TRPV1 function is modulated by Cdk5-mediated phosphorylation: insights into the molecular mechanism of nociception

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TRPV1 is a polymodally activated cation channel acting as key receptor in nociceptive neurons. Its function is strongly affected by kinase-mediated phosphorylation leading to hyperalgesia and allodynia. We present behavioral and molecular data indicating that TRPV1 is strongly modulated by Cdk5-mediated phosphorylation at position threonine-407 (mouse)/T406 (rat). Increasing or decreasing Cdk5 activity in genetically engineered mice has severe consequences on TRPV1-mediated pain perception leading to altered capsaicin consumption and sensitivity to heat. To understand the molecular and structural/functional consequences of TRPV1 phosphorylation, we generated various rTRPV1T406 receptor variants to mimic phosphorylated or dephosphorylated receptor protein. We performed detailed functional characterization by means of electrophysiological whole-cell and single-channel recordings as well as Ca2+-imaging and challenged recombinant rTRPV1 receptors with capsaicin, low pH, or heat. We found that position T406 is critical for the function of TRPV1 by modulating ligand-sensitivity, activation, and desensitization kinetics as well as voltage-dependence. Based on high resolution structures of TRPV1, we discuss T406 being involved in the molecular transition pathway, its phosphorylation leading to a conformational change and influencing the gating of the receptor. Cdk5-mediated phosphorylation of T406 can be regarded as an important molecular switch modulating TRPV1-related behavior and pain sensitivity.

The Transient receptor potential vanilloid 1 (TRPV1) is a ligand-gated non-selective cation channel which is prominently expressed in sensory nociceptive C- and Aδ fibers of trigeminal and dorsal root ganglia neurons1,2. Common for all TRP channels is a tetrameric structure, with each subunit including six transmembrane domains (TMD1-TMD6) and extensive intracellular amino and carboxyl termini3. As a polymodal receptor, TRPV1 is activated by various exogenous and endogenous stimuli such as the vanilloid capsaicin, heat2, and protons4, as well as the endocannabinoid anandamide5. During inflammation, sensory neurons are sensitized by inflammatory mediators, which activate several signal transduction pathways, leading to protein kinase-mediated phosphorylation of TRPV16,7. Several protein kinases such as PKA8–10, PKC11–13, CaMKII14, and c-Src kinase15, are known to phosphorylate TRPV1 at various serine and threonine residues, leading to sensitization of receptor function. In contrast, dephosphorylation of TRPV1 by the Ca2+–dependent phosphatase calcineurin leads to the desensitization of the receptor16.

In 2007, Pareek et al. reported that cyclin-dependent kinase 5 (Cdk5) mediated phosphorylation of TRPV1 regulates Ca2+ influx through this channel. They demonstrated that Cdk5 deficiency in sensory neurons of mice abrogated TRPV1 phosphorylation and induced thermal hypoalgesia. Analyzing the amino acid sequence of TRPV1 revealed three different potential consensus sites for Cdk5-mediated phosphorylation: threonine-108 (T108), threonine-407 (T407), and serine-612 (S612). Of the three sites, T407 is highly conserved and the preferred target site for Cdk5-dependent phosphorylation17. In order to further investigate pain signal transduction in nociceptive neurons, we functionally characterized the impact of Cdk5-mediated phosphorylation on nociceptive neurons.
Cdk5 activity regulates aversion to oral capsaicin in mice. We have previously reported that Cdk5 is able to phosphorylate TRPV1, which, in turn, influences thermal nociception. Conditional deletion of Cdk5 in nociceptive neurons abrogates phosphorylation of TRPV1, which possibly contributes to the thermal hypoalgesia seen in these mice. To further examine the interaction between Cdk5 activity and TRPV1 function, we tested the sensitivity of our mice to capsaicin (15 μM), a specific activator of TRPV1. Oral administration of capsaicin causes an unpleasant burning sensation, so we used the lickometer to measure aversion to water containing this compound. The behavior is expressed as a % of the baseline licking responses for plain water as compared to capsaicin. Increased aversion and hypersensitivity to capsaicin was evident in Tgp35 mice as compared to wild-type mice (WT) controls (One-way ANOVA followed by Dunnett’s multiple comparisons test, p < 0.0001). Data are presented as mean ± SEM from four animals during five different measurements (a). Effect of temperature activation of TRPV1 in mutant animals. An orofacial pain assessment device was used to measure the responses of the mice to hot facial stimulation. All mice showed similar consumption of the reward (sucrose) at 37 °C (b). Tgp35 mice displayed an aversive behavior to the increased temperature of the thermodes as noted by significantly decreased licking behavior (unpaired t-test, p = 0.0002), whereas p35KO mice displayed significantly increased number of licks compared to wild-type controls (One-way ANOVA followed by Dunnett’s multiple comparisons test, p < 0.05) (c). Data are presented as mean ± SEM from four animals measured five times using 37 °C and three times at 45 °C.
CONTROL, which show no difference in their capsaicin consumption. These results confirm that Cdk5 activity modulates oral pain related responses transduced via the TRPV1 channel.

**Cdk5 activity modulates thermal nociceptive signaling in orofacial area.** Since TRPV1 is a poly-modal ion channel, we additionally wanted to examine thermal nociception at a temperature known to activate TRPV1. To measure orofacial thermosensitivity, we used the OPAD system (Orofacial Pain Assessment Device, Stoelting), an operant behavioral testing device that provides an automated measurement of both hot and cold-induced noxious orofacial stimuli. We observed that both p35KO and transgenic p35 mice exhibit altered responses to thermal stimulation. We did not observe any difference in 30% sucrose consumption at 37 °C between the genotypes (Fig. 1b). However, we observed increased aversion to facial contact with thermodes set at 45 °C in mice overexpressing p35, as was evidenced by the decreasing number of attempts the mice made to access the reward (unpaired t-test, \( p = 0.0002 \), Fig. 1c). The number of reward licking/facial contact events was also significantly decreased in these mice when the test temperature was increased. To the contrary, mice with decreased Cdk5 activity displayed thermal hypoalgesia (One-way ANOVA followed by Dunnett’s multiple comparison test, \( p < 0.05 \), Fig. 1c). Using the lickometer and OPAD behavioral devices, we were able to test two known activators of TRPV1, capsaicin and noxious heat in our genetically engineered mouse models with altered Cdk5 activity. Our behavioral studies indicate that Cdk5 activity modulates TRPV1 channel activity in vivo, and that increased TRPV1 sensitivity is probably a result of direct phosphorylation of TRPV1 by Cdk5.

**Ca++-induced desensitization of TRPV1 is modulated by co-expression of Cdk5 and p35.** Next, we wanted to determine in more detail how Cdk5-mediated phosphorylation affects TRPV1 channel function by using the patch-clamp technique. To this end, we tested TRPV1 activity in CHO cells co-expressing rTRPV1 and GFP, or co-expressing a combination of rTRPV1, Cdk5-mCherry, and p35-CFP. The fluorescent tags were used to visually select CHO cells expressing the respective proteins for whole-cell voltage-clamp recordings. Voltage-ramp protocols (−100 mV to +100 mV) were applied in order to analyze inward and outward currents. To induce TRPV1-mediated currents, 3.3 μM capsaicin was applied for 200 s in Ca++-containing Ringer’s solution (solution A). Capsaicin induced fast activating TRPV1-mediated currents characterized by a strong acute desensitization in both inward and outward direction (Fig. 2a,b). The desensitization of currents could be prevented by removing extracellular Ca++ (solution B) (Fig. 2e,f). Interestingly, 3.3 μM capsaicin also induced non-desensitizing currents even in presence of Ca++, with slightly reduced activation kinetics, after the co-expression of TRPV1, Cdk5 and p35 (Fig. 2c,d), suggesting that Cdk5-mediated phosphorylation of TRPV1 is responsible for changing TRPV1 activity into a non-desensitizing state. Moreover, we investigated the effect of Cdk5-mediated phosphorylation on the TRPV1 capsaicin concentration-response relationship under Ca++-free non-desensitizing conditions. Therefore, CHO cells expressing TRPV1 or co-expressing TRPV1, Cdk5 and p35 were recorded in the whole-cell configuration and voltage-ramp protocols were used to characterize TRPV1-mediated currents evoked by various capsaicin concentrations. Supplemental Fig. 1 shows representative recordings of capsaicin-induced currents of CHO cells expressing TRPV1 or TRPV1, Cdk5 and p35. The TRPV1-mediated currents were normalized by referring the respective current amplitudes to the current evoked by application of 3.3 μM capsaicin in the same cell, and the Hill-equation (Equation 1) was used to calculate the EC50. For TRPV1 measurements, EC50 values were calculated to 0.25 ± 0.05 μM (out) and 0.63 ± 0.13 μM (in), whereas co-expression of TRPV1, Cdk5 and p35 resulted in EC50 values of 0.28 ± 0.04 μM (out) and 0.55 ± 0.08 μM (in). The statistical analysis revealed no significant Cdk5-mediated sensitization of capsaicin-induced TRPV1-mediated currents. As previously shown, the Cdk5-mediated phosphorylation of TRPV1 has severe physiological consequences on the perception and transduction of noxious stimuli. Therefore, we hypothesize that the reduced Ca++-dependent desensitization found in cells co-expressing TRPV1, Cdk5 and p35 leads to increased receptor efficacy and promotes the development of allodynia and hyperalgesia in sensory neurons.

**TRPV1 T406 mutagenesis affects the Ca++-induced desensitization.** Pareek et al. demonstrated that the conditional deletion of Cdk5 in pain-sensing neurons in mice abrogates TRPV1 phosphorylation at T40717 and our results demonstrate that the co-expression of TRPV1, Cdk5 and p35 leads to the reduction of Ca++-dependent desensitization. Based on these observations, we set out to further characterize the functional consequences of steric and electrical alterations at this particular position on functional parameters of the TRPV1 protein. Therefore, the corresponding threonine of the rat TRPV1 (T406) was replaced by different amino acids comprising different polar, nonpolar, aromatic, or charged characteristics. Transfected CHO cells were recorded in the presence of 2 mM Ca++ (solution A) and TRPV1-mediated currents were induced by applying 3.3 μM capsaicin for 200 s. Mutations that alter steric and electrical properties at position 406 differentially affected the TRPV1 ion channel properties such as kinetics, amplitude and desensitization. Since the size of the recorded cells was consistent at 24.9 ± 2.4 μm (n = 91), we consider that the observed variability of the induced TRPV1-mediated currents were due to different expression rates (Supplemental Fig. 2a). The activation kinetics of mutant receptors carrying aspartate-406, or glutamate-406, as well as lysine-406 or proline-406 were found to be reduced (Supplemental Fig. 2b). Moreover, the desensitization was reduced or even eliminated by the exchange of T406 to negatively charged amino acids, as well as lysine, histidine and proline (Supplemental Fig. 2c). Due to its structure, aspartic acid is the most appropriate amino acid to mimic phosphorylation of proteins20. Currents of TRPV1 receptor variants were induced by 0.3 μM capsaicin in the same manner as capsaicin- and cold-induced noxious orofacial stimuli. We observed that both p35KO and transgenic p35 mice exhibit altered responses to thermal stimulation. We did not observe any difference in 30% sucrose consumption at 37 °C between the genotypes (Fig. 1b). However, we observed increased aversion to facial contact with thermodes set at 45 °C in mice overexpressing p35, as was evidenced by the decreasing number of attempts the mice made to access the reward (unpaired t-test, \( p = 0.0002 \), Fig. 1c). The number of reward licking/facial contact events was also significantly decreased in these mice when the test temperature was increased. To the contrary, mice with decreased Cdk5 activity displayed thermal hypoalgesia (One-way ANOVA followed by Dunnett’s multiple comparison test, \( p < 0.05 \), Fig. 1c). Using the lickometer and OPAD behavioral devices, we were able to test two known activators of TRPV1, capsaicin and noxious heat in our genetically engineered mouse models with altered Cdk5 activity. Our behavioral studies indicate that Cdk5 activity modulates TRPV1 channel activity in vivo, and that increased TRPV1 sensitivity is probably a result of direct phosphorylation of TRPV1 by Cdk5.
Figure 2. Cdk5-mediated phosphorylation of TRPV1 prevents desensitization to capsaicin. TRPV1-mediated inward (−100 mV) and outward (+100 mV) currents in transiently transfected CHO cells induced by 3.3 μM capsaicin in the presence or absence of extracellular Ca2+. Left panels show I/V relationships corresponding to the representative recording traces on the right. In the presence of extracellular Ca2+, application of capsaicin for 200 s established a desensitized steady state in TRPV1 expressing cells (a,b). Co-expression of TRPV1, Cdk5-mCherry and p35-CFP inhibits the Ca2+-induced desensitization (c,d), similar to the capsaicin-induced currents of TRPV1 in absence of extracellular Ca2+ (e,f). Maximal induced currents (g), time to half-maximal response represented as t_{50} (h) and desensitization as ratio I_{steady}/I_{maximal} (i) of n = 11–22 independent measurements. Asterisk (*) indicates significant differences compared to the corresponding TRPV1 value at Ca2+-containing conditions (unpaired WR-test, p < 0.05).
Interestingly, a second application of the identical stimulus after priming the cells with a high capsaicin concentration induced a fast activating response of TRPV1 T406D with $t_{50}$ values similar to TRPV1 WT and TRPV1 T406A (TRPV1 WT: $3.0 \pm 0.3$ s; TRPV1 T406A: $3.5 \pm 0.9$ s TRPV1 T406D: $2.4 \pm 0.2$ s) (Fig. 3d,e) suggesting a use-dependent behavior of the TRPV1 T406D receptor mutant. Similar to the phosphorylated TRPV1 (after co-expression of TRPV1, Cdk5, and p35), TRPV1 T406D did not show any desensitization. The desensitization of inward currents (measured as $I_{\text{steady}}/I_{\text{maximal}}$) induced by $0.3 \mu M$ capsaicin was on average $0.50 \pm 0.05$ in TRPV1 WT and $0.94 \pm 0.01$ in TRPV1 T406D (Fig. 3f). Interestingly, in TRPV1-mediated responses of cells co-expressing TRPV1 T406D, Cdk5, and p35, we found no difference in activation and desensitization kinetics compared to cells only expressing TRPV1 T406D (Supplemental Fig. 3). Next we set out to investigate the use-dependent behavior of TRPV1 T406D in detail. In order to analyze the molecular mechanisms that lead to the altered activation kinetics of TRPV1 T406D and to address the question whether changes in response kinetics might be dependent on dynamic receptor trafficking, we monitored the dynamics of membrane localization of directly C-terminally GFP-tagged TRPV1 T406 receptor variants. Electrophysiological recordings of GFP-TRPV1 T406D showed that they were fully functional and showed the characteristic functional behavior of the respective untagged receptors (Supplemental Fig. 4). Using TIRF microscopy to visualize membrane expression of the fluorescent TRPV1 receptors, we could not detect any change in activation and desensitization kinetics compared to cells only expressing TRPV1 T406D (Supplemental Fig. 5), indicating that the density/efficiency of TRPV1 receptors in the membrane is not altered during capsaicin treatment. Interestingly, in addition to the markedly altered channel activation and desensitization kinetics, we observed that the TRPV1 T406D-mediated outward currents at $+100$ mV after maximal stimulation did not reach baseline, but stayed at a higher activation level (Fig. 3b), which is suggestive of an altered voltage-dependence.

**Targeted mutagenesis affects the voltage-dependence of TRPV1.** To investigate the voltage-dependence of TRPV1 T406D, voltage-induced currents were measured before, during, and after $3.3 \mu M$ capsaicin stimulation under Ca$^{2+}$-free conditions (solution B) by applying defined voltage-steps ($-120$ mV to $+160$ mV).
TRPV1WT and TRPV1T406A exhibited the characteristic outward rectifying voltage-activated currents, whereas hardly any currents could be recorded in TRPV1T406D mutants (Fig. 4a). However, application of 3.3 μM capsaicin resulted in robust inward and outward currents in CHO cells expressing TRPV1WT, TRPV1T406A, or TRPV1T406D (Fig. 4b). Voltage-activated currents evoked one minute after washout of capsaicin revealed an increased voltage-dependence of TRPV1T406D whereas the voltage-induced currents of TRPV1WT and TRPV1T406A recover to the same level as under Ringer's conditions (c). Normalized conductance G/Gmax of n = 6–7 independent measurements of TRPV1WT (d), TRPV1T406A (e), and TRPV1T406D (f). Sigmoidal fit was used to calculate V_1/2. Asterisk (*) indicates significant decrease of V_1/2 of TRPV1T406D after priming with 3.3 μM capsaicin (paired WR-test, p < 0.05).

Figure 4. Voltage-dependence of TRPV1 is altered in the T406D mutant. Voltage-dependence of TRPV1WT and TRPV1T406D mutants in transiently transfected CHO cells measured by voltage step protocols with depolarizing pulses from −120 mV to +160 mV. In Ca^{2+}-free ringer solution, voltage-dependent currents are detected in TRPV1WT and TRPV1T406A, but not in TRPV1T406D (a). Application of 3.3 μM capsaicin induces robust voltage-dependent currents in CHO cells expressing TRPV1WT, TRPV1T406A, or TRPV1T406D (b). Voltage-activated currents evoked one minute after washout of capsaicin revealed an increased voltage-dependence of TRPV1T406D whereas the voltage-induced currents of TRPV1WT and TRPV1T406A recover to the same level as under Ringer's conditions (c). Normalized conductance G/Gmax of n = 6–7 independent measurements of TRPV1WT (d), TRPV1T406A (e), and TRPV1T406D (f). Sigmoidal fit was used to calculate V_1/2. Asterisk (*) indicates significant decrease of V_1/2 of TRPV1T406D after priming with 3.3 μM capsaicin (paired WR-test, p < 0.05).
TRPV1 T406 mutations modify the sensitivity to capsaicin. In order to investigate the effect of T406 mutation on receptor sensitivity to capsaicin, we analyzed the concentration/response-relationship of TRPV1 WT and TRPV1 T406A mutants by applying various capsaicin concentrations (0.05 to 3.3 µM). Currents were recorded under Ca2+-free conditions to prevent desensitization of the receptors during repetitive agonist applications. Due to the use-dependent activation pattern observed for TRPV1 T406D, we analyzed the capsaicin concentration/response-relationship in two consecutive sets of application. Before priming of TRPV1 T406D with 3.3 µM capsaicin, responses were small and not evaluable. Challenging cells with capsaicin (3.3 µM) led to significant increase in sensitivity and allowed the TRPV1 T406D receptor to then respond to lower concentrations (paired t-test, p < 0.05). EC50 values obtained during the second series of capsaicin application (outward: TRPV1 WT 0.28 ± 0.06 µM; TRPV1 T406A 0.19 ± 0.02 µM; TRPV1 T406D 0.11 ± 0.01 µM; inward: TRPV1 WT 0.66 ± 0.14 µM; TRPV1 T406A 0.40 ± 0.07 µM; TRPV1 T406D 0.26 ± 0.03 µM). n = 6–10 independent measurements were performed for each receptor variant.

Figure 5. Sensitivity of TRPV1 to capsaicin is altered in the T406D mutant. Analysis of TRPV1 concentration/response-relationships in transiently transfected CHO cells using voltage-ramp protocols in the absence of extracellular Ca2+. Inward and outward currents of TRPV1 WT (a), TRPV1 T406A (b) and TRPV1 T406D (c) induced by 0.05, 0.1, 0.3, 1 and 3.3 µM capsaicin. Concentration/response-relationships of outward (d) or inward (e) currents of TRPV1 WT and TRPV1 T406A show no difference between first and second series of application. Before priming of TRPV1 T406D with 3.3 µM capsaicin, responses were small and not evaluable. Challenging cells with capsaicin (3.3 µM) led to significant increase in sensitivity and allowed the TRPV1 T406D receptor to then respond to lower concentrations (paired t-test, p < 0.05). EC50 values obtained during the second series of capsaicin application (outward: TRPV1 WT 0.28 ± 0.06 µM; TRPV1 T406A 0.19 ± 0.02 µM; TRPV1 T406D 0.11 ± 0.01 µM; inward: TRPV1 WT 0.66 ± 0.14 µM; TRPV1 T406A 0.40 ± 0.07 µM; TRPV1 T406D 0.26 ± 0.03 µM). n = 6–10 independent measurements were performed for each receptor variant.
detectable current responses to low capsaicin concentrations during the first set, the accurate estimation of the EC50 is not applicable. However, the second set of applications induced currents that correlated with capsaicin concentration and allowed the calculation of EC50 values for TRPV1WT and TRPV1T406D. At +100 mV, the EC50 was significantly reduced in TRPV1T406D compared to TRPV1WT (0.11 ± 0.01 μM vs. 0.28 ± 0.00 μM; p < 0.05) (Fig. 5d,e). Based on these findings, we conclude that electrical and ionic properties of the amino acid residue at position 406 strongly influence the capsaicin sensitivity of TRPV1.

**TRPV1T406D mutagenesis also affects proton and heat activation.** Since the TRPV1 receptor is activated by various noxious stimuli, such as voltage, capsaicin, heat, or protons, we set out to study the dependence of TRPV1WT on the modality of the activating stimulus. To this end, cells expressing the respective receptor variants were challenged with two consecutive sets of applications of pH 6, as well as 0.3 μM and 3.3 μM capsaicin under Ca2+-free conditions (Fig. 6a–c). While TRPV1WT and TRPV1T406D responded to pH 6, 0.3 μM, and 3.3 μM capsaicin during the first and second set of application, TRPV1T406D responded during the first set only to 3.3 μM capsaicin, but gained full responsiveness to low pH and capsaicin during the second set, again reflecting a use-dependent activation pattern. The ratio of the first and second response to the same stimulus is a measure of sensitization or desensitization of the receptor (Fig. 6d and Supplemental Fig. 2d).

In order to investigate the sensitivity of TRPV1 receptor mutants to heat, we performed Fura-2 Ca2+ imaging of HEK293T cells expressing TRPV1 WT or TRPV1T406D by making use of the high Ca2+ permeability of the TRPV1 ion channel. We evoked TRPV1-mediated Ca2+ fluxes by stimulating the cells with 3.3 μM capsaicin at room temperature, or by perfusing the recording chamber with physiological Ringer’s solution (solution A) heated up to >42°C. Figure 7 represents characteristic Ca2+ imaging measurements of HEK293T cells expressing the TRPV1WT or TRPV1T406D, respectively. Cells were challenged by heating solution A solution by application of 3.3 μM capsaicin at room temperature (24°C). The second heat activation was performed 15 min after the washout of 3.3 μM capsaicin (Fig. 7a–d). The functional properties of TRPV1 were analyzed by evaluating the ΔF ratio (F340/F380), as a measure for the heat or capsaicin-induced TRPV1-mediated Ca2+ influx. In TRPV1WT measurements, no difference between the first and second Ca2+ response to heat was detected, whereas in recordings of TRPV1T406D expressing cells, a decreased response to the first and an increased response to the second heat stimulus was observed (Fig. 7e). The increased efficacy of TRPV1T406D was paralleled by an acceleration in activation kinetics (Fig. 7f). Our Ca2+ imaging results are in line with our previous electrophysiological data on TRPV1T406D function and suggest that mutation of T406 affects the polymodal activation properties of TRPV1, probably by altering the voltage-dependence of the receptor.

**Single-channel characteristics of TRPV1T406D are different from those of TRPV1WT.** In order to study the functional effects of the TRPV1T406D mutation at the level of single protein level, we analyzed the biophysical properties such as single-channel amplitude, open probability, and gating. To this end, we performed cell-attached recordings, equalized the membrane potential by using high [K+] extracellular solution and filled the patch pipettes with a solution containing 10 mM BaCl2 (solution E) to block endogenous K+ ion channels. Ion channel openings and closings were recorded for periods of >2 min. At least three independent single-channel measurements were conducted for every experimental setting (Fig. 8a,b). In general, single-channel amplitudes are dependent on both the electrochemical gradient and the ion channel pore characteristics. In both TRPV1WT and TRPV1T406D expressing CHO cells, we found single-channel amplitudes of 5–8 pA at −60 mV pipette potential (resulting in a membrane potential of +60 mV). Remarkably, the open state of the individual events did not show constant amplitudes, but tend to decrease from an initial high to a lower plateau conductance state. This behavior indicates a dynamic process within the pore, leading to alteration of conductance/permeability during gating. However, the mechanism behind this observation is not clear at the moment, but will be addressed in future studies. In order to analyze the open probability (NP0) of TRPV1WT and TRPV1T406D, ion channels amplitude histograms were extracted from the data (Fig. 8c,d). Adding 0.3 μM capsaicin to the pipette solution increased the average open probability (NP0) of TRPV1WT from 16.5 to 60.6% and the NP0 of TRPV1T406D from 1.5 to 4.8%, demonstrating the low activity of TRPV1T406D under these conditions (Fig. 8c,d). Pre-treating TRPV1T406D expressing cells for two minutes with 3.3 μM capsaicin and adding 0.3 μM capsaicin to the pipette solution (after washout) led to priming of the receptor and increased the open probability to 88.8% (Fig. 8d), again reflecting the use-dependent activation of TRPV1T406D. The statistical analysis of the open and closed states of TRPV1WT and TRPV1T406D revealed that TRPV1T406D spent significantly less time in the open state at +60 mV in the presence of 0.3 μM capsaicin, but priming TRPV1T406D with 3.3 μM capsaicin shifted the open probability similar to TRPV1WT (about 90%). These findings were in line with our previous whole-cell measurements, indicating that the use-dependent activation of TRPV1T406D might be based on the engagement of different conformations.

In order to determine the state time constants of the open and closed states, the recorded single-channel events were further analyzed by calculating the open dwell-time distribution. Therefore the root square of the frequency versus the natural logarithm of time was plotted and fitted with polynomial functions to discriminate between the different open or closed states. Supplemental Fig. 7a,b shows representative dwell-time histograms for the activity of TRPV1WT and TRPV1T406D. Similar to previous studies, we found three open states and four closed states. Although the time constants of open states as well as the relative contribution of the states O1 to O3 seem to slightly vary with different experimental conditions (0.3 μM, with/without priming with 3.3 μM capsaicin), the open states were not significantly different between TRPV1T406D mutant and TRPV1WT. The detailed analysis of the closed states C1 to C4 revealed an (compared with TRPV1WT) increased long time constant C4 in the TRPV1T406D mutant, which is markedly reduced in both, time and relative contribution in the presence of 0.3 μM capsaicin after priming. However, closed states of TRPV1WT are only slightly modulated by capsaicin (Supplemental Fig. 7c,d). The analysis of the gating properties point to the involvement of closed state
modulation of TRPV1T406D resulting in the observed use-dependent behavior. In summary, we have demonstrated in our mouse models that the oral pain responses mediated by TRPV1 receptors are modulated by Cdk5 activity and that co-expression of Cdk5, p35 and TRPV1 modulates ion channel desensitization in vitro. By mutating rTRPV1T406 (corresponds to T407 in mouse sequence) we were able to characterize TRPV1 ion channel properties in more detail. We investigated the functional parameters on the whole-cell (sensitivity, desensitization, voltage-dependence, activation kinetics), as well as on the single-channel level (open probability, open

Figure 6. Sensitivity of TRPV1 to pH 6 is altered in the T406D mutant. pH 6, 0.3 μM and 3.3 μM capsaicin-induced currents of TRPV1WT and TRPV1T406A mutants in the absence of extracellular Ca2+. Application of Ringer's solution at pH 6 or 0.3 μM capsaicin induces currents in TRPV1WT(a) and TRPV1T406A(b) receptor variants. TRPV1T406D expressing cells respond to pH 6 or 0.3 μM capsaicin only after priming the cells with 3.3 μM capsaicin (c). Ratio between first and second response indicates sensitization (<1) or desensitization (>1) during 3.3 μM capsaicin priming (d). Analysis of n = 10–15 independent measurements shows that TRPV1T406D is sensitized by priming with 3.3 μM capsaicin (unpaired WR-test, p < 0.05).
dwell-time distribution, and gating). Based on the altered TRPV1 ion channel function, we conclude that T406 (rat sequence) is crucial for TRPV1 receptor function and that the Cdk5-mediated phosphorylation of TRPV1 at position T406 has severe consequences on the transduction of potential painful stimuli.

**Discussion**

Previous *in vivo* studies have demonstrated that genetically engineered Cdk5 mice have altered peripheral thermal and mechanical nociception. The direct phosphorylation of transducers of noxious stimuli by Cdk5 may account for the alterations in nociception in these mice. Under this premise, the thermosensitive cation channel TRPV1 was identified to be a substrate for Cdk5 and inhibition of Cdk5 activity attenuates TRPV1-mediated Ca\(^{2+}\) influx in cultured DRG neurons, suggesting that Cdk5 modulates TRPV1 activity. This hypothesis was tested *in vivo* using nociceptor specific Cdk5 conditional knockout mice, where abrogation of Cdk5-mediated phosphorylation of TRPV1 appeared to correlate with thermal hypoalgesia, as measured by increased paw and tail withdrawal latency. In the current study, we have extended our *in vivo* analysis of Cdk5 activity and TRPV1-mediated pain responses by examining orofacial aversion to both the specific TRPV1 agonist capsaicin and to noxious heat (45 °C). Our results clearly demonstrate a correlation between Cdk5 activity and TRPV1-mediated pain transduction. Increased Cdk5 activity was correlated with less aversion to capsaicin (hypalgesia). Thus, our findings indicate that Cdk5 phosphorylation of TRPV1 plays a crucial role in the mechanism of altered behavior and nociception in our Cdk5 mouse models.

In order to investigate the functional consequences of Cdk5-mediated TRPV1 phosphorylation on a molecular level, we used CHO cells co-expressing rTRPV1, Cdk5 and the neuron specific Cdk5 activator p35. We could show that in the presence of Cdk5 and p35, TRPV1-mediated currents induced by high concentrations of capsaicin (3.3 μM) did not desensitize, while the sensitivity to capsaicin was unaffected. As a functional consequence, a lack of TRPV1 desensitization most likely leads to a prolonged neuronal activity of sensory neurons, which then may cause hyperalgesia and/or allodynia. Similarly, Bhave et al. mutated potential PKA phosphorylation sites of the TRPV1 receptor to mimic or inhibit PKA-dependent phosphorylation. Replacing the target serine or threonine with aspartate or alanine, led to an altered receptor desensitization. Also Numazaki and colleagues confirmed the involvement of two serine residues in protein kinase C-dependent TRPV1 potentiation.

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**Figure 7.** Response of TRPV1 to heat is altered in the T406D mutant. HEK293T cells expressing TRPV1\_WT or TRPV1\_T406D in Fura-2 Ca\(^{2+}\) imaging experiments. Representative ratio image and time course of TRPV1 \(n = 131\) (a,b) and TRPV1\_T406D \(n = 136\) (c,d) measurements. Mean ± SEM of the TRPV1-mediated responses (Δ ratio \((F_{380}/F_{340})\)), induced by 42 °C or 3.3 μM capsaicin (e). Responses to first and second heat stimuli were equal in TRPV1\_WT expressing cells. Compared to TRPV1\_WT, the initial response of TRPV1\_T406D to heat (42 °C) is significantly lower, but increases after priming with 3.3 μM capsaicin (paired *t*-test, *p* < 0.05). The activation kinetics (rise time from 10 to 90% = \(t_{10–90}\)) of TRPV1\_T406D is significantly accelerated in the second heat-induced responses (1st \(51.1 ± 2.2\) s, 2nd \(31.3 ± 0.8\) s) (paired *t*-test, *p* < 0.05) (f).
by mutating S502 and S800 to alanine. Similar mutagenesis studies were performed to identify the phosphorylation sites for CaMKII and c-Src kinase. Pareek et al. were the first to describe a role of Cdk5 in phosphorylating TRPV1 leading to sensitization of its nociceptor function. However, no data on the molecular impact of TRPV1 phosphorylation by Cdk5 were available so far. We generated several TRPV1 T406 mutants aiming to mimic the phosphorylated or de-phosphorylated state of the receptor protein and to study the effect of amino acid residues owning different size and charge on the function of mutated TRPV1 ion channels. We could show that the introduction of both, negatively but also positively charged amino acid residues (as well as proline), reduced or even inhibited the Ca²⁺-dependent desensitization. Interestingly, TRPV1T406D and TRPV1T406E mutants exhibited a use-dependent behavior with low stimulus sensitivity and very slow kinetics at an initial activation, which was markedly enhanced and accelerated after priming with a high capsaicin concentration. In 2012, Xing et al. and in 2015, Liu et al. showed that Cdk5 positively regulates the TRPV1 membrane trafficking in nociceptors. They found that the Cdk5-dependent phosphorylation of the motor protein KIF13B promotes the TRPV1 trafficking process. Furthermore they showed that this regulatory mechanism contributes to inflammatory heat

Figure 8. TRPV1 single-channel properties are altered in the T406D mutant. TRPV1 single-channel events measured in cell-attached configuration of transiently transfected CHO cells at −60 mV pipette potential (equivalent to +60 mV membrane potential). Extracellular solution with elevated K⁺ concentration was used to adjust the resting potential of the cell to 0 mV. Pipette solution contained 10 mM Ba²⁺ to silence endogenous K⁺ channels. Representative gating events of TRPV1WT (a) and TRPV1T406D (b) at −60 mV (i) induced by 0.3 μM capsaicin (ii) and by 0.3 μM capsaicin after 2 min incubation with 3.3 μM capsaicin (iii). Representative event distribution histograms showing the open and closed probability (NP₀/C) of TRPV1WT (c) and TRPV1T406D (d). Statistical analysis of open and close probability revealed that the open probability of TRPV1T406D was significantly reduced at −60 mV (i), and −60 mV + 0.3 μM capsaicin, whereas the pretreatment with 3.3 μM capsaicin induces similar NP₀ of TRPV1T406D as the TRPV1WT (e). n = 3–10 independent recordings (unpaired WR-test, p < 0.05).
T406) generated by crossing Cdk5 f/f mice with SNS-Cre mice 17. Transgenic p35 (Tgp35) mice were bred in FVBN background 11. Age-matched wild-type mice served as controls. All animals were housed in standard cages in climate- and light-controlled rooms with free access to food and water. All experimental procedures were approved by the Animal Care and Use Committee of the National Institute of Dental and Craniofacial Research, National Institutes of Health and adhered to the guidelines of the IASP Committee for Research and Ethical Issue 31. Age-matched wild-type mice served as controls. All animals were housed in standard cages in climate- and light-controlled rooms with free access to food and water. All experimental procedures were approved by the Animal Care and Use Committee of the National Institute of Dental and Craniofacial Research, National Institutes of Health and adhered to the guidelines of the IASP Committee for Research and Ethical Issue 32.

Methods

Generation of transgenic mice. p35 knockout (p35KO) and Cdk5 conditional knockout (Cdk5 CoKo) mice were maintained in C57BL/6J backgrounds and genotyped as described 27,30. Cdk5 CoKo mice were generated by crossing Cdk5f/f mice with SNS-Cre mice 17. Transgenic p35 (Tgp35) mice were bred in FVB/N background 11. Age-matched wild-type mice served as controls. All animals were housed in standard cages in climate- and light-controlled rooms with free access to food and water. All experimental procedures were approved by the Animal Care and Use Committee of the National Institute of Dental and Craniofacial Research, National Institutes of Health and adhered to the guidelines of the IASP Committee for Research and Ethical Issue 32.

Mouse operant lickometer test. An operant lickometer test was used to test nociceptive responses to hot taste stimuli. Nociceptive sensitization was induced by 15 µM concentration of the TRPV1 agonist capsaicin (Sigma-Aldrich, St. Louis, MO, USA). Mice were deprived of water overnight (15 hrs), then placed in the lickometer cages (Habitest system, Coulbourn Instruments, USA). A computer-operated system monitored their licking events for 1 hour. Initially, the animals were tested with water (n = 5 sessions). Then, consumption/aversion to water with 15 µM capsaicin was monitored (n = 5 sessions). All mice were tested at the same time each day and then retested under the same conditions every other day.

Mouse thermal operant behavioral assay. Assessment of thermal sensitivity was measured using an Orofacial Pain Assessment device (OPAD) (Stoelting) 25,26. This device measures the changes in nociceptive behavior in trigeminal area after thermal stimulation. First, mice were trained to drink a sucrose (30%) reward while contacting two Pelletier-based thermodes (set to non-painful temperature 37 °C) to reach the reward. After completing 5 baseline training sessions, the animals were retested three times using heated thermodes to 45 °C. Mice were deprived of food and water overnight (for 12–15 hours) prior to testing to increase the incentive for sucrose acquisition. The Anymaze Software automatically tracked the number of licks or contacts with the thermodes and the time animals spent with the licking of the reward.

Molecular biological methods. All cDNAs coding for the protein of interest were sub-cloned in pCDNA3 vectors. To identify co-transfected cells Cdk5 and p35 were C-terminally tagged with either mCherry (a red fluorescent protein) or CFP (cyan fluorescent protein). TRPV1 T406 was replaced by 11 different native amino acids using overlap extension PCR or site-directed mutagenesis. Positively as well as negatively charged, aromatic, and non polar amino acids were inserted to mimic or inhibit the Cdk5-mediated phosphorylation. Additionally,
proline-407 was also replaced by alanine. For TIRF microscopy, TRPV1<sub>WT</sub>, TRPV1<sub>C406A</sub> and TRPV1<sub>C406P</sub> were C-terminally tagged with GFP. For each residue, mutagenesis primers were designed including the desired mutation. Mutagenesis PCR was performed using PFU DNA polymerase (Agilent Technologies, Santa Clara, CA, USA) to prevent unwanted mutations. All mutants were confirmed and checked for mutations by DNA sequencing.

**Cell culture and transfection.** Chinese hamster ovary (CHO) cells were cultured in MEM (PAN-Biotech, Aidenbach, Germany) and HEK293T (Human embryonic kidney) cells were grown in DMEM (Life Technologies, Darmstadt, Germany). Both media were supplemented with 10% (v/v) fetal calf serum and 1% (v/v) anti/anti (Sigma-Aldrich, Taufkirchen, Germany). Cells were cultured at 37 °C and 5% CO<sub>2</sub> in Ø 10 cm cell culture dishes (TPP, Trasadingen, Switzerland). For patch-clamp experiments, 50–100 k cells were seeded on Ø 3 cm cell culture dishes (Sarstedt, Nümbrecht, Germany). For Ca<sup>2+</sup>-imaging measurements, 200 k cells were seeded on Ø 2.5 cm glass coverslips (Menzel, Braunschweig, Germany). After 6 to 32 hours, CHO or HEK293T cells were transfected with 4–6 μg of coding plasmid DNA. Transfection was performed via calcium phosphate precipitation method as described before<sup>35</sup>. 12–24h after the transfection, CHO and HEK293T cells were used for electrophysiological and Ca<sup>2+</sup> imaging experiments.

**Solutions and chemicals.** In electrophysiological and Ca<sup>2+</sup> imaging experiments following bath solutions were used (in mM): (A) NaCl 140, KCl 5, CaCl<sub>2</sub> 2, MgCl<sub>2</sub> 2, (B) NaCl 140, KCl 5, EGTA 5, MgCl<sub>2</sub> 2, (C) KCl 140, MgCl<sub>2</sub> 1, CaCl<sub>2</sub> 0.1, EGTA 5. Utilized pipette solutions were (in mM): (D) CsCl 145, MgCl<sub>2</sub> 2, CaCl<sub>2</sub> 1, EGTA 11 and (E) CsCl 140, BaCl<sub>2</sub> 10, MgCl<sub>2</sub> 1, CaCl<sub>2</sub> 0.1, EGTA 5. All solutions contained 10 mM HEPES and HCl were used to adjust the pH to 7.35. For low pH stimuli, solutions were adjusted to pH 6. If necessary, mannitol was added to adjust the osmolarity to 290 ± 10 mOsm for bath solutions and 300 ± 10 mOsm for pipette solutions. All chemical products were purchased at ROTH, Karlsruhe, Germany or Sigma-Aldrich. Capsaicin (Alomone, Jerusalem, Israel) was solved in DMSO to a final stock concentration of 33.3 mM to prepare various capsaicin dilutions (0.05–3.3 μM).

**Electrophysiological measurements.** CHO cells were co-transfected with TRPV1 receptor variants and GFP or with TRPV1, Cdk5-mCherry and p35-CFP. Patch-clamp experiments were performed with an inverse microscope with 40× objectives (Zeiss, Jena, Germany). A micro manipulator (Scientifica, Uckfield, UK) was used to place the patch pipette on transfected cells for whole-cell or cell-attached configuration. Patch pipettes were pulled from borosilicate glass (Science Products, Hofheim, Germany) by means of a horizontal pipette puller (Zeitz Instruments, Munich, Germany) and fire-polished to obtain a series resistance of 3–5 MΩ for whole-cell configurations. Patch pipettes were pulled from borosilicate glass (Science Products, Hofheim, Germany) and fire-polished to obtain a series resistance of 3–5 MΩ for whole-cell or 6–8 MΩ for cell-attached configuration. Capacitance and liquid junction potentials were adjusted using the built-in compensation algorithm of the amplifier. Between 60% and 90% of the series resistance was compensated. Patch-clamp recordings were performed at room temperature (22–24 °C) using a HEKA EPC10 amplifier (HEKA, Lambrecht, Germany) and HEKA Patchmaster software was used for data acquisition.

**Whole-cell recordings.** In order to perform whole-cell recordings, transfected CHO cells were clamped at their assumed resting potential of −60 mV. Voltage ramp protocols were applied continuously every second, beginning with −60 mV for 100 ms, followed by a voltage step to −100 mV for 100 ms in order to record inward directed currents. A 500 ms long linear ramp segment from −100 mV to +100 mV was applied and held for 100 ms in order to conduct outward directed currents. The voltage ramp protocol was finished by a last step back to −60 mV. Voltage step protocols began with an initial constant segment at 0 mV for 20 ms, followed by steps from −120 mV to +160 mV in 20 mV increments ending with a step to +60 mV for 20 ms. Data of whole-cell voltage ramp and voltage step measurements were collected with a sample rate of 2 kHz or 50 kHz, respectively, and low pass-filtered at 2.9 kHz. To induce TRPV1-mediated currents, an air pressure driven 8 in 1 application system (ALA Scientific Instruments, Farmingdale, NY, USA) was used to apply different stimuli, such as capsaicin or low pH, directly onto the recorded cell.

**Cell-attached single channel recordings.** Single TRPV1 channels were recorded in cell-attached configuration. Only patches with a series resistance >1 GΩ and leak currents <$50 pA were recorded. To equilibrate the membrane potential to zero the bath solution contained 140 mM K<sup>+</sup> instead of Na<sup>+</sup> (solution C). Additionally to Cs<sup>+</sup>, the pipette solution contained Ba<sup>2+</sup> in order to block endogenous K<sup>+</sup> ion channels (solution E). Depending on the experimental approach, 0.3 μM capsaicin was added to the pipette solution. In a second approach, cells were pretreated with 3.3 μM capsaicin in the bath solution for two minutes prior to the measurement. Pipette potential was clamped at −60 mV and data were collected for >2 min with a sampling rate of 10 kHz filtered with a 2.9 kHz Bessel filter. To illustrate single channel recordings, a 1 kHz low-pass filter was added.

**TIRF microscopy.** For the TIRF microscopy experiments CHO cells were seeded on glass cover slips and were transiently transfected with the respective TRPV1-GFP plasmids. The GFP fluorescence (488 nm) of the transfected cells was monitored 24 h later in a Leica AF 6000LX stem using a HCX PL APO 100×/1.47 oil objective. To gain the best signal to noise ratio the penetration depth was set to 90 nm. GFP fluorescence was imaged in Ca<sup>2+</sup>-containing Ringer's solution and 5 min after the application of capsaicin, which leads to a final capsaicin concentration of 6.6 μM. The selected cells were used to analyze the time course of the fluorescence by capturing images every 5 s.

**Fura-2 Ca<sup>2+</sup> imaging.** Ca<sup>2+</sup> imaging experiments were performed using a ZEISS live cell imaging setup based on an Observer Z.1 (Zeiss, Jena, Germany) and images were recorded by using 40× and 20× objective lens as described before<sup>36</sup>. Temperature and capsaicin stimuli were applied via an 8 in 1 inline solution heater (Warner Scientific Reports | 6:22007 | DOI: 10.1038/srep22007
Instruments, Hamden, USA). 30 min before the measurement, cells were loaded with 2 μM Fura-2/AM in cell culture medium at 37 °C. The cell culture medium was replaced by Ringer’s solution (solution A). Illumination control and image recording were performed using a Lambda DG4 high-speed wavelength switcher (Sutter instruments, Novato, USA) and the Zen imaging software (Zeiss, Jena, Germany). Ca2+ signals were expressed as ratio of the fluorescence intensity during excitation with 340 nm or 380 nm (F340/F380).

**Statistical analysis.** All data are expressed as mean ± SEM. The statistical evaluation of mouse behavioral tests was done with GraphPad Prism software, version 6 (GraphPad, San Diego, CA, USA). Statistical differences between the WT and p35 overexpressing mice (FVBN background) were assessed by unpaired t-test. Statistical differences between the WT and p35 downregulated mice (C57BL6 background) were assessed by One-way ANOVA followed by Dunnett’s multiple comparisons test. Data of electrophysiological and Ca2+ imaging experiments are presented as mean ± SEM (n = number of cells). Data were visualized and analyzed using Igor Professional 6.37 (Wavemetrics, Portland, USA), TAC × 4.3.3 and TACFit (Bruxton Corporation, Seattle, USA), Microsoft Office (Microsoft Corporation, Redmond, USA) and CorelDraw X6 (Corel Corporation, Ottawa, Canada). Data were tested for normal distribution prior to the statistical analysis. Parametric data were tested with Student’s t-test and nonparametric data with the Wilcoxon signed-rank test. Level of significance was set at p < 0.05. The analysis of the capsaicin concentration/response-relationship and of the conductance/voltage-relationship, in order to calculate EC50 or V1/2, was performed by the Wavemetrics Igor Professional 6.37 software.

**Hill’s Equation (Equation 1).**

$$\frac{I}{I_{\text{max}}} = \frac{[x]^n}{[x]^n + \text{EC}_{50}^n}$$

I = current, I_{max} = maximal current at saturating concentration, x = concentration of tested agonist, EC_{50} = the calculated concentration that elicits 50% of maximal current, and n = Hill coefficient.

**Sigmoidal function (Equation 2).**

$$G = \frac{G_{\text{max}} - G_{\text{min}}}{1 + \exp \left( \frac{V_{1/2} - V}{V_{\text{slope}}} \right)}$$

G = conductance, G_{max} = maximal conductance, G_{min} = minimal conductance, V = applied voltage, V_{1/2} = voltage at half maximal conductance and V_{slope} = slope of the activation curve.

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**Author Contributions**

T.J., M.P., B.H., A.B.K. and C.H.W. designed research; T.J., M.P., B.H., V.M.M., G.C.N. and M.S. performed experiments; T.J., M.P., G.C.N. and B.H. analyzed data; T.J., A.B.K. and C.H.W. wrote the paper.

**Additional Information**

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