LETTERS

Spider toxins activate the capsaicin receptor to produce inflammatory pain

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Bites and stings from venomous creatures can produce pain and inflammation as part of their defensive strategy to ward off predators or competitors[1,2]. Molecules accounting for lethal effects of venoms have been extensively characterized, but less is known about the mechanisms by which they produce pain. Venoms from spiders, snakes, cone snails or scorpions contain a pharmacopoeia of peptide toxins that block receptor or channel activation as a means of producing shock, paralysis or death[3–5]. We examined whether these venoms also contain toxins that activate (rather than inhibit) excitatory channels on somatosensory neurons to produce a noxious sensation in mammals. Here we show that venom from a tarantula that is native to the West Indies contains three inhibitor cysteine knot (ICK) peptides that target the capsaicin receptor (TRPV1), an excitatory channel expressed by sensory neurons of the pain pathway[6]. In contrast with the predominant role of ICK toxins as channel inhibitors[5,7], these previously unknown 'vanillotoxins' function as TRPV1 agonists, providing new tools for understanding mechanisms of TRP channel gating. Some vanillotoxins also inhibit voltage-gated potassium channels, supporting potential similarities between TRP and voltage-gated channel structures. TRP channels can now be included among the targets of peptide toxins, showing that animals, like plants (for example, chilli peppers), avert predators by activating TRP channels on sensory nerve fibres to elicit pain and inflammation.

To identify previously unknown activators of sensory neurons, we examined venoms from 22 spider and scorpion species whose bites are known to produce pain. Screening targets included three members of the TRP ion-channel family whose activation leads to the direct depolarization of primary afferent neurons, consequently producing irritation or pain. These include TRPV1, TRPA1 and TRPM8, which are activated by plant-derived irritants such as capsaicin, mustard oil and menthol, respectively[8,9]. Using calcium imaging as a functional readout, we found that robust and reproducible signals were obtained when crude venom from *Psalmopoeus cambridgei*, a tarantula native to the West Indies, was applied to TRPV1-expressing HEK-293 cells. In contrast, no activity was observed with cells expressing TRPA1 or TRPM8 (Fig. 1a), nor did the venom inhibit activation of these channels by their cognate chemical agonists (not shown).

Reverse-phase chromatographic methods were used to purify the active component(s) from *P. cambridgei* venom. Three fractions with TRPV1 agonist activity were identified (Supplementary Fig. 1) and presumed to consist of peptides on the basis of retention times and molecular masses (observed monoisotopic masses were 4,008.5, 3,842 and 4,179 Da, respectively). Indeed, Edman sequencing revealed a family of three closely related peptides, which we have named vanillotoxins (VaTx) 1, 2 and 3. Each peptide consisted of 34 or 35 amino acid residues plus an amidated carboxy terminus (Fig. 1b), with calculated molecular masses (4,008.5, 3,842.5 and 4,179.6 Da, respectively) matching the observed masses noted above.

Vanillotoxins are new members of the extended family of ICK peptides from spiders and cone snails[10]. ICK toxins are widely recognized as blockers of cationic channels, perhaps best characterized by the interaction of hanatoxins (HaTx1 and HaTx2) with voltage-gated (Kv) potassium channels[7,11]. The typical ICK toxin is a 30–40-residue peptide in which three disulphide bridges form a cysteine knot, constraining the molecule into a rigid and compact structure. A short (five to seven residues) hypervariable β-hairpin turn protruding from this compact core is believed to be critical for specifying toxin–ion-channel interactions[12–14]. Interestingly, VaTx1 and VaTx2 are identical at five of the six residues in this hypervariable turn, whereas VaTx3 shows significant diversity within this region (Fig. 1b). VaTx1 and VaTx2 show greatest identity to heteroscodratoxin 1 (HmTx1) (Fig. 1c), an inhibitor of Kv2-type voltage-gated potassium channels[15]. VaTx3 is more closely related to the spider toxin, huwentoxin 5 (HwTx-V), from *Ornithoctonus huwena*, for which a molecular target is not known[16]. Intrigued by the striking similarity between these latter two toxins, we examined whether crude venom from *O. huwena* could activate the capsaicin receptor. Indeed, application of highly diluted (1:4,000) venom to TRPV1-expressing HEK-293 cells produced large calcium responses that were attenuated by ruthenium red, a non-selective blocker of numerous TRP channels (Fig. 1d). In contrast, no response was observed with TRPA1-expressing cells. These results show that *P. cambridgei* is not unique in targeting TRPV1, indicating that VaTx-like peptides might contribute to the pain-producing actions of other spider venoms.

To show unequivocally that VaTx peptides account for TRPV1 activation by *P. cambridgei* venom, we examined whether synthetic VaTx1 peptide could recapitulate the actions of native material. Synthetic VaTx1 was denatured, refolded in glutathione-based redox buffer and subjected to C_8 reverse-phase chromatography, revealing a series of overlapping peaks whose most prominent species had a retention time matching that of native VaTx1 peptide (Supplementary Fig. 2). Refolded synthetic material elicited robust TRPV1 activation, confirming the identity of VaTx1 as an active constituent of *P. cambridgei* venom.

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As observed with crude venom, each of the purified vanillotoxins selectively activated HEK-293 cells expressing TRPV1, but not TRPA1 or TRPM8 (Supplementary Fig. 3). Dose–response analysis by calcium imaging revealed a rank order potency of VaTx3 > VaTx2 > VaTx1, with half-maximal effective concentrations (EC_{50}) of 0.45 ± 0.04, 1.35 ± 0.15 and 9.9 ± 0.97 μM (means ± s.e.m.), respectively (Fig. 2a). We found that vanillotoxins are present in crude venom at concentrations greatly exceeding their potency at TRPV1, such that toxin concentration and potency are inversely correlated, with VaTx3 being least abundant (180 μM) and VaTx1 most prevalent (1,700 μM). Because TRPV1 is a heat-sensitive channel[6], the most physiologically relevant measure of toxin potency may be that assessed at skin temperature, corresponding to the site of venom injection. Under these conditions (34 °C), the EC_{50} for VaTx3 was substantially enhanced, to 0.2 ± 0.03 μM. Taken together, our findings demonstrate that TRPV1 is a specific and physiologically relevant target for vanillotoxins.

Whole-cell patch-clamp analysis of TRPV1-transfected HEK-293 cells showed that vanillotoxin-evoked currents are characterized by pronounced outward rectification, resembling that observed with capsaicin and other TRPV1 agonists (Fig. 2b, and Supplementary Fig. 4a). Thermosensitive TRP channels, such as TRPV1 and TRPM8, also show modest voltage dependence, a property that might contribute to gating by chemical or thermal stimuli[17]. In this regard, we analysed tail currents from TRPV1-expressing HEK-293 cells (Supplementary Fig. 4b) and found that conductance–voltage relations for capsaicin and VaTx3 were essentially superimposable except for a small but significant difference in slope (0.6 ± 0.0 and 0.9 ± 0.2 for capsaicin and VaTx3, respectively; $P = 0.0017$, Student's t-test) (Fig. 2b). Thus we conclude that these agonists gate TRPV1 by a similar mechanism, although the toxin may have a somewhat greater effect on voltage dependence of gating.

Capsaicin binds to residues on TRPV1 that face the cytoplasm or reside within the inner leaflet of the lipid bilayer[18–20]. In contrast, ICK toxins have been proposed to interact with their targets by binding to an exposed extracellular loop[21] or partitioning into the outer leaflet of the plasma membrane to contact residues within the bilayer[11,22,23]. It

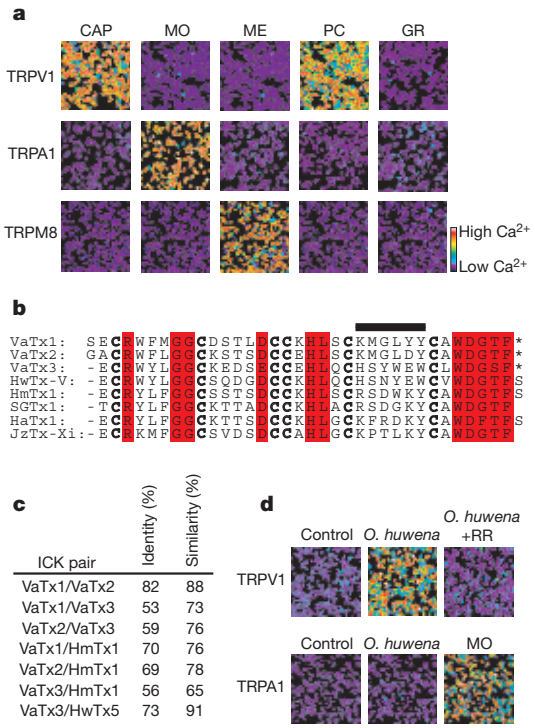

Figure 1 | A subfamily of peptides from tarantula venom activates TRPV1. **a**, HEK-293 cells expressing TRPV1, TRPM8 or TRPA1 were challenged with capsaicin (CAP; 40 nM), allyl isothiocyanate (mustard oil, MO; 100 μM), menthol (ME; 75 μM) or crude *P. cambridgei* (PC) or *Grammostola rosae* (GR) venom (diluted 1:1,000), as indicated. Channel activation was assessed by ratiometric calcium imaging (the colour bar indicates relative calcium levels). Similar results were obtained with nine independent venom lots ($n \geq 400$ cells per visual field). **b**, Alignment of *P. cambridgei* vanillotoxins (VaTx1, VaTx2 and VaTx3) with related ICK peptides from *O. huwena* (HwTx-V), *H. maculata* (HmTx1), *Scodra griseipes* (SGTx1), *Grammostola spatulata* (HaTx1) and *Chilobrachys jingzhao* (JzTx-Xi) spiders. Cysteine residues characteristic of ICK peptides are shown in bold and other highly conserved residues are highlighted in red. The black bar indicates the hypervariable turn. Asterisks denote C-terminal amidation in vanillotoxins. **c**, Pairwise comparison of ICK peptides showing percentage amino acid identities and similarities. **d**, Venom from *O. huwena* (1:4,000) elicited calcium influx into HEK-293 cells expressing TRPV1 (top) that was blocked by ruthenium red (+RR; 10 μM). Cells expressing TRPA1 were not affected by *O. huwena* venom but responded to mustard oil (MO; 100 μM) ($n \geq 400$ cells per visual field).

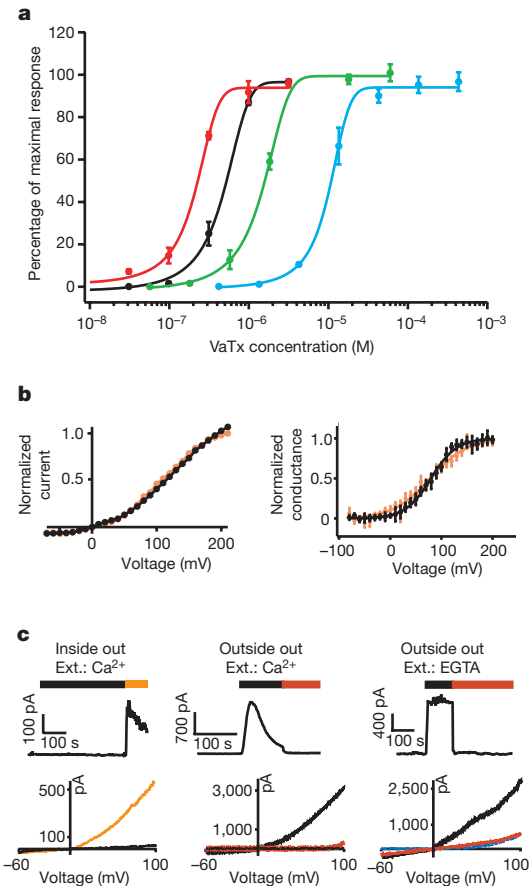

Figure 2 | Functional properties of purified vanillotoxins. **a**, Dose–response analysis of vanillotoxins (blue, VaTx1; green, VaTx2; black, VaTx3) by ratiometric calcium imaging of TRPV1-transfected HEK-293 cells. Responses at each toxin concentration were determined at room temperature (24 °C) and normalized to the response at saturating capsaicin concentration (5 μM). VaTx3 potency was also determined at 34 °C (red) ($n = 3$ trials per concentration). **b**, Currents evoked by capsaicin (0.2 μM; orange) and VaTx3 (5 μM; black) were recorded from TRPV1-transfected HEK-293 cells in whole-cell configuration, from which current–voltage (left) and conductance–voltage (right) relationships were derived. Error bars represent s.e.m.; $n = 4$ for VaTx3 and $n = 5$ for capsaicin. Plots were generated from current traces shown in Supplementary Fig. 4. **c**, Inside-out (left) and outside-out (middle and right) patches were excised from TRPV1-expressing HEK-293 cells, exposed to VaTx3 (black bar, 0.5 μM), capsaicin (orange bar, 2 μM) or ruthenium red (red bar, 10 μM) as indicated, and currents were recorded at +60 mV in calcium (1.5 mM)-containing or calcium-free (1.0 mM EGTA) bath solution. Current–voltage relations are shown below each trace (blue curve indicates no agonist control) ($n \geq 5$ patches per configuration).

therefore seems likely that vanillotoxins and capsaicin interact with TRPV1 at opposite faces of the membrane. To address this question, we examined whether VaTx3 could activate channels when applied to inside-out versus outside-out membrane patches excised from TRPV1-expressing HEK-293 cells. Indeed, the toxin elicited responses in outside-out patches only, whereas capsaicin was effective in either configuration (Fig. 2c, and not shown). Parenthetically, VaTx3-evoked currents showed calcium-dependent desensitization (Fig. 2c) resembling that observed with capsaicin[24]. Taken together, our observations indicate that capsaicin and vanillotoxins share similarities in their mechanisms of TRPV1 activation, but probably interact with distinct regions of the channel. Consistent with this was our observation that vanillotoxins activate avian TRPV1 channels (not shown), which are insensitive to capsaicin and structurally related agonists[19].

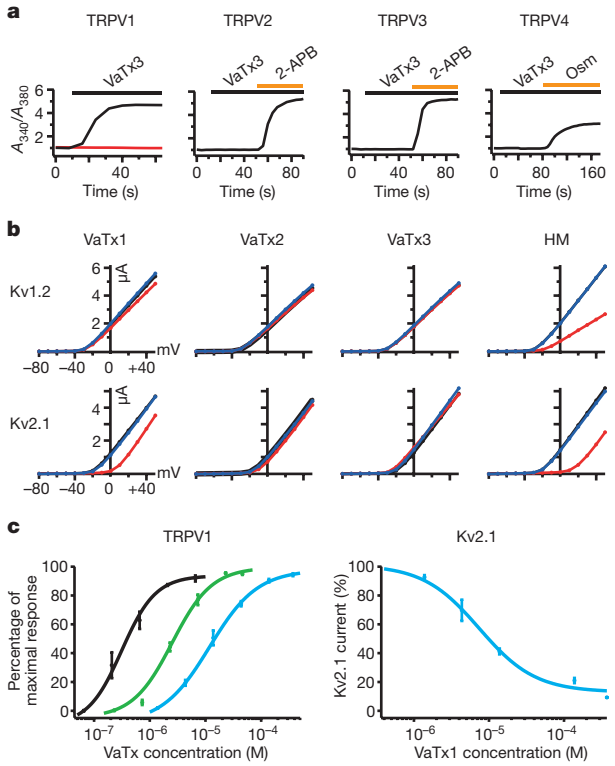

Figure 3 | Analysis of vanillotoxin specificity. a, VaTx3 activates TRPV1 but not the related thermosensitive or osmosensitive channels TRPV2, TRPV3 or TRPV4. HEK-293 cells expressing indicated channels were exposed to VaTx3 (0.5 μM) and responses were analysed by ratiometric calcium imaging. To control for functional expression, TRPV2, TRPV3 and TRPV4 were subsequently challenged with the broad-spectrum agonist 2-aminoethyl diphenylborinate (2-APB; 250 μM), or hypo-osmotic Ringer solution (Osm; 200 mOsm), as indicated. The red trace shows blockade of toxin response by ruthenium red (10 μM). Traces represent an average of $n \geq 80$ cells. **b**, Currents were elicited in oocytes expressing voltage-gated potassium channels, Kv1.2 or Kv2.1, by applying depolarizing pulses (−100 to +50 mV) from a −80 mV holding potential in 10-mV steps (each lasting 500 ms). Current–voltage relationships were plotted before the addition (black), after the addition (red) and after the wash-out (blue) of the respective toxin (VaTx1, 20 μM; VaTx2, 5 μM; VaTx3, 0.75 μM). *H. maculata* (HM) venom (dilution 1:200) served as positive control for Kv2.1 and Kv1.2 inhibition ($n = 4$ independent experiments per toxin). **c**, Left: dose–response analysis by whole-cell voltage-clamp recording of TRPV1-expressing oocytes (+80 mV) revealed EC_{50} values of 11.9 ± 1.4, 2.53 ± 0.02 and 0.32 ± 0.09 μM for VaTx1 (blue), VaTx2 (green) and VaTx3 (black), respectively. All values are in excellent agreement with those obtained by calcium imaging (see Fig. 2a). Right: toxin-mediated inhibition of Kv2.1 (at 0 mV) was assessed in oocytes. The full dose–response is shown for VaTx1 ($IC_{50} = 7.4 \pm 1.9$ μM). Error bars represent s.e.m.; $n \geq 3$ trials for each toxin concentration.

To address vanillotoxin specificity further, we examined their effects on other TRPV family members, including TRPV2, TRPV3 and TRPV4. Neither VaTx1, VaTx2 nor VaTx3 activated HEK-293 cells expressing these channels (Fig. 3a, and not shown). In light of the well-established interactions between ICK peptides (for example hanatoxins and heteroscodratoxins) and voltage-gated potassium channels, we also examined whether vanillotoxins modulate the activity of Kv1.2, Kv2.1 or Kv4.2 channels, representing Shaker, Shab and Shal subfamilies, respectively. Each of these Kv channels was expressed in oocytes and gating in response to voltage steps (−100 to +50 mV) assessed in the absence or presence of purified vanillotoxins. Significant inhibition of Kv2.1 was observed with VaTx1 (Fig. 3b, and Supplementary Fig. 5). Moreover, tail current analysis revealed that VaTx1 shifted Kv2.1 activation to higher voltages (Supplementary Fig. 5), resembling the inhibitory effects of hanatoxins and heteroscodratoxins[11,15]. Detailed dose–response analysis showed that VaTx1 is equally potent as a TRPV1 agonist or a Kv2.1 antagonist ($EC_{50} = 11.9 \pm 1.4$ μM and $IC_{50} = 7.4 \pm 1.9$ μM, respectively) (Fig. 3c). VaTx2 also inhibited Kv2.1, but only at concentrations severalfold exceeding its EC_{50} at TRPV1. VaTx3 showed greatest selectivity for TRPV1, producing little or no inhibition of Kv channels at concentrations 10–100-fold exceeding its EC_{50} at TRPV1 (Supplementary Fig. 5). Conversely, *Heteroscodra maculata* venom neither activated nor inhibited TRPV1 (Supplementary Fig. 6), demonstrating specificity of these ICK toxins for their respective channel targets.

To assess vanillotoxin selectivity *in vivo*, we first examined their actions on dissociated sensory neurons from mouse trigeminal ganglia. In cultures from wild-type mice, VaTx2 or VaTx3 produced robust calcium increases among the subpopulation of capsaicin-sensitive cells (Fig. 4a, b, and not shown), whereas neurons from TRPV1-deficient mice were completely unresponsive. Moreover, even crude venom (at 1:500 dilution) produced rapid and robust calcium increases in capsaicin-sensitive neurons (Supplementary Fig. 7). A significantly smaller response was observed in all other neurons, as well as in non-excitable cells such as fibroblasts, presumably reflecting the effects of other peptides, proteases, lipases, and so on, present in crude venom[25]. Consistent with this was our observation that neurons from TRPV1-deficient mice showed only this smaller 'background' calcium response (Supplementary Fig. 7). Together with our biochemical analysis, these results show that crude *P. cambridgei* venom contains ample vanillotoxin to activate sensory neurons and that most (but not all) of this response is mediated through the activation of TRPV1.

In wild-type mice, injection of capsaicin into the hind paw elicits pain-related behaviours, such as licking and flinching of the affected limb. Injection of purified VaTx3 produced similar responses in wild-type mice but not in TRPV1-deficient littermates (Fig. 4c), further demonstrating specificity of the toxin for the capsaicin receptor *in vivo*. We also measured paw thickness as an indication of neurogenic inflammation resulting from robust activation of sensory nerve fibres. Indeed, the hind paws of toxin-injected wild-type mice showed substantial oedema, whereas TRPV1-deficient animals displayed only minimal swelling akin to that observed with vehicle-injected wild-type controls (Fig. 4d). Injection of crude venom into the paw produced equivalent behaviour and swelling that was independent of genotype, but this was expected in light of our cellular studies showing a small but finite TRPV1-independent effect of crude venom on sensory neurons. Taken together, these data demonstrate that VaTx peptides are agonists for recombinant or native TRPV1, eliciting pain and inflammation through activation of this excitatory channel.

ICK toxins from spiders, scorpions and predatory cone snails target a variety of cationic channels that are gated by membrane voltage or extracellular ligands. Our findings show that TRP channels can now be included as molecular targets of ICK toxins, or, for that matter, any peptide toxin. With the exception of two scorpion ICK peptides that activate intracellular ryanodine receptors, all other

known toxins of the ICK group function as receptor or channel antagonists[10]. Thus, vanillotoxins are unique in serving as TRPV1 agonists, a property that enables them to excite sensory nerve endings to produce pain and inflammation, as commonly associated with bites or stings from venomous creatures. Our results with *O. huwena* venom indicate that a variety of spider species might use ICK toxins to target sensory TRP channels as a strategy to ward off predators or competitors. Indeed, this would parallel the mechanisms adopted by numerous plant species to deter predatory mammals through the production of chemical irritants (such as capsaicin or isothiocyanates) that also target TRP channels on sensory neurons of the pain pathway.

The sequence similarity between vanillotoxins and heteroscodratoxins or hanatoxins is consistent with the idea that TRP channels resemble voltage-gated potassium channels in their overall transmembrane topology and tetrameric subunit organization[26]. VaTx1 and VaTx2, which produce significant inhibition of Kv2.1, show greater sequence similarity to each other or to HmTx1 than they do to VaTx3 (Fig. 1b, c), presumably reflecting their relative activities as Kv antagonists and/or TRPV1 agonists. Biochemical and mutagenesis studies indicate that inhibition of Kv channels by hanatoxin might involve interaction of the toxin with regions of the third and fourth transmembrane helices that constitute the voltage sensor domain[11,27]. Perhaps vanillotoxins interact with a region of TRPV1 that is topologically equivalent to the voltage sensor domain in Kv channels, which may be similarly important in modulating the transition between open and closed states of the TRPV1 channel complex. Although TRP channels display only modest voltage-dependent behaviour in comparison with Kv channels[9,17], our conductance–voltage analysis indicates that vanillotoxins, like capsaicin, exert some effect on the voltage dependence of gating. Future biochemical and structure–function studies will be required to delineate the exact nature of vanillotoxin–channel interactions and determine how this alters the voltage sensitivity of TRP or Kv channels. Vanillotoxins provide new pharmacological tools for addressing these and other questions relating to TRP channel structure and gating mechanisms.

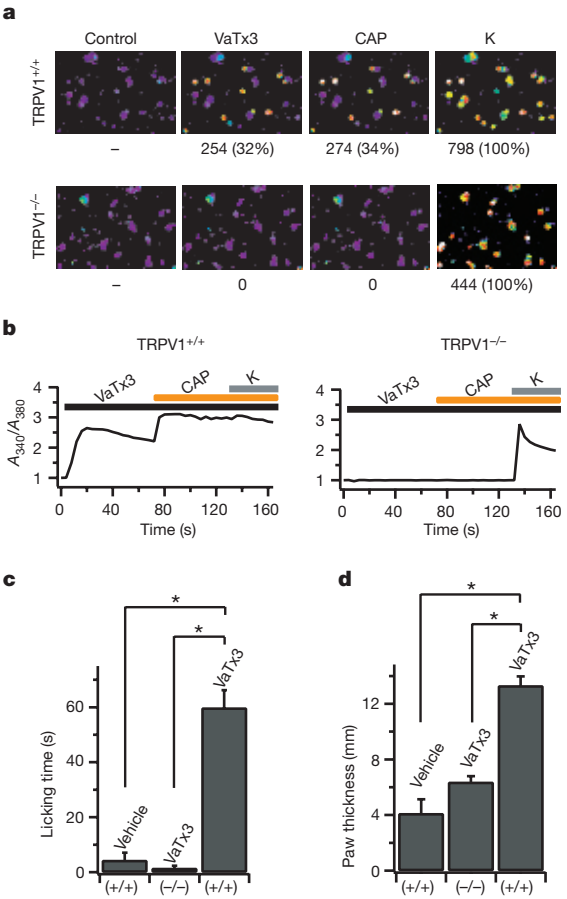

Figure 4 | VaTx3 activates capsaicin-sensitive trigeminal neurons and elicits TRPV1-dependent pain responses in mice. **a**, Trigeminal neurons from wild-type (TRPV1$^{+/+}$) or TRPV1-deficient (TRPV1$^{-/-}$) mice were challenged with VaTx3 (1 μM), capsaicin (CAP; 3 μM), then high-potassium buffer (K; 100 mM), as indicated, and analysed by ratiometric calcium imaging. The total number of responsive neurons (percentage of excitable cells) is shown below each image. **b**, Average calcium responses for wild-type ($n = 20$) and TRPV1-deficient ($n = 40$) neurons shown in **a**. VaTx3-evoked peak ratiometric responses were 2.6 ± 0.05 and 1.0 ± 0.02, respectively. **c**, **d**, Hind paws of wild-type (+/+) or TRPV1-deficient (−/−) littermates were injected with VaTx3 (40 μM in 20 μl of PBS with 0.5% dimethylsulphoxide) or vehicle alone. Total time spent licking the injected paw was recorded over 5 min (**c**) and change in paw thickness was measured before and 30 min after injection of toxin or vehicle (**d**). Error bars represent s.e.m. ($n = 5$ animals per trial; asterisk, $P \leq 0.0003$, ANOVA single-factor analysis). No significant differences were observed between vehicle-injected *TRPV1*$^{+/+}$ and toxin-injected *TRPV1*$^{-/-}$ mice. All measurements were performed blind to genotype.

METHODS

Cell lines and plasmid constructs. HEK-293 cells stably expressing rat TRPV1, human TRPA1 or rat TRPM8 were generated with inducible (pCDNA4/TO) or non-inducible (pcDNA3) vectors (Invitrogen). Human TRPV1, rat TRPV2 and human TRPV3 were transiently expressed in HEK-293 cells with the use of Lipofectamine 2000 (Invitrogen). Kv1.2, Kv4.2 and Kv2.1 plasmids were linearized and transcribed *in vitro* with T7 or T3 polymerase (Ambion).

Toxin purification and peptide chemistry. Crude spider venom was obtained from Spider Pharm. Detailed protocols describing vanillotoxin purification and biochemical characterization are provided in Supplementary Fig. 1. Protocols for solid-phase synthesis and refolding of VaTx1 are provided in Supplementary Fig. 2.

Calcium imaging and electrophysiology. Culturing and imaging of sensory neurons was performed as described[28]. Imaging of HEK-293 cells was performed in 10-μl adhesive silicone isolators (Invitrogen) attached to poly-(D-lysine)-coated microscope slides (Fisher). Fluorescent images were acquired with Metafluor Software (Molecular Devices) and analysed with automated routines written in Igor Pro (Wavemetrics). All experiments were performed at room temperature (24 °C) except dose–response analysis of VaTx3, which was performed at 34 °C.

Patch-clamp recordings in TRPV1-expressing HEK-293 cells were performed in whole-cell mode or excised-patch configurations as described previously[29]. Currents were recorded with an Axon 200B amplifier and a Digidata 1321A interface and acquired with pClamp software (Axon Instruments). Pipette resistance ranged from 0.9 to 1.5 MΩ. For excised patch experiments, no series resistance compensation was performed, because series resistance was generally less than 3 MΩ. Signals were filtered at 5 kHz and digitized at 25 μs. Standard bath and pipette solutions contained (in mM) 140 NaCl, 5 KCl, 2 $MgCl_2$, 2 EGTA, 10 HEPES, and 10 D-glucose (adjusted to pH 7.4 with NaOH). For the whole-cell tail current analysis, conductance–voltage curves were normalized to the maximal conductance at 200 mV and fitted with a Boltzmann equation ($G = G_{max}/(1 + \exp[-zF(V - V_{1/2})/RT])$). Oocytes were prepared and subjected to two-electrode voltage-clamp analysis as described previously[19]. Cells were bathed in normal ND96 solution for the analysis of Kv channels, or in calcium-free solution (120 mM CsCl, 1 mM EGTA, 10 mM HEPES, 2 mM $MgCl_2$ pH 7.4) for the analysis of TRPV1 channels.

Behaviour. Mice (20–30 g) were housed with a 12 h light/12 h dark cycle at 21 °C, and experiments were performed under the policies of the International Association for the Study of Pain and UCSF Animal Care and Use Committee. Nocifensive licking responses were measured for a period of 5 min after injection of vehicle or toxin (20 μl), as described[30]. Hindpaw thickness was determined with a spring-loaded caliper before and 30 min after injection. Experiments were performed with *TRPV1*$^{+/+}$ and *TRPV1*$^{-/-}$ littermates while blind to genotype. Data were analysed with ANOVA single-factor statistical analysis for pairwise comparisons.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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