Glutamate, Substance P, and Calcitonin Gene-Related Peptide Cooperate in Inflammation-Induced Heat Hyperalgesia

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ABSTRACT

The transient receptor potential cation channel subfamily V member 1 (TRPV1) is known as a thermosensor and integrator of inflammation-induced hyperalgesia. TRPV1 is expressed in a subpopulation of primary afferent neurons that express several different neurotransmitters. The role of the TRPV1 channel in the development of hyperalgesia is established, but the role of the neurotransmitter glutamate, used partially by the same neuronal population and thus probably mediating the response, is still under investigation. We have used a Trpv1-Cre mouse line in which we either ablated Trpv1-Cre expressing neurons or induced vesicular glutamate transporter 2 (VGLUT2) deficiency in Trpv1-Cre expressing neurons and investigated specific states of hyperalgesia after persistent inflammation. Furthermore, by pharmacologic inhibition of substance P (SP) or calcitonin gene-related peptide (CGRP) signaling in Vglut2-deficient mice, we also evaluated the contribution of SP or CGRP to inflammation-induced hyperalgesia, with or without the presence of vesicular glutamate transporter 2 (VGLUT2)-mediated glutamatergic transmission in Trpv1-Cre neurons. This examination, together with c-Fos analyses, showed that VGLUT2-mediated glutamatergic transmission in Trpv1-Cre afferents together with SP or CGRP is essential for the development of the heat hyperalgesia associated with persistent inflammation. Additionally, SP-, CGRP-, and VGLUT2-mediated transmission together were found to play a role in the development of mechanical hyperalgesia after persistent inflammation.

Introduction

Tissue injury can lead to peripheral inflammatory hyperalgesia, a troublesome condition for the affected patient. This sensitized state is caused by a reduction of the nociceptive threshold and an increase in peripheral afferent reactivity. The transient receptor potential cation channel subfamily V member 1 (TRPV1), which is expressed in a heterogeneous population of primary afferent neurons (Tominaga et al., 1998), has been shown to have a central role in inflammation-induced heat hyperalgesia (Caterina et al., 2000; Davis et al., 2000; Mishra et al., 2011). The TRPV1 population is also required for the development of thermal and mechanical hyperalgesia after complete Freund’s adjuvant (CFA) injections (Okun et al., 2011; Brenneis et al., 2013). However, the neurotransmission behind these processes is still under investigation.

One of the transmitters suggested to play a central role in peripheral inflammation is glutamate. The evidence supporting this theory include increased levels of glutamate in peripheral tissues in response to inflammation (Omote et al., 1998; deGroot et al., 2000) and reduced hyperalgesia after peripheral inflammation in response to various metabotropic glutamate receptors antagonists (Walker et al., 2001; Lee et al., 2007). The TRPV1 population of primary afferent neurons and trigeminal neurons have been shown to overlap with markers for glutamatergic transmission (Hwang et al., 2004; Lagerström et al., 2010, 2011), and TRPV1 antagonists are known to inhibit glutamatergic transmission after peripheral inflammation (Lappin et al., 2006). It is however not known how glutamate contributes to TRPV1-associated hyperalgesia after peripheral inflammation, presumably because studies of glutamate in specific neuronal populations have been lacking efficient tools.

This study focuses on the role of vesicular glutamate transporter 2 (VGLUT2) in TRPV1 neurons in peripheral hyperalgesia and also investigates whether glutamatergic transmission in the TRPV1 neurons cooperates with substance P (SP) and calcitonin-gene related peptide (CGRP) signaling in the development of peripheral hyperalgesia, neurotransmitters that have been associated with TRPV1-expressing primary afferent neurons (Tominaga et al., 1998;
Bae et al., 2004; Bernardini et al., 2004; Ziegelmayer et al., 2008). We first established the functional role of the Trpv1-Cre expressing population in persistent inflammatory pain transmission using the Gt(Rosa)26Sor<sup>tm1(DTA)Jpbm</sup> (R26<sup>DTA</sup>) line (Ivanova et al., 2005) and compared the results with Vglut2<sup>flu</sup>Trpv1-Cre mice to define the role of VGLUT2-mediated glutamatergic signaling in the neurons expressing Trpv1-Cre. Then, SP or CGRP antagonists were used individually or together in control and Vglut2<sup>flu</sup>Trpv1-Cre mice to reveal the potential contribution of neuropeptide transmitters to glutamatergic transmission mediated by the Trpv1-Cre-expressing neurons in states of inflammation-induced hyperalgesia.

### Materials and Methods

#### Generation of Transgenic Animals

Mice heterozygous for the for diphtheria toxin allele R26<sup>DTA</sup> (Ivanova et al., 2005) were crossed with mice heterozygous for the Trpv1-Cre allele (Lagerström et al., 2010) to generate mice lacking TRPV1-positive neurons (R26<sup>DTAflu</sup>; Trpv1-Cre<sup>het</sup>) and controls (R26<sup>DTAflu</sup>Trpv1-Cre<sup>het</sup> or R26<sup>DTAflu</sup>Trpv1-Cre<sup>hom</sup>). The Trpv1-Cre mice were also crossed with Vglut2<sup>flu</sup> mice (Wallen-Mackenzie et al., 2006) to generate mice in which Vglut2 was selectively removed from Trpv1-Cre-expressing neurons (R26<sup>DTAflu</sup>Trpv1-Cre). For expression analyses, crossings between Trpv1-Cre line and the reporter line tdTomato (Gt(Rosa)26Sor<sup>tm1(CAG-tdTomato)Hze</sup>; Allen Brain Institute) were performed.

#### Genotyping by Polymerase Chain Reaction

First, 1–2 mm of tail was incubated in 75 µl of buffer consisting of 25 mM NaOH and 200 µM EDTA at 96°C for 45 minutes and placed on ice before the sample was neutralized with 75 µl of Tris·HCl (40 mM), pH 8.0. The mice were genotyped for the presence of the Trpv1-Cre, the R26<sup>DTA</sup> allele, the Vglut2<sup>flu</sup> allele, and tdTomato. Trpv1-Cre was analyzed using the following primers: 5′-GGAGGACACGCTCTGAAAAC (forward), 5′-GTCGCAAAGTAAAGAGGGC (forward), 5′-TCTCCCTACATCTCCATTGTC (reverse), and 5′-CCAGATCATCACCATTCTCCT (reverse). R26<sup>DTA</sup> was analyzed using 5′-GTATGACTTAAAGGAGGTCAGTG, 5′-AAGACGCCGAGAAGTTCGTC (reversed), and 5′-GCGCGATCAGAAGAATTTTT (forward). Vglut2<sup>flu</sup> was analyzed using 5′-CTGTCTCAGGCTTTGTCCTC (forward), 5′-GACTTCAACAGCACGAAGAATACC (forward), and 5′-CCAGATCATCACCATTCTCCT (reverse).

#### Quantitative Polymerase Chain Reaction

Quantitative real-time polymerase chain reaction (PCR) on homogenate from dissected dorsal root ganglia (R26<sup>DTAflu</sup>Trpv1-Cre) and control mice was performed as described previously elsewhere (Olaszewski et al., 2009) with the following exceptions. The mRNA was prepared using the RNeasy kit (Qiagen, Valencia, CA), three housekeeping genes were used (β-actin, H3B [histocompatibility 3b], and RPL19 [ribosomal protein L19]), and the primer sequences for Trpv1 were 5′-CTG CAG CAG TTT GTC AAT G 3′ (forward) and 5′-TTC AAT GGC AAT GTG AAT G ATG G 3′ (reverse); for Sp 5′-TTGTTGCCC AAA TTA GTC CAA TA-3′ (forward) and 5′-AAG CCT CAG TCC TTC TTT GG 3′ (reverse); for Cgrp 5′-CAG GAG CAA GAT GAT GCC CGA AA-3′ (forward) and 5′-CCA GTC TCA GGA TCA AGA GT-3′ (reverse); and for TrkA 5′-GGCTTCTCCGATGCTTCTC (forward) and 5′-ACATGTTTCTG TGC TGTGAG (reverse). No outliers were detected using the Grubbs test, the control animal level of Trpv1, Sp, Cgrp, or TrkA mRNA was set to 1, and the statistics were performed using Mann–Whitney test (Prism version 5.01; GraphPad Software, San Diego, CA).

#### Tissue Preparation

Mice (>7 weeks old) were perfused as previously described in Gezelius et al., 2006. Dorsal root ganglia (DRG) and lumbar and sacral spinal cord were isolated for immunohistochemistry. The isolated tissue was fixed in fresh 4% phosphonoformic acid (PFA) for 2 hours shaking on ice followed by a graded series of sucrose solutions ending with 30% sucrose before the tissue was mounted and frozen in O.C. T. compound (Sakura Fineteck, Zoeterwoude, The Netherlands) at ~80°C. Sections of 14 µm were cut and mounted on Superfrost glass (Menzel-Gläser, Braunschweig, Germany) and stored at ~8°C.

#### Immunohistochemistry

The following criteria were used for immunohistochemical analysis: sections were briefly rinsed in 1× Tris-buffered saline (TBS), followed by an incubation in 10% methanol and 3%–4% H<sub>2</sub>O<sub>2</sub> in 1× TBS, before the sections were rinsed in 1× TBS and incubated overnight in 4°C in blocking buffer with 0.5% gelatin and 0.05% Triton X-100 in TBS supplemented with primary antibodies.

The primary antibodies used were rabbit anti-CGRP 1:1000 (Peninsula Laboratories, San Carlos, CA), rabbit anti-TRPV1 1:1000 (Abcam, Cambridge, UK), rabbit anti-TrkA 1:2000 (gift from Professors Finn Hallböök and Louis Reichardt), anti-iBA 1:50 (Invitrogen, Stockholm, Sweden), guinea pig anti-SP 1:200 (Abcam), rabbit anti-β-tubulin 1:500 (Nordic BioSite, Plymouth Meeting, PA), and goat anti-c fos 1:200 (Santa Cruz Biotechnology, Santa Cruz, CA). The c-Fos primary antibody was incubated for 48 hours in 4°C. The sections were then rinsed repeatedly in 1× TBS followed by incubation for 1 hour at room temperature in blocking buffer with 0.5% gelatin and 0.01% Triton X-100 in TBS supplemented with secondary antibodies (Invitrogen): goat anti-chicken Alexa 488 1:400, goat anti-rabbit Alexa 594 1:800, and goat anti guinea-pig Alexa 647 1:200 (Invitrogen).

The VGLUT2 analysis was performed differently: the sections were rinsed 3 times in phosphate-buffered saline, followed by 3 hours of incubation in blocking solution (5% goat serum, 1% Triton X-100, 0.01% NaAz). The sections were then incubated overnight with rabbit or guinea pig anti-VGLUT2 1:200 (Frontier Institute Co. Ltd., Hokkaido, Japan) and then rinsed in phosphate-buffered saline. The secondary antibody used for visualizing fluorescent signal was goat anti-guinea pig Alexa 488 1:647 (Invitrogen) or Alexa goat anti rabbit 488 1:200 (Invitrogen).

#### Imaging

Fluorescent images were viewed in an Olympus BX61WI microscope (Olympus, Stockholm, Sweden) and were analyzed using the Velocity software (Improvision, Lexington, MA). The Mirax software was used (Carl Zeiss MicroImaging GmbH, Jena, Germany) for embryo analysis.

#### General Behavior

All behavioral tests were performed on adult (>7-week-old) female and male mice. Control mice were littermates and gender matched. All behavior analyses were performed in a controlled environment of 20–24°C, 45%–65% humidity, and 12-hour day/night cycle. All animal procedures were approved by the local ethics committee in Uppsala (C79/9, C269/9, C194/8, C105/10, C248/11, and C269/11) and following the Directive 2010/63/EU of the European Parliament and of the Council, the Swedish Animal Welfare Act and Animal Welfare Ordinance (http://www.riksdagen.se/se/sv/Dokument-Lagar/Lagar/Svenskforfattningssamling/Djurkysd-slag-1988534_sfs-1988-534/) and the provisions regarding the use of animals for scientific purposes (DFS, 2004:15 [http://www.jordbruksverket.se/download/18.2434227f1212ececc74b08000878/12420468299017DFS_2004-15.pdf]; SJFVFS, 2012:26 [http://www.jordbruksverket.se/download/18.3c1967a13aaf3e1eb880020406/2012_026.pdf]).

#### Hargreaves Test

The mice were tested and acclimatized in transparent Plexiglas chambers with a glass floor for ~30–60 minutes or until no exploratory behavior was observed. The Hargreaves heat source (ITTC Life Science, Woodland Hills, CA) was placed with the guide light pointing toward the plantar surface of a hind paw, and the thermal beam was started. A paw withdrawal would stop the test, and the time was monitored. The cutoff time was set to 20 seconds. The test was repeated at least 3 times per animal, allowing at least 5 minutes in between each test. The result was expressed as the mean withdrawal latency time for each animal and group ± S.E.M.
Von Frey Test. The mice were acclimatized in elevated transparent chambers with a metal mesh floor for 60–90 minutes or until any exploratory behavior had stopped. The von Frey filaments (Scientific Marketing Associates, Barnet, UK) were applied using the Chaplan et al. (1994) up-and-down paradigm. Response to a hair filament would be the withdrawal of the paw from the applied hair. The lack of response to the application of one hair filament would lead to the application of a thicker hair; likewise, a response would lead to the next application being of a thinner size. The experiment would stop when six responses had been monitored around the 50% threshold. All experiments were initiated using a filament of 0.6 g. The 50% threshold was calculated using the Dixon (1980) method, and the result was expressed as the mean value for each animal and group ± S.E.M.

Nerve Growth Factor- and Carrageenan-Evoked Hyperalgesia. First, baseline measurements were performed using the Hotplate test or von Frey filaments as previously described. Then, the mice were injected with 50 ng human recombinant β-nerve growth factor (NGF) dissolved in 20 μl 0.9% saline or 2% Carrageenan (dissolved in 20 μl 0.9% saline) into the plantar surface of the right paw. For heat hyperalgesia, Hargreaves measurements were repeated at 30 minutes and at 1, 2, 4, 6, and 24 hours after the NGF injection or at 1, 2, 3, 4, and 6 hours after the Carrageenan injection to follow the development of hyperalgesia. To measure punctuate hyperalgesia, von Frey filaments were used, and the measurements were repeated at the same time points as for the assessment of heat hyperalgesia. The results were expressed as the mean withdrawal latency time (Hargreaves) or 50% threshold (von Frey) for each animal and group ± S.E.M.

For treatment with Win51708 (17β-hydroxy-17α-ethynyl-5α-androstan-3β,2-β-pyrimido[1,2-α]benzimidazol-2-one, BN4096BS (1-piperidino)carbonyl-L-tyrosyl-L-lysyl-L-prolinol-OH, 10% dimethylsulfoxide (DMSO) (10 mg/ml) 10 minutes before each measurement or/and in case of BIBN4096BS (Boehringer Ingelheim Pharma KG, Biberach, Germany) dissolved in 10% DMSO (the dose was based on Doods et al., 2000; Just et al., 2005; Starr et al., 2008) 30 minutes before each measurement using the Hargreaves setup and von Frey filaments. No injections of Win51708 or BIBN4096BS were made before the 30-minute measurement or/and in case of BIBN4096BS 30 minutes before each measurement. No injections of Win51708 or BIBN4096BS were made before the 30-minute measurement or/and in case of BIBN4096BS 30 minutes before each measurement.

Development of hyperalgesia was expressed as the difference between the mean withdrawal latency time (Hargreaves) or 50% threshold (von Frey) before the NGF injection versus the average of the 30 minutes and the 1, 2, 4, 6, and 24 hours withdrawal latency times (Hargreaves) or the 50% threshold (von Frey) measurements after the injection. In case of Carrageenan, development of hyperalgesia was expressed as the difference between the mean withdrawal latency time (Hargreaves) or the 50% threshold (von Frey) measurements after the injection.

c-Fos Activity after NGF-Induced Heat Hyperalgesia. For assessment of c-Fos activity, the mice were acclimatized in the behavioral setup before exposure to the Hargreaves test (as described earlier), and the basal response was measured. Then, the mice were injected with 50 ng of human recombinant β-NGF dissolved in 20 μl 0.9% saline into the plantar surface of the right paw. The Hargreaves measurements were performed after 30 minutes, 1 hour, and 2 hours after the NGF injection, or at 1 hour and 2 hours for Carrageenan, with 5 minutes between each thermal stimulation. The animals were then left in the behavioral setup for 55 minutes after the last thermal stimulation before they were perfused. The spinal cords were dissected out and prepared for subsequent cryosectioning (as described earlier). Sections from caudal L1 to rostral L3 were included (Watson et al., 2009). The animals that underwent Win51708 or BIBN4096BS treatment were injected with Win51708 10 minutes before each measurement or BIBN4096BS 30 minutes before each measurement. No injections of Win51708 or BIBN4096BS were made before the 30-minute measurement.

Statistics. Nonparametric calculations of P values between two groups were conducted using the Mann–Whitney test (Prism version 5.01), which was the standard test used unless otherwise noted. The NGF and Carrageenan data were also analyzed using two-way repeated measures analysis of variance (Prism version 5.01) using the Bonferroni post hoc test. Comparison of the degree of hyperalgesia (Vglut2Cre;Trpv1-Cre and control mice in heat hyperalgesia after NGF provocation and for concomitantly treated Vglut2Cre;Trpv1-Cre and control mice in mechanical hyperalgesia) was calculated by subtracting the average hyperalgesic response from the basal response (response before the injection) and dividing the sum with the basal response. The average change/genotype was then calculated and compared using Mann–Whitney test (Prism version 5.01). For negative results, the power of the test was calculated, and if necessary the number of samples was increased.

Results

TRPV1 Neurons Are Lost in R26DTA/w;Trpv1-Cre Mice. To define the precise role of the TRPV1-expressing neurons and their transmitters in hyperalgesia induced by persistent inflammation, we used Trpv1-Cre mice that showed prominent expression of Cre recombinase in TRPV1-expressing DRG neurons and very limited expression in the central nervous system (Lagerström et al., 2010). The Trpv1-Cre mice were crossed with the R26DTA line (Ivanova et al., 2005) to excise a floxed stop cassette upstream of the diphertheria toxin A (DTA) gene, generating mice where the Trpv1-Cre expressing neurons were exclusively deleted (R26DTA/w;Trpv1-Cre). The R26DTA/w;Trpv1-Cre mice were born with a normal Mendelian ratio, were viable, and did not show any obvious phenotypic abnormalities. The ablation of the Trpv1-Cre expressing neurons resulted in a loss of 38.2%–41.8% of the primary afferent neurons, analyzed per section or per DRG (128.6 ± 6.0 cells/section or 115.3 ± 76.3 neurons/DRG remained in R26DTA/w;Trpv1-Cre compared with 207.9 ± 14.1 cells/section or 198.8 ± 91.8 neurons/DRG in control mice, respectively), quantified using the pan-neuronal marker β-tubulin (n = 3; genotype, 53 versus 31 sections counted; n = 3, 3 DRGs/genotype, 58 versus 72 sections counted). More specifically, 91.2% of the TRPV1-immunopositive neurons were lost in the R26DTA/w;Trpv1-Cre mice compared with controls (7.3 ± 0.5 TRPV1-positive cells/section remained in R26DTA/w;Trpv1-Cre mice compared with 82.6 ± 8.0 cells/section in controls mice) (Fig. 1, A and B), which corresponded well with the total loss in cell numbers. The ablation of the TRPV1 population was also confirmed using quantitative PCR, which demonstrated that the expression of Trpe1 mRNA was reduced to 4.0% ± 2.2% in DRGs from R26DTA/w;Trpv1-Cre mice compared with control littermates (P = 0.00099) (Fig. 1C). Hence, by using the R26DTA line in combination with the Trpv1-Cre line, the TRPV1 population was close to completely ablated from the DRGs without affecting the viability of the resulting mouse line.

The Trpv1-Cre Population Expresses Markers for Glutamatergic, SP-, CGRP-, and NGF-Mediated Transmission. We next set out to analyze the transmitter phenotype of the Trpv1-Cre subpopulation of DRG neurons using the
reporter line tdTomato in combination with immunohistochemical markers for different neurotransmitters. In addition, DRG sections and cDNA from R26<sup>DTA/wt</sup>;Trpv1-Cre mice and controls were analyzed. VGLUT2 is the most common glutamatergic vesicular transporter in DRGs (Brumovsky et al., 2007). Our immunohistochemical analyses showed that more than half of the Trpv1-Cre;tdTomato population, 53.2% ± 1.7%, overlapped with VGLUT2, which corresponds to 67.7% ± 1.4% of the VGLUT2 population (Fig. 1, P–U). Accordingly, only 36.4% of the VGLUT2-immunopositive population remained in the R26<sup>DTA/wt</sup>;Trpv1-Cre mice compared with control littermates (Fig. 1, D and E). Quantitative PCR analyses confirmed the loss of VGLUT2-expressing cells, which showed that the expression of Vglut2 mRNA was reduced to 30.8% ± 3.4% in R26<sup>DTA/wt</sup>;Trpv1-Cre mice compared with control mice (Fig. 1F).

Further analyses showed that approximately one quarter, 26.9% ± 1.6%, of the Trpv1-Cre;tdTomato population overlapped with the SP population, which corresponds to 71.5% ± 1.9% of the SP-immunonegative cells (Fig. 1, P and S). This finding was corroborated by a reduction of SP-immunopositive cells to 29.6% in the R26<sup>DTA/wt</sup>;Trpv1-Cre mice (Fig. 1, G and H) and a loss of Sp mRNA expression to 57.5% ± 2.1% in R26<sup>DTA/wt</sup>;Trpv1-Cre mice compared with control mice (Fig. 1I).

The Trpv1-Cre;tdTomato population also overlapped with cells immunopositive for CGRP. Here, 34.7% ± 1.1% of Trpv1-Cre;tdTomato population overlapped with the CGRP-immunopositive cells, which corresponds to 72.2% ± 2.0% of the CGRP population; consequently, only a small fraction, 16.5%, of the CGRP population remained in the R26<sup>DTA/wt</sup>;,

Fig. 1. The Trpv1-Cre population contains markers for glutamatergic, SP-, CGRP-, and NGF-mediated transmission. Ablation of the Trpv1-Cre population results in a loss of peptidergic and glutamatergic sensory neurons. (A and B) A significant reduction of TRPV1 immunoreactive cells were observed in R26<sup>DTA/wt</sup>;Trpv1-Cre mice compared with controls (27 sections/control; 30 sections/R26<sup>DTA/wt</sup>;Trpv1-Cre mice counted, n = 3/genotype). (C) The expression of Trpv1 mRNA was 96.0% ± 2.2% lower in DRGs from R26<sup>DTA/wt</sup>;Trpv1-Cre mice compared with control littermates evaluated by quantitative PCR in relation to three housekeeping genes (β-tubulin, β-actin, ribosomal protein L19 [RPL19]) (n = 5/genotype). (D and E) In R26<sup>DTA/wt</sup>;Trpv1-Cre mice, 23.3 ± 2.7 VGLUT2-positive cells/section remained compared with 64.0 ± 3.8 cells/section in control mice (n = 3/genotype; 30 sections/control, 46 sections/R26<sup>DTA/wt</sup>;Trpv1-Cre<sup>Cre<sub>tm</sub></sup>). (F) The expression of Vglut2 mRNA was significantly lower in DRGs from R26<sup>D T A/wt</sup>;Trpv1-Cre mice compared with control littermates evaluated by quantitative PCR (n = 3/genotype). (G and H) In R26<sup>DTA/wt</sup>;Trpv1-Cre mice, 12.4 ± 0.6 SP-positive cells/section remained compared with 41.8 ± 2.1 cells/section in control mice (n = 3/genotype; 31 sections/control, 44 sections/R26<sup>DTA/wt</sup>;Trpv1-Cre mice). (I) The expression of Sp mRNA was significantly lower in DRGs from R26<sup>DTA/wt</sup>;Trpv1-Cre mice compared with control littermates evaluated by quantitative PCR (n = 3/genotype). (J and K) In R26<sup>DTA/wt</sup>;Trpv1-Cre mice, 13.3 ± 1.0 of CGRP-positive cells/section remained compared with 80.5 ± 2.9 cells/section in control mice (n = 3/genotype). (L) The expression of Cgrp mRNA was significantly lower in DRGs from R26<sup>DTA/wt</sup>;Trpv1-Cre mice compared with control littermates evaluated by quantitative PCR (n = 3/genotype). (M and N) In R26<sup>DTA/wt</sup>;Trpv1-Cre mice, 18.4 ± 1.5 TrkA-positive cells/section remained compared with 70.1 ± 3.0 cells/sections in control mice (n = 3/genotype, 28 sections/control, 26 sections/R26<sup>DTA/wt</sup>;Trpv1-Cre mice). (O) The expression of TrkA mRNA was significantly lower in DRGs from R26<sup>DTA/wt</sup>;Trpv1-Cre mice compared with control littermates evaluated by quantitative PCR (n = 4/genotype). (P–U) Out of 66.8 ± 3.6 VGLUT2-positive cells/section, 44.8 ± 2.3 cells overlap with Trpv1-Cre;Tomato (n = 6, 60 sections). (P and S) Out of 31.7 ± 1.8 SP-positive cells/section, 22.6 ± 1.4 cells overlap with Trpv1-Cre;tdTomato (n = 3, 30 sections/genotype). (Q–T) Out of 44.9 ± 2.0 CGRP-positive cells/section, 31.5 ± 1.4 cells overlap with Trpv1-Cre;tdTomato (n = 5, 30 sections/genotype). (R–U) Out of 53.2 ± 1.5 TrkA-positive cells/sections, 21.1 ± 1.1 cells overlap with Trpv1-Cre;tdTomato cells/section (n = 3, 30 sections/genotype). *P < 0.05; **P < 0.01; ***P < 0.001, Mann–Whitney test, two-tailed. Scale bars: 60 μm. Black/white arrows indicate a triple-labeled cell. Green arrow indicates a cell singularly labeled with VGLUT2, and a small white arrow the peptidergic marker in question. A red arrow highlights a Trpv1-Cre;tdTomato single labeled cell. Venn-diagrams were made with BioInforFx (Madison, WI) online software (http://bioinforfx.com/free/bxarrays/venn-diagram.php).
Trpv1-Cre mice (Fig. 1, J, K, Q, and T). This corresponded well with the quantitative PCR analysis, where a reduction of Cgrp mRNA to 23.8% ± 5.9% in R26<sup>DTA/w</sup>;Trpv1-Cre mice compared with control mice was observed (Fig. 1L).

Additionally, we analyzed the overlap of Trpv1-Cre–positive neurons with TrkA and IB4, markers for NGF binding and nonpeptidergic neurons, respectively. The analyses showed that 38.2% ± 2.3% of the Trpv1-Cre;tdTomato population overlapped with the TrkA population, which corresponds to 64.3% ± 2.1% of TrkA-immunopositive cells (Fig. 1, R and U). This finding was corroborated by a reduction of TrkA-immunopositive cells to 26.2% in the R26<sup>DTA/w</sup>;Trpv1-Cre mice (Fig. 1, M and N) and a loss of TrkA mRNA expression to 47.8% ± 15.5% in R26<sup>DTA/w</sup>;Trpv1-Cre mice compared with control mice (Fig. 1O). The IB4 analyses showed that 28.3% of IB4-positive neurons were deleted in R26<sup>DTA/w</sup>;Trpv1-Cre mice (Supplemental Fig. 1), indicating that a subpopulation of Trpv1-Cre neurons are nonpeptidergic.

In conclusion, the Trpv1-Cre population of primary afferent neurons stains positively for the neuropeptides SP and CGRP and markers for glutamatergic transmission (VGLUT2). Thus, these transmitters are potentially involved in pain transmission mediated by primary afferents and the Trpv1-Cre subpopulation in particular. Additionally, the extensive overlap between VGLUT2, TrkA, and TRPV1 indicate a role for glutamate in the TRPV1 population in response to the proinflammatory agent NGF which is central for inflammation-induced hyperalgesia.

**Glutamatergic Signaling from the Trpv1-Cre Population Together with SP or CGRP Is Essential for the Transmission of Inflammation-Associated Heat Hyperalgesia.** To define the role of the Trpv1-Cre expressing neurons and the potential cooperative roles of transmitters in hyperalgesic states associated with peripheral inflammation, we investigated the behavioral phenotype of R26<sup>DTA/w</sup>;Trpv1-Cre mice and controls. They were also compared with the phenotype of Vglut2<sup>ES</sup>;Trpv1-Cre mice (Wallen-Mackenzie et al., 2006; Lagerström et al., 2010) to determine the role of VGLUT2-mediated glutamatergic transmission in the Trpv1-Cre population. The Vglut2<sup>ES</sup>;Trpv1-Cre line also allowed us to determine the contribution of SP and CGRP to inflammation-induced hyperalgesia when a possible compensatory effect of glutamate was unmasked from the Trpv1-Cre population.

We injected the TrkA ligand NGF into the plantar surface of the right paw and followed the development of heat and punctate hyperalgesia for 24 hours using the Hargreaves and von Frey tests, respectively. Provocations with NGF induced heat hyperalgesia in controls 8.3 ± 0.5 seconds before NGF injection (P = 0.007) but not in R26<sup>DTA/w</sup>;Trpv1-Cre mice, 18.1 ± 0.5 seconds versus 17.9 ± 0.2 seconds (P = 0.75, power of test = 0.97) (Fig. 2A-B). Interestingly, mice in which Vglut2 had been deleted from the Trpv1-Cre population developed NGF-induced heat hyperalgesia, 16.1 ± 1.0 seconds versus 9.2 ± 0.4 seconds (P = 0.004), comparable to control mice (P = 0.8), 8.7 ± 0.6 seconds versus 5.6 ± 0.4 (P = 0.003) (Fig. 2, C and D). This suggests that VGLUT2-mediated glutamatergic transmission in the Trpv1-Cre population is not essential for the development and transmission of heat hyperalgesia. The basal difference between control mice and R26<sup>DTA/w</sup>;Trpv1-Cre or Vglut2<sup>ES</sup>;Trpv1-Cre mice in response to noxious heat corroborates previous findings and demonstrates the role of the Trpv1-Cre population and VGLUT2 in acute heat transmission (Lagerström et al., 2010; Mishra et al., 2011).

To investigate a possible contribution from SP to NGF-induced hyperalgesia, with or without intact glutamatergic transmission in the TRPV1 population, we injected the non-peptidergic SP antagonist Win51708 (Venepalli et al., 1992) in Vglut2<sup>ES</sup>;Trpv1-Cre mice and control littermates 10 minutes before each hourly measurement. Under these conditions, Vglut2<sup>ES</sup>;Trpv1-Cre mice were resistant to the development of heat hyperalgesia, 16.7 ± 0.8 seconds versus 18.0 ± 0.4 seconds (P = 0.13, power of test = 0.98), whereas control littermates developed heat hyperalgesia, 7.8 ± 0.8 versus 4.4 ± 0.5 seconds (P = 0.004), to a level comparable with untreated Vglut2<sup>ES</sup>;Trpv1-Cre control mice (P = 0.06, power of test = 0.84) (Fig. 2, E and F).

Further, Vglut2<sup>ES</sup>;Trpv1-Cre mice and littermate controls were treated with the CGRP antagonist BIBN4096BS (Doeds et al., 2000) to evaluate the contribution of CGRP to the development of heat hyperalgesia. Treated control mice developed heat hyperalgesia, 11.1 ± 0.7 versus 4.4 ± 0.5 seconds (P = 0.002), comparable to untreated controls (P = 0.09, power of test = 0.81) whereas Vglut2<sup>ES</sup>;Trpv1-Cre conditional knockout mice were resistant to the development of heat hyperalgesia, 18.7 ± 0.85 versus 19.1 ± 0.20 seconds (P = 0.57, power of test = 0.86) (Fig. 2, G and H). Interestingly, concomitant injection of Win51708 and BIBN4096BS prevented both Vglut2<sup>ES</sup>;Trpv1-Cre mice and control mice from developing heat hyperalgesia: 16.4 ± 1.0 versus 16.8 ± 0.7 seconds (P = 0.81, power of test = 0.91), and 10.4 ± 1.2 versus 14.6 ± 1.4 seconds (P = 0.0411), respectively (Fig. 2, I and J).

To analyze whether the absence of heat hyperalgesia was due to an attenuated peripheral inflammatory process, we analyzed the thickness of paw edema and number of infiltrated cells in the inflamed paw after NGF injection (Supplemental Fig. 2). The analysis showed no differences between the groups except a small attenuation in paw edema between untreated and BIBN4096BS-treated control mice (P = 0.041, from 671.0 ± 49.8 μm to 638.9 ± 26.0 μm).

Thus, our data suggest that heat hyperalgesia after peripheral inflammation develops despite the loss of VGLUT2-mediated glutamatergic transmission in TRPV1 neurons or the blockage of SP or CGRP-mediated transmission. However, VGLUT2-mediated transmission, together with SP or CGRP, or concomitant blockage of SP and CGRP transmission is enough to prevent the development of heat hyperalgesia after peripheral inflammation, displaying the cooperative roles of these three neurotransmitters in peripheral hyperalgesia. Our data also show that the targeted ablation of VGLUT2-mediated glutamatergic signaling and blockade of SP and CGRP transmission did not markedly affect the peripheral inflammatory process initiated by the NGF injections, which suggests that their actions in the development of heat hyperalgesia are mainly nociceptive.

We next used another proinflammatory agent, Carrageenan, which mainly acts through a TrkA-NGF pathway to induce hyperalgesia (McMahon et al., 1995), and we followed the development of heat hyperalgesia during 6 hours. Like with NGF, Carrageenan provocations resulted in profound heat hyperalgesia in control mice, 9.2 ± 0.4 versus 3.3 ± 0.2 seconds (P = 0.002), whereas R26<sup>DTA/w</sup>;Trpv1-Cre mice did not develop heat hyperalgesia, 18.0 ± 0.5 versus 18.7 ± 0.3 seconds (P = 0.48, power of test = 0.95) (Fig. 3, A and B),
again demonstrating the important role of the TRPV1 population in heat hyperalgesia and corroborating the data shown by Mishra et al. (2011). Next, we examined the role of VGLUT2-mediated glutamatergic transmission in the Trpv1-Cre population by repeating the experimental procedures on the Vglut2f/f,Trpv1-Cre line. Similar to NGF-induced inflammation, both Vglut2f/f,Trpv1-Cre and control mice developed heat hyperalgesia: 17.8 ± 0.8 versus 3.8 ± 0.3 seconds (P = 0.005) and 7.7 ± 0.7 versus 3.4 ± 0.2 seconds (P = 0.002), respectively (Fig. 3, C and D). This reiterates that VGLUT2-mediated transmission alone is not central for the development of heat hyperalgesia via the Trpv1-Cre population.

We next evaluated the role of SP signaling in Carrageenan-induced inflammation by Win51798-mediated blocking of SP transmission in Vglut2f/f,Trpv1-Cre and control mice. Win51708 treatment did not prevent development of heat hyperalgesia in control mice, 8.8 ± 0.3 versus 4.0 ± 0.1 seconds (P = 0.002), whereas in Vglut2f/f,Trpv1-Cre mice the heat hyperalgesia was absent, 17.1 ± 0.7 versus 16.5 ± 0.3 seconds (P = 0.13, power of test = 0.93) (Fig. 3, E and F). Thus, VGLUT2-mediated glutamatergic release from the Trpv1-Cre population together with SP mediates Carrageenan-induced heat hyperalgesia.

We also investigated the role of CGRP transmission in hyperalgesia associated with Carrageenan-induced inflammation by blocking CGRP transmission in Vglut2f/f,Trpv1-Cre and control mice. BIBN4096BS-treated control mice developed heat hyperalgesia, 11.6 ± 0.9 versus 5.3 ± 0.2 seconds (P = 0.002), whereas heat hyperalgesia in Vglut2f/f,Trpv1-Cre mice was absent, 18.9 ± 0.4 versus 19.7 ± 0.1 seconds (P = 0.13, power of test=0.91). This demonstrates that VGLUT2-mediated glutamatergic release from the Trpv1-Cre population together with CGRP is central for Carrageenan-induced heat hyperalgesia (Fig. 3, G and H). Concomitant injections of Win51708 and BIBN4096BS prevented both Vglut2f/f,Trpv1-Cre conditional knockout mice and control littermates from developing heat hyperalgesia, 17.4 ± 0.8 versus 18.8 ± 0.3 seconds (P = 0.3939, power of test=0.82) and 9.7 ± 0.7 versus 17.9 ± 0.6 seconds (P = 0.0022) (Fig. 3, I and J), corroborating the data from the NGF analysis.

Taken together, these results show that the Trpv1-Cre population is central for the development of heat hyperalgesia associated with peripheral inflammation, induced by NGF or Carrageenan, and that VGLUT2-mediated glutamatergic transmission from the Trpv1-Cre population together with SP or CGRP-dependent transmission is crucial for the development and mediation of heat hyperalgesia.

Fig. 2. Glutamatergic transmission from the Trpv1-Cre population is, together with SP and/or CGRP, essential for the development of inflammation-associated heat hyperalgesia. NGF-induced heat in R26DTA/wt; Trpv1-Cre mice, Vglut2f/f;Trpv1-Cre mice or control littermates were studied using intraplantar injections of 50 ng of NGF. (A and B) Development of hyperalgesia in controls (white bars), R26DTA/wt;Trpv1-Cre (gray bars), and Vglut2f/f;Trpv1-Cre mice (black bars) before the injection of NGF (the basal level of responses to heat stimulation) and after the injection of NGF (the average of all responses at different time points after NGF injection). (A and B) Heat hyperalgesia developed in control mice but not in R26DTA/wt;Trpv1-Cre mice after NGF injections (n = 8/genotype). (C and D) whereas both control and Vglut2f/f;Trpv1-Cre mice developed heat hyperalgesia (n = 12/genotype). (E and F) Intraperitoneal injections of the SP antagonist Win51708, 10 minutes before each measurement (except at 30 minutes) could not inhibit heat hyperalgesia from developing in control mice but rendered Vglut2f/f;Trpv1-Cre mice resistant to the development of heat hyperalgesia (n = 6/genotype). (G and H) Pretreatment with the CGRP antagonist BIBN4096BS prevented heat hyperalgesia from developing in Vglut2f/f,Trpv1-Cre mice but not in control mice (n = 6/ genotype). (I and J) Concomitant pretreatment with both antagonists Win51708 and BIBN4096BS prevented the development of heat hyperalgesia both in Vglut2f/f,Trpv1-Cre mice and in control animals (n = 6/ genotype). *P < 0.05; **P < 0.01; ***P < 0.001. Data represent mean ± S.E.M. Vglut2f/f,Trpv1-Cre (black) and R26DTA/wt;Trpv1-Cre (gray), respective controls (white). Mann–Whitney test, two-tailed (A, C, E, G, I). Two-way repeated measures analysis of variance, Bonferroni post hoc test (B, D, F, H, J).
VGLUT2-Mediated Transmission from the Trpv1-Cre Population, SP and CGRP Together Attenuates Mechanical Hyperalgesia after Peripheral Inflammation.

Analyses of punctuate hyperalgesia using von Frey filaments showed that both R26<sup>DTA/wt</sup> Trpv1-Cre mice and littermate controls developed a pronounced state of punctuate hyperalgesia after NGF injections: 0.50 ± 0.09 versus 0.03 ± 0.003 g (P = 0.005) and 0.54 ± 0.06 versus 0.003 ± 0.005 g (P = 0.002), respectively (Fig. 4, A and B). Also, Vglut2<sup>DTA/wt</sup> Trpv1-Cre mice and littermates developed punctuate hyperalgesia to a similar extent: 0.83 ± 0.08 versus 0.04 ± 0.001 g (P = 0.005) and 0.81 ± 0.06 versus 0.04 ± 0.006 g (P = 0.005), respectively (Fig. 4, C and D). This suggests that neither the Trpv1-Cre population nor VGLUT2-mediated glutamatergic transmission in the Trpv1-Cre population is required for the development of punctuate hyperalgesia.

Moreover, systemic treatments with the SP antagonist Win51708 or the CGRP antagonist BIBN4096BS did not prevent a hyperalgesic state toward punctuate stimuli to develop in either control nor Vglut2<sup>DTA/wt</sup> Trpv1-Cre mice: 0.75 ± 0.03 versus 0.05 ± 0.002 g (P = 0.004), 0.71 ± 0.15 versus 0.07 ± 0.02 g (P = 0.002), 0.76 ± 0.09 versus 0.07 ± 0.007 g (P = 0.005), and 0.65 ± 0.06 versus 0.06 ± 0.004 g (P = 0.005), respectively (Fig. 4, E–H). However, concomitant administration of both antagonists resulted in a difference in the development of hyperalgesia between Vglut2<sup>DTA/wt</sup> Trpv1-Cre and control mice (Fig. 4, I and J). Vglut2<sup>DTA/wt</sup> Trpv1-Cre and control littersmates both developed punctuate hyperalgesia despite treatment with Win51708 and BIBN4096BS: 0.92 ± 0.07 versus 0.06 g (P = 0.0048) and 0.96 ± 0.09 versus 0.22 ± 0.02 g (P = 0.0047), respectively. However, Vglut2<sup>DTA/wt</sup> Trpv1-Cre mice treated with Win51708 and BIBN4096BS developed a decreased level of hyperalgesia compared with control mice (P = 0.0022).

A similar behavioral analysis using von Frey filaments showed that punctuate hyperalgesia after Carrageenan injections developed both in controls and R26<sup>DTA/wt</sup> Trpv1-Cre mice after Carrageenan injections: 0.65 ± 0.12 versus 0.04 ± 0.003 g (P = 0.002) and 0.93 ± 0.17 versus 0.04 ± 0.002 g (P = 0.005), respectively (Fig. 5, A and B). Vglut2<sup>DTA/wt</sup> Trpv1-Cre and control mice also developed punctuate hyperalgesia: 0.73 ± 0.09 versus 0.04 ± 0.004 g (P = 0.004) and 0.75 ± 0.03 versus 0.04 ± 0.004 g (P = 0.005), respectively (Fig. 5, C and D). Neither Win51708 nor BIBN4096BS treatment prevented the development of punctuate hyperalgesia in control mice or in Vglut2<sup>DTA/wt</sup> Trpv1-Cre mice: 0.65 ± 0.12 versus 0.07 ± 0.008 g (P = 0.005) and 0.63 ± 0.08 versus 0.06 ± 0.006 g (P = 0.005) (Fig. 5, E and F), 0.79 ± 0.08 versus 0.05 ± 0.002 g (P = 0.005) and 0.73 ± 0.04 versus 0.04 ± 0.003 g (P = 0.004) (Fig. 5, G and H).

However, when both antagonists were administrated concomitantly, there was a difference observed in the development of hyperalgesia between treated Vglut2<sup>DTA/wt</sup> Trpv1-Cre and control mice (Fig. 5, I and J). Even though treated Vglut2<sup>DTA/wt</sup> genotypes. (I and J) Concomitant pretreatment with both antagonists Win51708 and BIBN4096BS prevented development of heat hyperalgesia in Vglut2<sup>DTA/wt</sup> Trpv1-Cre mice but as well in control animals (n = 6/ genotype). **P < 0.01, ***P < 0.001. Data represent mean ± S.E.M. Vglut2<sup>DTA/wt</sup> Trpv1-Cre (black) and R26<sup>DTA/wt</sup> Trpv1-Cre (gray), respective controls (white). Mann–Whitney test, two-tailed (A, C, E, G, and I). Two-way repeated measures analysis of variance, Bonferroni post hoc test (B, D, F, H, J).
hyperalgesia in R26 to together with SP and CGRP. Nerve growth factor-induced punctuate VGLUT2-mediated glutamatergic transmission from Trpv1-Cre neurons.

Fig. 4. NGF-induced punctuate hyperalgesia is partially mediated by VGLUT2 from Trpv1-Cre neurons together with SP and CGRP. Nerve growth factor-induced punctuate hyperalgesia in R26/Trpv1-Cre mice. Vglut2f/f;Trpv1-Cre mice or control littersmates were studied using intraplantar injections of 50 ng NGF, followed by von Frey test. (A and B) Punctuate hyperalgesia developed both in control and R26/Trpv1-Cre mice (n = 6 genotype). (C and D) Similarly, both controls and Vglut2f/f;Trpv1-Cre mice developed punctuate hyperalgesia (n = 6 genotype). (E and F) Pretreatment with the SP antagonist Win51708 did not prevent the development of punctuate hyperalgesia in Vglut2f/f;Trpv1-Cre mice or in control mice (n = 6 genotype). (I and J) Concomitant pretreatment with both antagonists Win51708 and BIBN4096BS led to a decreased development of punctuate hyperalgesia in Vglut2f/f;Trpv1-Cre mice compared with control animals (n = 6 genotype). Data represent mean ± S.E.M. **P < 0.01; ***P < 0.001. Mann–Whitney test, two-tailed (A, C, E, G, and I). Two-way repeated measures analysis of variance, Bonferroni post hoc test (B, D, F, H, and J).

In summary, SP and CGRP together have a role for mediating punctuate hyperalgesia associated with peripheral inflammation, as was most clearly detectable in the VGLUT2-reduced background.

The Role of Glutamate, SP, and CGRP in Persistent Inflammatory Pain Is Mainly Mediated by Primary Afferent Neurons. We next analyzed the onset of c-Fos protein expression in the dorsal horn that followed upon NGF or Carrageenan injection and heat stimulation using the Hargreaves setup. To determine the origin of the decreased heat hyperalgesia, we compared ablation of the Trpv1-Cre population, deletion of Vglut2 from the Trpv1-Cre population, and/or blocking of SP or CGRP transmission affected activation of second-order neurons in the spinal cord dorsal horn (Fig. 6, A–H). The analyses revealed a significant decrease in the number of c-Fos–positive nuclei in lamina I–III in segment L2 in R26/Trpv1-Cre mice compared with control littersmates (P < 0.0001). Because the expression of Trpv1-Cre is predominantly limited to the primary afferents and hence deletion of Trpv1 population neurons in R26/Trpv1-Cre mice primarily affects this area of the pain pathway, the reduced expression of c-Fos implies that the hyperalgesic phenotype originates from the primary afferent Trpv1-Cre population.

Although not apparent in their pain response (Fig. 2D), Vglut2f/f;Trpv1-Cre mice displayed a small but significant decrease in the number of c-Fos–positive nuclei compared with littermate controls at the same spinal level (P = 0.0028) (Fig. 6B). This reduction in activation of second-order interneurons might reflect a reduced level of glutamatergic transmission from the Trpv1-Cre afferents. A drastic reduction of c-Fos–positive cells was observed in Win51708-treated Vglut2f/f;Trpv1-Cre mice, especially in lamina I–III, compared with Win51708-treated control animals (P < 0.0001). The reduced heat hyperalgesia in Vglut2f/f;Trpv1-Cre mice during systemic blockade of SP probably also originates from loss of VGLUT2-mediated transmission from primary afferents based on the observed reduction in c-Fos expression in the dorsal spinal cord (Fig. 6C). Similarly, treatment of Vglut2f/f;Trpv1-Cre mice with BIBN4096BS resulted in significant reduction of c-Fos–positive cells in lamina I–III, compared with BIBN4096BS-treated controls (Fig. 6D). Thus, these results support a role for SP or CGRP transmission together with glutamatergic transmission from primary afferent neurons in NGF-induced heat hyperalgesia (P < 0.0001).

Heat stimulation of animals with Carrageenan-induced hyperalgesia resulted in an onset of c-Fos protein expression...
in the dorsal horn similar to NGF provocations. Hargreaves stimulation of Carrageenan injected R26\textsuperscript{DTA/\text{WT}};Trpv1-Cre and control mice revealed a significant decrease in the number of c-Fos–positive nuclei in lamina I–III in segment L2 in R26\textsuperscript{DTA/\text{WT}}, Trpv1-Cre mice compared with control littermates (P < 0.0001) (Fig. 6E). Also, stimulation of Vglut2\textsuperscript{f/f};Trpv1-Cre mice did not show any significant decrease in the number of c-Fos–positive nuclei compared with littermate controls at the same spinal level (P = 0.7, power of test = 0.93), which corresponds well with the behavior phenotype (Fig. 4, C and D; Fig. 6F). Further, a considerable reduction of c-Fos–positive cells was observed in Win51708-treated Vglut2\textsuperscript{f/f};Trpv1-Cre mice compared with Win51708-treated control animals (P < 0.0001) (Fig. 6G). Similarly, treatment of Vglut2\textsuperscript{f/f};Trpv1-Cre mice with BIBN4096BS resulted in significant reduction of c-Fos–positive cells compared with BIBN4096BS-treated controls (P < 0.0001) (Fig. 6H).

Hence, VGLUT2-mediated glutamatergic transmission from peripheral Trpv1-Cre neurons together with systemic effects of SP and CGRP are central for the development and transmission of heat hyperalgesia. The reduced VGLUT2-mediated transmission in combination with antagonistic blockage of SP or CGRP led to less activation of second-order interneurons in the dorsal horn, which tentatively explains the observed absence of heat hyperalgesia.

Discussion

We here evaluated the transmission accounting for persistent inflammatory pain states mediated by TRPV1 neurons by two different approaches: deletion of the TRPV1 neurons and genetic attenuation of glutamatergic transmission in the TRPV1 neurons. We have also analyzed the effects of SP or CGRP antagonists separately or together in control or glutamate-deficient mice.

The Trpv1-Cre population is central for transmitting heat hyperalgesia associated with peripheral inflammation because R26\textsuperscript{DTA/\text{WT}};Trpv1-Cre mice did not develop heat hyperalgesia after Carrageenan or NGF provocations. In contrast, punctuate hyperalgesia developed normally in R26\textsuperscript{DTA/\text{WT}};Trpv1-Cre mice compared with control mice, showing that the Trpv1-Cre population is not required for mediating punctuate hyperalgesia associated with peripheral inflammation. Our experiments also reveal a cooperative role for VGLUT2-mediated glutamatergic transmission from Trpv1-Cre neurons together with either SP or CGRP, or SP together with CGRP, in inflammation-induced heat hyperalgesia. The pain response in each transgenic line and after pharmacologic treatment was accompanied by a similar change in the number of c-Fos–responding spinal cord second-order neurons. Analyses of paw edema showed that the genetic and pharmacologic manipulations did not affect the development of those aspects of the peripheral inflammation and although effects from with both antagonists Win51708 and BIBN4096BS led to a decreased development of heat hyperalgesia in Vglut2\textsuperscript{f/f};Trpv1-Cre mice compared with control animals (n = 6/genotype). 

Fig. 5. VGLUT2-dependent transmission from Trpv1-Cre neurons together with SP and CGRP play a role in the development of Carrageenan-induced punctuate hyperalgesia. Punctuate hyperalgesia was followed for six hours after an intraplantar injection of 2% Carrageenan using the von Frey test. (A and B) Both control and R26\textsuperscript{DTA/\text{WT}};Trpv1-Cre mice develop punctuate hyperalgesia (n = 6/genotype). (C and D) Similarly, Vglut2\textsuperscript{f/f};Trpv1-Cre mice and controls developed a state of punctuate hyperalgesia (n = 6/genotype). (E and F) Pretreatment with Win51708 did not prevent the development of punctuate hyperalgesia in Vglut2\textsuperscript{f/f};Trpv1-Cre mice or control littermates (n = 6/genotype). (G and H) Similarly, there was no difference observed in the development of punctuate hyperalgesia (n = 6/genotype) after pretreatment with the CGRP antagonist BIBN4096BS 30 minutes before measurements. (I and J) Concomitant pretreatment with both antagonists Win51708 and BIBN4096BS led to a decreased development of heat hyperalgesia in Vglut2\textsuperscript{f/f};Trpv1-Cre mice compared with control animals (n = 6/genotype). 

Data represent mean ± S.E.M. Vglut2\textsuperscript{f/f};Trpv1-Cre (black) and R26\textsuperscript{DTA/\text{WT}}, Trpv1-Cre (gray), respective controls (white). Mann–Whitney test, two-tailed (A, C, E, G, and I). Two-way two-way repeated measures analysis of variance, Bonferroni post hoc test (B, D, F, H, and J).

**P < 0.01; ***P < 0.001.
Fig. 6. The contribution of VGLUT2, SP and CGRP to the development and transmission of heat hyperalgesia after NGF or Carrageenan injection is apparent at the second-order neuron level in the spinal cord. (A) Ablation of the Trpv1-Cre population resulted in a striking reduction of c-Fos–expressing spinal cord neurons after NGF injection and subsequent Hargreaves stimulation throughout lamina I–III at spinal segment L2 in R26\textsuperscript{DTA/w};Trpv1-Cre mice compared with control mice (n = 2/genotype, 58 sections/control, 65 sections/R26\textsuperscript{DTA/w};Trpv1-Cre). (B) Genetic ablation of Vglut2 in the Trpv1-Cre population also resulted in a reduction of the number of activated second-order neurons, although the change was less profound (n = 2/genotype, sections = 75/control; 53/Vglut2\textsuperscript{f/f};Trpv1-Cre\textsuperscript{wt}). (C) Win51708 treatment resulted in a drastic reduction of c-Fos–activated neurons in lamina I–III (n = 2/genotype, sections = 45/control; 78/Vglut2\textsuperscript{f/f};Trpv1-Cre). (D) Similarly, pretreatment with BIBN4096BS led to decreased number of c-Fos–positive cells in lamina I–III (P < 0.0001, n = 2/genotype, 28 sections/control and 29 sections/Vglut2\textsuperscript{f/f};Trpv1-Cre). (E) Carrageenan injection and subsequent Hargreaves stimulation resulted in a striking reduction of c-Fos–expressing spinal cord neurons throughout lamina I–III at spinal segment L2 in R26\textsuperscript{DTA/w};Trpv1-Cre mice compared with control mice (n = 2/genotype, 55 sections/control, 60 sections/R26\textsuperscript{DTA/w};Trpv1-Cre). (F) Ablation of Vglut2 in the Trpv1-Cre population did not result in any change in the number of activated second-order neurons (n = 2/genotype, sections = 75/control; 76/Vglut2\textsuperscript{f/f};Trpv1-Cre\textsuperscript{wt}). (G) Win51708 treatment resulted in a drastic reduction of c-Fos–activated neurons in lamina I–III (n = 2/genotype, sections =
higher brain areas cannot be excluded, this suggests that the phenotypic differences reside predominantly in altered transmission in the first synapse in the dorsal horn.

Cre-line specificity is a potential caveat of this study; expression of Trpv1-Cre is not entirely restricted to primary afferent neurons. However, general central nervous system expression is scarce and the sensory areas that potentially could affect the experiments performed in this study either show no expression of Trpv1-Cre (somatosensory cortex, thalamus, or locus coeruleus) or show expression in between 0.1%–3.0% of the neurons such as spinal cord interneurons or neurons of the raphe magnus and the periaqueductal gray (Lagerström et al., 2010). We therefore consider the Trpv1-Cre mouse line used here to be a sufficiently restricted and relevant tool to study the role of the TRPV1-expressing neurons of the primary afferents in inflammation-associated hyperalgesia.

Another potential confounding factor is the DMSO used to dissolve Win51708 and BINB4096BS. In our control experiments with DMSO compared with saline, we saw no effect on heat hyperalgesia; the mean baseline response of the saline group before Carrageenan injection was 8.8 ± 0.8 seconds and for the DMSO group 8.2 ± 0.6 seconds (P = 0.69, power of test = 0.93), whereas the mean response time after the injection was 2.7 ± 0.6 seconds for the saline group and 2.8 ± 0.5 seconds for the DMSO group (P = 0.94, power of test = 0.97).

**Peptidergic and Glutamatergic Transmission Mediated Persistent States of Inflammation-Associated Heat Hyperalgesia.** Our data show that the TRPV1 population is crucial for the development and transmission of heat hyperalgesia. This is consistent with previous findings where acute blockade of the TRPV1 population using the sodium channel blocker QX-314 [2-(2,6-dimethylphenylamino)-N,N,N-triethyl-2-oxoethanaminium] in combination with capsaicin attenuated the development of hyperalgesia after complete CFA injections (Brenneis et al., 2013). Moreover, desensitization of TRPV1-positive afferents using the capsaicin analog resiniferatoxin fully blocked CFA-induced thermal hypersensitivity (Okun et al., 2011). In another study, genetic deletion of the Trpv1 population attenuated Carrageenan-induced heat hyperalgesia (Mishra et al., 2011). Our data support and extend these observations to also show the role of VGLUT2-mediated transmission from the Trpv1-Cre population.

Glutamate is used by most, if not all, primary afferents and is packed into vesicles by VGLUTs (1–3). VGLUT2, which is the predominant transporter in the nociceptive primary afferent population (Brumovsky et al., 2007; Lagerström et al., 2010, 2011; Scherrer et al., 2010; Rogoz et al., 2012), overlaps to a large extent with the TRPV1 population (Fig. 1) (Hwang et al., 2004; Lagerström et al., 2010). Moreover, activation of TRPV1 fibers by capsaicin (Medvedeva et al., 2008) or by increased temperature (Shoudai et al., 2010) results in release of glutamate. Interestingly, when we removed Vglut2 specifically from the Trpv1-Cre neurons, the Vglut2f/f;Trpv1-Cre mice still developed inflammation-associated hyperalgesia with only a small reduction compared with control mice. Because activation of glutamatergic receptors is important for the development and transmission of heat hyperalgesia (Nakayama et al., 2010; Jin et al., 2012), we find it likely that other players compensate for the loss of VGLUT2-mediated glutamatergic transmission in the Trpv1-Cre population. VGLUT3 shows very little, if any, overlap with the TRPV1 population (Seal et al., 2009), but the TRPV1 population may coexpress VGLUT1, which could uphold a glutamatergic tone from the TRPV1 neurons. In addition, neuropeptides expressed in the primary afferent neurons may compensate for the loss of glutamatergic signaling in Vglut2f/f;Trpv1-Cre mice.

Our analysis showed that primary afferents, and the Trpv1-Cre population in particular, contains both SP and CGRP. SP is one of the main pain modulators (Randic et al., 1990; Hokfelt et al., 1994), and previous research has suggested an important role for neuropeptides in the development of peripheral and central sensitization in persistent states of pain (Lembeck et al., 1981; Morton et al., 1989; Dray, 1996). CGRP is involved in pain neurotransmission at different levels of the nervous system and acts via potentiation of the excitatory effect induced by diverse noxious stimuli (Biella et al., 1991). The role of CGRP in nociception has been demonstrated in studies of migraine (Benemei et al., 2009), noxious heat (Mogil et al., 2005), mechanical hyperalgesia (Hirsch et al., 2013), and secondary hyperalgesia (Zhang et al., 2001). Moreover, blockade of the TRPV1 receptor reduces the release of both glutamate and CGRP (Puttfarcken et al., 2010), and stimulation by capsaicin, noxious heat, or protons increased CGRP release from TRPV1 neurons (Sauer et al., 2001; Fischer et al., 2003; Bernardini et al., 2004). Our data indeed show that pharmacologic blockade of either SP or CGRP transmission together with genetic ablation of VGLUT2-mediated glutamatergic signaling from Trpv1-Cre neurons prevents the development of heat hyperalgesia.

Although the genetic abrogation of VGLUT2-mediated neurotransmission from the Trpv1-Cre population is involved in inflammation-induced hyperalgesia, we cannot conclude that the role for SP and CGRP in hyperalgesia is mediated exclusively by the Trpv1-Cre population because we base our findings on systemic treatments. Other Trpv1-Cre negative primary afferent populations may therefore have additional effects on the observed phenotypes, and the use of pharmacologic agents can result in additional off-target effects. However, the major part of the Trpv1-Cre population does express CGRP and SP, and activation of Trpv1 primary afferents leads to release of both neuropeptides, suggesting cotransmission.

To better understand the systemic effects, we included control groups consisting of wild-type mice that were provoked by systemic treatments of the peptide antagonists Win51708 and BINB4096BS, either alone or in combination. Single antagonist administration never led to any detectable change in the development of hyperalgesia, which could explain why hyperalgesia associated with peripheral inflammation can develop in the absence of the precursor for SP (Cao et al., 1998) or the neurokinin 1 receptor (De Felice et al., 1998). In contrast, we observed a decreased pain response only when the antagonist administration was combined with the Trpv1-Cre population specific deletion of Vglut2.
Surprisingly, the combined administration of peptide antagonists prevented the control group from developing hyperalgesia (Figs. 2I and 3I). This observation raises two important points. First, it begins to suggest that the combined activity of peptides can transmit inflammatory heat pain without involvement of glutamate. Second, the counterintuitive decrease in pain response after pain provocation (hypalgesia, see Figs. 2, I and J, and 3, I and J) suggests that the peptide antagonists may display an additive effect on analgesia over time. To verify this, we performed sham treatments in which we analyzed the response to heat after the injections of each antagonist separately or in combination but without inducing peripheral inflammation (Supplemental Fig. 3). When delivered separately, neither the SP nor the CGRP antagonist affected the response latency over time. However, when the two antagonists were injected concomitantly, the response latency to heat increased after the second round of injections. However, the pain-reducing effect from the concomitant injections was less pronounced compared with the pain-reducing effect after the introduction of Carragenan-induced inflammation (see Fig. 3, I and J), also in this case most pronounced after the second round of injections. This result is somehow puzzling because it indicates that the injury itself induces an analgesic effect through mechanisms unknown to us. Notably, to our knowledge, this is the first time where the combination of two peptidergic inhibitors has been shown to cause hypalgesia in both healthy and diseased states.

The TRPV1 population has previously been found to be redundant for the development of punctuate hyperalgesia after peripheral inflammation (Mishra et al., 2011), which is consistent with our findings with the R26DTA/w2,Trpv1-Cre line. However, our data imply that in a background of reduced peptidergic signaling cascade may therefore become therapeutic targets for the treatment of hyperalgesia. Furthermore, it becomes therapeutic targets for the treatment of hitherto unknown requirements for the development of punctuate hyperalgesia. Primary afferent tachykinins are required to experience moderate to intense pain. Nature 392:390–394.


Lagerström MC, Rogo K, Abrahamson J, Persson E, Nordenkamp K, Oland C, Smith C, Mendez JA, and Chen ZF et al. (2010) VGLUT2-dependent glutamatergic transmission from Trpv1-Cre neurons do contribute to the development of mechanical hyperalgesia, thus presenting a more complex situation and an expanded role for the Trpv1 population.

In summary, Trpv1-Cre–positive neurons are necessary for developing heat hyperalgesia associated with states of persistent inflammation. We show that these pain states are mediated by cooperative actions of glutamate, CGRP, and SP, uncovering hitherto unknown requirements for the development of hyperalgesia. Future identification of peripheral or spinal-specific components of the VGLUT2-dependent signaling cascade may therefore become therapeutic targets for the treatment of acute and persistent states of pain, especially if combined with reduction of peptidergic signaling.

Authorship Contributions

Participated in research design: Rogoz, Kullander, Lagerström.

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References


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