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Selective Sensory Axon Reinnervation and TRPV1 Activation

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Abstract

Current strategies to enhance regeneration of peripheral neurons involve broad activation of sensory, autonomic, and motor axons. Peripheral neuron regeneration is limited in persons with damage or disease of peripheral axons. Here, we provide evidence that subtoxic activation of TRPV1 channels in sensory neurons is associated with activation of growth and subtle changes in skin reinnervation. We identify a bidirectional, dose-related impact of capsaicin, a TRPV1 agonist, on sensory neurons and their axons with rises in their outgrowth plasticity at low doses and toxic neurodegeneration at high doses. Moreover, its impact on growth added to that of preconditioning. Neither outcome was observed in TRPV1 null neurons. We confirmed that low dose activation was associated with rises in neuronal calcium, as well as rises in TRPV1 mRNA transcripts. In mice with a sciatic nerve crush followed by a single application of capsaicin directly to the injury site, there was no impact on motor or myelinated axon recovery but there was evidence of better recovery of thermal sensation toward baseline with hyperalgesia. Moreover, skin reinnervation by epidermal axons approached contralateral levels. TRPV1 null mice displayed loss of thermal sensation during later recovery. In sensory axons innervating the pinna of the ear, local capsaicin rendered early axon loss followed by later hyperinnervation. Taken together, TRPV1 activation alters the regenerative behavior of adult neurons and their axons both in vitro and during epidermal reinnervation in vivo. The findings identify a selective manipulation that augments cutaneous innervation by thermosensitive axons.

Keywords Peripheral nerve · Regeneration · Capsaicin · TRPV1 · Sensory neurons

Selective restoration of sensory innervation of the skin may be an important strategy for encouraging sensory recovery following neuropathy. Many neuropathies target subclasses of sensory neurons, as also observed following capsaicin neurotoxicity. Whereas activation of TRPV1 receptors using capsaicin is linked to sensory axon loss, it is unclear whether the neurotoxicity first targets axons or perikarya. Woo et al. [1] demonstrated that sensory neuron axons degenerated without loss of perikarya in vitro following capsaicin. Wang et al. [2] identified capsaicin axonopathy using compartmentalized cultures that left perikarya intact. Targeted axon damage may involve mitochondrial toxicity, the calcium-dependent protease calpain 2 [2] and microtubular disassembly [3]. In vivo, capsaicin axonopathy, visualized serially in fluorescent skin axons of thy1-YFP transgenic mice [1], was followed by gradual regrowth and recovery. Similarly, in human studies, topical application of capsaicin depletes the skin of a large proportion of its axon innervation, attributed to toxic axonopathy [4]. This intervention has been used to estimate subsequent regeneration in persons with neuropathy.

Graded influx of calcium following exogenous electrical stimulation of injured adult nerves enhances peripheral axon regeneration [5]. Moreover, depolarization and calcium influx are linked to growth cone extension [6–9]. We wondered if similar but unexpected impacts that benefit regeneration might be observed through selective but titrated activation of TRPV1 axons. Supporting this idea, during long-term follow-up studies of capsaicin exposed skin in thy-1 YFP mice; we noted instances of unexpected hyperinnervation (unpublished data). Following the presentation of our preliminary findings [Poitras, Zochodne et al., 2017 JPNS abstract 22: 359], Frey et al. [10] also identified enhanced regeneration,

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using a compound screen, in small caliber TRPV1 neurons exposed to capsaicin.

Given these data, we surmised that there may be a continuum of neuronal responses to TRPV1 activation from neurotoxicity to growth enhancement. An apparent overlap of neuronal growth and death programs occurs in other situations, such as the DNA damage and BRCA1 activation response [11]. We investigated TRPV1 activation in both naïve and neurons already injury preconditioned. Moreover, we analyzed whether the apparent in vitro and early outgrowth data might translate into changes to how skin reinnervation might occur. We identified a biphasic impact of capsaicin on sensory neuron properties that required expression of the TRPV1 channel, occurred in both naïve and preconditioned neurons and involved rises in cytosolic calcium. Moreover, TRPV1 activation, in sublethal doses enhanced CHOP mRNA expression, a key player in the unfolded protein response [12, 13] and augmented expression of its own TRPV1 transcripts. In vivo, early TRPV1 activation altered aspects of later skin reinnervation by epidermal axons.

Methods

Animals and Surgery

The strains of mice used were CD1 mice from Charles River and a colony of TRPV1 homozygous null mice (B6.129X1-*Trpv1*^{tm1Jul}/J) from a breeding pair of the Jackson Laboratory with recommended controls 000664 C57BL/6. Rats were male Sprague-Dawley. Mice used were adult males of weight 25–40 g housed separately in individually ventilated cages with enrichment, maintained under a 12-h (hour) light:dark cycle, given food and water ad libitum. The protocols were reviewed and approved by the Animal Care Committee University of Alberta, adhering to guidelines of the Canadian Council of Animal Care.

Adult CD1 mice were anesthetized using isoflurane and the sciatic nerve was lesioned by crushing with forceps. Varying doses of capsaicin or the vehicle was applied directly to the site of injury for 1-h post-crush.

In Vitro Studies

Uninjured or previously axotomized (3d earlier) adult rat or mouse L4, 5, and 6 DRGs were dissociated and used for the sensory neuron culture protocol as described previously with minor modifications [14]. Harvested neurons were then utilized for two main aims: (i) evaluation of outgrowth in response to graded doses of capsaicin: after exposure to capsaicin or carrier, outgrowth was assessed in neurons processed for immunohistochemistry as below; (ii) analysis of calcium influx (see below).

For outgrowth studies, the cells were incubated for 18–24 h then underwent a media change (1:100 of N2 and penicillin, streptomycin each,100 ng/ml NGF, and 10 microM mitotic inhibitors (cytosine beta-D-arabinofuranoside)) with the addition of capsaicin (0–1000 μM in 1.0% ethanol), incubated for another 18–24 h and then fixed and processed for immunocytochemistry with anti-Nf 200 antibody (mouse,1:800, Sigma). Cells were also fixed after 24 h of capsaicin/carrier addition and stained for rabbit polyclonal Trpv1(RA14113, Neuromics, EDINA) (1:1000), mouse monoclonal β tubulin III (T8660, Sigma-Aldrich, St. Louis, MO USA) (1:1000), rabbit polyclonal PGP 9.5 (AB_2210932, EnCor Biotechologies Inc., FL USA)(1: 400) or for colabelling, and mouse monoclonal Nf-200 (N0142, Sigma-Aldrich, St. Loius, MO USA) (1:400). Secondary antibodies were goat anti-rabbit Alexa Fluor 488(A-11034, Life Technologies, CA USA) (1:100) (for PGP) and anti-mouse IgG Cy3 antibody (C2181, Sigma-Aldrich, St. Loius, MO USA) (1:100) (for Nf-200). Slides were viewed with a fluorescent microscope (Zeiss, Axioskop, Zeiss Canada, Toronto, Canada). Colabeling studies estimated that approximately 60% of cultured neurons also labeled intensely with an antibody to TRPV1. Outgrowth and survival were analyzed as previously described [14]. Neurite outgrowth was evaluated by culturing adult DRG neurons from Sprague-Dawley rats and treating cultures with varying doses of capsaicin. For calcium sequestering studies, we used 1 mM EGTA (Ethylene glycol-bis [β-aminoethyl ether]-N,N,NN'-tetraacetic acid)(E-3889, Sigma-Aldrich, St. Louis, MO USA). Quantification of outgrowth was evaluated using WIS-NeuroMath software (http://www.cs.weizmann.ac.il/~vision/NeuroMath/index.html). For any given intervention, at least 100 neurons were analyzed (data provided in figure legends) but *n* numbers were based on the number of independent experiments.

Calcium Imaging

Cultured DRG neurons 1 day following harvesting were exposed to 5 μM Fluo-8Lacetoxymethyl ester then live-cell imaged using a confocal microscope, equipped with an argon (488 nm) laser, emission band pass filter (490–540 nm), and 20× XLUMPlanF1, NA 0.95 objective as described [15]. Increases in fluorescence intensity of Fluo-8 L corresponded to an increase in intraneuronal cytosolic calcium. DRG cultures were continuously superfused with extracellular solution containing artificial cerebral spinal fluid (ACSF) containing 127 mM sodium chloride (Fischer), 2.5 mM potassium chloride (EMD, Darmstadt, Germany), 25 mM dextrose (Fischer), 1.3 mM magnesium sulfate septahydrate (EMD), 2.5 mM calcium chloride (EMD), 25 mM sodium bicarbonate (Fischer), and 1.2 mM sodium phosphate monohydrate (Anachemia, Edmonton, Canada). The ACSF was bubbled with 95% O_2 and 5% CO_2. Bath application of ACSF containing 30 mM

KCl for 30 s depolarized neurons and subsequently induced a calcium rise (positive control). Adult rat uninjured sensory neurons after 1 day in culture were studied in duplicate experiments using brief 30 mM KCl exposure for 30 s as a positive control experiment during each study. After 5 min of washing out the KCl, when cytosolic calcium levels had returned to baseline, the same neurons were exposed to 10 μM capsaicin for 10 s. ACSF containing 10 μM capsaicin was added to DRG cultures for 10 s and then washed out by resuming ACSF superfusion. Full frame images (512 × 512 pixels) were acquired at a scanning time of 3 s per frame and time course traces of change in fluorescence intensity were generated with FluoView software.

Immunohistochemistry

Footpad and pinna skin samples were harvested after sciatic nerve crush using a skin punch and immunohistochemistry as described previously [16]. Briefly, samples were fixed in 2% PLP (paraformaldehyde (2%), l-lysine, and sodium periodate) for 18 h at 4 °C and cryoprotected overnight in 20% glycerol/0.1 M Sorensen phosphate buffer at 4 °C. Skin sections were of 25 μM thickness and washed in PBS, 1% Triton X, blocked in 10% goat serum (1% BSA, 0.05%NaN3, 0.3% Triton X100, 0.05% Tween20/1× PBS) for 1 h at room temperature. The primary antibody was PGP 9.5 (1:800, EnCor Biotechologies Inc. Fla) followed by goat anti-rabbit Cy3; 1:100 (Jackson Immunoresearch, USA) secondary for 1 h at room temperature. Axons were visualized using a Leica TCS SP5 confocal microscope. Epidermal innervation was studied by the same, blinded examiner, by counting numbers of either vertically directed ($>45^{\circ}$ angle from the dermal-epidermal junction) axons that traversed the dermal-epidermal junction or total axon profiles in the epidermis irrespective of trajectory [17, 18].

qRT-PCR Analysis

Total RNA was extracted from rat DRG neurons studied in vitro and exposed to varying doses of capsaicin or carrier using Trizol reagent as per the manufacturer's instructions (Invitrogen). One microgram of total RNA was treated with DNase (Promega) and processed to cDNA synthesis using a cDNA reverse transcription kit (Applied Biosystems). Primer Express 2.0 software (Applied Biosystems) was used to design primers and amplification of the products was done using SYBR® Green master mix (Life Technologies, Burlington, ON). Relative expression of mRNAs was calculated using the comparative CT method ($2^{-\Delta\Delta CT}$) where all genes of interest were normalized to the average expression of 18Sr and RPLPO [19]. Primer sequences are shown below:

Sense	Anti-sense
1. RPLP F: 5′TACCTGCT CAGAACACCGGTCT3′	RPLP R: 5′GCACATCG CTCAGGATTTCAA3 ′
2. 18S F: 5′TCCCTAGTGATCCC CGAGAAGT3′	18S R: 5′CCCTTAATGGCAGT GATAGCGA3′
3. TRP V1 F: 5′GTGGACAG CTACAGTGAGATAC3′	TRPV1 R: 5′CACCATGG AAGCCACATACT3′
4. TRPV2 F: 5′TGCATACA CAGAAGGCTCCA3′	TRPV2 R: 5′ CCGGAATC CTTGTCAATCTG3′
5. TRPV3 F: 5′ CACCCCCA CCAAGAAGAGT3′	TRPV3 R: 5′ ACAGTTGC CAGAGAGGCACT3′
6. TRPV4 F: 5′ AGAAAGCG CCCATGGATT3′	TRPV4 R: 5′ TCTGTGGC TGCTTCTCTACG3′
7. TRPA1 F: 5′TCCTATAC TGGAAGCAGCGA3′	TRPA1 R: 5′ CTCCTGAT TGCCATCGACT3′
8. TRPM2 F: 5′GAAGGAAA GAGGGGGTGTG3′	TRPM2 R: 5′CATTGGTG ATGGCGTTGTAG3′
9. TRPM5 F: 5′CATCTCCT TCAGTGAGGATGC3′	TRPM5 R: 5′ CTTCTCCA ATTGGCCACCAT 3′
10. TRPM8 F: 5′GCAGTGGT ACATGAACGGAGT3′	TRPM8 R: 5′ TGAAGAGT GAAGCCGGAATAC3′
11. Cacna2d1 F: 5′CTATGAGG GCTCAACCATAGTG3′	Cacna2d1 R: 5′CCACAGCA ATGTAGGGTCTT3′
12. CHOP F: 5′CTCCAGAT TCCAGTCAGAGTT3′	CHOP R: 5′TCTCCTTC ATGCGCTGTTT3′

Electrophysiology and Sensory Behavioral Testing

Multifiber motor and sensory conduction studies were carried out as previously described [16] under isoflurane anesthesia at a near nerve temperature of 37.0 ± 1.0 °C. For sensory conduction velocity measurements, recording electrodes were placed at the popliteal fossa, and stimulating electrodes were applied to digital sensory nerves in the hindpaw. Motor conduction was performed by stimulating at the sciatic notch and the knee and recording from tibial innervated interosseous muscle endplates in the dorsum of the hindpaw. Measurements included baseline preinjury studies and 28 days post-crush.

Over identical time periods, we tested for thermal sensation using the Hargreave's apparatus [19] as previously described [16]. Mechanical sensitivity on the CD1 background mice was tested using North Coast (Morgan Hill, California) Touch Test Von Frey filaments with filaments ranging from 0.006 to 4 g of force. Mice were placed on a metal mesh platform and fibers were pressed against the plantar surface of the animal's hindpaw until either the fiber flexed, or a withdrawal reflex was elicited. Animals were tested five times with each filament until a 60% withdrawal response was observed. The force required to generate this level of response was recorded. The TRPV1 knockout animals and C57 controls had mechanical sensitivity tested using the Ugo Basile (Gemonio, Italy) electronic Von Frey apparatus. Animals were placed on a metal mesh platform and the filament was pressed against the

plantar surface of the animal's hindpaw until a withdrawal reflex was observed. Given the small size of the mouse hindpaw, selective stimulation of one plantar territory for either test was not possible, an important issue reported by Cobianchi and Navarro [20]. Given this, we cannot exclude that some of the behavioral data was influenced by saphenous nerve collateral sprouting. Each animal was tested in triplicates to give an average force required for a response.

Pinna Skin Studies

As in previous work [1], mice underwent application of capsaicin 0.2% (6.5 mM) on one ear and carrier on the contralateral ear. Topical 0.2% capsaicin (Sigma-Aldrich, USA) diluted in 10% Tween 80 (Sigma-Aldrich, USA) and 10% ethanol were applied for 2 h for each of two consecutive using a protocol known to eliminate cutaneous axons in humans [4]. A small capsaicin-soaked cotton swab was placed on the inner surface of the mouse ear covering the full pinna and held in place for 2 h under continuous anesthesia. Without further intervention, 6 weeks later, the ears were harvested and immunostained for PGP 9.5 as above. Axons were visualized using a Leica TCS SP5 confocal microscope. Epidermal innervation was studied by the same, blinded examiner, by counting numbers axon profiles in the epidermis.

Analysis

Results were calculated as means ± sem. Groups were compared with one-way ANOVA and post hoc Tukey's multiple comparisons or Student's *t* tests as appropriate. The null hypothesis was accepted as $p \leq 0.05$. In graphical data, asterisks were not applied to nonsignificant differences.

Results

A Dose-Related Biphasic Impact of TRPV1 Channel Activation on Sensory Neurons In Vitro

We assumed that activation of TRPV1 sensory neurons by capsaicin would lead to toxic neurodegeneration of axons, then neurons [1]. To address the impact of activating TRPV1 sensory neurons using capsaicin, we examined neurite outgrowth of adult rat and mouse sensory neurons in response to capsaicin (0, 10,100, and 1000 μM), for 24 h followed by assessment of growth. We used neurofilament (Nf200) labeling as a robust marker for neuronal perikarya and their axons estimating that 60% of these neurons had evidence of significant TRPV1 expression. We also analyzed what proportion of overall neurons harvested and analyzed in vitro were also labeled with Nf200. Overall pan-neuronal staining was established using PGP 9.5 as the gold standard. In four replicates, the proportion of overall PGP 9.5 neurons stained with Nf200 was 92.9 ± 1.3% in those exposed to carrier and 93.3 ± 0.5% in neurons exposed to capsaicin. The findings confirmed that Nf200 staining captures a large proportion of DRG sensory neurons in vitro, including those of larger and smaller caliber.

We observed a rise in neurite outgrowth after exposure to 10 μM capsaicin, a trend toward increased outgrowth at 100 μM and a decline after exposure to 1000 μM [Fig. 1a–d]. Moreover, this pattern was observed in both naïve uninjured neurons and preconditioned neurons having undergone axotomy injury 3d prior [Fig. 1e–h]. The magnitude of rise after 10 μM capsaicin of injured neurons exceeded that of naïve neurons. Findings were similar in neurons labeled with an antibody directed to an alternative pan-neuronal marker for neurons, βIII tubulin at 10 μM capsaicin, indicating a rise in neurite outgrowth [Fig. 1i].

Experiments were repeated using mouse sensory neurons from TRPV1 null animals and littermate controls [Fig. 2]. There was no difference in basal outgrowth. As in rats, the littermate control mice had heightened outgrowth after exposure to 10 μM capsaicin and evidence of toxicity at 1000 μM. However, mice lacking TRPV1 had no response to capsaicin excepting a nonsignificant decline at 1000 μM.

Taken together, these studies indicated that sensory neurons respond to capsaicin with heightened plasticity at lower doses and neurodegeneration at high doses (hormesis). This pattern required expression of TRPV1 channels. Since preinjured neurons had the most robust outgrowth response, capsaicin was superimposed on the preconditioning response.

Regeneration-Associated TRPV1 Activation Is Associated with Rises in Cytosolic Calcium Within Sensory Neurons

Given the role of TRPV1 as a calcium ionophore, we wondered if its low-dose activation increased cytosolic calcium in harvested adult rat neurons in vitro. As described, only neurons with demonstrated, but transient calcium signals, as expected to develop following KCl exposure were analyzed in response to capsaicin. Not all neurons that responded to KCl also responded to capsaicin and the increase in cytosolic calcium following the capsaicin treatment was not as high as that following KCl [Fig. 3a, b]. Following capsaicin exposure, the cytosolic calcium levels did not return to baseline, indicating a prolonged action. Since we were chiefly interested in whether growth promotion was associated with an expected capsaicin-evoked calcium response, we did not expand these studies to ranges involving its known toxicity. To further address the role of calcium in eliciting an outgrowth response, adult rat neurons were exposed, as above to capsaicin 10 μM in the presence of the calcium chelator EGTA. The addition of EGTA prevented the rise in neurite outgrowth in response to low-dose capsaicin [Fig. 3c].

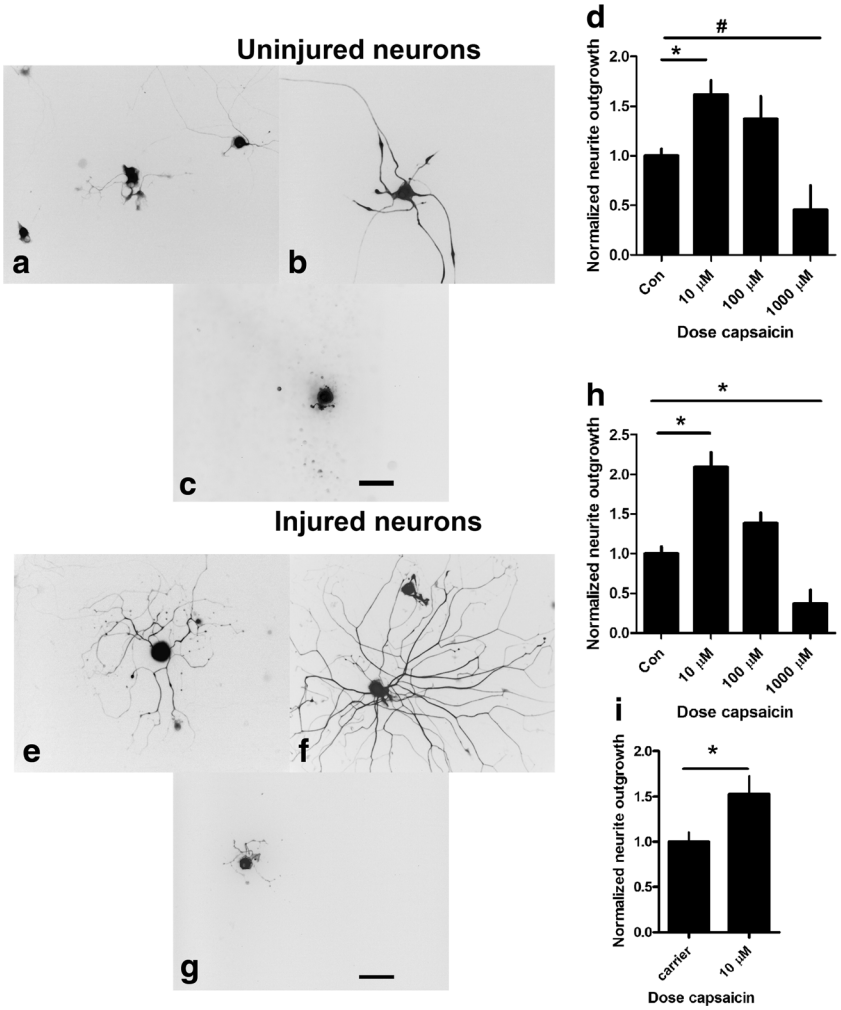


Fig. 1 Capsaicin is associated with a biphasic response in adult sensory neurons. Examples of adult previously uninjured dissociated rat DRG sensory neurons exposed to varying concentrations over 24 h: carrier (con; **a**), 10 μM capsaicin (**b**), 1000 μM capsaicin (**c**) with quantitation of outgrowth (**d**). Previously axotomized rat DRG sensory neurons (3d earlier) were exposed to carrier (con; **e**), 10 μM capsaicin (**f**), 1000 μM capsaicin (**g**) with quantitation of outgrowth (**h**). Previously uninjured rat DRG sensory neurons were exposed to carrier or 10 μM capsaicin but labeled with alternative neuronal marker βIII-tubulin (**i**). [**d**, One-way ANOVA $p = 0.01$; post hoc one-tailed Student's t test carrier vs.10000 μM #$p = 0.05$; Student's t test carrier vs. 10 μM *$p = 0.017$; $n =$ 3/group. **h**, One-way ANOVA $p = 0.0003$; post hoc Student's t test carrier vs. 1000 μM *$p = 0.031$; Student's t test carrier vs. 10 μM *$p = 0.007$; $n = 3$ for all groups excepting 1000 μM ($n = 2$). **i**, *$p = 0.036$ paired Student's t test]. Neurons are labeled with an antibody to Nf200.Total number of neurons examined as graphed were 295, 262, 216, 148 (**d**), 441, 440, 326, 104 (**h**), 530, and 526 (**i**). Bar = 100 μM for **a**–**c**, **e**–**g**

In anticipation of regeneration work described below, we asked whether graded doses of capsaicin might alter overall sensory properties of axons through the expression profile of TRP channels in sensory neurons. While not an exploration of how TRPV1 activation impacts growth, we wondered if its activation might alter how axons behave during later regrowth studies in vivo. After exposure to 10 or 100 μM capsaicin, neurons were probed with specific primers to TRPV1, V2, V3, V4, A1, M2, M4, M5, and M8. There was higher TRPV1 mRNA expression at 10 μM but inconsistent trends of the other TRP subtypes [Fig. 4]. TRPV2 mRNA levels were significantly reduced by capsaicin at 100 μM. There was no impact on the expression of calcium α2γ channel mRNA. Given the known toxicity of higher doses of capsaicin, we also analyzed CHOP mRNA levels, measures of the unfolded protein response. These transcripts had a trend toward elevation at 10 μM and a significant rise at 100 μM. This confirmed that nontoxic doses of capsaicin also likely unleash the unfolded protein response, linked by Ying, Verge, and colleagues to regeneration [12, 13]. Despite this important finding, further exploration of CHOP was beyond the scope of this work.

Recovery of Thermal, but Not Mechanical Sensation Is Altered by TRPV1 Activation In Vivo

In outbred CD1 mice, thermal and mechanical sensation were serially studied before and after a single application

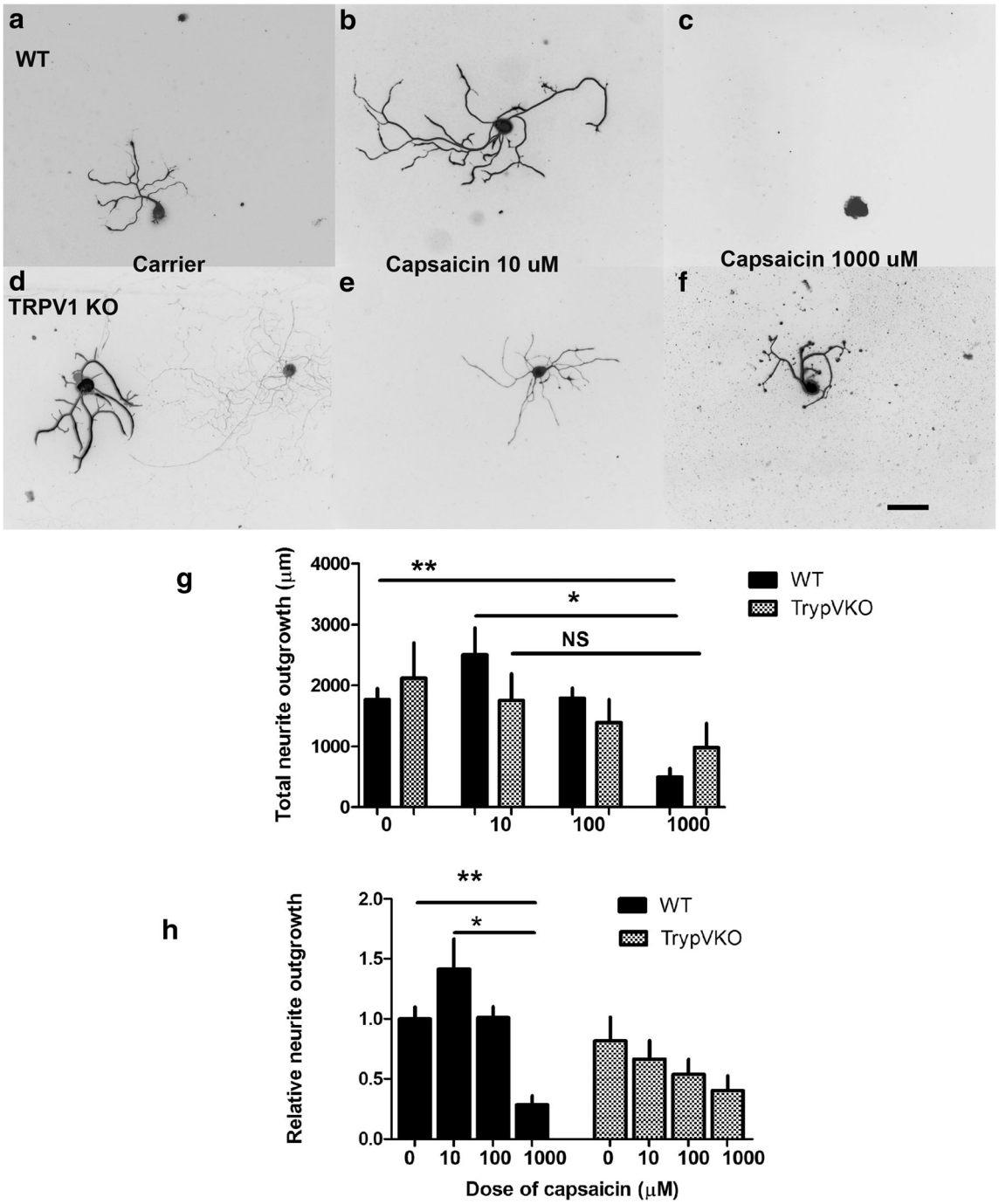


Fig. 2 TRPV1 null mouse sensory neurons lack a biphasic response to capsaicin. Examples of adult previously uninjured dissociated mouse DRG sensory neurons from littermate wild type (WT; (**a**–**c**)) or TRPV1 null (KO; (**d**–**f**)) mice exposed to capsaicin: carrier (**a**, **d**), 10 μM (**b**, **e**), or 1000 μM (**c**, **f**) with quantitation of outgrowth as raw growth (**g**) or normalized (**h**). [**g**, One-way ANOVA $p = 0.0005$; post hoc Student's t test WT 10 μM vs. 1000 μM $^{*}p = 0.012$; post hoc Student's t test WT carrier vs. 1000 μM $^{**}p = 0.005$. **h**, One-way ANOVA $p = 0.0002$; post hoc Student's t test WT carrier vs. 1000 μM $^{**}p = 0.005$; Student's t test WT 10 μM vs. 1000 μM $^{*}p = 0.013$; $n = 3$ WT, 4 TVPV1null/group]. Neurons are labeled with and antibody to Nf200. Total number of neurons examined as graphed were 236, 887, 233, 814, 212, 981, 104, 158 (**g**, **h**). Bar = 100 μM **a**–**f**

of capsaicin at 0 (carrier), 100, and 1000 μM at the injury site, left in situ for 1 h prior to wound closure. Since whole nerve exposure to capsaicin may be limited by diffusion and penetration into the endoneurium, we chose higher ranges of doses than studied in vitro. Given the inherent variability of behavioral assessments, particularly during regeneration, we chose to compare results to our baseline data. As expected after injury, by 14 days,

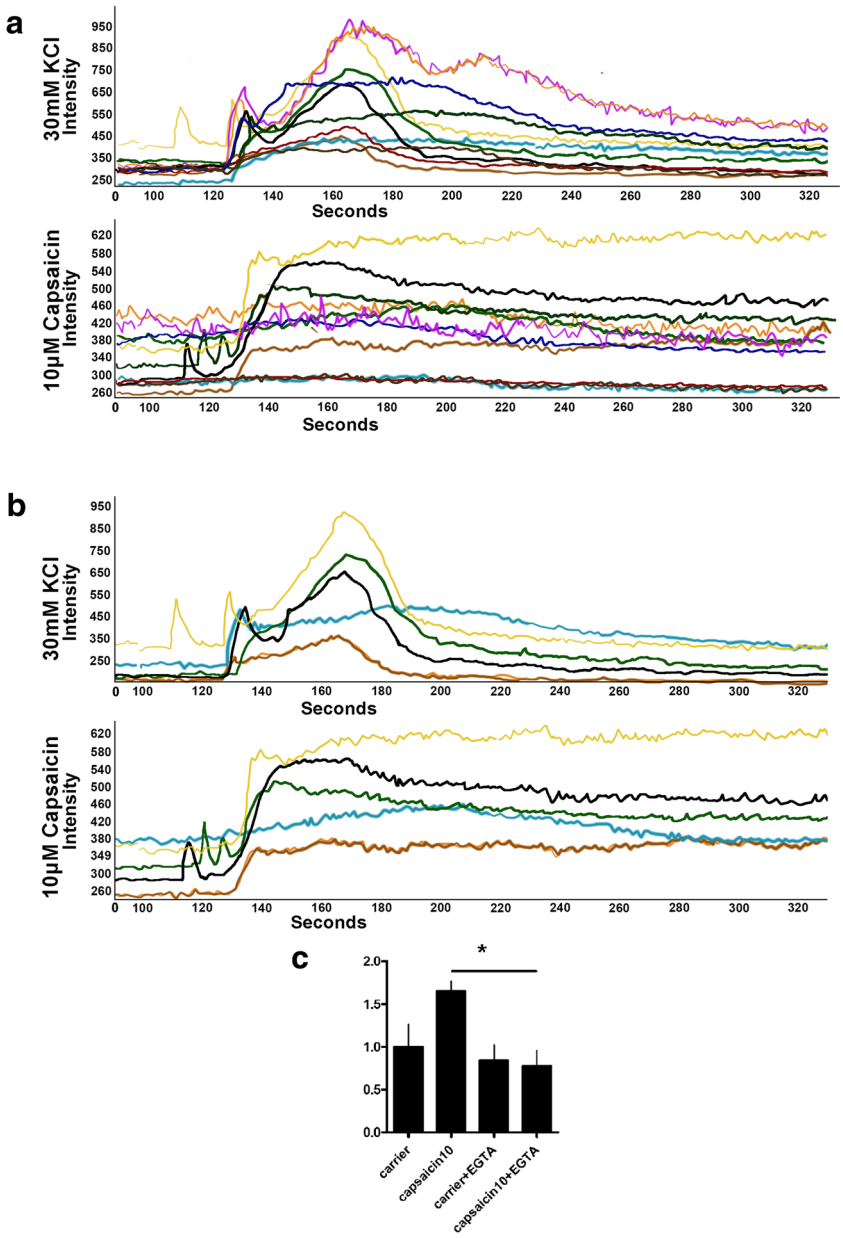


Fig. 3 Low dose capsaicin, associated with enhanced growth, is associated with a prolonged calcium signal. Imaging of cytosolic calcium in adult sensory neurons 1 day following dissociation. **a** Represents ensembles of neurons exposed first to 30 mM KCl exposure for 30 s (top traces). Five minutes later, after the intracelluar calcium levels as identified by their fluorescence reporter, had returned to baseline, the same neurons were exposed to 10-μM capsaicin for 10 s. Not all the neurons that responded to KCl also responded to capsaicin. While the increase in intracellular calcium following the capsaicin treatment was less intense than with KCl, it was more prolonged. In **b,** only the traces of the neurons that responded to capsaicin (paired with the bottom trace to the corresponding neurons following KCl) are shown. The experiments were carried out in duplicate (*y*-axis is fluorescence intensity, *x*-axis is time in seconds). In **c**, previously uninjured rat DRG sensory neurons were exposed to carrier or 10-μM capsaicin with or without addition of the calcium chelator EGTA. EGTA prevented the rise in neurite outgrowth associated with 10 μM capsaicin. [**c** $*p = 0.029$, one-way ANOVA; post hoc capsaicin 10 μM without versus with EGTA $**p = 0.008$ unpaired two-tailed Student's *t* test; total number of neurons examined as graphed were 654, 563, 583, and 628]

there was a loss of thermal sensitivity in nerves exposed to carrier. However, both capsaicin groups had apparent thermal sensation comparable to baseline values. By 28 days, mice exposed to 100 μM, but not 1000 μM, had evidence of hyperalgesia with withdrawal latencies shorter than those at baseline [Fig. 5a, b]. Intragroup comparisons at 14 and 28 days were not significant. There was no impact on the mechanical threshold with any of the interventions.

We next tested serial sensory behavioral indices after a nerve crush injury in TRPV1 null mice and their C57 littermate controls. At baseline, there was a trend, albeit not significant toward less thermal sensation in TRPV1 KO mice. There were limited numbers of WT littermate mice available for study ($n = 3$ at 14 days, $n = 2$ at 28 days) in this analysis but they demonstrated a similar pattern of behavior compared with outbred mice with apparent improvement in sensation in capsaicin exposed nerves. However, in TRPV1 null mice, there was no early (14 days) impact of capsaicin or carrier on thermal sensation. In contrast, at 28 days, there was a loss of thermal sensitivity in TRPV1 KO mice independent of capsaicin exposure [Fig. 5c, d]. There were no differences among the groups in the response to mechanical testing after injury at either time point.

Taken together, the findings indicated that capsaicin treatment after a nerve injury appeared to improve thermal sensitivity by 14 days and at 100 μM was associated with hyperalgesia at 28 days. TRPV1 mice exhibited impaired

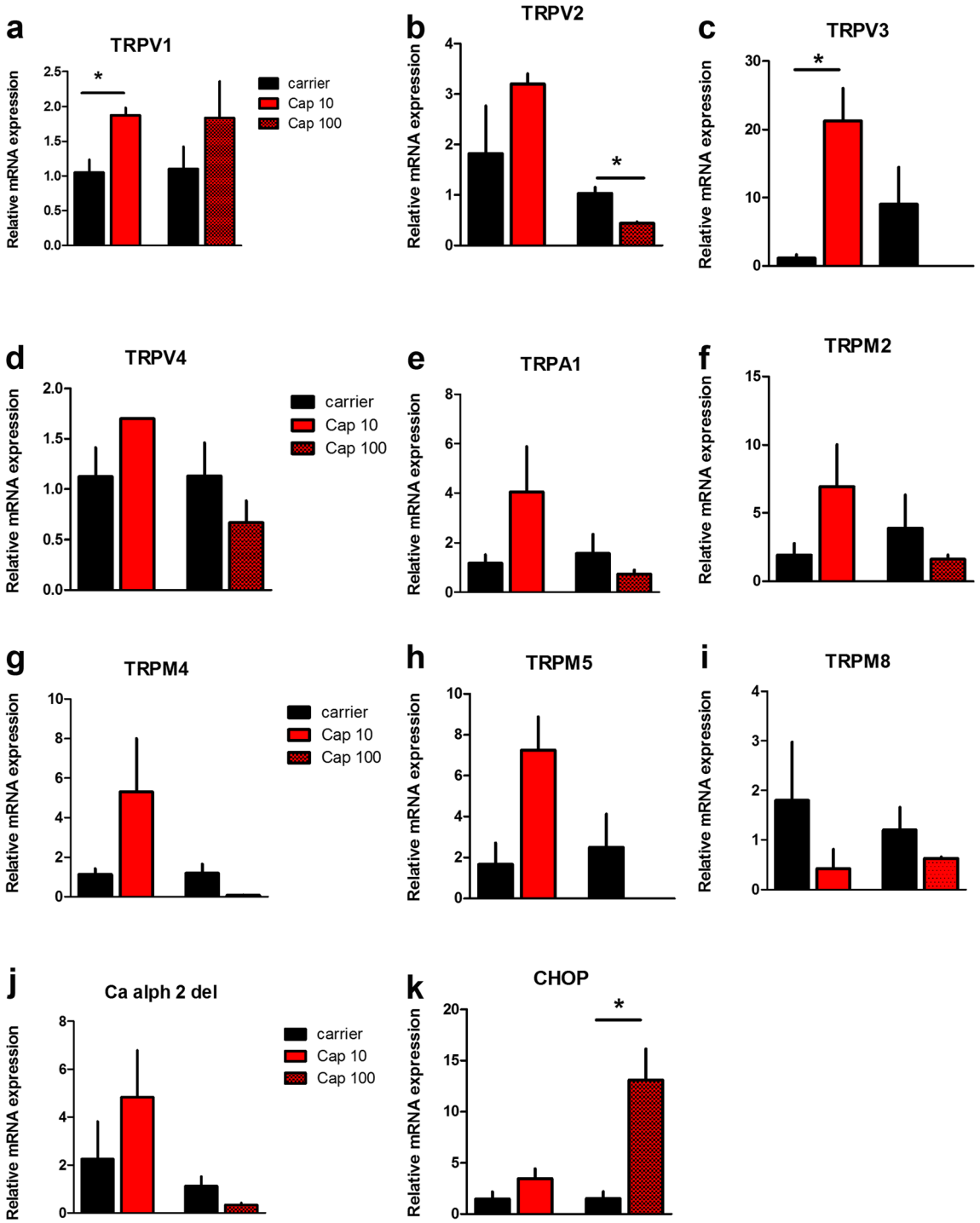


Fig. 4 **a**–**k** Relative mRNAs from neurons exposed to capsaicin show an upregulation of TRPV1 transcripts. qRT-PCR data from harvested adult rat sensory neurons exposed over 24 h to carrier, 10 or 100 μM capsaicin [**a**, carrier vs. cap $*p = 0.009$; **b**, carrier vs. cap $*p = 0.009$; **c**, carrier vs. cap $*p = 0.049$; **k**, carrier vs. cap $*p = 0.02$; $n = 2$–4/group]

thermal sensation at 28 days during recovery, independent of capsaicin exposure, despite no such trend earlier. The findings support the possibility that capsaicin heightens the sensitivity of regenerating sensory axons and that TRPV1 deletion diminishes it, but only during later regeneration.

Capsaicin Selectively targets TRPV1 Neurons During Regeneration: No Impact on Motor Axons or Remyelination

It was possible that capsaicin might impact wider targets than TRPV1, including non-neuronal cells, indirectly supporting

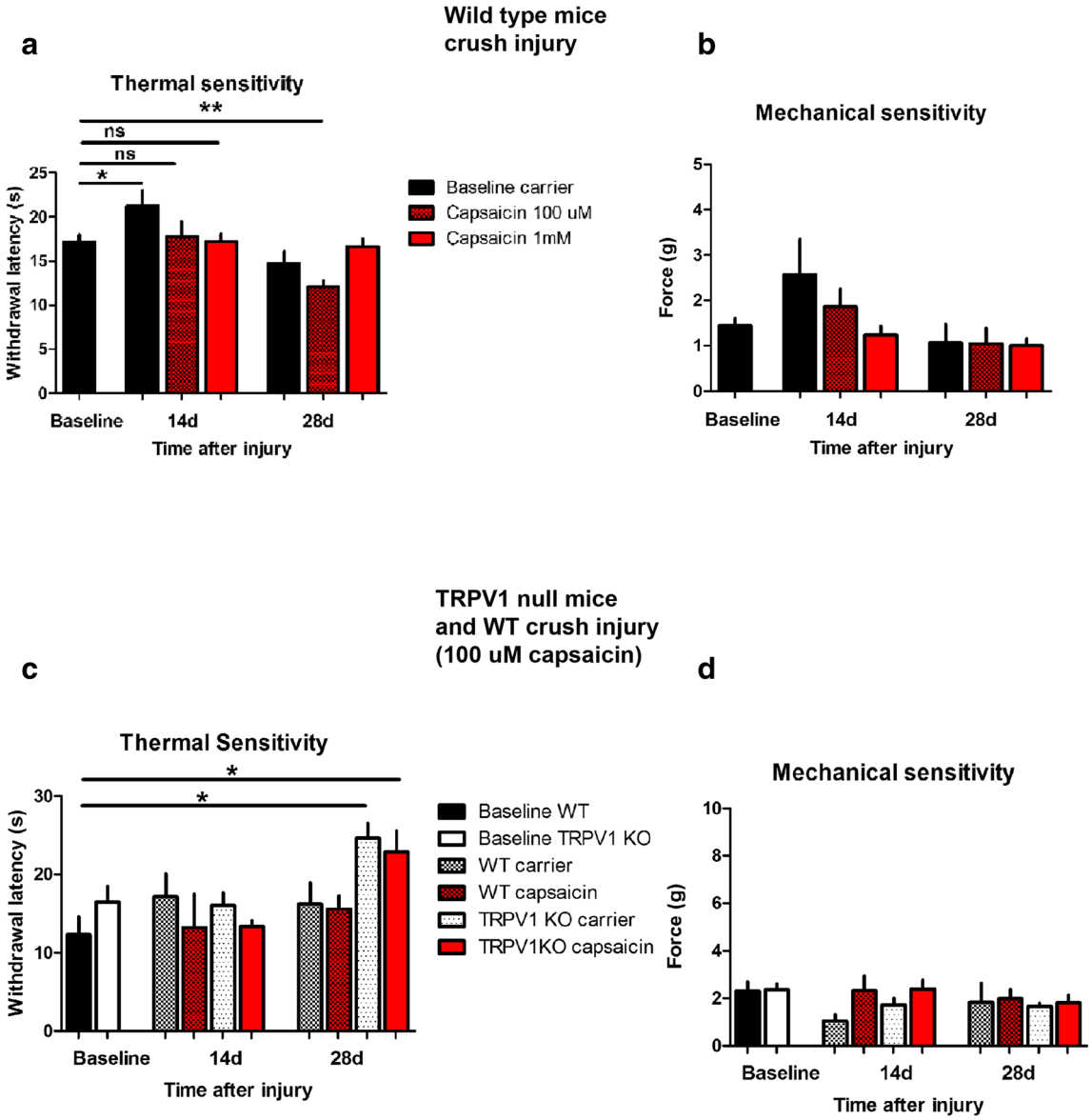
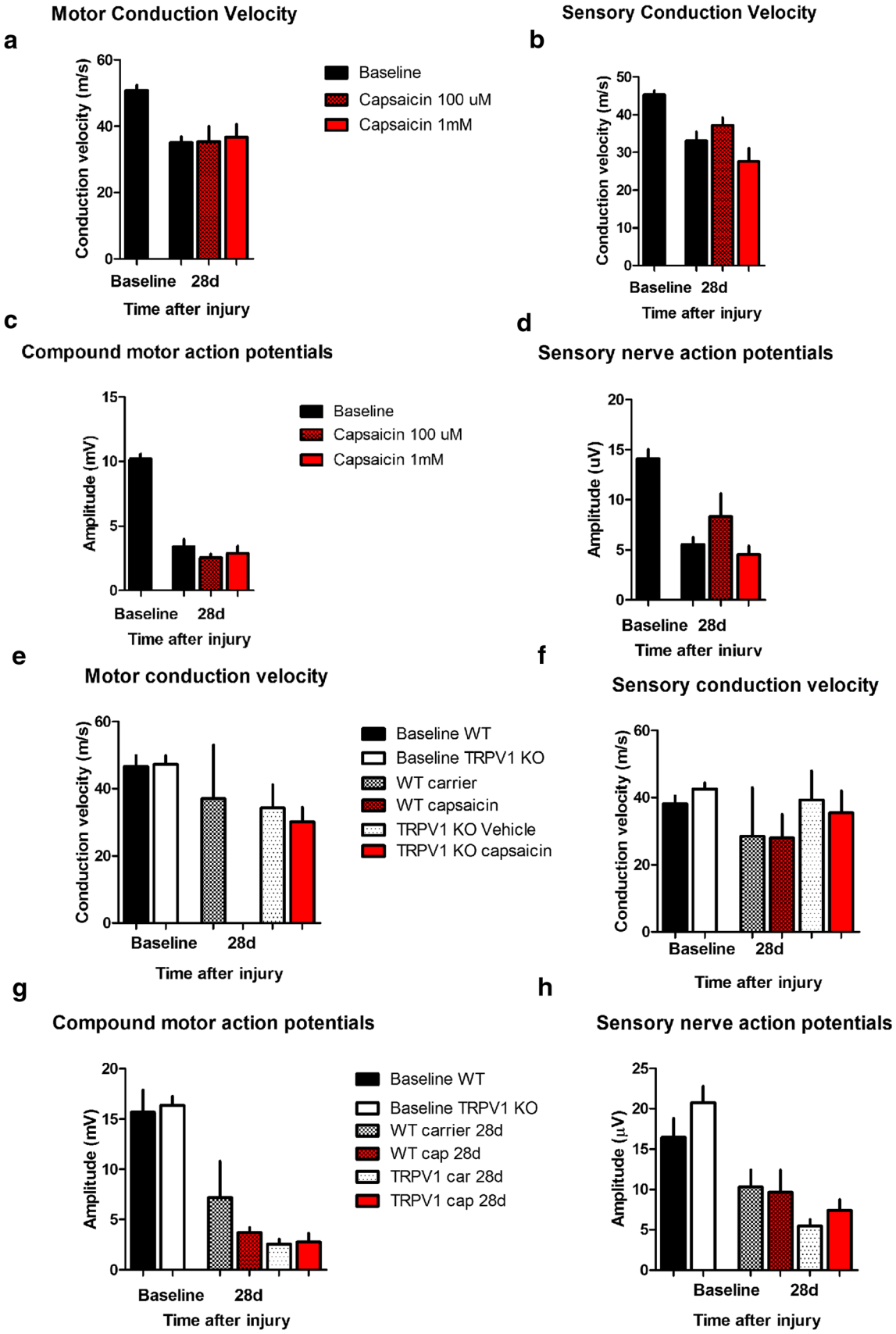


Fig. 5 TRPV1 activation is associated with altered thermal sensation in regenerating axons. Behavioral data assessing thermal sensation (Hargreave's hindpaw thermal latencies (**a**, **c**)) and mechanical (manual von Frey (**b**) or electronic mechanical testing (**d**)) before and serially following nerve crush in outbred mice (**a**, **b**) exposed to carrier, 100 μM or 1 mM capsaicin for 1 h following crush. TRPV1 mice (C, D) and littermate controls were exposed to 100 μM capsaicin as above after crush. [**a**, One-way ANOVA $p < 0.001$; post hoc Student's t test baseline vs. 14d carrier $^{*}p = 0.024$; Mann-Whitney baseline vs. 28d 100 μM capsaicin $^{**}p = 0.0007$; $n = 31$, 13, 10, 10, 13, 10, 7 for baseline, 14d car, 14d 100 μM, 14d 1 mM, 28d car, 28d 100 μM, 28d 1 mM. **c**, One-way ANOVA $p = 0.014$; post hoc Student's t test baseline WT vs. 28d TRV1 KO carrier $^{*}p = 0.003$; baseline WT vs. 28d TRV1 KO capsaicin $^{*}p = 0.01$; $n = 7$, 13, 3, 4, 5, 6, 2, 4, 5, 6 for baseline WT, baseline TRPV1 KO, 14d WT car, 14d WT cap, 14d TRPV1KO car, 14d TRPV1KO cap, 28d WT car, 28d WT cap, 28d TRPV1KO car, 28d TRPV1KO cap]

regeneration. To test this, we followed serial multifiber motor and sensory electrophysiology in regenerating axons after injury. These indices measure reconnection of motor axons to muscles (CMAPs), regenerating myelinated axon maturation (motor and sensory CVs) and sensory axon regrowth (SNAPs). As expected, all of these indices were impacted by injury and there was recovery by the 28-day timepoint [Fig. 6a–h]. However, neither capsaicin at 100 μM or 1000 μM nor TRPV1 null mice had an impact on their recovery. The findings confirmed the specificity of the capsaicin impact in vivo without an impact on motor axons or myelinated sensory axons.

Epidermal Reinnervation and TRPV1 Activation Following Nerve Injury

We next examined epidermal reinnervation of the skin by (i) axon innervation normalized to the length of skin, as routinely used in human studies [19], (ii) epidermal axon density as


a
Motor Conduction Velocity
Conduction velocity (m/s)
60
40
20
0
Baseline
28d
Time after injury
Baseline
Capsaicin 100 uM
Capsaicin 1mM
b
Sensory Conduction Velocity
Conduction velocity (m/s)
50
40
30
20
10
0
Baseline 28d
Time after injury
c
Compound motor action potentials
Amplitude (mV)
15
10
5
0
Baseline
28d
Time after injury
Baseline
Capsaicin 100 uM
Capsaicin 1mM
d
Sensory nerve action potentials
Amplitude (uV)
20
15
10
5
0
Baseline 28d
Time after iniury
e
Motor conduction velocity
Conduction velocity (m/s)
60
40
20
0
Baseline
28d
Time after injury
Baseline WT
Baseline TRPV1 KO
WT carrier
WT capsaicin
TRPV1 KO Vehicle
TRPV1 KO capsaicin
f
Sensory conduction velocity
Conduction velocity (m/s)
60
40
20
0
Baseline
28d
Time after injury
g
Compound motor action potentials
Amplitude (mV)
20
15
10
5
0
Baseline
28d
Time after injury
Baseline WT
Baseline TRPV1 KO
WT carrier 28d
WT cap 28d
TRPV1 car 28d
TRPV1 cap 28d
h
Sensory nerve action potentials
Amplitude (μV)
25
20
15
10
5
0
Baseline
28d
Time after injury

◀ **Fig. 6** TRPV1 activation does not impact recovery of myelinated motor or sensory axons. Electrophysiological data assessing motor nerve conduction (**a**, **e** conduction velocity; **c**, **g** amplitudes of compound muscle action potentials, CMAPs) and sensory nerve conduction (**b**, **f** conduction velocity; **d**, **h** amplitudes of sensory nerve action potentials, SNAPs) before and serially following nerve crush in outbred mice (**a**–**d**) exposed to carrier, 100 μM or 1 mM capsaicin for 1 h following crush. TRPV1 mice (**e**–**h**) and littermate controls were exposed to 100 μM capsaicin as above after crush. [**a**–**d**, $n = 35, 13, 10, 7$ for motor groups (reliable CMAPs not recorded at 14d) and 34, 12, 9, 4, 13, 10, 7 for sensory groups. **e**–**h**, $n = 7, 13, 2, 1, 3, 3, 2, 0, 5, 6$ for motor groups and 6, 13, 2, 1, 2, 3, 2, 3, 4, 5] for sensory groups]

previously described in our work [21, 22], and (iii) vertical (axons directed 45° angle or more from the dermal/epidermal junction) normalized to skin length. For outbred mice, there was a large, expected decline in innervation as assessed 28 days following injury with vehicle treatment. For all three forms of measurement, comparing contralateral and ipsilateral innervation, there was an apparent, but subtle differences in reinnervation favoring the highest dose of capsaicin. These results were not normalized, but contralateral paws after capsaicin had lower levels of innervation. Given this, at 100 μM, there was evidence that ipsilateral innervation remained significantly lower, albeit not dramatic, than contralateral. At 1 mM, capsaicin innervation was no longer different between ipsilateral and contralateral paws. [Fig. 7a–e]. However, despite these comparisons with contralateral innervation, there were no significant differences among the ipsilateral counts. Baseline innervation was comparable between TRPV1 null mice and littermate controls of identical background. There was no significant impact of TRPV1 deletion on baseline density or reinnervation and no response to capsaicin [Fig. 7f–j].

Epidermal Hyperinnervation Follows Recovery from TRPV1 Axonopathy

We next asked whether activation of TRPV1 channels might alter the innervation pattern of skin in the absence of a discrete nerve injury. In previously published work [1], we noted that topical capsaicin induces a reversible axonopathy followed by gradual regrowth of the original innervation architecture. We analyzed cutaneous innervation of the ear pinna of mice 6 weeks following exposure to 0.2% (6.5 mM) capsaicin. Since the model did not involve skin dissection or nerve exposure, higher doses of capsaicin were required to penetrate and access cutaneous nerves. We confirmed the loss of axons at 9 days after capsaicin then identified a rise in overall epidermal innervation of the pinna in ears 6 weeks after their exposure to capsaicin compared with contralateral ears exposed to carrier [Fig. 8]. The findings indicated that activation of TRPV1 channels induces early axonopathy followed by enhanced reinnervation.

Discussion

The major findings from our work were (i) adult sensory neurons undergo biphasic responses to capsaicin activation: low-dose enhancement of growth and toxicity at higher doses; (ii) this pattern is not observed in TRPV1 null mice; (iii) at a capsaicin dose that fosters growth, there are rises in calcium levels of sensory neurons; (iv) exposure to capsaicin by injured and regenerating axons in vivo alters the recovery of thermal but not mechanical sensation nor myelinated motor or sensory function; (v) TRPV1 null mice had late impairment of thermal sensation in their regenerating axons; (vi) there were subtle improvements in axon reinnervation by 28 days in outbred mice exposed to capsaicin during regeneration; and (vii) mice with cutaneous capsaicin axonopathy had late hyper-reinnervation. Taken together, the findings identify a remarkable and potent impact of TRPV1 channel activation on the regenerative plasticity of thermal sensitive sensory neurons.

Since our initial identification of biphasic responses to capsaicin, Frey et al. [10] reported increased outgrowth of axons in vitro following a screen of approximately 480 compounds. No impact on nerve trunk outgrowth after in vivo injury was identified but behavioral recovery and skin innervation were not examined. Unlike that report studying βIII tubulin-labeled neurons, we argue that neurofilament (Nf) staining, used here, is appropriate for studying the full spectrum of sensory neurons in vitro. In fact, all sensory neurons express the Nf polymer, an essential feature of the axon structural scaffold. We noted robust rises in growth and evidence of degeneration in this unselected population, estimating that, like the DRGs that housed them, approximately 40–60% are probably TRPV1 positive based on immunohistochemistry and in situ hybridization [23, 24] although proportions as low as 27% have also been reported [25]. However, it is possible that low-level expression leads to an underestimate of TRPV1 localization by immunohistochemistry. Here, we identified intense TRPV1 expression in approximately 60% of harvested neurons. Since TRPV1-labeled neurons were not used and not suitable as a gold standard for assessing neurite outgrowth, it was not possible to identify changes in neurite outgrowth with these neurons. However, with neurons labeled with βIII tubulin, an undisputed marker of all neuron subtypes, there was a similar outgrowth responsiveness to 10 μM capsaicin.

For the same reasons, our analysis of skin innervation emphasized the robust marker PGP 9.5, a well-validated marker of epidermal axons. While not convincingly addressed in previous literature, the proportion of epidermal axons that express TRPV1 in skin samples is likely to be high. This is also supported by the near universal loss of epidermal axons in skin exposed to capsaicin [4]. TRPV1 negative neurons in the DRG include joint and other large fiber afferents not present in the epidermis. Strictly epidermal axons that are both peptidergic

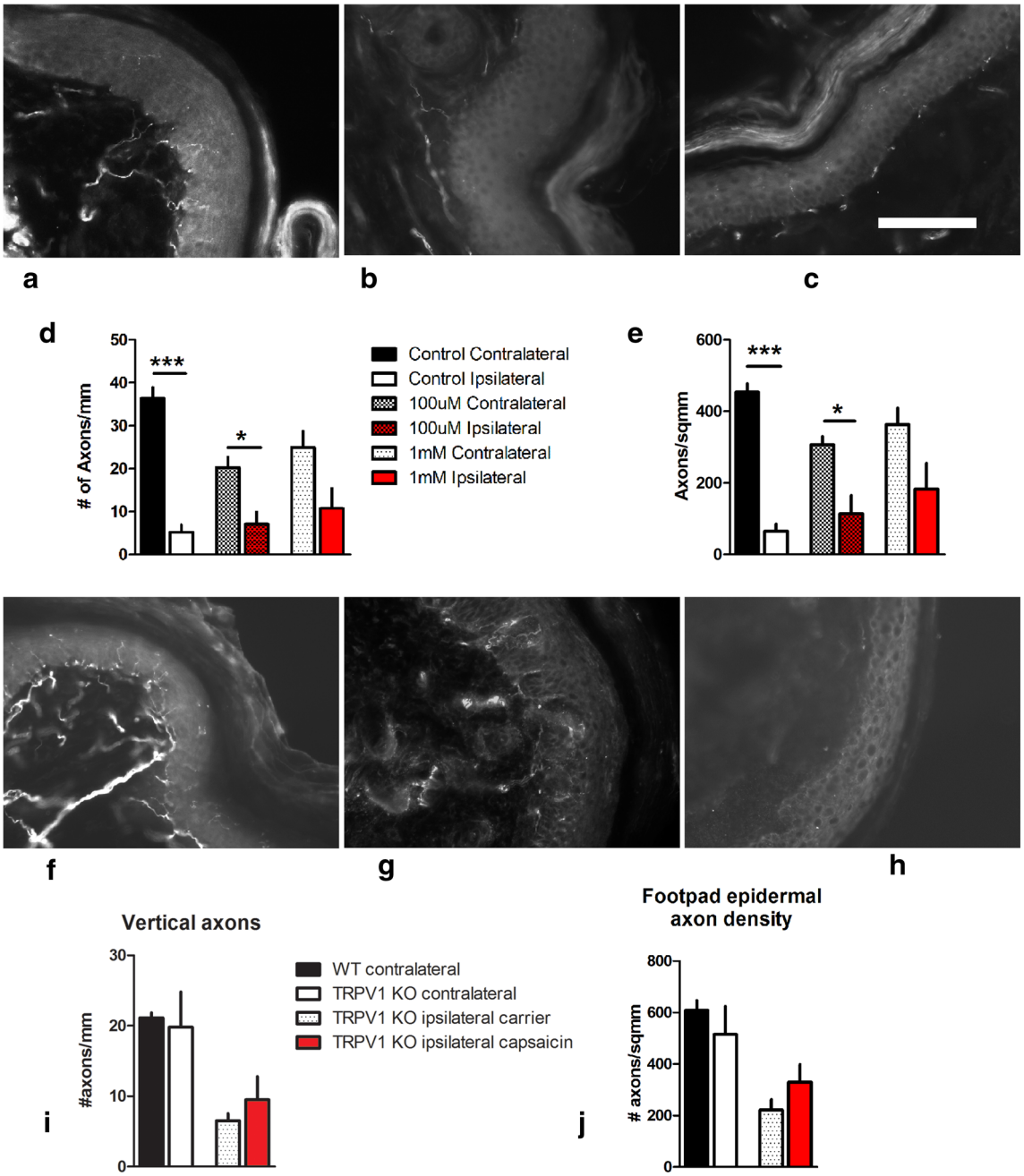


Fig. 7 TRPV1 activation alters reinnervation of the foot pad skin following nerve injury in comparison to contralateral paws. Examples of hindpaw footpad sections labeled with PGP 9.5 for epidermal axons from contralateral (to sciatic nerve crush) paws without injury (**a**), ipsilateral paws with injury and carrier treatment (**b**), and ipsilateral paws with injury and capsaicin (1 mM) treatment (**c**). Quantitative axon counting data assessing numbers of axons for a given length of epidermis (**d**, **i**) or axon profile densities (**e**, **j**) before and serially following nerve crush in outbred mice (**d**, **e**) exposed to carrier, 100 μM or 1 mM capsaicin for 1 h following crush. Hindpaw footpad sections labeled with PGP 9.5 for epidermal axons of wild type mice (WT) from contralateral (to sciatic nerve crush) paws without injury (**f**), TRPV1 KO mice contralateral without injury (**g**), and ipsilateral paws with injury from TRPV1 KO mice (**h**) and capsaicin (100 μM) treatment (quantitation **i**, **j**).[**d**, One-way ANOVA $p < 0.0001$; post hoc Student's t test contralateral vs. ipsi carrier ***$p < 0.0001$; contralateral vs. ipsilateral 100 μM capsaicin *$p = 0.017$; $n = 3$–4/group. **e**, One-way ANOVA $p < 0.0001$; post hoc Student's t test contralateral vs. ipsi carrier ***$p = 0.0004$, contralateral vs. ipsilateral capsaicin 100 μM *$p = 0.017$; $n = 3$–4/group for groups. **i**, **j**, $n = 6, 4, 3, 4$ for groups]

and nonpeptidergic likely express TRPV1 [24]. However, TRPV1 terminal epidermal axons are notoriously difficult to label. Similarly, reliable quantitation of nonpeptidergic fiber populations in epidermal samples has been problematic. Thus, we did not identify staining in our epidermal samples of sufficient resolution that would allow analysis of these fiber classes alone, a problem also observed by others [25]. Mice with a GFP fluorophore expressed under a TRPV1 promoter

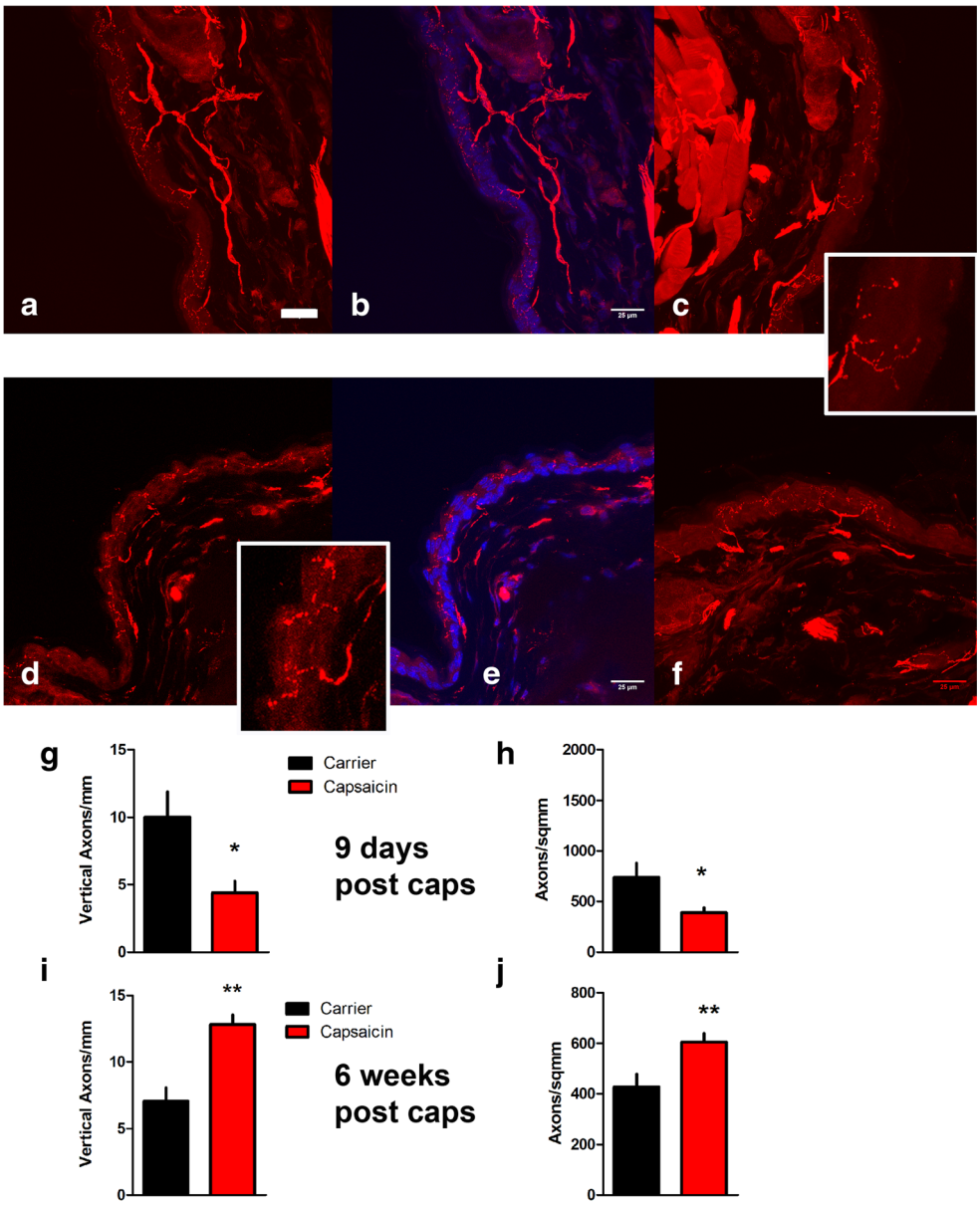


Fig. 8 Capsaicin axonopathy is associated with early axonopathy followed by long-term epidermal hyper-reinnervation. Examples of ear pinna sections labeled with PGP 9.5 for epidermal axons (and DAPI for nuclei, (**b**, **e**)) from contralateral (to capsaicin) ears without axonopathy (**a**–**c**, with higher power inset of **c**), ipsilateral ears 6 weeks following capsaicin axonopathy (**d**–**f**, with higher power inset of **d**). Quantitative axon counting data assessing numbers of vertical axons (> 45° from the dermal-epidermal junction and crossing it (**g**, **i**) or total profile densities (**h**, **j**) at 9 days after capsaicin (indicating depletion of axons from capsaicin) and 6 weeks post capsaicin (indicating regeneration) [**g**–**j**, paired Student's *t* tests $^{**}p \leq 0.001$; $^{*}p < 0.05$, $n = 5$/group]

described by Wang et al. [2] had a depletion of epidermal axons following capsaicin that was limited to 40% probably because of loss of lineage expression during development. Thus, it remains uncertain how these numbers related to overall expression using the PGP 9.5 gold standard. In humans, the proportion of TRPV1 axons in the epidermis was reported at approximately 16% [26]. One difficulty is that most of these estimates would not exclude lower level expression below the threshold for detection.

Our preconditioned and preinjured neurons had a more robust response to capsaicin, beyond that induced by preconditioning [27]. This indicates that preconditioning does not fully ramp up plasticity programs in neurons, leaving room to coax greater outgrowth as observed in knockdown of either PTEN (phosphatase and tensin homolog deleted on chromosome 10) or Rb1 (retinoblastoma 1) [14, 28]. A limitation of the work is in understanding how exactly the differing biological impacts of lower dose and higher dose capsaicin are related to the degree of TRPV1 channel activation. It may be that activation extent or duration differs between the doses to account for the findings.

As in neurons activated by exogenous brief electrical stimulation (ES), we identified rises in intraneuronal calcium that we believe are linked with their growth response [29]. Like the responses recorded here, these ES responses were similarly prolonged, providing a broad window to activate a series of calcium-related growth programs. Unlike ES that enhances the regrowth of sensory and motor axons, the response here is confined to TRPV1 neurons. However, given the identification of a calcium signal, it seems likely that intracellular

growth mechanisms activated by capsaicin are wider than the PKA pathway role identified recently [10]. The ES response instead has been linked to activation of BDNF [29–31] as well as other mechanisms including declines in PTEN and rises in Shh and GAP43 [32]. Neither our growth studies nor calcium studies had enough resolution to address whether the doses of capsaicin applied to neurons or axons saturated receptors. To address receptor saturation, occupancy, and desensitization, not done here, would require a differing biophysical approach with a challenge of translating these data into downstream growth or toxicity impacts.

We find it remarkable that only a single exposure to capsaicin was associated with lasting, albeit subtle alterations in the regenerative behavior of skin axons up to 28 days later. This alteration in regenerative trajectory is more selective, but not unlike that observed using only a 1 h exposure of nerves to exogenous ES [29, 30, 32–34]. At 14 days, nerves exposed to capsaicin in both doses had normal thermal sensitivity whereas controls had hypalgesia. Moreover, at 28 days, the mice exposed to low-dose capsaicin had evidence of hyperalgesia. Both findings may relate to enhanced regrowth of TRPV1 axons, but may also indicate that individual regenerating axons have heightened signaling from TRPV1 activation. There was no impact on the recovery of mechanical sensitivity. Some degree of mechanical sensitivity has been linked to TRPV1 but it may depend on the type of mechanical stimulus [35]. For example, noxious pinching may involve TRPV1 neurons whereas simple von Frey touch, as in this work, may not. Classical electrophysiological indices of regrowth of myelinated motor and sensory axons were not impacted. In TRPV1 null mice, thermal sensitivity was not altered at 14 days but at 28 days, there was evidence of a remarkable loss of thermal sensation irrespective of the treatment group. These findings may inform us about what channels mediate sensation during earlier and later phases of regeneration. It may be that earlier regrowing fibers do not accurately transmit their sensory modalities or that our testing stimuli spread beyond the injured nerve itself, involving sprouted saphenous axons [20]. By 28 days in normal mice, when more axons reach their targets and mature, thermosensitive fibers may resume their contribution to the sensation. However, reinnervating axons lacking TRPV1 fail in this task. Late hypalgesia was not from failed epidermal reinnervation since an expected complement of axons was identified.

By 28 days, the numbers and density of reinnervating epidermal axons were limited. Despite this, there were behavioral sensory responses detected. Saphenous axons nearby undergo collateral sprouting as recently evaluated by Cobianchi, Navarro, and colleagues in the rat hindpaw [20, 36]. In the much smaller mouse hindpaw, differentiating sensory territories is much more challenging, such that thermal stimuli routinely spread beyond strict sciatic innervation from saphenous fibers. While epidermal axon density measures may capture additional fibers that traverse in and out of the plane of section, some profiles may be branches. In TRPV1 null mice, these measures were variable at the 28-day timepoint without a significant impact of capsaicin.

One of the most remarkable findings here was that capsaicin axonopathy was followed by late hyper-reinnervation of the epidermis. This supports the concept that there is a long-term facilitation of regrowth by activation of TRPV1 channels. Whether hyper-reinnervation might also follow lower level TRPV1 activation without initial axonal degeneration is unknown. Similarly, it is unclear whether this unusual outcome, an override of usual constraints on the density of innervation, arises from distal or more proximal afferent branches.

In summary, the limitations of this work were that relatively short regenerative time lines were studied and that epidermal-specific axon subtypes were challenging to accurately map. The small hindpaw territory of the mouse, despite its more rapid reinnervation than in larger rodents such as the rat, precludes exclusion of input from collaterally sprouted nearby axons. Our changes in thermal sensation and epidermal innervation after sciatic injury were modest and subtle. While we have identified some insights into how low-dose capsaicin might activate sensory neurons during regeneration, including mediation by calcium, the full repertoire of downstream actors in this growth has yet to be determined. Finally, the range of effective doses for in vivo work needs additional attention.

Like ES, capsaicin facilitation of regeneration offers a simple, relatively noninvasive approach to enhance neurological recovery from neuropathies that target thermosensitive fibers. It is possible that a similar, but unexplored window of sensory neuron activation exists in non TRPV1 neurons by manipulating their intracellular calcium activation.

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Compliance with Ethical Standards

The protocols were reviewed and approved by the Animal Care Committee University of Alberta, adhering to guidelines of the Canadian Council of Animal Care.

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