

Resiniferatoxin Mediated Ablation of TRPV1+ Neurons Removes TRPA1 as Well

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ABSTRACT: Objectives: Resiniferatoxin, the most potent agonist of inflammatory pain/vanilloid receptor/cation channel (TRPV1) can be used for neuron subtype specific ablation of pain generating cells at the level of the peripheral nervous system by Ca²⁺-excitotoxicity. Molecular neurosurgery is an emerging technology either to alleviate severe pain in cancer or treat/prevent different local neuropathies. Our aim was determining sensory modalities that may be lost after resiniferatoxin treatment. **Methods:** Newborn or adult mice were treated with resiniferatoxin, then changes in chemical and heat sensitivity were correlated with alterations of the cell composition of sensory ganglions. **Results:** Only mice treated at adult age became less sensitive to heat stimuli, while both treatment groups lost sensitivity to specific vanilloid agonists of TRPV1 and, interestingly, to allyl-isothiocyanate, a selective agonist of TRPA1. Our *in vivo* and *post mortem* analytical results confirmed that TRPV1 and TRPA1 function together and resiniferatoxin-mediated neurosurgery removes both sensor molecules. **Discussion:** In adult mice resiniferatoxin causes: i) desensitization to heat and ii) sensitization to cold. Cold hyperalgesia, an imbalance in thermosensation, might be conferred by a prominent cold receptor that is expressed in surviving resiniferatoxin-resistant sensory neurons and compensates for pain signals lost with TRPA1 and TRPV1 double positive cells in the peripheral nervous system.

RÉSUMÉ: L'ablation de neurones TRPV1+ par la résiniférotaxine élimine aussi les neurones TRPA1. Objectifs : La résiniférotaxine, l'agoniste le plus puissant de la douleur inflammatoire/du récepteur vanilloïde/du canal cationique (TRPV1), peut être utilisée pour l'ablation spécifique par excitotoxicité Ca²⁺, d'un sous-type de neurones faisant partie des cellules génératrices de douleur au niveau du système nerveux périphérique. La neurochirurgie moléculaire est une technologie émergente, pour soulager la douleur cancéreuse sévère ou pour traiter ou prévenir différentes neuropathies locales. Notre but était de déterminer les pertes sensibles suite au traitement par la résiniférotaxine. **Méthodes :** Des souris ont été traitées par la résiniférotaxine à la période néonatale ou adulte et les changements de la sensibilité chimique et calorique ont été corrélés aux altérations de la composition cellulaire des ganglions sensitifs. **Résultats :** Seules les souris traitées à l'âge adulte sont devenues moins sensibles aux stimuli caloriques alors que les deux groupes traités ont perdu la sensibilité aux agonistes vanilloïdes spécifiques de TRPV1 et, ce qui est fort intéressant, à l'allyl-isothiocyanate, un agoniste sélectif de TRPA1. Nos résultats d'analyses *in vivo* et *post mortem* ont confirmé que TRPV1 et TRPA1 fonctionnent conjointement et que la neurochirurgie au moyen de la résiniférotaxine élimine les deux molécules détectrices. **Discussion :** Chez les souris adultes la résiniférotaxine cause : 1) une désensibilisation à la chaleur et 2) une sensibilisation au froid. Une hyperalgésie au froid, qui est un déséquilibre dans la thermosensibilité, pourrait être médiée par un récepteur important au froid qui est exprimé dans les neurones sensitifs résistants à la résiniférotaxine survivants et qui compense pour les signaux douloureux perdus des cellules doublement positives pour TRPA1 et TRPV1 dans le système nerveux périphérique.

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Mammalian temperature sensors of the transient receptor potential (TRP) family are tetrameric 6-transmembrane cation channels gated by heat, cold, as well as either endogenous or exogenous agonists^{1,2}. The vanilloid receptor (VR1/TRPV1) is a non-selective ligand-gated Na⁺/Ca²⁺-channel³⁻⁶. TRPV1 is selectively activated by heat above 42°C, pH < 6.3⁷, endogenous lipid mediators such as anandamide and oleoylethanolamide^{4,8,9}, intracellular signaling molecules^{10,11} and plant toxins, including capsaicin (CAPS) and resiniferatoxin (RTX).

TRPA1 has recently been suggested to serve as receptor of noxious cold temperature¹², and it transduces pain induced by

isothiocyanate pharmacophore of the pungent ingredients of mustard oil¹¹ and garlic¹³. However, various observations^{11,14,15}

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now call into question that TRPA1 is a bona fide cold sensor^{12,16,17}, that prompted functional investigations, such as ours described here.

Resiniferatoxin, the most potent natural diterpene agonist of TRPV1, is present in latexes of various *Euphorbia* species, such as *E. resinifera*, *E. poissoni* and *E. unispina*¹⁸. Bioassays rank RTX as a 3-4 orders of magnitude more potent agonist of TRPV1 than CAPS, the active ingredient of chili pepper¹⁹. One of us proposed exploitation of the Ca²⁺-excitotoxic potential of RTX for pain management, using it as an ablative agent specific for TRPV1 expressing cells. *In vivo* RTX treatment eliminates TRPV1+ nociceptors in the peripheral nervous system while bystander neurons and their functions are spared^{4,20}. Indeed, determination of specificity *in vitro* and *in vivo* in different animal models and human dorsal root ganglion cultures validated RTX as a “nano-scalpel” capable of “molecular neurosurgery” of specific inflammatory pain cells without affecting the other functionalities of the peripheral and central nervous system^{4,5,21}.

Although vanilloids had been used previously *in vivo*²², we started to employ RTX in various routes of applications, such as systemic, epidural, intra-nerve and intra-ganglionic injections in adult^{5,23,24} as well as newborn rats (Olah, Karai, Iadarola, unpublished observation). Systematic studies with a therapeutic mechanisms in mind shifted the desensitization paradigm and identification of the vanilloid receptor have helped interpretation of long-term loss of response to thermal and stimuli evoked by noxious chemicals. Our selective ablation pain management technology by usurpation of nociceptive neurons for elimination of hyperalgesia and neurogenic inflammation is distinct from the desensitization theory proposed previously²⁵. Resiniferatoxin is now a validated drug lead to remove TRPV1+ inflammatory pain generator cells. We demonstrated that RTX preserves bystander sensory cells and motoneurons in rats, dogs, monkeys and human embryonic dorsal root ganglion (DRG) cultures^{4,5,26,27}.

Resiniferatoxin, although extremely specific to its target, TRPV1, can delete sensation modalities conferred by bystander sensor molecules, including other TRP channels co-expressed in the same neuron. Therefore, the specific aim of this study was to determine potential loss/gain of sensations that RTX-mediated molecular surgery may cause. Furthermore, previous to discovery of TRPV1 and TRPA1, it was routine to evaluate the pharmacological effects of vanilloids by mustard oil test. The discovery that allyl isothiocyanate is a cognate ligand of TRPA1 and not TRPV1 implies that this test only can work due to co-expression of these TRP channels. Since both TRPV1 and TRPA1 can evoke sterile inflammation, visualized by Evans Blue extravasation^{5,27}, their functional loss induced by potent vanilloids could not be easily distinguished²⁸. Story et al proposed that TRPA1 might be another cation channel co-expressed with TRPV1¹², but no systematic study has been carried out to verify this notion and reveal the functional consequences of co-deletion. According to our data in this paper, allyl isothiocyanate, the pungent ingredient in mustard oil, selectively targets TRPA1 only, and has no agonist side effect on TRPV1. In addition we also described the ununexpected effects of RTX treatment on cold sensitivity of the treated animals.

MATERIAL AND METHODS

Reagents

Resiniferatoxin (LC Laboratories, Woburn, MA) and capsaicin (Sigma, St. Louis, MO) were dissolved in ethanol and further diluted in physiological saline solution. Allyl isothiocyanate (AITC) (Sigma, St. Louis, MO) was diluted in physiological salt solution and dispersed by ultrasonication before testing.

⁴⁵Ca²⁺ uptake assay

One day before the assay, HaCaT cells permanently expressing rat TRPV1⁶ were seeded in 96-well flat bottom plates (Orange Scientific, Braine-l'Alleud, Belgium) at a density of 20,000 cells/well. Assays were performed using a BioMek 1000 robotized liquid handler (Beckman Instruments, Fullerton, CA). The plates were washed three times with assay medium (Ca²⁺- and Mg²⁺-free Hanks' balanced salt solution supplemented with 0.8 mM MgCl₂ and with 25 mM TRIS-HCl, pH=7.4). The serial dilutions of reagents containing ⁴⁵Ca²⁺ were prepared using the robot. The ⁴⁵Ca²⁺-uptake assay was performed for ten minutes (m) at 24°C using 1.33 µCi/ml of ⁴⁵Ca²⁺ (Amersham Biosciences, Buckinghamshire, UK) in 100 µl final volume/well. To stop ⁴⁵Ca²⁺ uptake and remove the free isotope, cells were washed three additional times and then lysed in 80 µl/well lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 5 mM EDTA) for 30 minutes. Seventy µl aliquots of the solubilized cell extracts were mixed with 100 µl aliquots of Optiphase Supermix scintillation cocktail (Perkin Elmer, Wellesley, MA) and counted in a Perkin Elmer liquid scintillation counter.

RTX treatment

The studies were carried out on male CD/1 mice. One mg RTX was dissolved in 500 µl 96% ethanol and diluted in physiological saline and injected s.c into the scruff of the neck in a volume of about 100 µl. The RTX was applied at the dosage of 50 µg/kg body weight under light ether anesthesia to avoid unnecessary pain. Control mice received vehicle. In the case of adult treatment, two to three months old adult mice weighing about 30 g at the start of the experiments were tested ten days after RTX treatment. Mice treated on the second day of life were tested at two months of age. In the case of chronic RTX treatment RTX was added at 20 µg/kg body weight daily for three days.

All animal experimental protocols were approved by our institutional review committee, then the responsible governmental agency.

Cold tail-flick test

Animals were held over ice cold water (~5°C) and their tails submerged approximately half length into the bath. The time from immersion to tail removal or flicking was measured.

Cold plate test

A microplate thermostate set at 5°C was used to generate a cold surface. Tested mice were placed on the plate, then time to

first response of forelimbs withdrawal and shivering was recorded.

Hot plate test

The animals were individually exposed to the hot plate maintained at 53°C. The time for forepaw licking was taken as reaction time.

Water consumption

The daily water consumption of individually housed mice was measured. Each bottle contained tap water with allyl isothiocyanate at a concentration of 0.1 mM.

Eye wipe test

The eye wipe test was performed in anaesthetized animals. The 100 µM capsaicin solution and the 1 mM AITC solution was dropped into the eye and the number of defensive wiping movements was counted.

Production of a monoclonal antibody recognizing an extracellular loop of intact TRPV1

BALB/c 3T3 cells expressing the rat TRPV1 driven by the metallothionein promoter of the pMET plasmid⁴ were induced with 75 µM Zn²⁺ overnight. The induction resulted in a twofold elevation of vanilloid-induced Ca-uptake measured in a robotized ⁴⁵Ca²⁺ uptake assay. The TRPV1 overexpressing cells were homogenized, the debris was removed by centrifuging, then the homogenization and centrifuging steps were repeated once more. The cell membrane fraction was recovered by ultracentrifuging the supernatant. After determination of the protein content, the aliquotted cell membrane suspension was stored at -85°C. BALB/c mice were injected with 100 µl membrane suspension containing 300 µg total protein mixed with 100 µl complete Freund's adjuvant, then the immunization was repeated three times using incomplete Freund's adjuvant. A last boost was given in the form of an ip injection of 100 µl membrane homogenate and three days later spleen cells of the immunized mice were fused with SP/2 myeloma cells. A hybridoma clone producing antibody to an extracellular loop of the intact TRPV1 molecule was selected by testing pooled, then individual hybridoma supernatants on intact TRPV1-expressing 3T3 cells by flow cytometry using a FITC labeled anti-mouse secondary antibody. The resulting hybridoma clone was verified to produce a monoclonal antibody recognizing the intact rat TRPV1 expressed by the transformed 3T3 cell line, as well as the naturally expressed molecule in rat or mouse trigeminal ganglion or the prostate. The monoclonal antibody was directly labeled with green fluorescence using Alexa Fluor 488 Monoclonal Antibody Labeling Kit (Invitrogen, Carlsbad, CA.).

Protein Extraction and Western Blot

Tissue samples were homogenized in modified RIPA buffer (50 mM Tris-HCl, 140 mM NaCl, 5 mM EDTA, 1% TritonX-100, Protease Inhibitor Cocktail - Roche, Mannheim, Germany) and incubated on ice for 15 minutes to let lysis proceed. All samples were precleared by centrifugation (15' 12000 g at 4°C) before determining protein concentrations using the BCA

method (Sigma, St. Louis, MO). PAGE was done as described in the Protein Electrophoresis technical manual of Amersham Biosciences (Buckinghamshire, UK). Protein samples were separated on 8% polyacrylamide gels, then transferred to Millipore Immobilon PVDF membrane using Tris-Glycine transfer buffer (0.192 M Glycine, 25 mM Tris, 20% MetOH). Transfer was followed by blocking of the membrane (30' at RT in 5% dry milk TBS-T), incubation with primary antibody (overnight at 4°C, in 0.5% BSA TBS-T), secondary antibody (2h at RT, in 1% dry milk TBS-T), results were revealed using the ECL method (SuperSignal West Chemiluminescent Substrate (Pierce Biotechnology Inc. Rockford, IL), Hyperfilm ECL - Amersham Biosciences, Buckinghamshire, UK). TBS: 50 mM Tris, 140 mM NaCl, pH 7.6; TBS-T: TBS with 0.5% Tween-20. Antibodies used and their dilutions: TRPV1 - 1:1000, ABR Bioreagents, PA1-747; TRPA-1 - 1:1000, Transgenic Inc. Kumamoto, Japan. #KM120; TRPM8 - 1:1000, Alomone Labs, Jerusalem, Israel #ACC-049, β-actin - 1:1000 (Sigma, St. Louis, MO #A5060), anti-mouse and anti-rabbit HRP - 1:10000 (Sigma, St. Louis, MO #A0168 and #A6154, respectively).

Immunohistochemistry

Over-anesthetized mice were perfused intracardially with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB, pH 7.4). Trigeminal ganglions were dissected out, and cryoprotected in 10% glycerol/30% sucrose) overnight at 4°C. Trigeminal ganglions were embedded in Tissue Tek OCT (Sakura Finetek, Torrance, CA) and frozen in dry ice. Frozen sections were cut on an Shandon Cryotome Fe (Thermo Fisher Middletown, VA) sliding microtome and collected in Superfrost slides kept at -20°C. Sections were pre-incubated in blocking solutions (5% goat serum, 0,1% Triton-X in PBS pH=7.4). After that the sections were incubated in primary antibody dilutions at 4°C for 24 hours then secondary antibody. Antibodies used and their dilutions: anti-TRPV1 1:50 (our monoclonal antibody directly labeled with Alexa Fluor 488), anti TRPA1 1:50 (rabbit polyclonal, Transgenic Inc. Kumamoto, Japan. #KM120) anti TRPM8 1:50 (rabbit polyclonal, Alomone Labs, Jerusalem, Israel #ACC-049) anti rabbit IgG - TRITC 1:400 (Sigma, St. Louis, MO) . Sections were mounted on standard glass slides and coverslipped with Dako Faramount Aqueous Mounting Medium (Dako, Glostrup, Denmark).

RESULTS

To determine the potential effect of allyl isothiocyanate *in vitro* we used a cell-based functional assay using a permanent cell line expressing TRPV1 but not TRPA1 ectopically. Resiniferatoxin and CAPS evoked ⁴⁵Ca-influx with an EC₅₀ of 2.6 nM and 78 nM, respectively (Figure 1). Contrary to the report of Ohta et al²⁹, however, we found that allyl isothiocyanate has no effect on TRPV1-expressing cells, since it did not evoke calcium influx applied at concentrations ranging from 125 nM to 2.5 mM. Lack of AITC effect on TRPV1 and selectivity of this ligand for TRPA1, has recently been shown by others too^{11,16}.

To dissect potential side effect(s) of molecular neurosurgery *in vivo*, mice were treated with RTX either at birth or at adult age then changes in behavioral, cellular, and molecular biomarkers

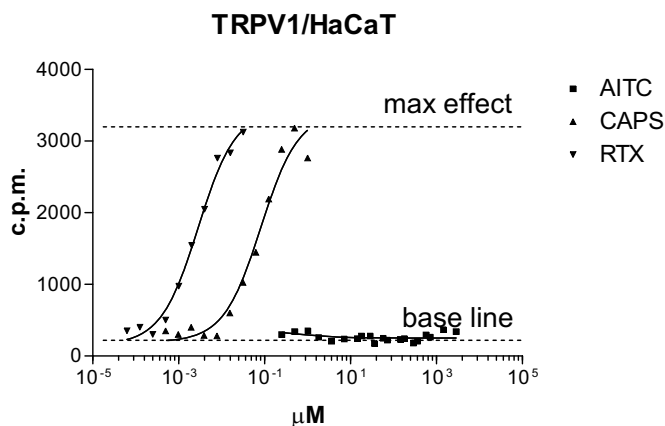


Figure 1: $^{45}\text{Ca}^{2+}$ -influx induced by resiniferatoxin (RTX), capsaicin (CAPS) and allyl isothiocyanate (AITC). HaCaT keratinocytes stably expressing rat TRPV1 were seeded on 96-well plates, then ten-minute agonist induced $^{45}\text{Ca}^{2+}$ -influx assays were performed. Cell-bound radioactivity was measured in a liquid scintillation counter. Only RTX and CAPS induced a Ca-influx, while AITC was totally ineffective. The experiment was repeated two additional times with similar results.

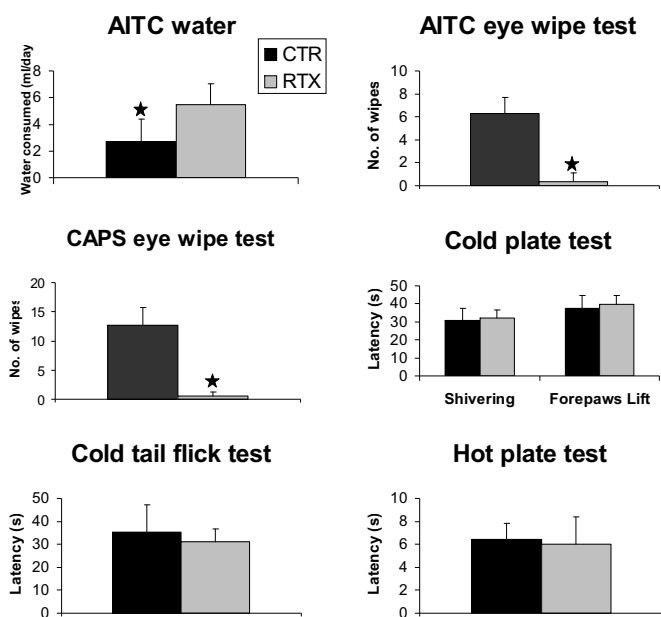


Figure 2: Changes in the chemical- and heat sensitivity of mice treated with resiniferatoxin at 50 $\mu\text{g}/\text{kg}$ body weight on the second day after birth. Mice were tested at second month of their life. Comparisons were made using age-matched control animals treated with vehicle only. Bars represent means of measured values plus standard deviations ($n=7$). The experiments were repeated two additional times with similar results. A.) Oral aversion to allyl isothiocyanate (AITC; mustard oil). Treated mice consumed significantly more allyl isothiocyanate containing drinking water than untreated mice in a 24 h test. $*P < 0.05$ vs. control B-C.) Eye-wipe response to corneal application of 100 μM capsaicin and 10 mM allyl isothiocyanate. Neonatal RTX administration significantly reduced the eye-wipe response to corneal application of capsaicin and also to allyl isothiocyanate. $*P < 0.05$ vs. control. D.) Cold plate test. Paw withdrawal latency (left) or time to first shiver (right) was measured following placement of mice on a plate maintained at 5°C. Responses were not significantly different. E.) Response latency in the tail immersion into 4°C cold water. No significant differences were seen between control and RTX treated animals. F) Licking latencies in the hot plate assay at 53°C. The responses were not significantly different.

(i.e. chemo-, and heat sensitivity, tissue specific expression/distribution of TRPV1 and TRPA1) were systematically compared.

As expected from previous studies⁵, mice treated with RTX two days post-natal and tested at the age of two months completely lost the sensitivity to vanilloids, since capsaicin-induced eye wipe, a behavioral marker of TRPV1 conferred pain signal, was completely abrogated. We have chosen an RTX dose at which no mortality occurred. In our experimental conditions applied in preliminary experiments, RTX-caused mortality was detected from 75 $\mu\text{g}/\text{kg}$ body weight, while the maximal dose used in the paper was 50 $\mu\text{g}/\text{kg}$ body weight. We also noted that mice were unresponsive in AITC-induced eye wipe tests as well, as the AITC-induced eye wipes were completely abrogated. In accordance with that fact, these animals consumed AITC containing drinking water in a significantly higher volume overnight than untreated mice (2.7 ± 1.7 ml vs 5.5 ± 1.5 ml), another signature of specific loss of this sensory modality. Interestingly, they showed no change in temperature sensitivity neither in cold plate, nor in hot plate assay (Figure 2).

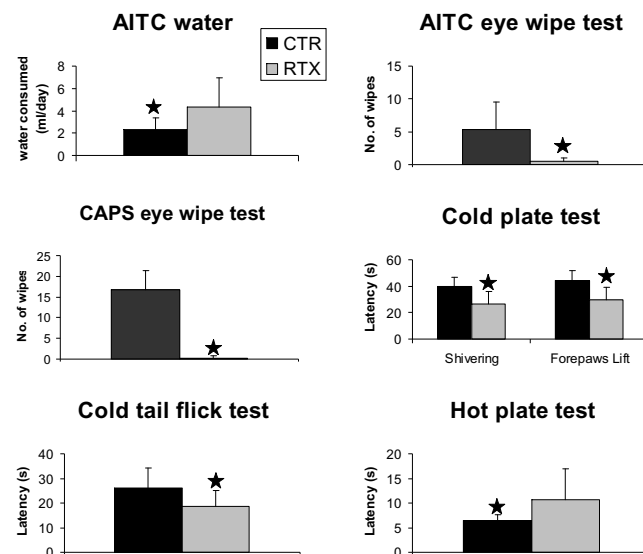


Figure 3: Changes in the chemical- and heat sensitivity of mice treated with 50 $\mu\text{g}/\text{kg}$ resiniferatoxin at adult age. Mice were tested two weeks later. Comparisons were made using age matched control animals treated with vehicle only. Bars represent means plus standard deviations ($n=7$). The experiments were repeated two additional times with similar results. A.) Oral aversion to allyl isothiocyanate (AITC). Treated mice consumed significantly more allyl isothiocyanate containing drinking water than untreated mice in 24 h tests. $*P < 0.05$ vs. control B-C.) Eye-wipe response to corneal application of 100 μM capsaicin and 10 mM allyl isothiocyanate. RTX administration significantly reduced the eye-wipe response to corneal application of capsaicin and allyl isothiocyanate. $*P < 0.05$ vs. control. D.) Cold plate test. Paw withdrawal latency (left) or time to first shiver (right) was measured following placement of mice on a plate maintained at 5°C. Treated mice showed more sensitivity to cold. $*P < 0.0001$ vs. control. E.) Response latency in the tail immersion into 4°C cold water. Significant differences were seen between control and RTX treated animals. $*P < 0.05$ vs. control. F) Licking latencies in the hot plate assay at 53°C Responses were significantly different. RTX treated animals had longer reaction times. $*p < 0.05$ vs. control.

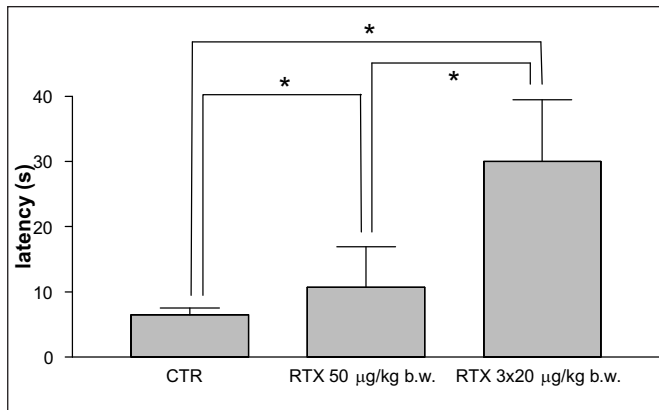


Figure 4: Comparison of acute and chronic RTX treatment. Chronic RTX (3 x 20 µg/kg body weight) is more effective than acute treatment (1 x 50 µg/kg body weight) as measured in hot plate assay. Bars represent means plus standard deviations. Statistics were made by LSD post hoc test following one-way ANOVA, * $P < 0.05$ vs. control.

Mice treated with one dose of RTX at adult age become similarly unresponsive to chemical stimuli, i.e., they did not respond either to CAPS or AITC in the eye wipe tests, and they were also able to consume AITC containing drinking water in high quantities. In addition, the animals that underwent RTX-mediated neurosurgery at adult age exhibited changes of heat sensitivity. As it was expected from previous observations⁵, loss of TRPV1-expressing neurons resulted in decreased heat sensitivity. Surprisingly, however, the RTX-treated animals also become more sensitive to cold, showing a marked reduction of reaction time (around 30%) on cold plate as measured in cold plate tail flick and cold plate shivering tests (Figure 3).

In the case of chronic RTX treatment (3 x 20 µg/kg body weight rather than 1 x 50 µg/kg body weight) mice become similarly unresponsive to CAPS. However these mice lost heat sensitivity to a higher extent compared to mice with acute treatment, so chronic treatment seemed to be more effective than a single treatment in this respect (Figure 4).

Immunohistological staining experiments performed on adult mice treated with 1 x 50 µg/kg body weight RTX confirmed that TRPV1 is indeed lost after the treatment. The staining was performed with a new monoclonal antibody developed in our laboratory. Our hybridoma cell line was established from a normal BALB/c mouse immunized previously with a crude plasma membrane fraction isolated from a BALB/c 3T3 cell line permanently expressing the rat TRPV1. By employing indirect fluorescent labeling technologies, the monoclonal antibody was verified to react with an extracellular loop of both rat and mouse TRPV1. Parallel to TRPV1, TRPA1 also disappeared from the trigeminal ganglions of the RTX treated animals (Figure 5), but the TRPM8-positive cells remained readily detectable. TRPA1 and TRPM8 were stained with commercially available antibodies.

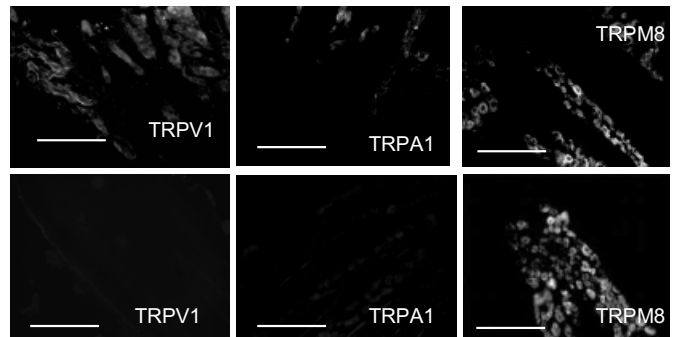


Figure 5: Microscopic images showing the effect of RTX on TRPV1-, TRPA1- and TRPM8-positive trigeminal ganglion neurons. Unlike TRPM8, both TRPV1 and TRPA1 immunoreactivity was lost in the treated animals. Bar represents: 0.2 mm.

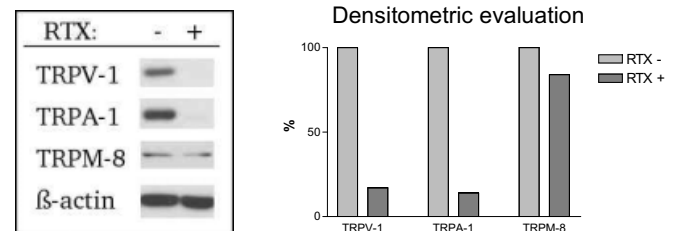


Figure 6: Western blot analysis. Western blot shows the level of TRPV1, TRPA1 and TRPM8 proteins in the lysates of pooled trigeminal ganglions obtained from control and RTX treated mice. Similarly to TRPV1, TRPA1 also disappears from the trigeminal ganglions of treated animals, while TRPM8 does not change. Sigma beta-actin was used in the Western blot as an internal control.

The correlation between TRPV1 and TRPA1 levels in the dorsal root ganglion and trigeminal ganglion with the changes in chemical and heat sensitivity in mice treated at adult age was tested in Western blot experiments. In this case, TRPV1, TRPA1 and TRPM8 were immunostained with commercially available polyclonal antibodies. Our Western blot experiments confirmed that, parallel to TRPV1, TRPA1 also disappeared from the trigeminal ganglions of the RTX treated animals; the decrease was 83% in the case of TRPV1, while 86% in the case of TRPA1, while the amount of TRPM8 barely, non-significantly changed (Figure 6).

DISCUSSION

Recently one of us proposed a novel use of RTX as pain killer drug⁵. Being at least a thousand fold more potent analogue of CAPS, RTX applied either intrathecally or intraganglionically can execute a highly specific TRPV1 targeted molecular

neurosurgery to eliminate cellular generator of inflammatory as well as cancer pain^{4,5}. According to literature data, 24 hours after CAPS treatment a population of primary sensory neurons of newborn³⁰ and adult rats³¹ show TUNEL staining, indicating that neurons are killed by apoptosis. Likewise CAPS, RTX, due to 3-4 magnitudes more potent agonist activity on TRPV1 can induce Ca²⁺-cytotoxicity and eventually ablates these neurons by rapid necrosis, followed by removal of the cell debris^{4,5,21,24}.

Resiniferatoxin has been shown to eliminate inflammatory pain by specific Ca²⁺-cytotoxicity in newborn²⁵ as well as in adult rats⁵, and it had an anti-nociceptive effect in adult mice³², monkeys²⁷ and dogs²⁶. A number of *in vitro* and *vivo* experiment has already validated safety and efficacy of molecular neurosurgery management of arthritic and cancer pain, even in large animals such as dogs and monkeys, promoting human trials and registration of RTX as an alternative drug in pain inflammation and various neuropathy indications^{26,27}. Contrary to TRPV1 antagonists, also in development as painkillers, intraganglionic or intrathecal RTX treatment would provide a permanent solution to incurable chronic pain.

Resiniferatoxin treatment manifests in mice, as in other experimental animals, in drastic loss of sensitivity to noxious chemicals and coincident drop out of TRPV1+ nociceptive neuronal bodies in sensory ganglia. Results in mice not only give another reassuring validation of the molecular neurosurgery technology^{4,5,24}, but prompted us to develop the first monoclonal antibody for better diagnosis of cell type specific drop out⁴. Convincing decrease in number of small and medium size TRPV1+ neuron counts has been determined in RTX treated mice also cross-validated specificity of our novel monoclonal antibody. Moreover, we found that RTX-treated mice also showed loss of TRPA1-dependent chemical sensitivity, confirming previous observations that TRPV1 indeed co-expresses with TRPA1 in a number of nociceptive neurons in the peripheral nervous system. The eventual effect of RTX on TRPA1 is excluded by previous publications. For example, cultured DRG neurons of TRPV1-/- mice did not show stimulus-evoked rise in intracellular free Ca in response to resiniferatoxin, proving that none of the remaining TRP receptors is vanilloid-sensitive³³. In addition, iodo-resiniferatoxin did not interfere with DRG neuron activation in response to TRPA1 agonists³⁴, confirming that TRPA1 does not bind vanilloids ligands. We can conclude that RTX-mediated molecular neurosurgery eliminates most or all TRPA1-containing neurons as well via TRPV1 activation, as it is indicated by and the almost complete loss specific immunoreactivity in sensory nociceptors.

The RNA diagnostics with RT-PCR technology in DRG samples from RTX-treated and untreated rats have shown significant decrease of both receptors³⁵. Observation in these studies extends and corroborates results of Ruparel and co-workers that allyl-isothiocyanate, a selective agonist of TRPA1, can heterologously inactivate TRPV1, probably located in the same nociceptor neurons³⁶. However, Ruparel et al interpreted their results as consequence of TRPV1 desensitization involving signaling events. Although transient, functional desensitization based on signalling events might also be induced by low dose vanilloids in other models^{37,38}, in our case RTX treatment obviously induces the loss of both TRPV1+ and TRPA1+ neurons. As we have confirmed, the treated animals become

insensitive to vanilloids for a lifetime, indicating that the mechanism is indeed deletion-, and not signaling-based. Since RTX treated mice are also insensitive to allyl-isothiocyanate, that only can happen if TRPA1 expression is coincident with TRPV1 expression in the vast majority of nociceptive neurons that normally confer pain signal to endogenous substances produced in pain and inflammation.

Co-ablation of TRPV1+ neurons with TRPA1/allyl-isothiocyanate receptors by the RTX treatment have been circumstantially verified in experiments carried out in TRPV1 knockouts. In concert with evidences here, TRPV1-/- genotyped mice still express TRPA1 in their inflammatory pain sensor neurons, thus preserve sensitivity to allyl-isothiocyanate³⁹ and behave like wild-types.

The neuron subtype specificity of TRPA1 was characterized by Story and co-workers as well in cell cultures¹² and they noted that TRPM8 is expressed in a distinct cell population. Moreover, vast majority of TRPA1-positive cells showed co-localization with the CGRP neuropeptide, another prominent biomarker of TRPV1+ inflammatory pain-sensing neurons. Based on these data, two markedly distinct populations of cold-responsive neurons can be distinguished: i) TRPM8-positive neurons that lack of TRPV1 and TRPA1 expression and resistant to RTX treatment and ii) TRPA1-positive neurons that express TRPV1 as well and can be deleted either with allyl-isothiocyanate or RTX. Adult mice, treated with RTX as newborn, had an impaired response to both CAPS and AITC in eye-wipe tests and agonist-laced water consumption assays. In hot-plate experiments, reaction time of the treated animals was not significantly different from that of normal controls. Similar to our results, however, Gamse et al have found that mice treated with CAPS as newborns had normal reaction times either on the hot plate or in tail withdrawal latency tests. In contrast, mice treated at adulthood had greatly prolonged latencies in both thermal sensitivity. Thus, responses to noxious chemicals in adulthood were only moderately inhibited in mice treated as newborn, but almost completely abolished if the vanilloid was administered to adults⁴⁰.

Nociceptive thresholds reported in neonatal CAPS-treated animals are apparently contradictory. Several groups have found small increase in thermal nociceptive threshold^{28,41} but others reported little if any effect in hot plate tests^{42,43}. It was reported that perineural resiniferatoxin selectively inhibits inflammatory hyperalgesia⁴⁴. We have determined in serial experiments that the heat threshold increase depends on the efficacy of the RTX treatment; heat insensitivity of the animal becomes more pronounced if RTX ablates TRPV1+ nociceptors almost completely. Chronic, repeated RTX gave the best results while neonatal treatments were less effective.

Based on comparative data collected from TRPV1-null mice (little³³ or no effect⁴⁵ in hot plate test), we can conclude that there might be additional heat receptors. The complete lack of signal transmitted by TRPV1 positive neurons leads to the most complete heat insensitivity in mice lacking TRPV1+ neurons rather than the TRPV1 receptor only.

Previously, Obata et al noted that inflammation and nerve injury increases expression of TRPA1, but not TRPM8, in DRG neurons. Occurrence of cold hyperalgesia coincided with nerve growth factor (NGF)-induced up-regulation of TRPA1 in distinct

set of sensory neurons⁴⁶. Moreover, Katsura et al with intrathecal administration of an anti-sense-oligo specific for TRPA1 could suppress the nerve-ligation-induced cold hyperalgesia, but anti-TRPM8 had little or any effect⁴⁷. Contrary to these observations we found that not TRPA1 but TRPM8 is the main cold sensor. To explain this unexpected finding, at least two alternative hypotheses can be made.

First, following to RTX-mediated neuronal drop out the intense arborization of resistant nerve endings can produce NGF, a validated exocrine transducer of gene inducer in nerve injury and might cause over-expression of TRPM8, a prominent cold pain signal transducer, as verified by others⁴⁸. In fact, upon inflammations and nerve injuries, nociceptors' and thermoceptors' NGF expression is known to induced that correlates with sensitization to cold and allodynia^{49,50}. Intraplantar injection of CAPS produces a transient thermal and mechanical hyperalgesia within three to six hrs, that disappears by 24 hours. Interestingly, CAPS injection upregulates proinflammatory cytokines and NGF within 1 hour, then cytokine levels return to control levels⁵¹. However, we have not found upregulation of TRPM8 channel in RTX treated mice using Western blot and RT-PCR. In accordance with that, DRG samples from RTX-treated and untreated rats have not shown significant change on the TRPM8 level³⁵.

On the other hand, sensory stimuli are known to "compete" with each other for representation at the level of brain, for example, blind individuals have better auditory ability than sighted subjects, a mechanism that apparently compensates for loss of vision⁵². Analogously, cold hypersensation might confer an imbalance in the representation of sensory stimuli signaling heat and cold at the levels of the CNS. Due to neuronal plasticity, mice treated at neonatal age most likely can compensate for the imbalance caused by that drop out of C-type afferents neurons, while effect of RTX treatment in adulthood can not be compensated by "fine-tuning".

Importantly, experimental data in this paper predict a shift in heat perception in humans following RTX injection, as conceptualized in the clinical trial protocol to treat severe cancer pain. Similar changes might be expected during application of reversible, specific inhibitors of TRPV1, currently in development by different pharmaceutical companies. Such behavioral change, however, was not obvious in cancer/arthritis patient dogs treated via the intrathecal rout of application²⁶, neither in monkeys, targeted with this molecular neurosurgery agent at the trigeminus ganglion²⁷. Nevertheless, clinical trial of RTX in cancer pain patients may provide the answers to this question via thorough monitoring of potential change in cold sensation if it may happen in human at all.

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