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RESEARCH ARTICLE

Nociception, neurogenic inflammation and thermoregulation in TRPV1 knockdown transgenic mice

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Abstract Transgenic mice with a small hairpin RNA construct interfering with the expression of transient receptor potential vanilloid 1 (TRPV1) were created by lentiviral transgenesis. TRPV1 expression level in transgenic mice was reduced to 8% while the expression of ankyrin repeat domain 1 (TRPA1) was unchanged. Ear oedema induced by topical application of TRPV1 agonist capsaicin was completely absent in TRPV1 knockdown mice. Thermoregulatory behaviour in relation to environmental thermopreference (30 vs. 35°C) was slightly impaired in male knockdown mice, but the reduction of TRPV1 function was not associated with enhanced hyperthermia. TRPV1 agonist resiniferatoxin induced hypothermia and tail vasodilatation was markedly inhibited in knockdown mice. In conclusion, shRNA-mediated knock down of the TRPV1 receptor in mice induced robust inhibition of the responses to TRPV1 agonists without altering the expression, gating function or neurogenic oedema provoked by TRPA1 activation. Thermoregulatory behaviour in response to heat was inhibited, but enhanced hyperthermia was not observed.

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Abbreviations

TRPV1	Transient receptor potential vanilloid 1
TRPA1	Transient receptor potential ankyrin repeat domain 1
shRNA	Small hairpin RNA
siRNA	Small interfering RNA
RTX	Resiniferatoxin
GFP	Green fluorescence protein

Introduction

The transient receptor potential vanilloid 1 (TRPV1) capsaicin receptor is a noxious heat-gated cation channel expressed in the largest subset of somatosensory neurons which supply nociceptors [1–4]. The importance of these TRPV1-expressing polymodal nociceptors is that they can be activated by chemically unrelated exogenous and endogenous compounds which are potentially damaging and elicit pain in humans under physiological and pathological conditions. Thus, these integrative chemoceptive nocisensors have become promising drug targets for developing the first class of analgesics that act selectively on nociceptive primary afferent neurons [2, 4–9].

Several selective TRPV1 antagonists are under clinical evaluation and in several preclinical studies TRPV1 knockout mice (TRPV1 KO) are utilized for target validation. However, an unexpected side effect of some drug candidates has appeared. Hyperthermia has been observed in both animals and humans and can be attributed to the

unique multifaceted gating function of this type of chemoceptive–thermoceptive cation channel [4, 5, 10, 11]. Thus, in order to extend the repertoire of gene-manipulated animals for evaluation of the role of TRPV1 receptor, the novel approach of silencing the TRPV1 gene by using small hairpin RNA (shRNA) in transgenic mice have recently been described [12].

The aim of this study was to create and extensively characterize TRPV1 knockdown mice. In these mice, besides verifying the loss of in vitro and in vivo responses to TRPV1 receptor agonists capsaicin and resiniferatoxin (RTX) was also extended by studies on the responses to mustard oil. Mustard oil selectively gates another thermosensitive transient receptor potential channel, which is also gated by various noxious chemicals but not by capsaicin, RTX or noxious heat. This transient receptor potential ankyrin repeat domain 1 (TRPA1) receptor is a noxious cold-gated integrative nocisensor coexpressed with TRPV1 [13–16]. The importance of these evaluations is underlined by the recently described interactions of these channels in the plasma membrane of nociceptors [17]. Further aims were to shed light on the thermoregulatory behaviour of TRPV1 knockdown mice and to obtain data in these animals on neurogenic inflammation evoked by agonists of TRPV1 and TRPA1 receptors.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (D-MEM), and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY). Collagenase (type XI), deoxyribonuclease I, poly-D-lysine, nerve growth factor, capsaicin, RTX, xylazine and a GeneElute mammalian total RNA kit (RTN-70) were purchased from Sigma (St. Louis, MO). Mustard oil was purchased from Merck (Darmstadt, Germany). The chemically synthesized siRNAs, phosphorylated oligonucleotides for shRNA construction, pU6Entry plasmid, and fura-2-acetoxy-methyl ester were purchased from Invitrogen (Carlsbad, CA). TransMessenger transfection reagent was purchased from Qiagen (Hilden, Germany). ExGen 500 and RevertAid H Minus first strand cDNA synthesis kit (K1632) were purchased from Fermentas (St. Leon-Rot, Germany). TaqMan assays were purchased from Applied Biosystems (San Francisco, CA). *Hae*III restriction endonuclease was purchased from Promega (Madison, WI). The primers for the genomic PCR and ligation-mediated PCR (LM-PCR) were purchased from IDT (Leuven, Belgium). Ketamine as Calypsol injection was purchased from Gedeon Richter (Budapest, Hungary). The lentiviral plasmid constructs (pWPTS-GFP, psPAX2 and pMD2G) were kindly provided by Patrick Salmon PharmD, PhD (Department of Neurosciences, Faculty of Medicine, University of Geneva, Switzerland). The rat dorsal root ganglion-derived cells (ND C) were provided by John N. Wood (Sandoz Institute for Medical Research, London, UK). HeLa cells were purchased from ATCC (clone CCL-2) and CHO/K1 cells were purchased from ECACC.

Transfection of ND C and CHO/K1 cells with small interfering RNAs (siRNAs)

The sequences of VsiR1 and VsiR1INV siRNAs have been published previously [18, 19]. VsiR1ST is a 25 base length Stealth variant of VsiR1 with the following sense sequence: GCGCAUCUUCUACUUCAACUUCUUC. The ND C cell line is a hybrid cell line derived from neonatal rat dorsal root ganglia neurons fused with mouse neuroblastoma N18Tg2 [20]. ND C cells and CHO/K1 cells were grown at 37°C under an atmosphere containing 5% CO_2 in D-MEM plus 10% FBS supplemented with penicillin and streptomycin. The day before transfection, the cells were trypsinized, diluted with fresh complete medium, and transferred to 24-well plates at 4×10^4 cells per well. RatTRPV1eGFP fusion protein expressing vector pZS5 (200 ng) [21] and siRNA (8 pmol) were cotransfected with TransMessenger transfection reagent according to the manufacturer's protocol.

After overnight incubation, the GFP fluorescence of transfected cells was measured by flow cytometry (Partec CyFlow space with FloMax software, version 2.4; Münster, Germany). GFP expression was calculated according to the following procedure. A region was marked which excluded the nontransfected autofluorescent cells but included all strongly fluorescent GFP-positive cells. The percent of GFP-positive cells and the mean fluorescence of these cells were multiplied to obtain a single composite value, representing the amount of GFP expression in the sample. One-way ANOVA and Dunnett's post hoc comparison were used to analyse differences between transfections.

Primary cultures of trigeminal ganglion neurons and fura-2 microfluorimetry

Trigeminal ganglion (TRG) neuron cultures were prepared from 2- to 4-day-old rat or mouse pups as described previously [22]. The trigeminal ganglia were dissected, incubated in phosphate-buffered saline solution (comprising 138 mM NaCl, 5.4 mM KCl, 7.8 mM $Na_2HPO_4 \cdot 2H_2O$, and 1.4 mM KH_2PO_4) containing 8 mg/ml collagenase (type XI) for 30 min. The sample was digested in phosphate-buffered saline containing 0.3 mg/ml deoxyribonuclease I and dissociated by trituration. TRG cells were plated on poly-D-lysine-coated glass coverslips and grown in D-MEM plus

10% FBS supplemented with 200 ng/ml nerve growth factor for 2–4 days. TRG neurons were stained at room temperature with 1 μM fluorescent Ca^{2+} indicator dye fura-2-acetoxy-methyl ester. Calcium transients were examined in extracellular solution (containing 160 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2{\cdot}2H_2O$, 2 mM $MgCl_2{\cdot}6H_2O$, 10 mM HEPES, and 10 mM glucose) under a fluorescence microscope. Cells were illuminated by light at alternately 340 and 380 nm generated by a monochromator (Polychrome II; Till Photonics, Munich, Germany) under the control of Axon Imaging Workbench 2.1 software (Axon Instruments, Union City, CA) and the light emitted at >510 nm was measured. The fluorescence ratio ($R = F_{340}/F_{380}$) was monitored continuously at a rate of 1 Hz. The background noise appeared as a change in fluorescence ratio (ΔR) of about 0.05 in our setup. To excite TRPV1 receptors a 10-s pulse of 330 nM capsaicin was applied, and TRPA1 receptors were stimulated with 100 μM mustard oil for 30 s. Cells showing a ΔR of >0.1 after agonist treatment were considered to be receptor-expressing cells. In siRNA experiments, before microfluorimetry TRG neurons were incubated overnight with 50 nM siRNA in the medium without any transfection reagent. Cells reacting to agonist treatment were counted in the samples and Fisher's exact test was used to analyse the data.

Building and testing of shRNA constructs

shRNAs were designed based on previously published principles [23]. The different shRNA constructs were built from phosphorylated oligonucleotides and inserted into an ampicillin-resistant version of pU6Entry plasmid behind the human U6 promoter. A derivative of this vector producing no shRNA was used as control in the transfection experiments.

ND C cells were plated at 10^5 cells per well into 24-well plates. Next day, pZS5 plasmid (250 ng) and shRNA vector (250 ng) were cotransfected into the cells using polyethylenimine transfection reagent ExGen 500, according to the manufacturer's protocol. Cells were incubated for 24 h and green fluorescence was measured by flow cytometry as described in the siRNA section. One-way ANOVA and Dunnett's post hoc comparison were used to analyse the transfection results.

Preparation of lentiviral particles

The U6 promoter and shRNA*a* construct were moved into the pWPTS-GFP lentiviral vector [24]. The U6 promoter-shRNA*a* construct sits between the WHV region and the 3′ self-inactivating long-terminal repeat (3′-LTR/SIN), and has the same orientation as the GFP transcription unit.

Lentiviral particles were produced as described previously [25]. Briefly, the transfer vector containing shRNA*a*, packaging plasmid psPAX2 and helper plasmid pMD2G were cotransfected into 293T cells using the calcium phosphate method and the viral supernatant was harvested after 48 h. Purification steps included centrifugation (2,000 rpm, 10 min, 4°C) and filtration (PVDF-coated 0.45-μm filters) to remove cells and large debris. For concentration the supernatant was layered onto 20% sucrose for ultracentrifugation (26,000 rpm, 90 min, 4°C). Viral particles concentrated 1,000-fold were resuspended in D-MEM. Biological titres of concentrated viral particles reached and exceeded 10^8 TU/ml as measured on HeLa cells by GFP flow cytometry.

Creation of transgenic animals

Sprague-Dawley rats and of FVB/Nhsd mice (from Harlan Laboratories, Indianapolis, IN) were kept in a conventional animal facility. Superovulation of donor females, embryo isolation and transfer into surrogate mothers were performed according to standard laboratory protocols. Lentiviral vector was injected based on the modified perivitelline space injection method to produce transgenic mice from low-titre lentiviral vector [26]. Approximately 300–500 pl medium containing about 10^8 infectious units/ml lentiviral vector was repeatedly injected into the perivitelline space of single-cell embryos.

Genotyping of transgenic animals

Animals were illuminated with at 455–495 nm wavelength blue light and GFP fluorescence was detected in vivo with a GFSP-5 headset (Biological Laboratory Instruments, Budapest, Hungary). Transgene integration was detected by GFP-specific genomic PCR using forward primer 5′-CTCGTGACCACCCTGACCTAC-3′ and reverse primer 5′-CATGATATAGACGTTGTGGCTGTT-3′ with the following amplification parameters: 95°C for 4 min (1 cycle); 95°C for 30 s, 61°C for 30 s, 72°C for 30 s (25 cycles); 72°C for 30 s (1 cycle). The size of the amplified product was 282 bp. The shRNA region-specific PCR used forward primer 5′-GAGGGCCTATTTCCCATGAT-3′ and reverse primer 5′-TAAAGGTACCTCGCGAATGC-3′ with the following amplification parameters: 95°C for 4 min (1 cycle); 95°C for 30 s, 61°C for 30 s, 72°C for 30 s (25 cycles); 72°C for 30 s (1 cycle). The amplification product was 327 bp.

The integration site of the transgene was determined by LM-PCR on HaeIII digested genomic DNA with provirus 3′-LTR/SIN-specific primer 1 (5′-CAGGGTACCTTTAAG ACCAATGAC-3′), primer 2 (5′-TTTGCTTGTACTGGG TCTCTCTGG-3′) and primer 3 (5′-TCAAGTAGTGTG

TGCCCGTCTG-3′) according to published protocols [27]. After running the PCR reaction on agarose gel the dominant fragment of about 500 bp was isolated and directly sequenced. Based on the sequence obtained, the integration site-specific primers Chr3A (5′-GCTTTAAATGCCT TCCTTGTTAAA-3′) and Chr3B (5′-TAACTGAGAAG CAAGGTTTTGTTG-3′) were used to amplify the whole integrated provirus of about 4.5 kb with the flanking chromosomal DNA, and the junctions were sequenced.

Quantitative PCR

TRG neurons were isolated from 4-week-old animals and total RNA was isolated with a GeneElute mammalian total RNA kit including on-column DNAse digestion. Total RNA (70 ng) was reverse-transcribed with a RevertAid H Minus first strand cDNA synthesis kit using oligo-dT primers according to the manufacturer's protocol. The TRPV1, TRPA1 and TRPV3 mRNAs were quantified using TaqMan assays Mm01246301_m1, Mm00625268_m1 and Mm00454996_m1, respectively. In each sample, the C_t values obtained were normalized against the geometrical mean of the C_t values of the housekeeping genes hypoxanthine phosphoribosyltransferase 1 (HPRT1) and β2-microglobulin to obtain the presented ΔC_t values. Two-way ANOVA and Bonferroni post hoc comparison were used to analyse the data.

In vivo experiments

Capsaicin-induced eye-wiping test Solution containing 10 μg/ml capsaicin, 0.01% ethanol and 0.01% Tween 80 in saline was instilled into the eyes of 3-week-old mice. The number of protective eye-wiping movements with the forelegs was counted during a 30-s observation period [28]. One-way ANOVA and Dunnett's post hoc comparison were used to analyse data.

Capsaicin-evoked nocifensive response on the paw Capsaicin (20 μl, 100 μg/ml) was injected into the sole of the left hindpaw of 4-week-old mice and the duration of paw licking and the number of paw flinches were recorded for 5 min. The day before, serving as a solvent control, 20 μl of saline containing 0.1% ethanol and 0.1% Tween 80 was injected in the right hindpaw of the same mice, and paw licking and flinching were similarly recorded. Nocifensive behaviour was quantified as a pain score by summing the duration of paw licking in seconds and the number of flinches. Two-way ANOVA and Bonferroni post hoc comparison were used to analyse data.

Measurement of capsaicin- or mustard oil-induced neurogenic oedema formation in the ear Mice were anaesthetized by intraperitoneal (i.p.) injection of a mixture of ketamine and xylazine (100 and 5 mg/kg, respectively), and 30 μl of 2.5% capsaicin dissolved in 96% ethanol or 3% mustard oil dissolved in paraffin oil was smeared topically onto both sides of the ears. The diameter of the ear was measured with an engineer's micrometer before the treatment and every hour during the 5-h examination period. Swelling was expressed as percent relative to the initial control values. Two-way ANOVA and Bonferroni post hoc comparison were used to analyse the data.

Temperature selection Thermopreference of the mice was studied by putting the animals into an apparatus in which they could choose between two ambient temperatures by locomotion. The thermostatically controlled apparatus consisted of two jacketed cylindrical compartments of different temperatures which were connected by a tube to allow free passage for the animals [29]. The mice were previously habituated to the experimental conditions by putting them in the apparatus for 1 h with both chambers kept at room temperature. On the day of the experiment no habituation was performed to avoid a learning phenomenon. The air temperature of the compartments was kept constant by means of two thermostats which heated the air to 30°C in one compartment and 35°C in the other one. The temperatures of the chambers were measured by digital thermometers placed 5 cm above the middle of the floor. The time spent in each compartment was recorded over a period of 40 min at 10-min intervals. Rectal temperature was measured before and after the exposure. Data for thermopreference and rectal temperature were analysed by two-way ANOVA and Bonferroni post hoc comparison.

RTX-induced hypothermia Rectal temperature of 8-week-old mice was measured using a digital thermometer before i.p. injection of 20 μg/kg RTX (diluted with saline from a 1 mg/ml stock solution in ethanol) and at 7.5, 15, 30 and 60 min after injection. As a solvent control, 0.2% solution of ethanol was administered i.p. (0.1 ml/10 g) the day before and body temperature was monitored in a similar fashion. Two-way ANOVA and Bonferroni post hoc comparison were used to analyse the data.

RTX-induced peripheral vasodilatation Transgenic (tg+) mice, nontransgenic (tg−) mice and TRPV1 KO mice (Jackson Laboratories, Sacramento, CA) were anaesthetized with urethane and injected i.p. with RTX (20 μg/kg). Blood flow in the tail of the mice was measured with a high-resolution PIM-2 laser Doppler perfusion imager (Perimed, Stockholm, Sweden). The parameter settings during the measurement were: scanning area, 40 × 60 mm; medium resolution; distance between the scanner head and tail,

25 cm. The images were analysed and perfusion values determined using the LDISOFT software package. Two-way ANOVA and Bonferroni post hoc comparison were used to analyse the data.

Ethics

Construction of transgenic animals was approved by the Animal Care and Ethics Committee of the Agricultural Biotechnology Center and complied with the Hungarian Code of Practice for the Care and Use of Animals for Scientific Purposes, including conditions for animal welfare and handling prior to slaughter. All in vivo experimental procedures were carried out according to the 1998/XXVIII Act of the Hungarian Parliament on Animal Protection and Consideration Decree of Scientific Procedures of Animal Experiments (243/1988). The in vivo experiments complied with the Ethical Guidelines of the International Association for the Study of Pain. The studies were approved by the Ethics Committee on Animal Research of Pécs University according to the Ethical Codex of Animal Experiments, and a licence was given (licence no. BA02/2000-16-2006).

Results

siRNA experiments

siRNAs were designed against the rat TRPV1 mRNA based on published data [18, 19]. VsiR1 is a previously described 21-bp long siRNA targeting position 1373 in TRPV1 mRNA. VsiR1ST is a 25-bp long nuclease-resistant (Invitrogen Stealth modification) double-stranded RNA targeting the same site. The third siRNA, VsiR1INV, was used as a control and has the reverse sequence to VsiR1. The effectiveness of these siRNA was tested in transient cotransfection experiments with pZS5 plasmid expressing the ratTRPV1eGFP fusion protein in the rat dorsal root ganglion-derived ND C cells. One day after transfection ratTRPV1eGFP expression was analysed by flow cytometry. Compared to transfection with pZS5 plasmid alone (100% expression), a significant decrease in GFP fluorescence was observed when the plasmid was cotransfected with either the VsiR1 or the VsiR1ST siRNA. The remaining expression levels were 15.41 ± 3.5% and 13.89 ± 0.3% for VsiR1 and VsiR1ST, respectively. As expected, TRPV1 knockdown activity was not observed with the control VsiR1INV siRNA (102.4 ± 9.4% expression).

To assess the effect of siRNAs on cells already expressing the native TRPV1 receptor, TRG neurons were isolated from newborn rats and grown overnight in medium containing 50 nM siRNA. The presence of functional TRPV1 receptor was tested by measuring capsaicin-induced Ca^{2+} influx by fura-2 microfluorimetry. In the untreated sample, 49.5% of the cells (47 out of 95) reacted ($\Delta R_{F340/F380} > 0.1$) to a 10-s exposure to 330 nM capsaicin. A significant decrease was observed after VsiR1 treatment, as only 0.6% of cells (8 out of 134) responded to capsaicin administration. In the remaining eight cells which reacted to capsaicin the peak values and time-course of Ca^{2+} influx were similar to the values in untreated cells, indicating similar TRPV1 expression, probably because these cells did not pick up the VsiR1 siRNA. As expected, after treatment with control VsiR1INV, the proportion of cells responding to capsaicin remained at 48% (38 out of 79) and there were no significant changes in the peak values or time-course of Ca^{2+} influx.

To confirm the selectivity of VsiR1 and to exclude nonspecific inhibition of receptor expression, mustard oil-induced TRPA1 receptor activation was also evaluated in TRG neurons. In the untreated sample, 23% of neurons (14 out of 60) reacted with Ca^{2+} influx to a 30-s exposure to 100 μM mustard oil. VsiR1 treatment had no effect on TRPA1 activation, as the percentage of mustard oil-sensitive cells remained at 23% (15 out of 66). These results confirmed the potent and selective TRPV1 knockdown effect of the VsiR1 and VsiR1ST siRNAs. We have tried to use these oligonucleotides to inhibit TRPV1 expression in vivo in the rat by intrathecal administration, but have not been able to achieve a significant TRPV1 knockdown effect.

shRNA experiments

shRNA constructs (14 including controls) targeting six sites on the rat TRPV1 mRNA were built into a plasmid vector behind the human U6 promoter (vectors named pshRNA*a* to pshRNA*n*). The structures and target sites (numbered according to GenBank entry NM_031982.1) of these shRNAs are shown in Table 1.

In a first set of experiments we tested the efficiency of six shRNAs targeting different sites on the rat TRPV1 mRNA. The plasmids expressing the shRNAs were cotransfected with ratTRPV1eGFP-expressing vector pZS5 into ND C cells, and green fluorescence was measured 24 h later by flow cytometry. The relative expression values in relation to an empty vector not expressing shRNA at all are shown in Table 1. Construct pshRNA*a* which was designed based on the previously tested VsiR1 siRNA almost completely inhibited the expression of ratTRPV1eGFP, with only 2 ± 1% remaining expression. A large degree of inhibition was also observed in the case of pshRNA*c* and pshRNA*e*, the remaining expression levels being 11 ± 2 and 8 ± 3%, respectively. In addition, pshRNA*f* moderately decreased the expression of ratTRPV1eGFP as well

Table 1 Structure and efficiency of shRNA constructs. Inhibition of ratTRPV1eGFP expression by different shRNA constructs in rat dorsal root ganglion-derived ND C cells

Name	Target site (start:length)	shRNA sequence (sense-loop-antisense)	TRPV1eGFP expression (mean±SD)
pshRNA*a*	1373:21	5′GCGCAUCUUCUACUUCAACUU^{C}G UUCGCGUAGAAGAUGAAGUUGAA$_{A}$A	2±1***
pshRNA*c*	1726:21	5′GGACCAACAUGCUCUACUAUA^{G}A UUCCUGGUUGUACGAGAUGAUAU$_{A}$G	11±2***
pshRNA*d*	1946:20	5′GCCAGGUAACUCUUACAACA^{C}G UUCGGUCCAUUGAGAAUGUUGU$_{A}$A	100±5
pshRNA*e*	1000:21	5′GCAUGUACAACGAGAUCUUGA^{A}A UUCGUACAUGUUGCUCUAGAACU$_{G}$C	8±3***
pshRNA*f*	2471:21	5′GGAUGCAAGCACUCGAGAUA^{A}A UUCCUACGUUCGUGAGCUCUAU$_{G}$C	43±6***
pshRNA*g*	802:20	5′GGAGGCCUGGCUUCUACUUU^{A}A UUCCUCCGGACCGAAGAUGAAA$_{G}$C	98±5
pshRNA*h*	scrambled control	5′GCACGAUCAUCGUCUACAAUA^{G}A UUCGUGCUAGUAGCAGAUGUUAU$_{A}$G	79±4***
pshRNA*b*	opposite loop	5′GAAGUUGAAGUAGAAGAUGCGC^{C}G UUCUUCAACUUCAUCUUCUACGCG$_{A}$A	37±9***
pshRNA*i*	mismatch	5′GCGCA**a**CUUCUACUUCAACUU^{C}G UUCGCGU**u**GAAGAUGAAGUUGAA$_{A}$A	9±1***
pshRNA*j*	mismatch	5′GCGC**ua**CUUCUACUUCAACUU^{C}G UUCGCG**au**GAAGAUGAAGUUGAA$_{A}$A	40±3***
pshRNA*k*	mismatch	5′GCGCA**a**CUUCUA**g**UUCAACUU^{C}G UUCGCGU**u**GAAGAU**c**AAGUUGAA$_{A}$A	81±3***
pshRNA*l*	big loop	5′GCGCAUCUUCUACUUCAAC^{UUAG}C UUCGCGUAGAAGAUGAAGUUG$_{GUC}$A	4±2***
pshRNA*m*	deletion	5′GCGCA-CUUCUACUUCAAC^{UUAG}C UUCGCGUAGAAGAUGAAGUUG$_{GUC}$A	15±2***
pshRNA*n*	inverted control	5′GCAACUUCAUCUUCUACGCGUU^{C}G UUCGUUGAAGUAGAAGAUGCGCAA$_{A}$A	101±3

Cells were cotransfected with plasmid expressing the ratTRPV1eGFP fusion protein and vectors with different shRNA constructs and GFP fluorescence was measured by flow cytometry. Expression values are presented relative to a control experiment in which a vector producing no shRNA was used (100%). The expression values presented are means ± SD of three experiments. Besides expression data, the name, TRPV1 mRNA target site and structure of each shRNA construct are shown. shRNA constructs below the black line target the same site as pshRNA*a* but carry various mutations. ***$P < 0.001$ compared with pshRNA*n* control, one-way ANOVA test, Dunnett's post hoc comparison

(43 ± 6%). In contrast, pshRNA*d* and pshRNA*g* did not influence the expression of ratTRPV1eGFP. Interestingly, pshRNA*h* which was designed as a scrambled control shRNA also showed a small (20%) but significant decrease in expression. The reason for this effect is not clear and was not investigated further.

In a second series of experiments we tested the efficiency of seven variants of shRNA*a* (shRNA*b*, shRNA*i*–shRNA*n*). In contrast to pshRNA*a*, which has a sense-loop-antisense structure, pshRNA*b* targets the same site, but has an antisense-loop-sense configuration and an extra G at the beginning to facilitate the expression from the U6 promoter. The pshRNA*b* construct, while decreasing ratTRPV1eGFP expression to 37 ± 9%, performed much less well than the original pshRNA*a* construct. The next three shRNAs (pshRNA*i* to pshRNA*k*) were designed to test the effect of mismatches on the efficiency of inhibition. All of them worked less well than the original pshRNA*a* construct, but the single mismatch variant pshRNA*i* was still very effective (9 ± 1% remaining expression). The tandem mismatch variant pshRNA*j* was moderately effective (40 ± 3%), while the separated double mismatch construct pshRNA*k* inhibited expression only by a small amount (81 ± 3%). A variant pshRNA*l* with a nine-base loop worked equally well in agreement with already published data [12]. A single nucleotide deletion in

the sense strand of pshRNA*m* decreased the efficiency of the construct, but still a large inhibition was observed (15 ± 2%). Finally, the inverted control pshRNA*n*, which mimics the previously tested siRNA VsiR1INV was ineffective, as expected. As shRNA*a* was the most efficient in these in vitro tests, it was used in the following experiments.

Lentiviral transgenesis

The human U6 promoter-shRNA*a* construct was moved from the pshRNA*a* plasmid into the pWPTS lentiviral vector. The obtained lentiviral transfer plasmid also contained the EF-1 promoter-driven GFP marker gene. This vector was used to produce lentiviral particles which were concentrated by ultracentrifugation to about 10^8 TU/ml and were injected into rat and mouse single-cell embryos. From 96 lentivirus-injected rat embryos, 24 newborn animals were obtained, but none of them showed visible GFP expression and none was positive in GFP-specific genomic PCR tests. From 60 injected mouse embryos, 43 newborn mice were obtained, of which 5 gave a positive GFP genomic PCR result and 2 had visible GFP expression. Two of the five GFP-PCR-positive founder animals (nos. 4 and 11) produced transgenic offspring when crossed with wild-type FVB/Nhsd mice in ratios corresponding to the Mendelian laws. The founders and their progeny transgenic mice were viable and largely indistinguishable from non-transgenic littermates.

GFP PCR-positive F1 litters were subjected to a simple capsaicin-induced eye-wiping test to evaluate TRPV1 function in vivo (Fig. 1). In wild type mice and in animals expressing GFP alone [25], the numbers of eye-wiping movements were 14.2 ± 0.89 and 14.95 ± 0.85, respectively. Blepharospasm was seen in all these control animals. In contrast, TRPV1 KO animals did not show blepharospasm and responded with zero or one eye-wiping movement. Most transgenic offspring displayed a phenotype between these two extremes, responding to similar treatment with fewer than five wiping movements. One offspring from each founder (nos. 9 and 19) showed normal sensitivity despite showing GFP fluorescence, probably indicating a nonfunctional shRNA*a* unit in these animals.

Based on these results, animal no. 10 was selected for establishing the transgenic mouse line for further experiments. Integration of the lentiviral vector was determined by LM-PCR and found to be a single copy in chromosome 3 at nucleotide 154300070 right behind the crystalline, zeta (GeneID 12972) gene. After weaning, the offspring of animal no. 10 were tested by genomic PCR, visible GFP fluorescence, and capsaicin sensitivity in the eye-wiping test. Based on these results, the animals were classified as having the functional transgene (tg+) or lacking the transgene (tg−). Littermates tg− were used as control animals in further experiments.

Analysis of TRPV1 expression in TRG neurons of transgenic mice

TaqMan quantitative PCR assays were used to determine TRPV1, TRPV3 and TRPA1 mRNA levels in TRG neurons of tg+ animals. TRPV3 mRNA levels were below the detection limit in both tg+ and tg− animals. Housekeeping genes HPRT1 and β2-microglobulin were used to normalize ΔC_t values in each sample. The normalized ΔC_t values indicating TRPV1 and TRPA1 expressions are shown in Fig. 2a. There was a significant difference in TRPV1 mRNA levels between tg− ($\Delta C_t = 4.4$) and tg+ ($\Delta C_t = 8.1$) animals. This ΔC_t difference of 3.71 indicates that compared to tg− mice the TRPV1 expression was reduced 13-fold, to around 8% in tg+ animals. On the other hand, there was no significant difference in TRPA1 mRNA expression levels

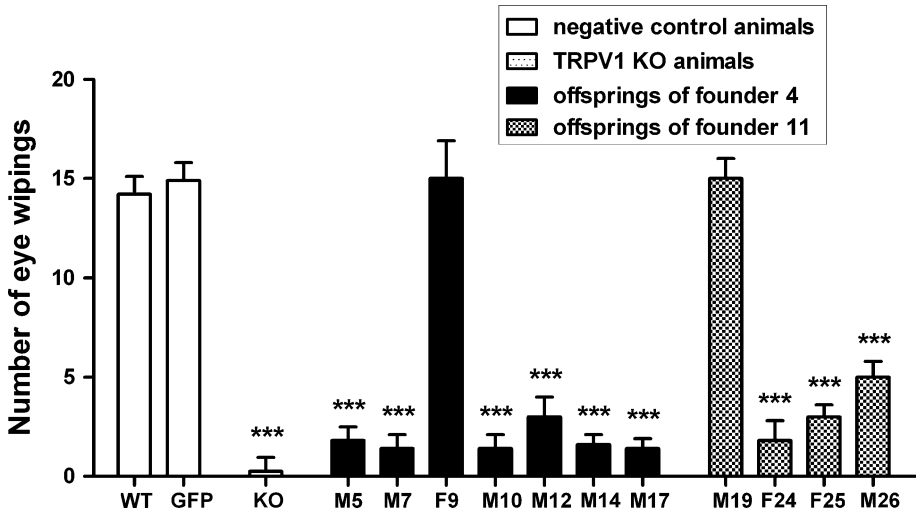

Fig. 1 Chemonociception induced by instillation of capsaicin (10 μg/ml in saline) into the eye of mice carrying the shRNA*a* transgene. The numbers of protective eye wiping movements of tg+ progeny of founder animals nos. 4 and 11 are shown. The bars represent mean ± SD values of five experiments. ***$P < 0.001$ compared with wild type (FVB/Nhsd) mice, one-way ANOVA test, Dunnett's post hoc comparison

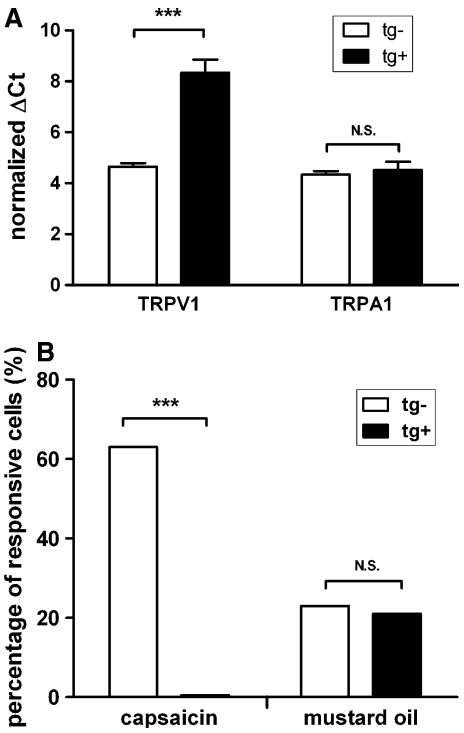

Fig. 2 **a** Measurement of TRPV1 and TRPA1 expression in TRG neurons of tg− and tg+ mice by TaqMan quantitative PCR assay. ΔC_t values normalized to housekeeping genes are shown. Bars represent mean ± SD values of three experiments. $^{***}P < 0.001$, two-way ANOVA test, Bonferroni post hoc comparison. **b** Capsaicin and mustard-oil sensitivity of trigeminal sensory neurons isolated from tg− and tg+ mice, measured by fura-2 microfluorimetry. The percentages of cells responsive ($\Delta R_{F340/F380} > 0.1$) to capsaicin or mustard oil treatment are shown. $^{***}P < 0.001$, Fisher's exact test

between the tg+ and tg− animals. These results indicate that the shRNA*a* transgene significantly and selectively reduced TRPV1 expression in the TRG neurons of tg+ animals.

The presence of functional TRPV1 and TRPA1 receptors in TRG neurons of tg+ mice was also determined by fura-2 microfluorimetry. Of the TRG neurons isolated from tg- animals, 63% (77 out of 123) reacted with Ca^{2+} influx to a 10-s exposure to 330 nM capsaicin (Fig. 2b). The average peak $\Delta R_{F340/F380}$ value was 1.11 ± 0.156. None of the TRG neurons from the tg+ animals (0 out of 134) responded to a similar capsaicin administration with a detectable fluorescent ratio change above the background noise. Assuming a linear relationship between the peak ΔR value and the number of opened TRPV1 receptors and considering the background fluorescent change (ΔR about 0.05), we can estimate that <5% of TRPV1 receptors are active in these cells, compared to TRG neurons from tg− animals. Mustard oil-induced TRPA1 receptor activation was also examined in TRG neurons obtained from tg+ and tg− mice. In tg− mice, 23% of neurons (20 out of 85) reacted to a 30-s exposure to 100 μM mustard oil. In tg+ animals similarly 21% of neurons (17 out of 81) reacted to mustard oil. These results indicate that the anti-TRPV1 shRNA transgene practically eliminated functional TRPV1 receptors, while had no effect on TRPA1 receptor expression.

Capsaicin-induced nocifensive response on the paw in transgenic mice

Intraplantar injection of capsaicin induced a marked nocifensive behaviour consisting of paw licking and flinching in tg− mice compared to solvent-induced behaviour (Fig. 3). The pain score was 84.5 ± 33.2 and 20.2 ± 22.0, respectively. In contrast, the reaction to the same capsaicin treatment was markedly reduced in tg+ animals. In these animals the pain score of capsaicin- and solvent-evoked nocifensive behaviour was 24.4 ± 26.2 and 12.0 ± 12.7, respectively. There was no significant difference between capsaicin and solvent treatment in tg+ mice, indicating that very little residual TRPV1 activity remained in the polymodal sensory neurons of the paw of tg+ animals.

Acute neurogenic oedema formation in the ears of transgenic mice

Oedema formation in the ears of tg+ mice was observed after topical application of capsaicin and mustard oil. Ear thickness increased within 1 h in response to 2.5% capsaicin in tg− mice (Fig. 4a). The swelling was most pronounced 2 h after treatment (42.6 ± 5.9%) and could be seen at all measurement time points. In contrast, oedema did not develop in the ears of tg+ animals after capsaicin treatment, as no significant increase in ear thickness was observed compared to solvent-treated animals.

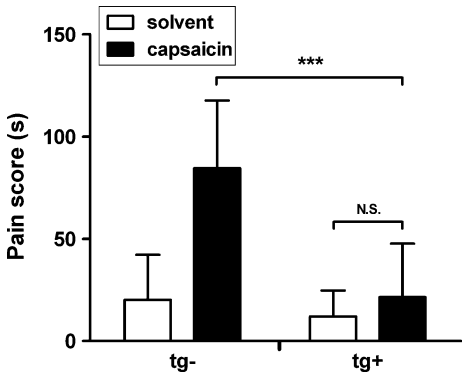

Fig. 3 Capsaicin-induced nocifensive behaviour on the paw of tg− and tg+ mice. Capsaicin (20 μl, 100 μg/ml) was injected into the sole of the left hindpaw and licking and flinches were observed and a composite pain score was calculated. Results are shown as means ± SD of the sum of pain score in seconds ($n = 8$–16 animals per group). In tg+ mice there was no significant difference between responses evoked by capsaicin or by the solvent ($P > 0.05$). $^{***}P < 0.001$, two-way ANOVA test, Bonferroni post hoc comparison

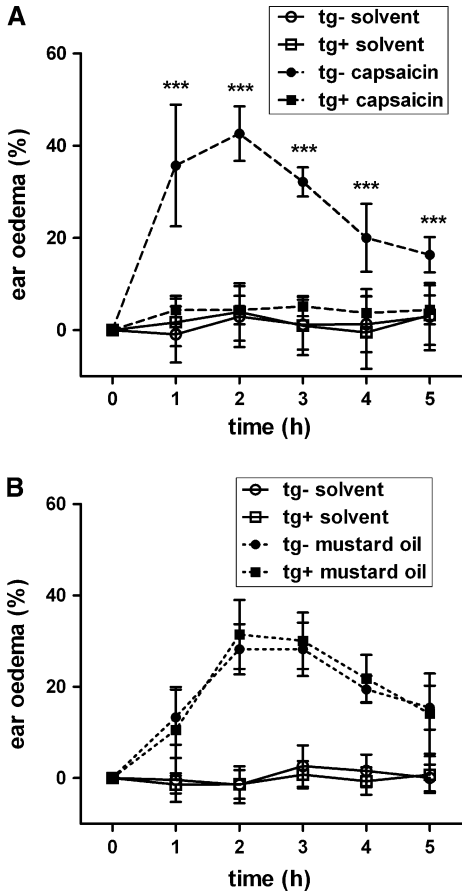

Fig. 4 Neurogenic ear oedema in tg− and tg+ mice. **a** Topical application of 2.5% capsaicin. The solvent of capsaicin (96% ethanol) was used as control. The results are expressed as percent swelling as compared to the initial (0-h) control. The data points represent the mean ± SD values of five to seven experiments. ***$P < 0.001$, capsaicin treatment, tg+ versus tg− animals, two-way ANOVA test, Bonferroni post hoc comparison. **b** Topical application of 1% mustard oil. The solvent of mustard oil (paraffin oil) was used as control. Results are expressed as percent swelling as compared to the initial (0-h) control. The data points represent the mean ± SD values of five to seven experiments. There was no significant difference between tg+ and tg− animals ($P > 0.05$, two-way ANOVA test, Bonferroni post hoc comparison)

In both tg− and tg+ animals, significant ear swelling was detected within 1 h of mustard oil treatment (Fig. 4b). The largest increase in ear thickness was observed 2 h after treatment and the increments were 28.2 ± 5.5 and 31.4 ± 7.5% in tg− and tg+ animals, respectively. Again solvent treatment did not cause a significant change in ear thickness in any of the animals. These results indicate that TRPA1 function was not impaired in tg+ animals.

Environmental temperature selection of transgenic mice

The temperature preference (30 or 35°C) of tg+ mice was investigated in this experiment. The average percentage of time that the four groups of mice (female/male, tg+/tg−) spent at 35°C in the first 10 min was around 30–38% (Fig. 5a). The percentage of time spent at the higher temperature gradually decreased over the next three 10-min intervals. There was no statistically significant difference between tg− and tg+ female mice in temperature preference or rectal temperature (Fig. 5). Tg+ male mice, however, spent slightly more time at 35°C than tg− male controls, but the difference was statistically significant only for the second interval or if the overall percentage of time at 35°C was taken (tg−, 21.1 ± 2.0%; tg+, 29.0 ± 2.7%). Surprisingly, although tg+ male mice spent slightly more time in the warmer environment than the tg− animals, elevation of their rectal temperature after the exposure was significantly lower (Fig. 5b).

Effect of RTX on body temperature and peripheral blood flow in transgenic mice

Rectal temperature of tg+ mice was measured after i.p. injection of RTX or solvent alone. The initial body temperature was 37.2 ± 0.2°C both in tg+ and in tg− animals,

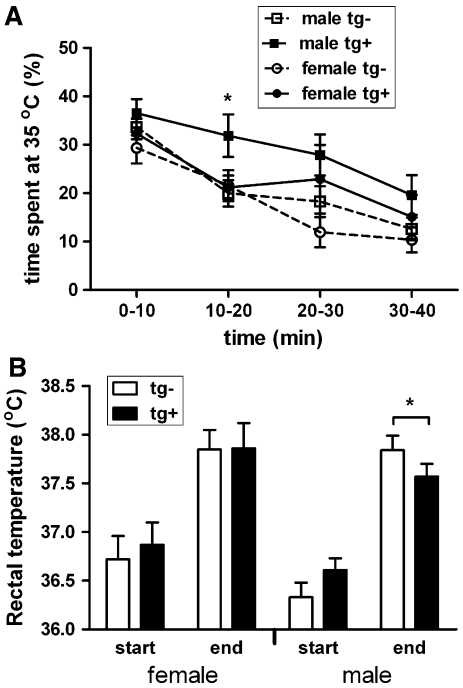

Fig. 5 **a** Temperature preference (30 or 35°C) of male and female tg− and tg+ mice was measured at 10-min intervals. The percentage of time spent at 35°C is shown. The data points represent the mean ± SD of eight mice in each group. *$P < 0.05$, male tg+ versus tg−, two-way ANOVA test, Bonferroni post hoc comparison. **b** Rectal temperatures of the animals measured at the beginning and at the end of the temperature preference experiment. The data points represent the mean ± SD of eight experiments. The only significant difference was observed in male mice at the end of the experiment (*$P < 0.05$, two-way ANOVA test, Bonferroni post hoc comparison)

and after solvent treatment, remained constant at a slightly higher level within 1 h (Fig. 6a).

RTX injection led to a significant decrease in temperature at all measurement time points in tg− mice. In tg+ mice the rectal temperature after injection was in the same range as before injection, but was clearly lower than that in the solvent-treated animals. The lowest body temperature was measured 30 min after injection (the values were 36.2 ± 0.2°C in tg+ mice and 33.3 ± 0.1°C in tg− mice).

The blood flow in the tail of tg−, tg+ and TRPV1 KO mice was monitored every third minute after i.p. injection of RTX. The treatment induced a significant increase in blood flow in the tail of tg− control mice which was most pronounced at 15 min, when the increase was 75.8 ± 25.9% as compared to the initial value (Fig. 6b). Only a modest and, up to 18 min, an identical change was observed in blood flow in the tail of tg+ and TRPV1 KO animals, the maximum increases reaching 35.5 ± 1.97% and 21.9 ± 18.5%, respectively, at 27 min. These results indicate that in the tail of tg+ animals vasodilatation following i.p. injection of RTX was markedly inhibited (Fig. 6B).

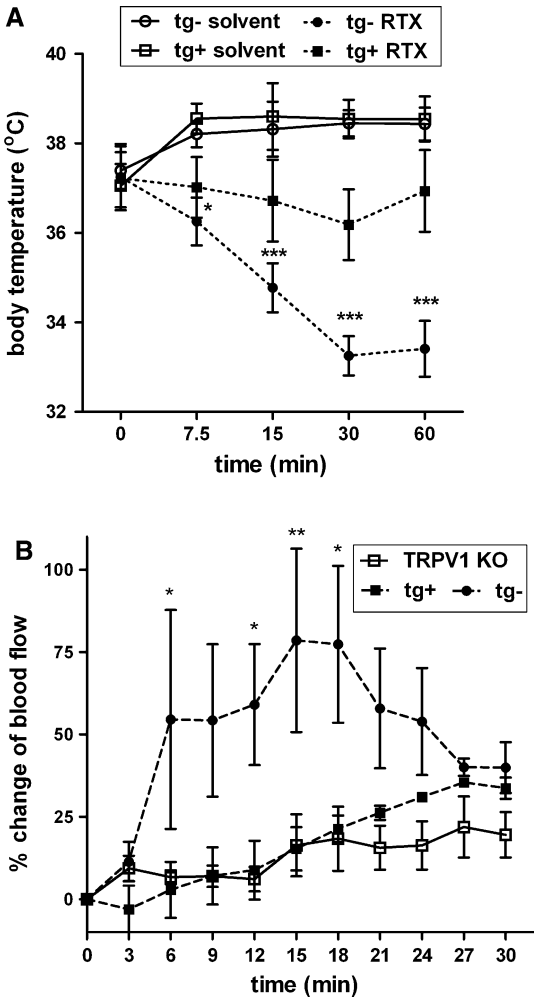

Fig. 6 **a** RTX-induced hypothermia in tg− and tg+ mice. Body temperature was measured before i.p. injection of 20 μg/kg RTX and at 7.5, 15, 30 and 60 min after injection. The solvent of RTX was used as control. The data points represent the mean ± SD of ten mice in each group. $*P < 0.05$, $***P < 0.001$, RTX-treated tg+ versus tg− mice, two-way ANOVA test, Bonferroni post hoc test. **b** RTX-induced change in blood flow in the tail of tg−, tg+ and TRPV1 KO mice. After induction of anaesthesia with urethane, mice received an i.p. injection of 20 μg/kg RTX and blood flow in the tail was monitored by laser doppler imaging every third minute. The percentage change in blood flow is shown compared to the initial values measured at the beginning of the experiment. $*P < 0.05$, $**P < 0.01$, tg+ versus tg− animals, two-way ANOVA test, Bonferroni post hoc test

Discussion

Studies on TRPV1 KO mice underlined the pivotal role of TRPV1 in thermo- and chemonociception [4, 30–32]. Consequently a burst of tremendous effort by the drug industry started [4, 5, 7–9] with the goal of diminishing pain signalling evoked by TRPV1-expressing capsaicin-sensitive nociceptors. The advent of a new generation of analgesics appeared and several compounds reached clinical trials. The major obstacle of hyperthermia appeared, however, in preclinical studies and also in some clinical trials [4, 5, 11]. This side effect resembled the long-term alteration in thermoregulation observed in capsaicin-pretreated rodents [3, 29, 33, 34]. Long-term effects of capsaicin pretreatment, however, is not related to a simple blockade or loss of its receptor, but is due to a massive influx of Ca^{2+} and Na^{+} through this cation channel leading to lasting loss in function of capsaicin-sensitive neurons [3, 35].

Inhibition of the TRPV1 gene by RNA interference is a complementary approach to the TRPV1 gene-deleted mice for testing the loss of function of this important drug target molecule in vivo. Recently published studies involving experiments in TRPV1 knockdown mice have sought further evidence for the importance of TRPV1 cation channels as drug targets for treatment of neuropathic pain [12], and differences were found between TRPV1 KO and knockdown animals in this respect. TRPV1 shRNA mice have also shown strongly diminished nociception following intraplantar injection of capsaicin, enhanced paw withdrawal latencies on hot plates (48°C, 58°C) and diminished tactile hypersensitivity and allodynia in an injury-induced neuropathic pain model [12].

In this study we used RNA interference to inhibit the expression of the potentially important drug target TRPV1 receptor in different experimental systems. Using different siRNAs, we were able to effectively and selectively knockdown TRPV1 expression in transfected cells and isolated rat TRG sensory neurons, in good agreement with previously published data [36]. However, in our hands,

single or multiple intrathecal administration of siRNAs, either by direct injection or through an implanted cannula, with or without different transfection agents, did not elicit a TRPV1 knockdown phenotype in rats as tested by capsaicin- or temperature-induced nocifensive behaviour on the paw. The apparent lack of effect in vivo was most probably due to the inability of VsiR1 to enter DRG neurons in sufficient quantity. An alternative hypothesis is that VsiR1 was able to knock down TRPV1 expression in the body of DRG neurons, but in the time-frame of our experiments this change was not yet reflected at the peripheral nerve termini in the paw, where the presence of TPRV1 function was actually tested.

Therefore, a transgenic mouse line carrying an effective anti-TRPV1 shRNA construct was created by lentiviral transgenesis. The expression level of the TRPV1 gene in the TRG neurons of tg+ animals was only about 8% compared to tg− animals, and tg+ mice showed a diminished sensitivity to capsaicin in eye-wiping and plantar nocifensive tests.

The effect of TRPV1 knockdown in the tg+ animals was investigated in parallel with the closely related TRPA1 receptor. TRPA1 is another chemonociceptive thermosensor cation channel gated by noxious cold and by diverse chemicals including mustard oil, cinnamaldehyde, acrolein and the endogenous ligand of 4-hydroxy-2-nonenal that is produced from membrane phospholipids under oxidative stress [13–16]. TRPA1 is expressed by capsaicin-sensitive neurons [15] and interaction between TRPV1 and TRPA1 in the plasma membrane has been described [17]. Antisense knockdown of TRPA1 alleviates cold hyperalgesia [37] and TRPA1 KO mice also display a behavioural deficit in response to mustard oil and to punctate mechanical stimuli, but not to hot stimuli [15, 38].

While TRPV1 expression was greatly diminished in our tg+ mice, the TRPA1 mRNA level was unchanged. Direct functional testing of the TRPV1 and TRPA1 receptors on isolated TRG neurons also confirmed the selective knockdown of TRPV1 expression. Furthermore, we showed in these animals that the profound inhibition of various responses in nociceptive and inflammatory tests in vivo was selective and was not accompanied by changes in the action of the TRPA1 agonist mustard oil. This selective action is in line with that reported for TRPV1 gene deleted mice [39]. These results indicate that neither the complete loss nor a profound reduction in TRPV1 cation channels resulted in compensatory enhanced expression or trafficking of TRPA1 channels to the plasma membrane of nociceptors.

The thermoregulatory behaviour of TRPV1 tg+ mice has been investigated in several models and, owing to the hyperthermia induced by some TRPV1 antagonist drug candidates, a putative tonic, predominant function of TRPV1 receptors in body temperature regulation has been suggested [6, 10]. Results obtained with our tg+ mice do not support this hypothesis.

1. As in TRPV1 KO mice [40], no hyperthermia was observed in tg+ mice at room temperature or after heat exposure. Furthermore, thermoregulatory behaviour of female tg+ mice, i.e., thermopreference for an ambient temperature of 30°C over a warmer environment (35°C) was indistinguishable from that of their tg− controls. Under similar experimental conditions rats pretreated from 1 week to 3 months before with 150 mg/kg capsaicin showed pronounced hyperthermia in a warm ambient temperature and did not avoid the warmer environment, indicating a loss of heat protective thermoregulatory behaviour [3, 29]. On the basis of several previous experiments it has been concluded [3, 29, 32–34] that capsaicin excites and induces a long-term impairment in central and peripheral warmth sensors leaving the thermoregulation in response to cold—including thermopreference—unimpaired.
2. Systemic injection of the TRPV1 agonist RTX elicits, as does capsaicin, a heat loss response with profound hypothermia accompanied by diminished oxygen consumption in cold environments [35, 41]. Here we showed that in TRPV1 knockdown mice the fall in colonic temperature and tail vasodilatation induced by i.p. injection of RTX was markedly inhibited. It is worth mentioning that abdominal thermosensors desensitized by RTX have been implicated in the tonic regulation of body temperature [11]. In the tg+ animals the ineffectiveness of RTX indicates a robust loss of TRPV1 channels including in the abdominal space. This state, however, was not accompanied by an impaired regulation in response to overheating of the body and these animals showed no sign of hyperthermia or impaired thermoregulatory behaviour in response to overheating of the body.

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