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# Nociceptive signaling through transient receptor potential vanilloid 1 is regulated by Cyclin Dependent Kinase 5-mediated phosphorylation of T407 in vivo

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## Abstract

Cyclin dependent kinase 5 (Cdk5) is a key neuronal kinase whose activity can modulate thermo-, mechano-, and chemo-nociception. Cdk5 can modulate nociceptor firing by phosphorylating pain transducing ion channels like the transient receptor potential vanilloid 1 (TRPV1), a thermoreceptor that is activated by noxious heat, acidity, and capsaicin. TRPV1 is phosphorylated by Cdk5 at threonine-407 (T407), which then inhibits $Ca^{2+}$ dependent desensitization. To explore the in vivo implications of Cdk5-mediated TRPV1 phosphorylation on pain perception, we engineered a phospho-null mouse where we replaced T407 with alanine (T407A). The T407A point mutation did not affect the expression of TRPV1 in nociceptors of the dorsal root ganglia and trigeminal ganglia (TG). However, behavioral tests showed that the $TRPV1^{T407A}$ knock-in mice have reduced aversion to oral capsaicin along with a trend towards decreased facial displays of pain after a subcutaneous injection of capsaicin into the vibrissal pad. In addition, the $TRPV1^{T407A}$ mice display basal thermal hypoalgesia with increased paw withdrawal latency while tested on a hot plate. These results indicate that phosphorylation of TRPV1 by Cdk5 can have important consequences on pain perception, as loss of the Cdk5 phosphorylation site reduced capsaicin- and heat-evoked pain behaviors in mice.

## Keywords

Cdk5, p35, TRPV1, knock-in mouse, pain, phosphorylation

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## Introduction

Inflammation can trigger neuroplastic alterations in nociceptors that promote hyperalgesia and allodynia. Inflammatory mediators trigger downstream signaling cascades that lead to the activation of protein kinases in nociceptors, including protein kinase A (PKA), protein kinase C (PKC), calcium/calmodulin-dependent protein kinase II (CaMKII), and cyclin dependent kinase 5 (Cdk5).[1,2] These protein kinases can then promote peripheral sensitization by modulating the activity of pain transducing ion channels like TRPV1. TRPV1 is a non-selective cation channel expressed in unmyelinated C fibers that is polymodally activated by

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noxious stimuli such as heat (>42°C), low pH, and capsaicin.[3] TRPV1 is also expressed in microglia and astrocytes and may additionally have a role in regulating neuro-immune functions.[4]

TRPV1 forms an ion channel from a homotetramer of four subunits, each containing six-helix (S1–S6) transmembrane domains.[5] The intracellular N-terminal domain of TRPV1 contains six ankyrin repeats domain that are thought to be involved in protein–ligand interactions, while the C-terminal domain contains a TRP box helix that may modulate TRPV1 gating. TRPV1 can be phosphorylated at various serine and threonine residues by kinases like PKA, PKC, CaMKII, and Cdk5, which often then modulate TRPV1 channel activity to promote nociceptor hyperexcitability.[6] Most of the protein kinases that phosphorylate TRPV1 are activated by second messenger signaling molecules such as cyclic AMP, diacylglycerol, and calcium. In contrast, Cdk5 requires the upregulated expression of its activator p35, which typically occurs downstream of ERK1/2 activation by neurotrophins and proalgesic cytokines.[7,8]

Cdk5 phosphorylates TRPV1 at threonine 407 (T407) within a flexible linker of the N-terminal domain that structurally could be involved in ion channel gating.[9,10] Our lab has shown that inhibition of Cdk5 activity in cultured dorsal root ganglia (DRG) neurons blocks TRPV1-mediated $Ca^{2+}$ influx induced by capsaicin. Later, we expanded on these findings to show that phosphorylation of TRPV1 by Cdk5 affects $Ca^{2+}$-induced desensitization leading to a non-desensitizing state. As a result, the degree of Cdk5 activity in mice can thereby regulate pain behavioral responses to capsaicin and heat, both of which are known to activate TRPV1. We have also described the making and testing of rat TRPV1 mutants in transfected CHO cells, where threonine-407 (406 in rat) is mutated either to an alanine, to block phosphorylation, or to an aspartate, to mimic phosphorylation.[10] Electrophysiological recordings showed that these TRPV1407 (T407) (406 in rat) mutants were fully functional. The rat T406 aspartate substitution, however, exhibits altered use-dependent activation kinetics, but the rat T406 alanine mutation behaved similarly to the wild type TRPV1.[10] Along with impacting $Ca^{2+}$-induced desensitization of TRPV1, Cdk5 activity has also been proposed to affect nociceptor sensitivity by regulating the localization of TRPV1 to the plasma membrane as well.[11]

Conditional deletion of Cdk5 in nociceptive neurons in mice results in reduced sensitivity to capsaicin and thermal hypoalgesia to noxious heat.[9,10] Our in vivo data suggests that these changes in pain behavior are mostly linked to Cdk5-mediated phosphorylation of TRPV1, yet Cdk5 can also affect nociception by phosphorylating other key substrates such as the collapsin response mediator protein 2 (CRMP2) and other known pain transducing cation channels.[12–14] To better understand the extent to which TRPV1-T407 phosphorylation can exert on pain signaling, we created a mouse with the T407 phosphorylation site mutated to a phospho-null alanine (T407A). The $TRPV1^{T407A}$ KI mice have less oral aversion to a low dose of capsaicin, show a trend towards reduced spontaneous behavioral signs of pain when injected with capsaicin in the vibrissal pad, and display thermal hypoalgesia in a hot plate test. These results demonstrate that Cdk5 plays a key role in modulating pain sensation through direct phosphorylation of TRPV1.

## Materials and methods

### *Generation of $TRPV1^{T407A}$ mice*

$TRPV1^{T407A}$ mice were generated using gene targeting techniques described previously.[15,43] Briefly, a single guide RNA (sgRNA) was designed to target the T407 site in exon six of the TRPV1 gene.[16] A sgRNA was in vitro transcribed consisting of the common guide backbone and the target sequence of AGCAGTGAGACCCCCGTGAG**TGG.** A 200bp donor DNA (IDT, Coralville, IA), depicted in Figure 1a, converts Threonine (ACC) to Alanine (GCC) and contains two additional point mutations, one for the NGG protospacer and another to delete a Eco RI site for genotyping purposes. The pronuclei from FVB/N donor zygotes (Envigo, Indianapolis, IN) were microinjected with the sgRNA, donor DNA, and Cas9 mRNA (Trilink, San Diego, CA). Three founder mice were derived, of which A8 was bred to homozygosity and used for further study. For genotyping, the region around the T407A site was amplified using the following PCR primers: Forward 5′-agagggagatccacgaacca-3′ and Reverse 5′-tgcagatagcttgccagtcc-3'. The subsequent 563bp PCR product was then digested with EcoRI. The knock-in allele is uncut due to the loss of the EcoRI site in the donor DNA, while the wild type allele was cleaved to produce 443 and 120 bp bands (Figure 1b).

### *qPCR of TRPV1*

The trigeminal ganglia (TG) were dissected from ~2-month-old wildtype (WT), $TRPV1^{T407A}$, and TRPV1 knockout (KO) (B6.129X1-*Trpv1*$^{tm1Jul}$/J, Stock No: 003,770, The Jackson Laboratory, Bar Harbor, ME) mice and homogenized (Precellys 24 tissue homogenizer, Bertin technologies) in Trizol (Thermo Scientific, Rockford, IL) in order to extract total RNA. RNA was subsequently converted into cDNA with a High Capacity cDNA Reverse Transcription Kit (Thermo Scientific, Rockford, IL). DNA sequencing (NIDCR Sequencing Core) was performed using a 5′-agagggagatccacgaacca-3′ forward primer to further verify the T407A knock-in point mutation within the Trpv1 gene (Figure 1c). Quantitative real-time PCR was performed as described in Prochazkova et al.[24] using Assays on Demand TaqMan primers for TRPV1 and GAPDH (Applied Biosystems, Foster City, CA, USA). Real-time PCR was run in duplicate on a 7500 Real-time PCR System (Applied Biosystems, Foster City, CA), where TRPV1 expression levels were

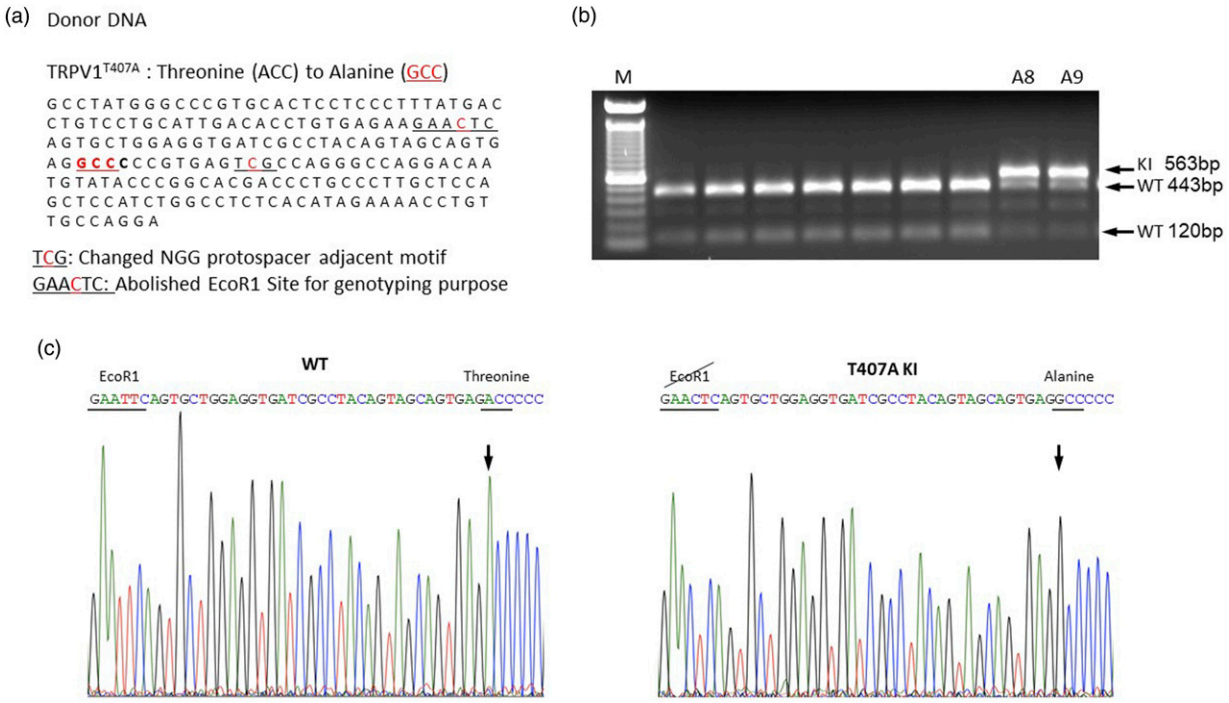


**Figure 1.** Generation of TRPV1$^{T407A}$ mice. a. A 200 bp donor DNA was microinjected along with the sgRNA to convert Threonine (ACC) to Alanine (GCC) in mice through CRISPR mediated recombination. In addition to the T407A point mutation, the NGG protospacer was also mutated and a EcoRI site was disrupted for genotyping purposes. b. A PCR was designed to amplify exon six of TRPV1 where T407 is located. For wild type mice (WT), the intact EcoRI site can be cut within the PCR product to create 443 and 120 bp bands. In contrast, the PCR product amplifying the TRPV1$^{T407A}$ modified allele remains uncut (563 bp) due to the introduced mutation to the EcoRI site in the donor DNA. c. Total RNA was extracted from the trigeminal ganglia (TG) from WT and TRPV1$^{T407A}$ mice, converted to cDNA, and used for DNA sequencing. DNA sequencing results further confirm the introduction of the T407A point mutation into the TRPV1 gene.

normalized to the levels of GAPDH using the comparative cycle threshold (Ct) method (Figure 2a). The expression of TRPV1 from TRPV1 T407A and TRPV1 KO mice were then compared to WT controls.

## *Western blot and immunofluorescence*

Western blot was performed as previously described in Prochazkova et al.[17] TG were dissected from ~4-month-old WT, TRPV1$^{T407A}$, and TRPV1 KO mice. Protein lysates were prepared using T-PER (Thermo Scientific, Rockford, IL) supplemented with protease and phosphatase inhibitors (Complete Mini and PhosSTOP, Roche, Indianapolis, IN). Then, 40 µg of protein was run on 4–20% Bis-Tris gel (GenScript, Piscataway, NJ), transferred onto a 0.45 µm nitrocellulose membrane (Thermo Scientific, Rockford, IL), and then immunoblotted with a TRPV1 polyclonal antibody at a 1:1000 dilution (N221/17, NeuroMab, Davis, CA). Membranes were later stripped and reblotted with an anti-actin antibody at a 1:2000 dilution (66,009, Proteintech, Rosemont, IL) as a loading control (Figure 2b).

Trigeminal ganglia (TG) and dorsal root ganglia (DRG) were carefully dissected from perfused ~2-month-old wild-type, TRPV1$^{T407A}$ and TRPV1 KO mice according to Malin et al.[18] The DRGs were embedded in OCT (Sakura® Finetek) and 5 µm sections were cut with a cryostat (Leica, Buffalo Grove, IL). Sections of TG and DRG were then fixed in 4% PFA, blocked with 1% BSA, and incubated overnight with a TRPV1 polyclonal anti-rabbit antibody (1:1000 dilution: RA14113, Neuromics, Edina, MN) along with an Anti-NeuN antibody as a neuronal marker (Anti-NeuN Antibody, clone A60, Alexa Fluor®488 conjugated). TRPV1 expressing neurons were subsequently visualized using a Rhodamine goat anti-rabbit secondary antibody (1:250 dilution: 111-295-144, Jackson ImmunoResearch Laboratories, West Grove, PA) and nuclei were stained with DAPI (Thermo Scientific, Rockford, IL). Images were captured on a Zeiss LSM 880 confocal microscope (Oberkochen, Germany) (Figure 2c).

## *Lickometer drinking test*

Oral aversion to the TRPV1 agonist capsaicin (MilliporeSigma, Burlington, MA) was measured using the EthoVision instrumented observation cage with the lickometer add-on (Noldus, Leesburg, VA). The lickometer records the drinking behavior of the mice by detecting changes in capacitance whenever the mouse makes contact with the metallic spout of the water bottle. TRPV1$^{T407A}$ and

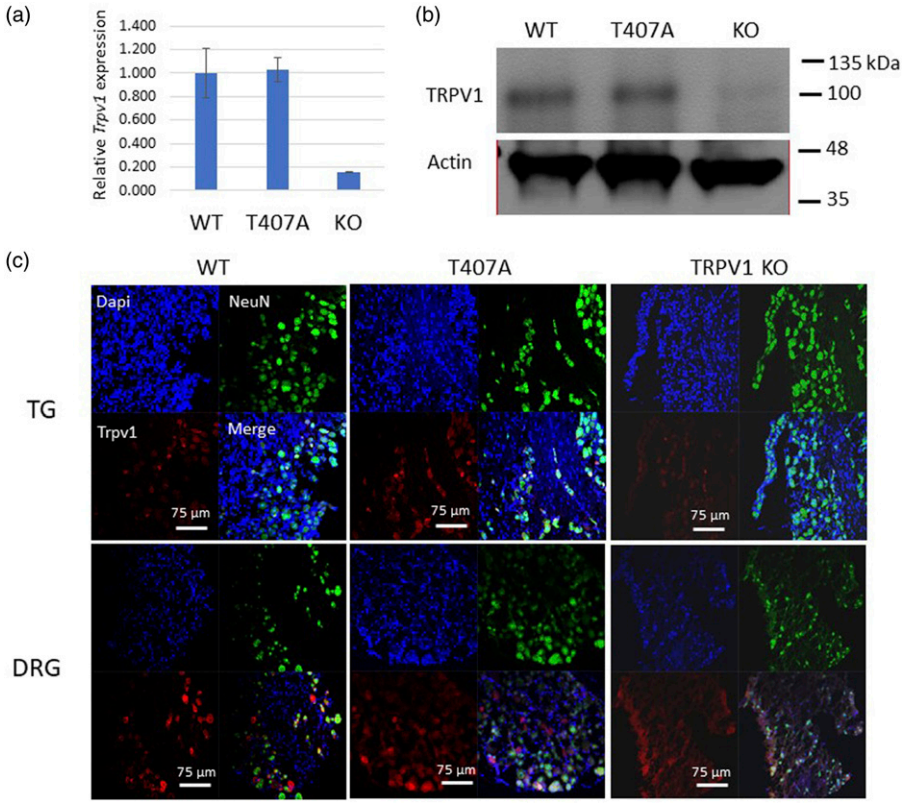


**Figure 2.** Expression of TRPV1$^{T407A}$. a. Real-time PCR was conducted using RNA from the TG of WT and TRPV1$^{T407A}$ mice. No change in mRNA expression was seen between WT and TRPV1$^{T407A}$ mice. TRPV1 knockout (KO) mice were used as a control that display affected TRPV1 expression resulting from the genetically targeted knockout mutation. b. Western blot was also performed using TG from WT and TRPV1$^{T407A}$ mice. Equivalent expression of TRPV1 was seen between the control and knock-in mice but no corresponding band was seen in TRPV1 KO. c. Immunofluorescent staining was performed on trigeminal ganglia (TG) and dorsal root ganglia (DRG) from WT, TRPV1$^{T407A}$, and TRPV1 KO mice. Images showing TRPV1 expression (red) in some DRG neurons from WT and TRPV1$^{T407A}$ while no real staining is seen with the TRPV1 knockouts. Blue: DAPI (Nucleus), Green: NeuN (Neuronal Marker), Red: TRPV1. Scale bar is shown in bar.

WT female mice ($N$ = 6; ~4-month-old) were deprived of water overnight. Mice were allowed to first drink plain water (4 trials) for 1 h to habituate the mice to the lick-ometer and establish a baseline (data not included). Once a baseline with water was established, mice were provided solutions of water containing 0.15, 1.5, or 15 µM capsaicin (3 trials).

## Mouse grimace scale test

Mouse grimace scale testing was conducted to examine facial expressions of pain in response to a subcutaneous injection of capsaicin. Facial displays of orbital tightening, nose bulge, cheek bulge, ear position, and whisker change are recorded and scored on a 0–2 scale.[19] First, TRPV1$^{T407A}$ and WT female mice ($N$ = 3; ~6-month-old) were acclimatized to a fabricated clear Lucite holder 3 days in a row prior to testing. The mice were then videotaped 15 min before and after an injection of 0.1% capsaicin into the vibrissal pad. The video was transferred onto the computer and pain was assessed blindly as detailed in Joseph et al.[21]

## Hot plate assay

Aversion to noxious heat was measured using the hot plate test (Ugo Basile, Model 35,150, Gemonio VA, Italy). TRPV1$^{T407A}$ and WT mice ($N$ = 4; ~4-month-old) were acclimatized to the hot plate 1 day before testing day. On testing day, the hot plate was set at 50°C. Mice were placed on the hot plate and observed for nocifensive behavior, where lifting of the hindpaw in reaction to the heated surface was timed. Testing was done three times and the results were averaged.

## Statistics

The statistical evaluation from the behavioral tests was calculated using GraphPad Prism software, version 5.0 (GraphPad, San Diego, CA, USA) with the data expressed as

a mean ± SEM. Statistical differences between the experiments were assessed by paired or unpaired t-test at a significance level set at $p < 0.05$.

## Results

### Development of TRPV1$^{T407A}$ mice

To explore how the T407A substitution in TRPV1 would impact nociception in vivo, we decided to use CRISPR mediated gene editing to create a Cdk5 phospho-null point mutation in the TRPV1 gene of a mouse. A sgRNA was designed to guide Cas9 to the T407 site within the TRPV1 locus and generate a double strand break that would then be repaired by an accompanying donor DNA (Figure 1a). Correct recombination with the 200 bp single stranded donor oligo would then convert nucleotides ACC to GCC for substitution of threonine to alanine. A mutation in the NGG protospacer adjacent motif site was also included to prevent the donor DNA from getting cut by Cas9, while an additional point mutation was added to abolish the Eco RI restriction site for genotyping purposes. Homologous recombination was identified within the resulting pups using a restriction fragment length polymorphism strategy, as any PCR product amplified from the T407A knock-in allele won't be cut by Eco RI (Figure 1b). Of three founder TRPV1$^{T407A}$ lines that were generated following microinjection, line A8 was bred to homozygosity and expanded for further analysis. To confirm subsequent expression of the TRPV1 T407A point mutation, total RNA was extracted from the trigeminal ganglia (TG) from WT and TRPV1$^{T407A}$ KI mice and the converted cDNA was then used for DNA sequencing. As shown in Figure 1c, DNA Sanger sequencing confirmed that threonine 407 was mutated to alanine in the TRPV1 allele through CRISPR mediated recombination.

### Normal expression and distribution of TRPV1$^{T407A}$

After generating the TRPV1$^{T407A}$ mice, we next wanted to ensure that the T407A point mutation did not interfere with the normal expression of TRPV1, particularly as TRPV1 is critical for body temperature homeostasis.[20] With RNA extracted from the TG of WT, TRPV1$^{T407A}$ KI and TRPV1 KO mice, we performed real-time PCR to determine if there might be any altered expression levels of TRPV1 resulting from the introduced point mutation. As seen in Figure 2a, the relative mRNA expression of TRPV1$^{T407A}$ is similar to the expression levels of TRPV1 in WT mice. We then examined TRPV1 expression by Western blot to confirm that the knock-in didn't block protein translation. Using protein lysates from the TG, we see that the TRPV1$^{T407A}$ is expressed at similar levels to the WT TRPV1 receptor, while no corresponding band is seen with the TRPV1 KO mice (Figure 2b). Lastly, we performed immunofluorescence staining on sections of dorsal root ganglia from WT, TRPV1$^{T407A}$, and TRPV1 KO mice. In Figure 2c, we detected comparable numbers of TRPV1$^{+}$ neurons in the WT and TRPV1$^{T407A}$ mice while no expression of TRPV1 was seen in the knockout mice. So, the CRISPR generated T407A mutation in TRPV1 does not appear to impact its expression in the primary afferent neurons of the TG and DRG, which is in line with other knock-in mouse models engineered with point mutations in TRPV1.[21,22]

### Reduced pain in TRPV1$^{T407A}$ mice

We have earlier shown that mice genetically modified to have Cdk5 hyperactivity showed more oral aversion to water containing capsaicin, while mice with engineered Cdk5 hypoactivity conversely licked more capsaicin than the wild-type controls.[10] This lickometer data demonstrates that Cdk5 is either directly or indirectly modifying TRPV1 mediated pain transduction in vivo. After confirming the adequate expression and localization of TRPV1$^{T407A}$, we then wanted to measure the aversion to oral capsaicin within the TRPV1$^{T407A}$ knock-in mice to better assess how direct phosphorylation of TRPV1 by Cdk5 affects nociceptor signaling. At high doses of capsaicin (15 and 1.5 μM), WT and TRPV1$^{T407A}$ mice showed no significant differences in licking behavior (data not shown). However, with a lower 0.15 μM concentration, we observed that the TRPV1$^{T407A}$ mice significantly licked more capsaicin than the controls, indicating that they had less aversion to capsaicin at this dose (Figure 3a). So, at saturating high doses of capsaicin, the loss of the T407 phosphorylation site in TRPV1 did not have an effect, but at a low concentration, a difference in the sensitivity to capsaicin could be observed.

To further study the interplay between Cdk5 and capsaicin-mediated TRPV1 channel activity, we next wanted to track the facial responses of mice to subcutaneous injection of capsaicin using the mouse grimace scale (MGS).[19,21] We selected this assay since Cdk5-mediated regulation of capsaicin-evoked responses in vivo were more apparent at low dose of capsaicin, and MGS would also likely report behavioral responses to the administration at a low dose of capsaicin. Essentially, mice were videotaped, and facial reactions to pain were scored on a 0–2 scale. First, baseline responses were established to saline injected into mouse vibrissal pad. The MGS score of the mice following saline injection showed no difference between the two genotypes (data not shown). With injection of a 0.1% capsaicin solution, we observed that the MGS score was trending higher in WT mice compared to the TRPV1$^{T407A}$ mice, although this was not statistically significant (Figure 3b). Still, between the lickometer data and the MGS test, there appears to be indications that the lack of the Cdk5 phosphorylation site in TRPV1 is impacting pain behavioral responses to capsaicin.

TRPV1 is a thermosensitive TRP channel that is also activated by noxious heat. As with capsaicin, we

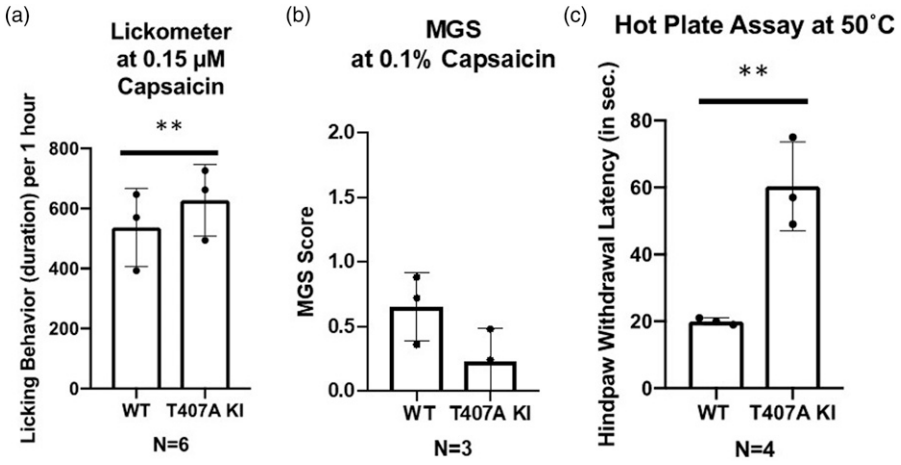


**Figure 3.** Behavioral Testing. a. Oral aversion to capsaicin was measured in the WT and TRPV1$^{T407A}$ mice using the lickometer. The duration of drinking behavior from the mice was recorded for 1 h. At 0.15 μM, TRPV1$^{T407A}$ show less aversion to capsaicin (3 trials using $N = 6$ mice), although at higher concentrations (1.5 and 15 μM) no significant difference in drinking behavior was seen. b. Mouse grimace scale was used to score facial expressions of pain and discomfort after injection of 0.1% Capsaicin injection into the whisker pad of the mouse. While not significant, TRPV1$^{T407A}$ show a trend towards reduced facial signs of pain as compared to WT littermate controls ($N = 3$). c. The hot plate assay was used to monitor basal nocifensive behaviors in the TRPV1$^{T407A}$ mice. The hot plate was set at 50°C and hindpaw withdrawal was timed ($N = 4$). TRPV1$^{T407A}$ mice display increased hindpaw withdrawal latency compared to WT mice, suggesting reduced peripheral aversion to noxious heat (3 trials).

previously showed that Cdk5 activity can also modulate basal thermal responses to heat in mice.[9,10,23] Therefore, we tested the nocifensive behavior of TRPV1$^{T407A}$ mice when placed on a hot plate. The hot plate was set to 50°C and hindpaw withdrawal was timed. The TRPV1$^{T407A}$ mice showed significantly increased hindpaw withdrawal as compared to wildtype mice, which demonstrates thermal hypoalgesia to painful heat (Figure 3c). So, the T407A mutation in TRPV1 appears to strongly affect heat tolerance in mice.

## Discussion

Cdk5 is a unique member of the cyclin-dependent kinase family that is not involved in the cell cycle, but, instead, is active in post-mitotic neurons. Cdk5 is not activated by cyclins but by binding with its regulatory subunits p35 or p39, both of which possess a cyclin-box. Although Cdk5 is ubiquitously expressed, its activity is primarily limited to neurons because of the restricted expression of its two activators. Cdk5/p39 activity has no effect on pain,[24] whereas the expression levels of p35 can, in contrast, modulate basal pain behavioral responses in mice.[2,10,17] Importantly, p35 expression is induced downstream of inflammation, particularly through sustained ERK1/2 activation via inflammatory mediators such as nerve growth factor and tumor necrosis factor-α.[7,8] Activated Cdk5/p35 is then thought to promote hyperalgesia by phosphorylating pain transducing ion channels like TRPV1, the transient receptor potential ankyrin 1 (TRPA1), and the purinergic receptor P2X2a.[9,13,14] Cdk5 activity may also impact pain signaling by either regulating NMDA receptor activity or by affecting the expression of the NMDA receptor subunits in the spinal cord and DRG.[25–28]

Using genetically engineered mice, we showed that Cdk5 activity modulates the sensitivity to noxious heat. Overexpressing p35 to induce Cdk5 activity causes heat hyperalgesia, while mice with decreased Cdk5 activity, such as p35 knockouts and Cdk5 conditional knockouts, show hypoalgesia.[2,9,10] With the Orofacial Pain Assessment Device, for example, wildtype mice showed reduced licking behavioral when making facial contact with a thermo-sensor set at 45°C, but the licking behavior of our mice with Cdk5 hypoactivity was essentially unchanged compared to a baseline of 37°C. Similarly, p35 knockouts and Cdk5 conditional knockouts also showed reduced oral aversion to capsaicin, a TRPV1 agonist that shares structural similarities with endogenous activators like 12-hydroperoxyeicosatetraenoic acid (12-HPETE).[29] With GCaMP6f imaging in mice, we also saw that Cdk5 hyperactivity both promotes higher fluorescent signaling intensities and increased numbers of activated neurons in the TG following facial application of heat and capsaicin.[30] Overall, this in vivo data suggest that TRPV1-mediated nociception can be regulated by Cdk5 activity.

Cdk5 activity has been proposed to control membrane trafficking of TRPV1 through either phosphorylation of the kinesin motor KIF13B or the clathrin adaptor protein AP2μ2.[31,32] Although we cannot exclude the possibility that reduced pain behaviors in T407A is attributable to the reduced membrane trafficking, we have alternatively shown that Cdk5 directly phosphorylates TRPV1 at T407 in a highly

conserved region of the N-terminal, which, in turn, affects ion channel activity.[9,10] Further electrophysiological and biochemical studies in sensory neurons may clarify the detailed mechanisms whereby TRPV1 T704A mutation contribute to behavioral phenotypes. The T407 is located in the membrane proximal domain that has been suggested to be involved heat sensing in TRP channels,[33] although other sites in TRPV1 are also thought to contribute to thermo-sensing.[34] Computer modeling has shown that the T407 site (T406 in rats) specifically resides in a flexible linker within a close proximity to the TRP box. The Cdk5 site, in particular, shows high van der Waals energy differences as the conformation of this linker changes between the TRPV1 open and closed state, all of which suggests that Cdk5 mediated phosphorylation could impact ion channel gating.[10,35] Direct phosphorylation of TRPV1 by protein kinases like PKA and PKC generally either increase the sensitivity of the channel[36,37] or decrease $Ca^{2+}$-dependent desensitization.[38] When tested in transfected CHO cells, we saw that Cdk5/p35 phosphorylation of TRPV1-T407 significantly reduced or even eliminated $Ca^{2+}$ dependent desensitization, yet sensitivity to capsaicin remained unaffected.[10,12] Mutations to the threonine residue further demonstrated the importance of the T407 Cdk5 site as substitution with a negatively charged aspartate residue dramatically altered the activation kinetics of TRPV1. This change in the aspartate mutant did not appear to be a consequence of altered membrane trafficking.

In our current study, we further examined the effects of Cdk5 on TRPV1-mediated nociception by mutating the phosphorylation site from threonine to an alanine. We showed that T407A mutation did not affect the expression of the TRPV1. Behavioral testing subsequently showed that the responses to capsaicin and heat were altered in $TRPV1^{T407A}$ mice. First, oral aversion to capsaicin was significantly reduced, although at a low concentration. Then, we saw a trend towards less facial actions of pain following subcutaneous injection of capsaicin into the vibrissal pad. Importantly, we saw that the T407A mutation influenced basal heat responses in mice. The $TRPV1^{T407A}$ mice have significantly reduced hindpaw withdrawal latency when place on a hot plate compared to the wild type controls. The use of blocking T406A peptides to prevent Cdk5 mediated phosphorylation of TRPV1 has been shown to cause reduced inflammatory thermal hyperalgesia in rats but did not seem to affect basal heat sensitivity.[39] Our results with site directed genomic mutation of the Cdk5 site, however, clearly had a stronger impact on acute heat nociception in contrast to interfering peptides. Of note, two independently generated TRPV1 knockout mice from different labs show behavioral differences in response to noxious heat. While both knockout mice show that TRPV1 is essential for inflammatory thermal hyperalgesia, the TRPV1 null mice by Caterina et al.[40] additionally display hypoalgesia to acute noxious thermal stimuli with increased latency responses to heat, whereas Davis et al.[41] reported that TRPV1 KO mice showed normal reactions to acute heat that were comparable to the wild type mice.[29] Our $TRPV1^{T407A}$ mice with just a single TRPV1 point mutation appear to show altered behavioral responses on a hot plate set at 50°C. Of note, $TRPV1^{T407A}$ mice are on the FVB/N strain background, commonly used for developing transgenic mice, which could impact pain sensitivity by exhibiting heightened pain reflexes.[42,43]

We suspect that the overall behavior of the $TRPV1^{T407A}$ mice to heat and capsaicin are due to Cdk5-mediated effects on TRPV1 activation and desensitization. Phosphorylation of TRPV1 has also been proposed to affect the localization of the receptor to plasma membrane via binding with the kinesin motor KIF13B.[39] However, we believe that phosphorylation of TRPV1 predominantly affects ion channel activity based on (1) the electrophysiological recordings of reduced $Ca^{2+}$-dependent desensitization, (2) the location of this T407 site, (3) mutation of threonine to a negatively charged aspartate residue (rat T406D), while not exactly correlating with Cdk5-mediated phosphorylation, dramatically altered the activation kinetics of TRPV1 without leading to agonist-induced (e.g. capsaicin) changes in membrane density/expression (the rat T406A mutation also coincidentally does not affect capsaicin-induced membrane trafficking, yet channel behavior remained like the unmodified wild type TRPV1).[10,44]

Pain has a huge impact on health, work productivity, and wellbeing. An estimated 20% of U.S. adults report chronic pain, including about 8% of individuals who categorize their pain as severe enough to interfere with their quality life, so new therapies to treat pain are needed.[45] Cdk5 has been shown to modulate nociception by both phosphorylating substrates linked with pain signaling and by influencing morphine tolerance.[12,28,46] Cdk5 has thereby become an attractive target for developing new analgesics. Intrathecal injection of the Cdk5 inhibitor roscovitine has already been shown to attenuate neuropathic and cancer pain.[26,27] A 24 amino acid peptide derived from p35 also displays analgesic properties. The peptide TFP5 can reduce Cdk5 hyperactivity back to normal physiological levels and is able to decrease neuronal hyperexcitability in the TG caused by injection of complete Freund's adjuvant.[30,47] Cdk5 has a phospho-regulatory role in influencing pain sensitivity by modulating nociceptive signaling through TRPV1. Cdk5 activity can affect $Ca^{2+}$-induced desensitization in TRPV1 while also shaping the extent of nociceptor firing in response to heat and capsaicin.[10,30] The development of the $TRPV1^{T407A}$ mice further establishes a direct link between Cdk5 phosphorylation and pain signaling through TRPV1. Our study shows that loss of the Cdk5 T407 phosphorylation site in TRPV1 in mice causes alterations in oral aversion to capsaicin, reduced facial expressions after the injection of capsaicin, and increased paw withdrawal latency to heat on a hot plate. These behavioral changes in the $TRPV1^{T407A}$ mice illustrate that the Cdk5 phosphorylation of T407 alone can impact TRPV1-mediated nociception and that the inhibition of Cdk5 has potential therapeutic value for deriving new analgesics.

Further studies utilizing TRPV1$^{T407A}$ mice in pathological pain should help such development.

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## Authors' contributions

AC, BEH, and ABK conceptualized the project, and drafted the manuscript. AC, BEH, ASL, and SW collected and analyzed data. AC, BEH, M-KC, and ABK drafted and edited the manuscript.

## Declaration of Conflicting Interests

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## References

1. Ji RR, Kawasaki Y, Zhuang ZY, Wen YR, Zhang YQ. Protein kinases as potential targets for the treatment of pathological pain. *Handb Exp Pharmacol* 2007; 177: 359–389.
2. Pareek TK, Keller J, Kesavapany S, Agarwal N, Kuner R, Pant HC, Iadarola MJ, Brady RO, Kulkarni AB. Cyclin-dependent kinase 5 activity regulates pain signaling. *Proc Natl Acad Sci USA* 2006; 103: 791–796.
3. Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D. The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* 1997; 389: 816–824.
4. Kong WL, Peng YY, Peng BW. Modulation of neuro-inflammation: role and therapeutic potential of TRPV1 in the neuro-immune axis. *Brain Behav Immun* 2017; 64: 354–366.
5. Zheng W, Qin F. A combined coarse-grained and all-atom simulation of TRPV1 channel gating and heat activation. *J Gen Physiol* 2015; 145: 443–456.
6. Utreras E, Futatsugi A, Pareek TK, Kulkarni AB. Molecular roles of Cdk5 in pain signaling. *Drug Discov Today Ther Strateg* 2009; 6: 105–111.
7. Harada T, Morooka T, Ogawa S, Nishida E. ERK induces p35, a neuron-specific activator of Cdk5, through induction of Egr1. *Nat Cel Biol* 2001; 3: 453–459.
8. Utreras E, Futatsugi A, Rudrabhatla P, Keller J, Iadarola MJ, Pant HC, Kulkarni AB. Tumor necrosis factor-alpha regulates cyclin-dependent kinase 5 activity during pain signaling through transcriptional activation of p35. *J Biol Chem* 2009; 284: 2275–2284.
9. Pareek TK, Keller J, Kesavapany S, Agarwal N, Kuner R, Pant HC, Iadarola MJ, Brady RO, Kulkarni AB. Cyclin-dependent kinase 5 modulates nociceptive signaling through direct phosphorylation of transient receptor potential vanilloid 1. *Proc Natl Acad Sci USA* 2007; 104: 660–665.
10. Jendryke T, Prochazkova M, Hall BE, Nordmann GC, Schladt M, Milenkovic VM, Kulkarni AB, Wetzel CH. TRPV1 function is modulated by Cdk5-mediated phosphorylation: insights into the molecular mechanism of nociception. *Sci Rep* 2016; 6: 22007.
11. Liu J, Du J, Yang Y, Wang Y. Phosphorylation of TRPV1 by cyclin-dependent kinase 5 promotes TRPV1 surface localization, leading to inflammatory thermal hyperalgesia. *Exp Neurol* 2015; 273: 253–262.
12. Gomez K, Vallecillo TGM, Moutal A, Perez-Miller S, Delgado-Lezama R, Felix R, Khanna R. The role of cyclin-dependent kinase 5 in neuropathic pain. *Pain* 2020; 161: 2674–2689.
13. Hall BE, Prochazkova M, Sapio MR, Minetos P, Kurochkina N, Binukumar BK, Amin ND, Terse A, Joseph J, Raithel SJ, Mannes AJ, Pant HC, Chung M-K, Iadarola MJ, Kulkarni AB. Phosphorylation of the transient receptor potential ankyrin 1 by cyclin-dependent kinase 5 affects chemo-nociception. *Sci Rep* 2018; 8: 1177.
14. Coddou C, Sandoval R, Castro P, Lazcano P, Hevia MJ, Rokic M, Hall B, Terse A, Gonzalez-Billault C, Kulkarni AB, Stojilkovic SS, Utreras E. Cyclin-dependent kinase 5 modulates the P2X2a receptor channel gating through phosphorylation of C-terminal threonine 372. *Pain* 2017; 158: 2155–2168.
15. Hall B, Cho A, Limaye A, Cho K, Khillan J, Kulkarni AB. Genome editing in mice using CRISPR/Cas9 technology. *Curr Protoc Cel Biol* 2018; 81: e57.
16. Hsu PD, Scott DA, Weinstein JA, Ran FA, Konermann S, Agarwala V, Li Y, Fine EJ, Wu X, Shalem O, Cradick TJ, Marraffini LA, Bao G, Zhang F. DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat Biotechnol* 2013; 31: 827–832.
17. Prochazkova M, Terse A, Amin ND, Hall B, Utreras E, Pant HC, Kulkarni AB. Activation of cyclin-dependent kinase 5 mediates orofacial mechanical hyperalgesia. *Mol Pain* 2013; 9: 66–69.
18. Malin SA, Davis BM, Molliver DC. Production of dissociated sensory neuron cultures and considerations for their use in studying neuronal function and plasticity. *Nat Protoc* 2007; 2: 152–160.
19. Langford DJ, Bailey AL, Chanda ML, Clarke SE, Drummond TE, Echois S, Glick S, Ingrao J, Klassen-Ross T, Lacroix-

Fralish ML, Matsumiya L, Sorge RE, Sotocinal SG, Tabaka JM, Wong D, van den Maagdenberg AMJM, Ferrari MD, Craig KD, Mogil JS. Coding of facial expressions of pain in the laboratory mouse. *Nat Methods* 2010; 7: 447–449.

20. Yonghak P, Miyata S, Kurganov E. TRPV1 is crucial for thermal homeostasis in the mouse by heat loss behaviors under warm ambient temperature. *Sci Rep* 2020; 10: 8799.
21. Joseph J, Qu L, Wang S, Kim M, Benett D, Ro J, Caterina MJ, Chung MK. Phosphorylation of TRPV1 S801 contributes to modality-specific hyperalgesia in mice. *J Neurosci* 2019; 39: 9954–9966.
22. Duo L, Hu L, Tian N, Cheng G, Wang H, Lin Z, Wang Y, Yang Y. TRPV1 gain-of-function mutation impairs pain and itch sensations in mice. *Mol Pain* 2018; 14: 1744806918762031.
23. Caterina MJ, Leffler A, Malmberg AB, Martin WJ, Trafton J, Petersen-Zeitz KR, Koltzenburg M, Basbaum AI, Julius D. Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science* 2000; 288: 306–313.
24. Prochazkova M, Hall B, Hu M, Okine T, Reukauf J, Binukumar BK, Amin ND, Roque E, Pant HC, Kulkarni A. Peripheral and orofacial pain sensation is unaffected by the loss of p39. *Mol Pain* 2017; 13: 1744806917737205.
25. Li BS, Sun MK, Zhang L, Takahashi S, Ma W, Vinade L, Kulkarni AB, Brady RO, Pant HC. Regulation of NMDA receptors by cyclin-dependent kinase-5. *Proc Natl Acad Sci USA* 2001; 98: 12742–12747.
26. Zhang R, Liu Y, Zhang J, Zheng Y, Gu X, Ma Z. Intrathecal administration of roscovitine attenuates cancer pain and inhibits the expression of NMDA receptor 2B subunit mRNA. *Pharmacol Biochem Behav* 2012; 102: 139–145.
27. Yang L, Gu X, Zhang W, Zhang J, Ma Z. Cdk5 inhibitor roscovitine alleviates neuropathic pain in the dorsal root ganglia by downregulating N-methyl-D-aspartate receptor subunit 2A. *Neurol Sci* 2014; 35: 1365–1371.
28. Pareek TK, Kulkarni AB. Cdk5: a new player in pain signaling. *Cell Cycle* 2006; 5: 585–588.
29. Suh YG, Oh U. Activation and activators of TRPV1 and their pharmaceutical implication. *Curr Pharm Des* 2005; 11: 2687–2698.
30. Hu M, Doyle AD, Yamada KM, Kulkarni AB. Visualization of trigeminal ganglion sensory neuronal signaling regulated by Cdk5. *Cel Rep* 2022; 38: 110458.
31. Xing BM, Yang YR, Du JX, Chen HJ, Qi C, Huang ZH, Zhang Y, Wang Y. Cyclin-dependent kinase 5 controls TRPV1 membrane trafficking and the heat sensitivity of nociceptors through KIF13B. *J Neurosci* 2012; 32: 14709–14721.
32. Liu J, Du J, Wang Y. CDK5 inhibits the clathrin-dependent internalization of TRPV1 by phosphorylating the clathrin adaptor protein AP2μ2. *Sci Signal* 2019; 12: eaaw2040.
33. Yao J, Liu B, Qin F. Modular thermal sensors in temperature-gated transient receptor potential (TRP) channels. *Proc Natl Acad Sci USA* 2011; 108: 11109–11114.
34. Kim M, Sisco NJ, Hilton JK, Montano CM, Castro MA, Cherry BR, Levitus M, Van Horn WD. Evidence that the TRPV1 S1-S4 membrane domain contributes to thermosensing. *Nat Commun* 2020; 11: 4169.
35. Zheng W, Qin F. A combined coarse-grained and all-atom simulation of TRPV1 channel gating and heat activation. *J Gen Physiol* 2015; 145: 443–456.
36. Rathee PK, Distler C, Obreja O, Neuhuber W, Wang GK, Wang GK, Wang SY, Nau C, Kress M. PKA/AKAP/VR-1 module: a common link of Gs-mediated signaling to thermal hyperalgesia. *J Neurosci* 2002; 22: 4740–4745.
37. Bhave G, Hu HJ, Glauner KS, Zhu W, Wang H, Brasier DJ, Oxford GS, Gereau RW 4th. Protein kinase C phosphorylation sensitizes but does not activate the capsaicin receptor transient receptor potential vanilloid 1 (TRPV1). *Proc Natl Acad Sci USA* 2003; 100: 12480–12485.
38. Mohapatra DP, Nau C. Desensitization of capsaicin-activated currents in the vanilloid receptor TRPV1 is decreased by the cyclic AMP-dependent protein kinase pathway. *J Biol Chem* 2003; 278: 50080–50090.
39. Liu J, Du J, Yang Y, Wang Y. Phosphorylation of TRPV1 by cyclin-dependent kinase 5 promotes TRPV1 surface localization, leading to inflammatory thermal hyperalgesia. *Exp Neurol* 2015; 273: 253–262.
40. Caterina MJ, Leffler A, Malmberg AB, Martin WJ, Trafton J, Petersen-Zeitz KR, Kolzenburg M, Basbaum AI, Julius D. Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science* 2000; 288: 306–313.
41. Davis JB, Gray J, Gunthorpe MJ, Hatcher JP, Davey PT, Overend P, Harries MH, Latcham J, Clapham C, Atkinson K, Hughes SA, Rance K, Grau E, Harper AJ, Pugh PL, Rogers DC, Bingham S, Randall A, Sheardown SA. Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia. *Nature* 2000; 405: 183–187.
42. Mogil JS, Wilson SG, Bon K, Lee SE, Chung K, Raber P, Pieper JO, Hain HS, Belknap JK, Hubert L, Elmer GI, Chung JM, Devor M. Heritability of nociception I: responses of 11 inbred mouse strains on 12 measures of nociception. *Pain* 1999; 80: 67–82.
43. Cho A, Haruyama N, Kulkarni AB. Generation of transgenic mice. *Curr Protoc Cel Biol* 2009; 42(1): 19.11.
44. Sanz-Salvador L, Andrés-Borderia A, Ferrer-Montiel A, Planells-Cases R. Agonist- and Ca2+-dependent desensitization of TRPV1 channel targets the receptor to lysosomes for degradation. *J Biol Chem* 2012; 287: 19462–19471.
45. Dahlhamer J, Lucas J, Zelaya C, Nahin R, Mackey S, Debar L, Kerns R, Von Korff M, Porter L, Helmick C. Prevalence of chronic pain and high-impact chronic pain among adults—United States, 2016. *MMWR Morb Mortal Wkly Rep* 2018; 67: 1001–1006.
46. Pareek TK, Zipp L, Letterio JJ. Cdk5: an emerging kinase in pain signaling. *Brain Disord Ther* 2013; 2013(Suppl 1): 003.
47. Zheng YL, Amin ND, Hu YF, Rudrabhatla P, Shukla V, Kanungo J, Kesavapany S, Grant P, Albers W, Pant HC. A 24-residue peptide (p5), derived from p35, the Cdk5 neuronal activator, specifically inhibits Cdk5-p25 hyperactivity and tau hyperphosphorylation. *J Biol Chem* 2010; 285: 34202–34212.