25% CFA (CFA was diluted with Incomplete Freund's Adjuvant) into the left hindpaw. The heat hyperalgesia and mechanical allodynia of the rats were assessed 1 h, 2 h, 6 h, and 1 d after the CFA injection. For the experiments assessing the effect of TAT-T406 peptide on basal heat sensitivity, the same doses of TAT peptides were injected intrathecally, and basal heat sensitivity was tested 1.5 h, 2.5 h, 6.5 h, and 1 d after the intrathecal injection.

2.10. Isolation of DRG neurons and calcium imaging

Isolation of DRG neurons and Fura-2-AM-based Ca^{2+} imaging experiments was performed. Rats were treated with intrathecal delivery of 10 μl of TAT-T406 peptide (1 μg/μl) or TAT-T406A peptide (1 μg/μl) for 30 min before the injection of 100 μl of 25% CFA into the left hindpaw. Two hours after the CFA injection, the left L4 and L5 DRGs were rapidly dissected out and treated with 1.5 mg/ml collagenase D (Sigma-Aldrich, St. Louis, Missouri, USA) for 45 min and with 0.125% trypsin (Sigma-Aldrich, St. Louis, Missouri, USA) for 9 min at 37 °C. Then, the ganglia were washed three times with extracellular solution (ES), containing 130 mM NaCl, 5 mM KCl, 2 mM KH_2PO_4, 1.5 mM $CaCl_2$, 6 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.2 (osmolarity, 305 mOsm), followed by gentle trituration with a flame-polished Pasteur pipette. Dissociated cells suspended in 100 μl of ES were plated on a poly-D-lysine-coated confocal dish, and 2 ml of ES was added to each dish 15 min later. Neurons were maintained at 37 °C for 1 h. Then, DRG neurons were loaded with 5 μM Fura-2 AM (Biotium, Hayward, California, USA) in ice-cold ES for 40 min at room temperature. After loading, the neurons were washed with ES and left in ES at room temperature in the dark for 1 h. For calcium imaging, an inverted fluorescence microscope equipped with a 340 and 380 nm excitation-filter changer (Olympus, Tokyo, Japan) and a computer with MetaFluor software were used. Fluorescence images and the F 340/F 380 ratio were acquired every 5 s. TRPV1 activation was evoked by the addition of 1 μM capsaicin, and the cells were considered to be responsive if the ratio increased by 20% after capsaicin application. Non-responsive neurons that also failed to respond to 25 mM KCl were discarded from the analysis. Cells derived from at least three separate isolations were analyzed.

2.11. Statistical analyses

Data are expressed as the mean ± SEM. Statistical analyses were performed using Prism 4.0 software. Differences between groups were compared using either Student's *t* test or one-way ANOVA followed by Newman–Keuls post-hoc tests or by two-way repeated-measures ANOVA followed by Bonferroni's post-hoc tests. Statistical significance was set at $P < 0.05$. For Western blot, the immunoreactive bands were scanned and analyzed quantitatively by densitometry with TotalLab software.

3. Results

3.1. Phosphorylation of TRPV1 at Thr-406 by Cdk5 regulates the interaction of TRPV1 and cytoskeletal elements

It is well-known that transmembrane protein synthesis is followed by screening and folding in the endoplasmic reticulum (ER). Furthermore, after modification in the Golgi apparatus, mature protein is transported to the plasma membrane in vesicles. Kinesin superfamily proteins are important molecular motors that directionally transport various cargos, including transmembrane receptors (Hirokawa and Takemura, 2004). Our previous study has demonstrated that Cdk5 positively regulates TRPV1 membrane trafficking by mediating the KIF13B-TRPV1 association and treatment with roscovitine substantially decreased the surface TRPV1 levels (Xing et al., 2012). Cdk5 has been reported to phosphorylate TRPV1 at Thr-407 in humans (Thr-406 in rats), which is thought to be involved in thermal hyperalgesia (Pareek et al., 2007). To explore whether this phosphorylation affects TRPV1 transport, HEK 293 cells were transfected with GFP-TRPV1 or GFP-TRPV1-T406A (with a mutation from threonine to alanine at amino acid 406) plasmids. The cells were lysed with 1% Triton X-100 in lysis buffer to extract the cytoskeletal proteins (including plasma membrane protein), which were in the insoluble fraction (pellet). Interestingly, we found that TRPV1 protein level in the pellet fraction was dramatically reduced in the GFP-TRPV1-T406A group compared with the WT group ($P = 0.0311$, $n = 4$) while total TRPV1 levels did not differ between the two groups ($P = 0.6098$, $n = 4$) (Fig. 1A). This result indicates that phosphorylation at Thr-406 could effectively promote the interaction between TRPV1 and the cytoskeletal elements, which is critical for TRPV1 translocation. As our previous study has shown, KIF13B, an N-kinesin motor protein, is important for TRPV1 anterograde transport, dependent on binding with TRPV1 and on phosphorylation at Thr-506 of KIF13B by Cdk5 (Xing et al., 2012). KIF13B has a forkhead-associated domain (FHA domain) which is a phosphor-specific protein–protein interaction motif (Durocher et al., 1999; Westerholm-Parvinen et al., 2000). We believe that this modular phosphopeptide recognition motif interacts with TRPV1 under conditions of TRPV1 Thr-406 phosphorylation. To confirm this hypothesis, coimmunoprecipitation assays were performed using HEK293 cells co-transfected with GFP-TRPV1/GFP-

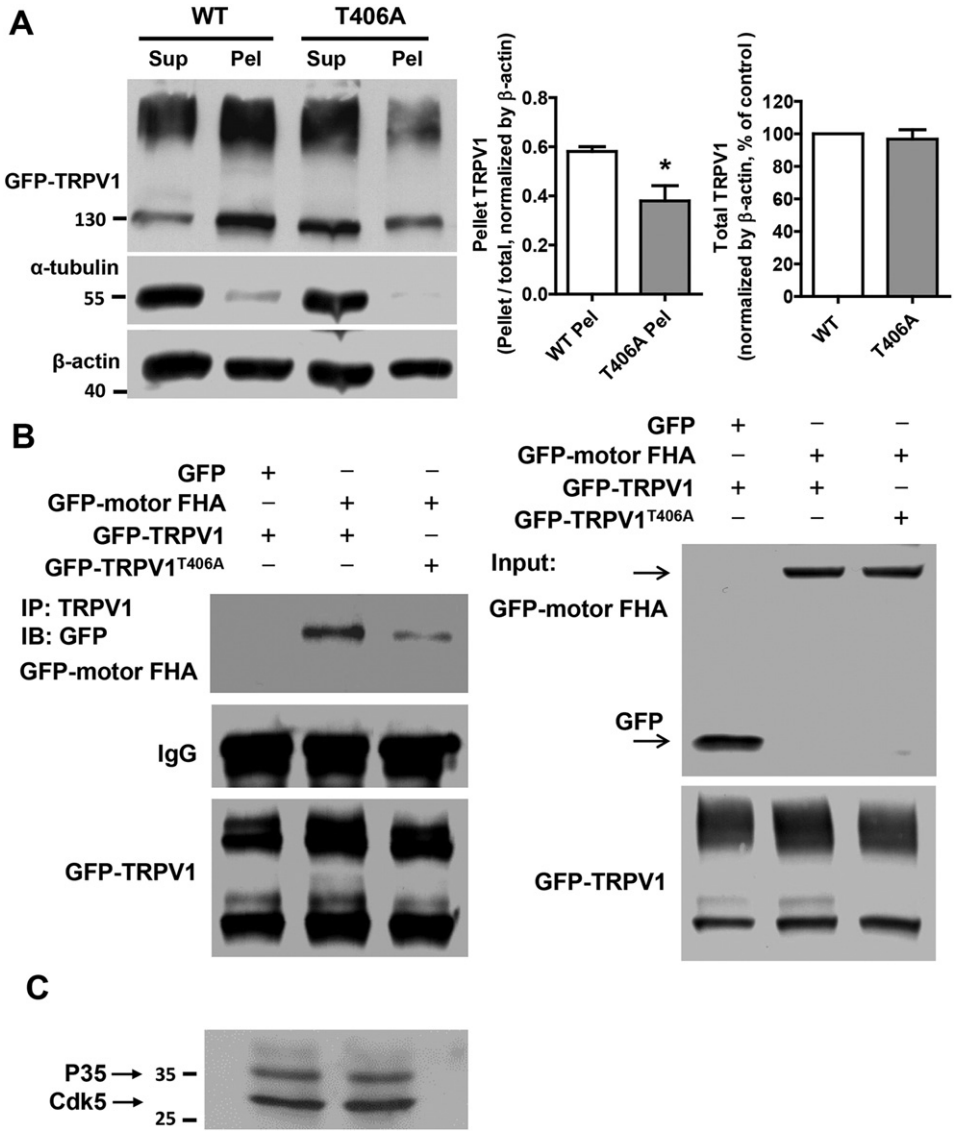

Fig. 1. Thr-406 phosphorylation promotes the interaction of TRPV1 with the cytoskeletal elements. (A) HEK 293 cells transfected with GFP-TRPV1 or GFP-TRPV1-T406A plasmids for 48 h were lysed with 1% Triton X-100 in lysis buffer and centrifuged for 14,000 ×*g* for 10 min. The cytoskeleton protein was in the insoluble section (pellet) and cytoplasmic protein was in the soluble section (supernatant). Mutation of T406 decreases the protein level of TRPV1 in the pellet fraction including constituents of the cytoskeleton. The quantitative analyses show the ratio of pellet TRPV1 to total TRPV1 normalized by β-actin and the total TRPV1 normalized by β-actin. Data were analyzed by two-tailed paired *t* test. $^{*}P < 0.05$, n = 4. Error bars indicate SEM. (B) HEK 293 cells were co-transfected with GFP-TRPV1 or GFP-TRPV1-T406A and GFP-motor-FHA or GFP vector plasmids. Cell lysates were immunoprecipitated with an anti-TRPV1 antibody, and the immunoblots were detected with an anti-GFP antibody. Input represents the direct immunoblotting of the lysates. (C) HEK293 cell lysates were immunoblotted with anti-Cdk5 and anti-p35 antibodies.

TRPV1-T406A and GFP vector/GFP-motor FHA (1–557 aa of KIF13B). Results showed that the mutation of Thr-406 to alanine remarkably reduced the binding of TRPV1 to the KIF13B motor FHA domain (Fig. 1B), suggesting that Thr-406 phosphorylation at least partially regulated TRPV1 transport. Considering the general concern about the low expression of Cdk5 and its activator p35 in HEK293 cells, we examined the protein levels of Cdk5 and p35, which were detectable (Fig. 1C), suggesting the existence of active Cdk5 in this cell line. The above results indicate that TRPV1 distribution and it's interaction with cytoskeletal elements, like KIF13B, are regulated by the Thr-406 phosphorylation of TRPV1 by Cdk5.

3.2. Phosphorylation of TRPV1 at Thr-406 by Cdk5 promotes surface localization of TRPV1

Since TRPV1 transport was regulated by phosphorylation at Thr-406, and our previous study has shown that the Cdk5 inhibitor roscovitine can significantly decrease TRPV1 surface levels and overexpression of Cdk5 increases and overexpression of D144N–Cdk5 decreases the levels of surface TRPV1 (Xing et al., 2012), we attempt to reveal direct evidence of contribution of this phosphorylation to the subcellular distribution of TRPV1. HEK 293 cells were transiently transfected with GFP-TRPV1 or GFP-TRPV1-T406A plasmids for 48 h, and a biotinylation assay was performed to detect the cell-surface protein level of TRPV1. The ratio of surface TRPV1 level to total TRPV1 level was reduced by 75% ($P = 0.0057$, n = 4) in cells transfected with GFP-TRPV1-T406A compared to the WT control, whereas the total amount of TRPV1 was not significantly altered ($P = 0.6942$, n = 4) (Fig. 2A). We also did the same experiment in F11 cells and observed a more pronounced decrease of surface TRPV1 protein level ($P < 0.0001$, n = 3) (Fig. 2B). We concurrently performed immunofluorescence staining experiments with an antibody specific to the extracellular domain of TRPV1. HEK 293 cells transiently transfected with GFP-TRPV1 or with GFP-TRPV1-T406A were incubated with anti-TRPV1e (extracellular) antibody at 4

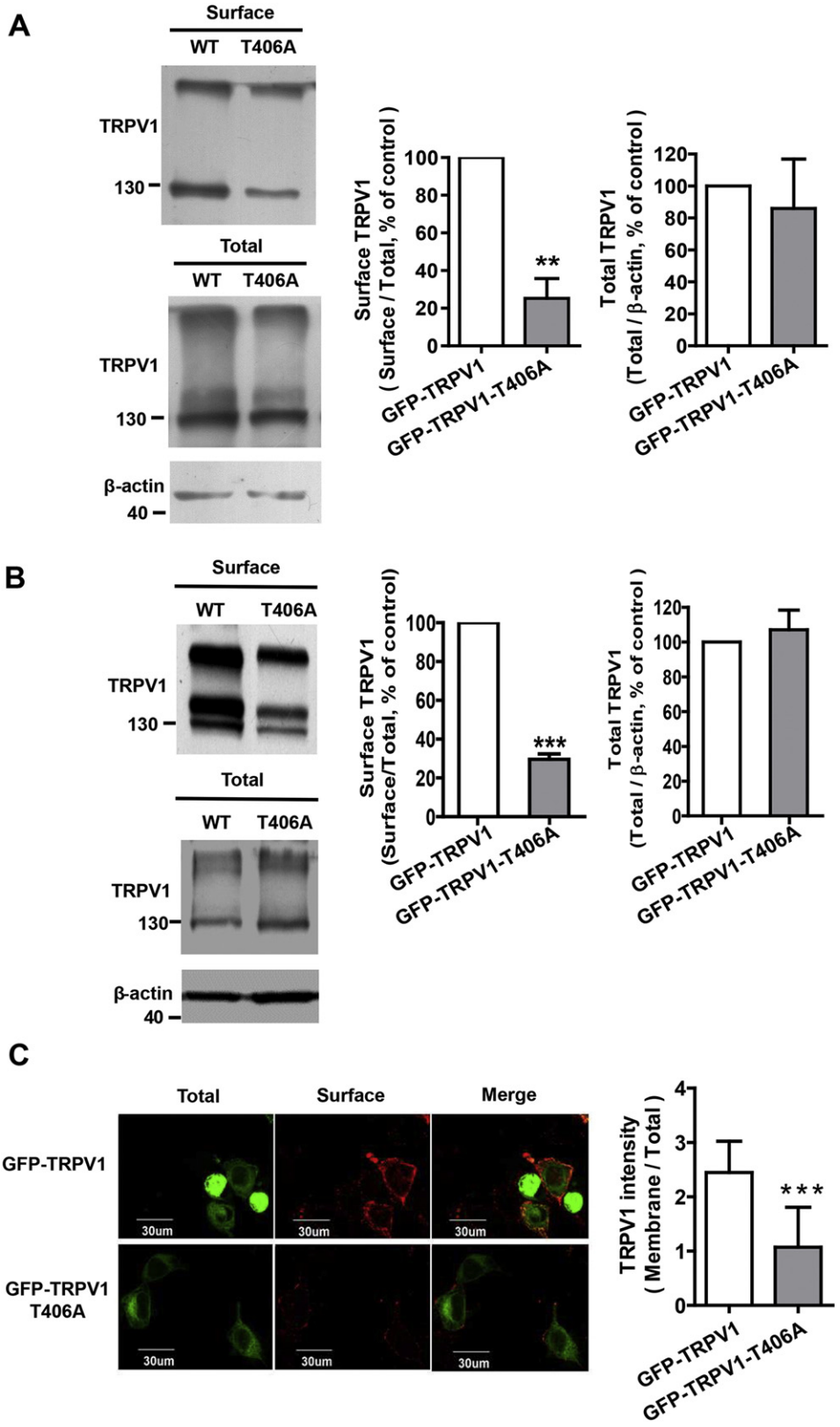

Fig. 2. Surface TRPV1 is decreased in GFP-TRPV1-T406A-transfected cells. (A) A surface biotinylation assay was performed (left), and a significant decrease in surface TRPV1 was found in the HEK 293 cells transiently transfected with GFP-TRPV1-T406A compared with cells in the GFP-TRPV1 group. $^{**}P < 0.01$, n = 4 (center). And there was no significant difference in the amount of total protein between these two groups (right). Data were analyzed by two-tailed paired *t* test. Error bars indicate SEM. (B) F11 cells transfected with GFP-TRPV1 or GFP-TRPV1-T406A were subjected to biotinylation assays (left). Quantitative analysis shows the ratio of surface to total TRPV1 normalized to the WT control (center) and the total TRPV1 protein levels normalized to the WT control (right). $^{***}P < 0.001$, n = 3. Data were analyzed by two-tailed paired *t* test. Error bars indicate SEM. (C) Representative images are shown. Green indicates total TRPV1 and red indicates cell-surface TRPV1 (left). A significant reduction in average fluorescence intensity was observed in cells transfected with GFP-TRPV1-T406A compared with cells transfected with GFP-TRPV1. $^{***}P < 0.001$; GFP-TRPV1, n = 41; GFP-TRPV1-T406A, n = 60. Error bars indicate SEM (right).

°C to limit internalization of the channel, followed by A594-labeled secondary antibody to label cell-surface TRPV1. GFP expression indicated the total amount of TRPV1 (Fig. 2C left). Quantitative analysis showed that the ratio of red fluorescence intensity to GFP intensity in the GFP-TRPV1-T406A group was approximately 55% lower than in the WT control group ($P < 0.0001$, WT-TRPV1, n = 41 vs TRPV1-T406A, n = 60) (Fig. 2C right). All the results described above indicate that phosphorylation of TRPV1 at Thr-406 profoundly and directly promotes the distribution of TRPV1 at the cell surface.

3.3. Disruption of Thr-406 phosphorylation efficiently reduces membrane levels and function of TRPV1

To further confirm the role of Thr-406 phosphorylation in TRPV1 membrane trafficking, we constructed a peptide comprising 15 aa of the TRPV1 N-terminal, including the Threonine 406 (the phosphorylation site of Cdk5), and rendered it cell permeable by fusing it with the HIV TAT protein sequence (RKKRRQRRR) (termed TAT-T406). The other researchers and our previous studies confirmed that the peptide generated by targeting the specific phosphorylation site by certain protein kinase could disrupt endogenous phosphorylation at this site in the targeting substrate (Pareek et al., 2007; Xie et al., 2009; Xing et al., 2012). Therefore, in this study, we used TAT-T406 peptide (targeting Thr-406 of TRPV1) to disrupt endogenous Thr-406 phosphorylation at TRPV1. As a control peptide, Thr-406 was replaced with alanine (termed TAT-T406A) (Fig. 3A). According to our previous study, the concentration of TAT fusion peptide increased in a time-dependent manner and peaking at 1 and 3 h after injection (Xie et al., 2009). HEK 293 cells transiently transfected with GFP-TRPV1 were treated with 10 μM TAT fusion peptide for 4 h. Subsequently, biotinylation assays showed that the TAT-T406 peptide significantly decreased the amount of surface TRPV1 without affecting total TRPV1 expression ($P = 0.009$, n = 4) (Fig. 3B). Thus, Thr-406 phosphorylation is indeed a critical determinant in TRPV1 trafficking to the membrane. To observe the channel function of TRPV1, primary cultured DRG neurons were pretreated with 3 μM TAT-T406 peptide for 5 h and then subjected to calcium imaging assays. DRG neurons responding to 25 mM KCl were identified and analyzed to confirm cell activity. The average capsaicin-induced calcium influx was effectively suppressed in cells treated with TAT-T406 peptide compared with the influx in cells treated with TAT-T406A peptide ($P = 0.0231$) (Fig. 3C left), indicating an attenuation of the activity of functional TRPV1. The number of capsaicin-sensitive neurons was greatly reduced by TAT-T406 peptide treatment ($P = 0.0115$, n = 4) (Fig. 3C right). For further exploration, the capsaicin-sensitive cells were analyzed separately, and the calcium influx exhibited an approximately 30 s delayed response to capsaicin after TAT-T406 peptide treatment, although there is no difference of the magnitude of the Ca^{2+} response between two groups (Fig. 3D left). Measurements of the area under the curve within 30 s after the beginning of the capsaicin stimulation revealed lower response of the TAT-T406 peptide-pretreated neurons to the capsaicin ($P = 0.0481$) (Fig. 3D right), suggesting an obvious inhibition of TRPV1 channel function by the TAT-T406 peptide. Thus, these results suggest that the TAT-T406 peptide could significantly suppress TRPV1 function by reducing the cell-surface protein level of TRPV1.

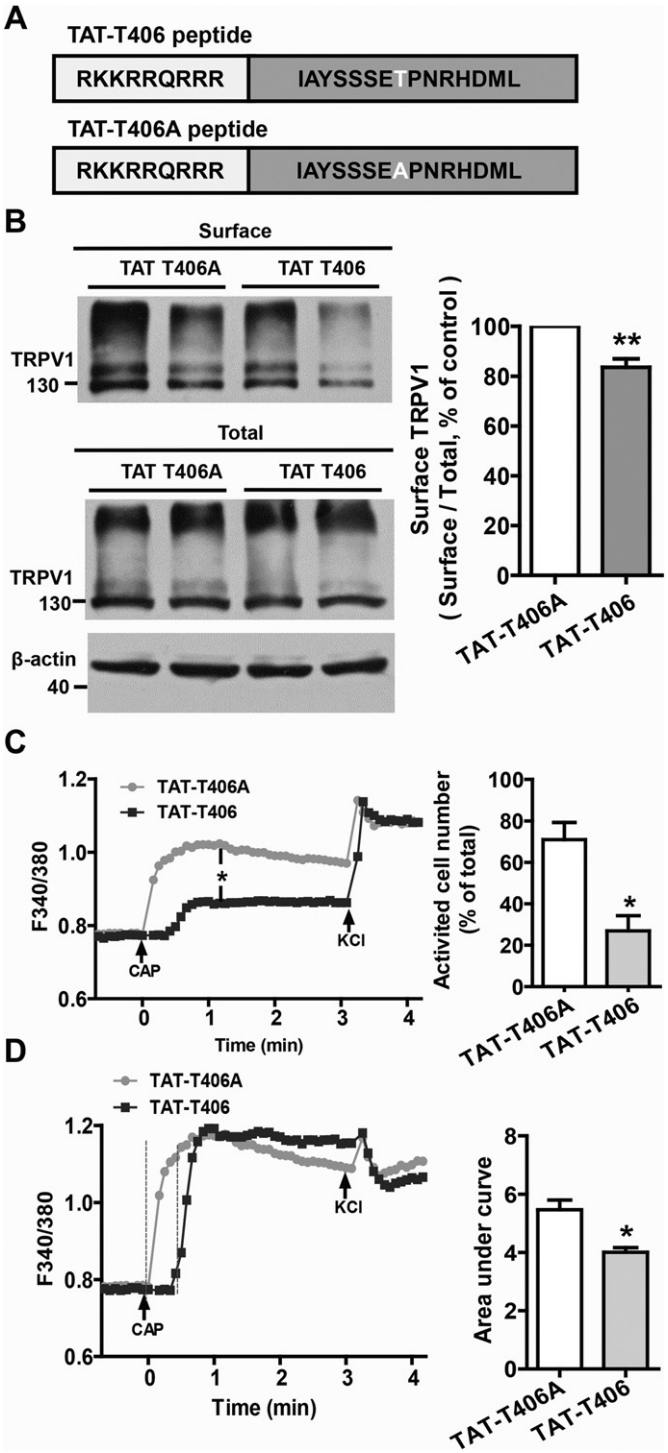

Fig. 3. TAT-T406 significantly attenuates the surface level and function of TRPV1 in HEK 293 cells and in primary cultures of DRG neurons. (A) Schematic diagram of the TAT-T406 peptide and TAT-T406A control peptide. (B) Transfected HEK 293 cells were pretreated with TAT-T406 or TAT-T406A peptide (10 μM) for 4 h and then subjected to biotinylation assays (left). The quantitative analysis shows the comparative levels of surface TRPV1 over the TAT-T406A control after normalizing to total TRPV1 which is normalized by β-actin. $^{**}P < 0.01$, n = 4. Error bars indicate SEM (right). (C) Representative traces show the typical average change in Ca^{2+} influx of DRG neurons in each group (left). Application of capsaicin or KCl is shown with arrows. For calculation, neurons from one of the four independent experiments (TAT-T406, n = 24 vs TAT-T406A, n = 34) were analyzed by two-way ANOVA, followed by Bonferroni's post-hoc tests. $^{*}P < 0.05$ (left). The quantitative analysis (right) shows the percentage of capsaicin-sensitive cell numbers of total cell numbers from four independent experiments (TAT-T406, n = 77 vs TAT-T406A, n = 93, "n" represents the total numbers). In primary cultured neurons treated with TAT-T406 peptide, fewer DRG neurons responded to 1 μM capsaicin compared with that treated with TAT-T406A peptide. $^{*}P < 0.05$, two-tailed paired *t* test. Error bars indicate SEM (right). (D) Representative traces show the typical average change in Ca^{2+} influx of capsaicin-sensitive neurons from each group (left). Quantification of the area under the curve for 30 s after capsaicin stimulation is shown on the right. In the cells activated by 1 μM capsaicin, the TAT-T406 peptide delayed the response by approximately 30 s. TAT-T406, n = 5 vs TAT-T406A, n = 21. $^{*}P < 0.05$, two-tailed unpaired *t* test. Error bars indicate SEM.

3.4. TAT-T406 attenuates functional TRPV1 surface targeting and the development of thermal hyperalgesia after inflammation

It has been reported that Cdk5 phosphorylates TRPV1 at Thr-407 (in humans) to regulate pain signaling and that C-fiber-specific Cdk5 conditional-knockout mice exhibit delayed withdrawal to a 50 °C water bath (Pareek et al., 2006). However, because of its multifunctional effects, side effects are possible if we inhibit Cdk5 activity with a chemical inhibitor or with a peptide that blocks p35 activity. The phosphorylation-inhibiting peptide TAT-T406 could be helpful in relieving thermal hyperalgesia after inflammation. To test this hypothesis, SD rats were intrathecally injected with the TAT-T406 or TAT-T406A peptide (10 μg) 30 min before the injection of CFA into a hindpaw. The basal heat-evoked paw-withdrawal latency of the rats treated with the TAT-T406 peptide was not significantly different from the latency of the control peptide-treated rats (Fig. 4A). There also was no significant difference in the withdrawal threshold to mechanical stimuli (von Frey filaments) after CFA injection between the two groups (Fig. 4B). Inflammation-induced thermal hyperalgesia was relatively attenuated in rats pretreated with the TAT-T406 peptide (###$P < 0.001$), especially at 2 h (***$P < 0.001$) and 6 h (*$P < 0.05$) after the CFA injection, and the inhibition effect disappeared 1 d after the induction of inflammation (Fig. 4C). The most efficient time point (2 h) was in accord with the timing of cellular uptake of TAT peptides following intrathecal injection which was peaking at 1 h and 3 h (Xie et al., 2009). To further investigate the specific mechanisms, L4 and L5 DRG neurons of SD rats pretreated with the TAT-T406 or TAT-T406A peptide were acutely dissociated 2 h after CFA injection and subjected to Fura-2 calcium imaging. Capsaicin (1 μM) evoked a rapid, robust increase in intracellular Ca^{2+} concentration in neurons pretreated with the control peptide, whereas neurons pretreated with the TAT-T406 peptide showed weaker maximum calcium signals ($P = 0.0269$, TAT-T406A, n = 12 vs TAT-T406, n = 50) (Fig. 4D). Acutely dissociated DRG neurons were also subjected to the surface biotinylation assay; the amount of surface TRPV1 was notably decreased, whereas the total TRPV1 level was not altered (Fig. 4E). These results suggest that disruption of Thr-406 phosphorylation with the TAT-T406 peptide during inflammation can alleviate thermal hyperalgesia but not mechanical allodynia after inflammation and that this treatment does not affect basal heat sensitivity.

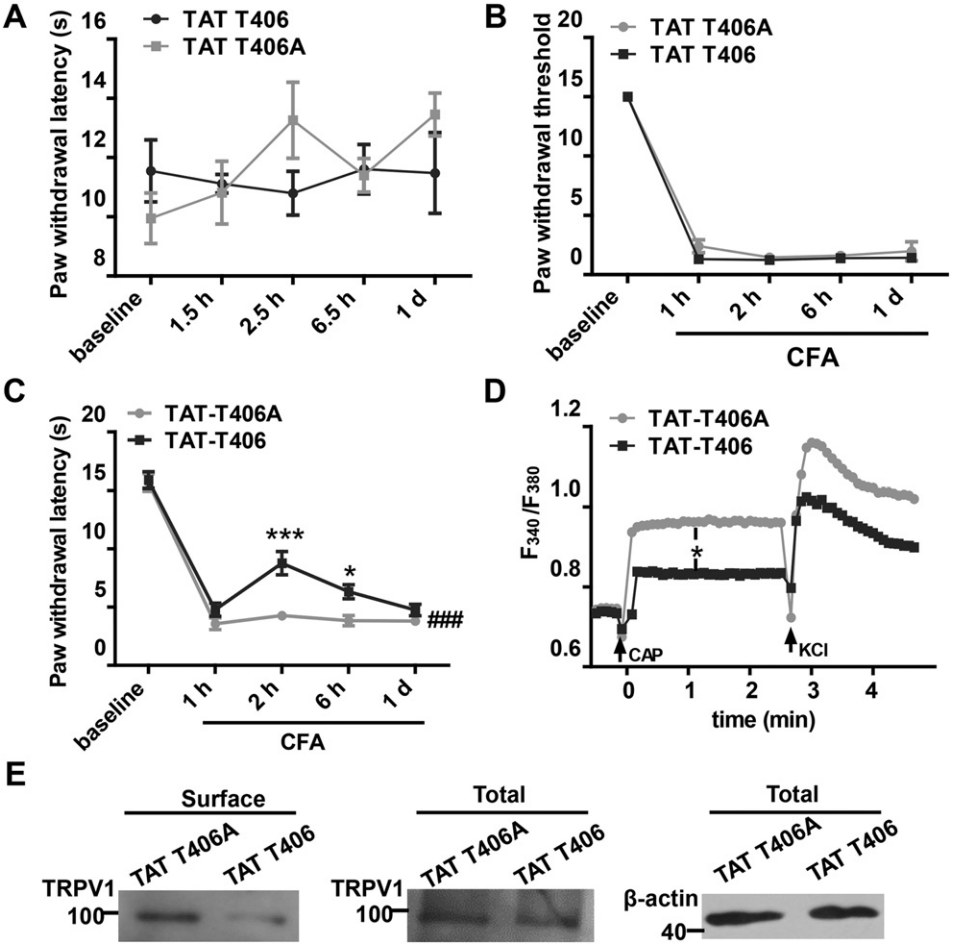

Fig. 4. The TAT-T406 fusion peptide attenuates the function of TRPV1 in the CFA-induced inflammatory pain model. (A) Time course of the basal paw-withdrawal latency (PWL) of the ipsilateral hind paw as measured by radiant heat stimuli before and after injection of 10 μg of TAT-T406 or TAT-T406A peptide, n = 4. Error bars indicate SEM. (B) Time course of the PWL of the ipsilateral hind paw as measured by von Frey filaments stimuli before and after injection of 25% CFA. Rats were pretreated with the TAT-T406 or TAT-T406A peptide (10 μg) 30 min before CFA injection, n = 4–5. Error bars indicate SEM. (C) Time course of the PWL of the ipsilateral hind paw as measured by radiant heat stimuli before and after injection of 25% CFA. Rats were pretreated with the TAT-T406 or TAT-T406A peptide (10 μg) 30 min before CFA injection. Data were analyzed by two-way repeated-measures ANOVA, followed by Bonferroni's post-hoc tests. *$P < 0.05$, ***$P < 0.001$ compared with the corresponding time point in the TAT-T406A peptide group. ###$P < 0.001$, two-way ANOVA, followed by Bonferroni posttests, compared with corresponding control groups, n = 10–11. Error bars indicate SEM. (D) The TAT-T406 fusion peptide attenuates the function of TRPV1 in acutely dissociated DRG neurons in the CFA-induced inflammatory pain model. The TAT-T406 peptide dramatically diminished the elevation of intracellular calcium signals in response to capsaicin (1 μM) (TAT-T406A, n = 12 vs TAT-T406, n = 50). *$P < 0.05$, two-way ANOVA, followed by Bonferroni's post-hoc tests, compared with corresponding control groups. Error bars indicate SEM. (E) Acutely dissociated DRG neurons were subjected to the surface biotinylation assay. The TAT-T406 peptide decreased the surface level of TRPV1 in the acutely dissociated DRG neurons in the CFA-induced inflammatory pain model, n = 2.

4. Discussion

In this study, we provided convincing evidence that TRPV1 Thr-406 phosphorylation by Cdk5 promotes the distribution of TRPV1 in the plasma membrane and that this process contributes to heat hyperalgesia during inflammation. This finding is crucial for further determining the potential role of Cdk5/p35 in nociception and pain transduction by TRPV1.

Cdk5 has been identified as a regulator of inflammatory pain signals (Pareek et al., 2007; Pareek et al., 2006). Increased calpain activity during inflammation results in the cleavage of p35 to p25, which forms a more stable complex with Cdk5 and leads to the elevation of Cdk5 activity as a result (Pareek et al., 2006). Mice that overexpress p35 exhibit elevated Cdk5 activity and show hyperalgesia, and in contrast, p35 knockout mice ($p35^{-/-}$), which exhibit significantly decreased Cdk5 activity, show delayed responses to painful thermal stimulation compared with WT controls (Pareek et al., 2006). Cdk5-mediated phosphorylation of TRPV1 at Thr-407 could modulate capsaicin-induced calcium influx, and nociceptor-specific Cdk5 conditional-knockout mice show reduced TRPV1 phosphorylation, resulting in significant hypoalgesia (Pareek et al., 2007). Unlike other posttranscriptional regulators of TRPV1, such as NGF and PKD1 (Ji et al., 2002; Zhu et al., 2008), Cdk5/p35 does not affect the total protein level of TRPV1; instead, it only regulates TRPV1 membrane trafficking. Our previous study reported that Cdk5 positively regulates TRPV1 membrane trafficking by association with and phosphorylation of the motor protein KIF13B (Xing et al., 2012), but did not assess the regulatory function of Cdk5-mediated phosphorylation of TRPV1 at Thr-406 in this process. Here, we found that TRPV1 Thr-406 phosphorylation by Cdk5 increased the interaction of TRPV1 with cytoskeletal elements (Fig. 1A). Then, that phosphorylation at Thr-406 of TRPV1 affects the interaction of TRPV1 and the KIF13B motor FHA domain was confirmed by our results (Fig. 1B). As a consequence of interactions with the cytoskeletal elements, the localization of TRPV1 to the cell surface is significantly promoted, while the converse was observed in the TRPV1-T406A mutation group (Fig. 2). Here, we provide the first report of the molecular biological effects of TRPV1 Thr-406 phosphorylation, which is necessary for further understanding of the function of Cdk5 in thermal sensation.

Pain is a severe clinical problem that reduces patient quality of life. Exogenous opioid receptor agonists are the most efficacious analgesic drugs used to relieve severe pain, but they may induce serious side effects, such as respiratory depression, mental clouding, altered consciousness, constipation, nausea and opioid tolerance (Sehgal et al., 2011). Capsaicin is widely used in the clinic as an analgesic treatment for different pain conditions, although it also has adverse effects, such as a burning sensation and neurotoxicity (Derry et al., 2013; Knotkova et al., 2008; O'Neill et al., 2012). Cdk5 is a notable target for the development of new pharmaceutical therapies for the treatment of several diseases including pain. Several classes of chemical inhibitors and Cdk5 inhibitor peptides (CIPs) have been identified (Bosutti et al., 2013; Kesavapany et al., 2004; Oumata et al., 2008; Sondhi et al., 2005; Sundaram et al., 2013; Utreras et al., 2009a); however, all of these are nonspecific for the treatment of inflammatory pain due to the multiple physiological functions of Cdk5. The Kulkarni group has synthesized an eight amino acid peptide (SSETPNRH) against the threonine 407 site of human TRPV1 to investigate the phosphorylation of this site by Cdk5 (Pareek et al., 2007), but there is no any further research about it. In this study, we constructed a different peptide specific against the same amino acid in rat TRPV1 to reveal the biological and physiological effects of Thr-406 phosphorylation. Our peptide included 15 amino acids and was rendered cell permeable by fusion with the HIV TAT protein sequence (RKKRRQRRR) (Fig. 3A). Compared to the control peptide, the TAT-T406 peptide dramatically reduced the cell-surface TRPV1 protein level but did not influence total TRPV1 levels (Fig. 3B). Furthermore, the TAT-T406 peptide also obviously attenuated the capsaicin-induced TRPV1 functional response in primary cultures of DRG neurons (Fig. 3C and D). In a rat model of CFA-induced inflammatory pain, the TAT-T406 peptide exhibited an obvious anti-hyperalgesic effect (Fig. 4C). As expected, cell-surface TRPV1 protein levels and capsaicin-induced functional calcium influx in acutely dissociated L4 and L5 DRG neurons were contemporaneously suppressed by the TAT-T406 peptide (Fig. 4D and E), suggesting that our cell-penetrating peptide holds promise as a novel therapeutic for the control of inflammatory pain that is specific to the Cdk5 phosphorylation site of TRPV1.

It is clear that in inflammatory hyperalgesia, cytoskeletal proteins such as KIF13B mediate TRPV1 trafficking to the cell membrane and that Cdk5 plays a crucial role in this process via phosphorylation of KIF13B at Thr-506, which enhances its interaction with TRPV1 (Xing et al., 2012). On the other hand, we believe that Cdk5-mediated phosphorylation of TRPV1 at Thr-406 may be a precondition for recognition of the KIF13B FHA domain and its binding to TRPV1 because it is a modular phosphopeptide recognition motif (Durocher et al., 1999; Westerholm-Parvinen et al., 2000). There is a balance between the insertion and endocytosis of cell-membrane proteins. Increased numbers of functional membrane proteins may be attributed to the promotion of anterograde transport or to reductions in internalization. In general, the distribution of metabolic receptors or of G protein-coupled receptors (GPCRs) in the cell membrane is dynamically regulated by rapid endocytosis and membrane recycling, whereas the distribution of ionic receptors is relatively stable. Nonetheless, it has recently been reported that ionic receptors are also regulated through endocytosis. Roche et al. demonstrated that NMDA receptors are internalized through the YEKL motif located on the distal C-terminal of GluR2B and that PSD-95 inhibits this process (Roche et al., 2001). It has also been reported that after exposure to RTX (Resiniferatoxin, an ultrapotent synthetic TRPV1 agonist)/capsaicin, both cells transfected with TRPV1 and DRG neurons exhibit a loss of membrane, followed by vesiculation and the subsequent internalization/expulsion of the vesicles, which is dependent on intracellular Ca^{2+} accumulation (Caudle et al., 2003). Recently, another study has indicated that TRPV1 agonists induce long term receptor downregulation by modulating channel expression levels through the promotion of receptor endocytosis and degradation and that cAMP-PKA signaling sensitizes nociceptors through several mechanisms (Sanz-Salvador et al., 2012). However, whether Cdk5 suppresses the endocytosis of TRPV1 to upregulate its surface levels as well as the roles of TRPV1 Thr-406 phosphorylation still need to be investigated.

Collectively, the results of our study revealed that phosphorylation of TRPV1 at Thr-406 by Cdk5 increases the amount of functional TRPV1 on the cell surface and consequently potentiates activation of TRPV1 channels in heat hyperalgesia. We also demonstrated that the TAT-T406 peptide, which significantly reduces the number of functional TRPV1 receptors on the plasma membrane in vitro and in vivo, could effectively ameliorate inflammatory pain-related behaviors, supporting the potential therapeutic value of this peptide for thermal hyperalgesia.

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