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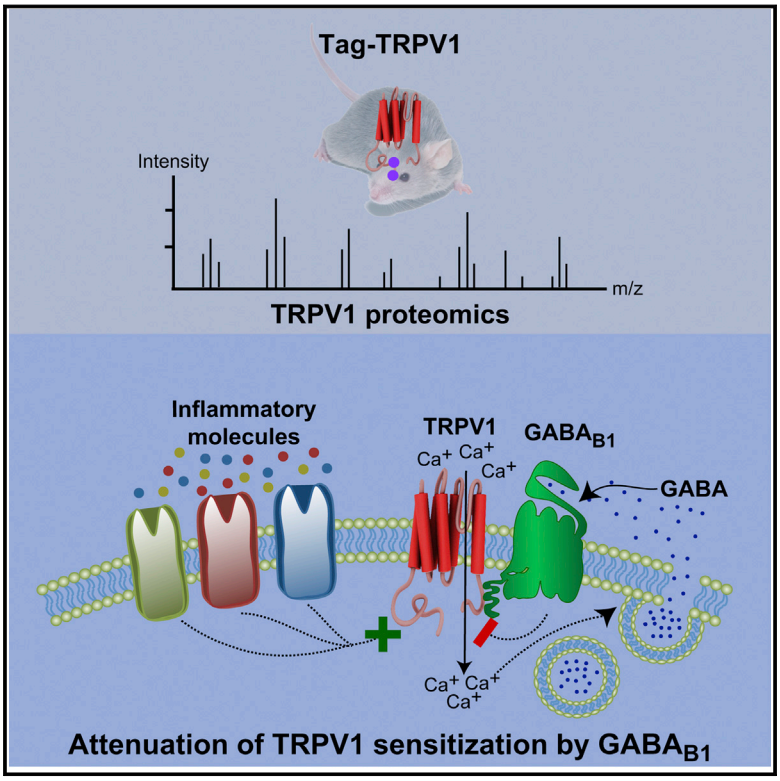
GABA Blocks Pathological but Not Acute TRPV1 Pain Signals

Graphical Abstract



Highlights

- GABA$_{B1}$ forms a complex with TRPV1 to counteract inflammatory pain
- GABA$_{B1}$ modulates TRPV1 via a non-canonical, GABA$_{B2}$-independent pathway
- TRPV1 activation triggers GABA release from peripheral nerve endings
- GABA serves as a modulator of nociceptor sensitization in the periphery

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In Brief

The neurotransmitter GABA is released upon stimulation of the pain receptor TRPV1 and engages a non-canonical signaling pathway that inhibits only hyperactive TRPV1, leaving homeostatic pain responses intact.

Hanack et al., 2015, Cell *160*, 759–770
February 12, 2015
http://dx.doi.org/10.1016/j.cell.2015.01.022

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GABA Blocks Pathological but Not Acute TRPV1 Pain Signals

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http://dx.doi.org/10.1016/j.cell.2015.01.022

SUMMARY

Sensitization of the capsaicin receptor TRPV1 is central to the initiation of pathological forms of pain, and multiple signaling cascades are known to enhance TRPV1 activity under inflammatory conditions. How might detrimental escalation of TRPV1 activity be counteracted? Using a genetic-proteomic approach, we identify the $GABA_{B1}$ receptor subunit as bona fide inhibitor of TRPV1 sensitization in the context of diverse inflammatory settings. We find that the endogenous $GABA_B$ agonist, GABA, is released from nociceptive nerve terminals, suggesting an autocrine feedback mechanism limiting TRPV1 sensitization. The effect of $GABA_B$ on TRPV1 is independent of canonical G protein signaling and rather relies on close juxtaposition of the $GABA_{B1}$ receptor subunit and TRPV1. Activating the $GABA_{B1}$ receptor subunit does not attenuate normal functioning of the capsaicin receptor but exclusively reverts its sensitized state. Thus, harnessing this mechanism for anti-pain therapy may prevent adverse effects associated with currently available TRPV1 blockers.

INTRODUCTION

Pathological forms of pain are usually triggered by injury or inflammation of peripheral sensory neurons of the pain pathway. A diverse set of inflammatory stimuli can sensitize nociceptive neurons to promote pain hypersensitivity. As a consequence, inhibition of individual inflammatory pathways as a means to attenuate pain is a problematic approach for drug development, as parallel signaling cascades are still able to drive pathological, pain-promoting sensitization (Gold and Gebhart, 2010).

One receptor that has been found to serve as downstream integrator of many inflammatory pathways and thus holds great hope for anti-pain therapy is the capsaicin receptor TRPV1 (Caterina et al., 1997; Tominaga et al., 1998).

Next to its physiological function as a detector of noxious stimuli, a large body of literature attests to a crucial pathological role for TRPV1. Importantly, inflammatory sensitization leads to dramatically reduced activation thresholds of TRPV1, producing hyperalgesia and pain hypersensitivity. Indeed, *Trpv1*$^{-/-}$ animals completely lack thermal hyperalgesia, confirming TRPV1's central role as integrator of disparate inflammatory pathways (Caterina et al., 2000; Davis et al., 2000).

TRPV1's central role in pathological forms of pain has sparked intense efforts to develop TRPV1 antagonists. However, blocking TRPV1 activity per se leads to impaired noxious heat sensation and produces hypothermia, attesting to a role of this temperature-gated receptor in homeostatic thermoregulation. Therefore, preventing and counteracting sensitization of the receptor while leaving basal TRPV1 activity untouched has been suggested to be a more promising avenue for pain therapy (Szallasi and Sheta, 2012; Vay et al., 2012; Woolf, 2010).

Combining mouse genetics with proteomics, here we report the identification of the $GABA_{B1}$ receptor as a modulator of TRPV1 sensitization.

Canonical $GABA_B$ signaling requires $GABA_{B1}$ and $GABA_{B2}$ receptor subunits to orchestrate activation of inhibitory G proteins (Gassmann et al., 2004; Jones et al., 1998; Kaupmann et al., 1998; Kuner et al., 1999; White et al., 1998). Surprisingly, the $GABA_{B2}$ subunit is dispensable for mediating inhibition of TRPV1 sensitization, demonstrating that the underlying mechanism diverges from canonical $GABA_B$ receptor signaling.

Collectively, our results establish an endogenous GABA/$GABA_{B1}$ feedback mechanism that keeps TRPV1-mediated pain hypersensitivity in check.

RESULTS

Transgenic Tag-TRPV1 Recapitulates Native TRPV1 Function

Given the complex integrative function of the capsaicin receptor, we reasoned that analysis of TRPV1 accessory proteins would shed light on unrecognized modulation of the receptor. Therefore, we utilized a genetic-biochemical approach to identify components of TRPV1 protein complexes isolated from native sensory tissue of transgenic animals.

To achieve efficient and specific biochemical purification of TRPV1 protein complexes for downstream analysis by mass spectrometry, we generated mice expressing an affinity-tagged

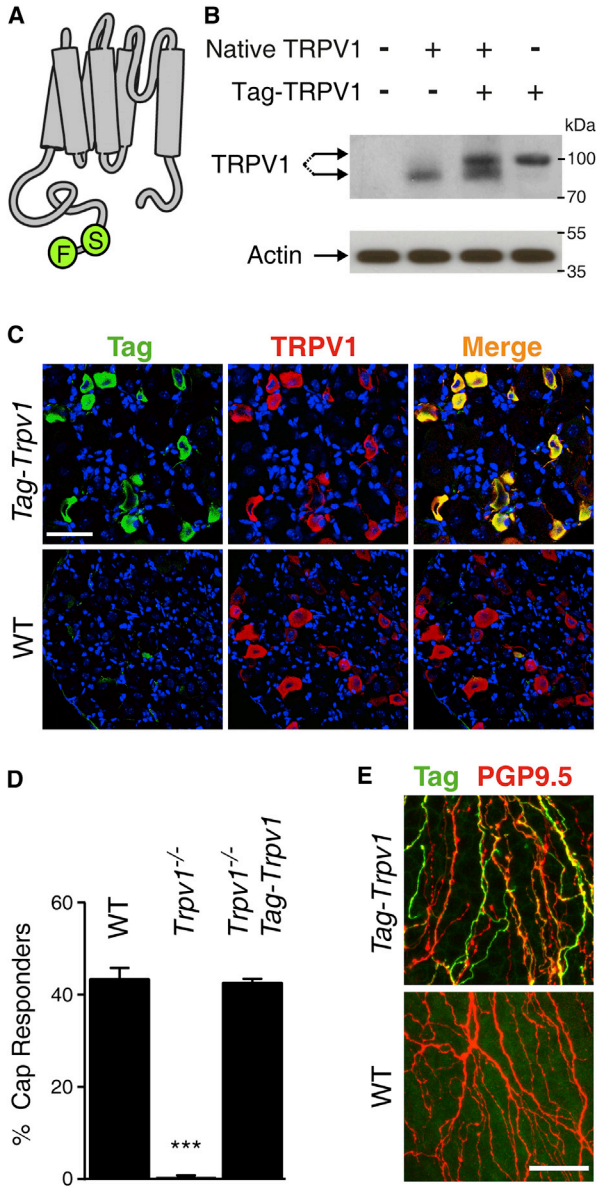

Figure 1. Tag-TRPV1 Reproduces Native TRPV1 Expression and Function

(A) Cartoon depicting Tag-TRPV1.

(B) Western blot of solubilized membrane fractions of DRG derived from WT, *Trpv1*$^{-/-}$, and *Trpv1*$^{-/-}$;*Tag-Trpv1* mice.

(C) Immunostaining of DRG derived from WT and *Tag-Trpv1* mice with anti-Flag (green) and anti-TRPV1 (red) antibodies reveals that Tag-TRPV1 recapitulates the expression profile of the native receptor.

(D) Tag-TRPV1 expression rescues capsaicin responses in *Trpv1*$^{-/-}$ mice. Cultured DRG neurons obtained from WT, *Trpv1*$^{-/-}$, and *Trpv1*$^{-/-}$;*Tag-Trpv1* mice were challenged with capsaicin and the proportion of responders assessed by calcium imaging. Error bars represent SEM.

(E) Immunostaining of Tag-TRPV1 (anti-Flag antibody, green) and the pan-neuronal marker PGP9.5 (red) of corneas derived from WT and *Tag-Trpv1* mice reveals that Tag-TRPV1 protein is transported to peripheral sensory endings. Scale bars, 50 μm.

See also Figure S1.

version of TRPV1 (Tag-TRPV1, Figure 1A). Western blotting of protein extracts derived from sensory ganglia (dorsal root ganglia, DRG) of transgenic animals revealed the expected size of the tagged receptor protein, which is slightly shifted toward higher molecular weight compared to native (untagged) TRPV1 (Figure 1B).

To assure identification of physiological relevant TRPV1 protein complex components, we first verified cell-type-specific expression and functionality of the tagged receptor in BAC transgenic animals. Tag-TRPV1 expression recapitulated the native profile in DRG (Figures 1C, S1A, and S1B). Accordingly, the tagged receptor rescued capsaicin responses in DRG sensory neurons derived from transgenic animals crossed onto the TRPV1 knockout background (Figures 1D and S1C). Importantly, the Tag did not impair trafficking of TRPV1 to peripheral and central nerve terminals (Figures 1E and S1D).

Finally, we tested TRPV1-dependent nociceptive behavior. Whereas we observed normal responses to heat stimuli in a transgenic mouse line expressing close to physiological levels of Tag-TRPV1, a second line, expressing high levels of the tagged receptor (Figure S1E), exhibited significantly reduced pain thresholds (Figure S1F). These findings not only prove the functionality of the tagged receptor in vivo but also underscore TRPV1's predominance in mediating heat hyperalgesia.

Mass Spectrometry Reveals the $GABA_{B1}$ Subunit as a Constituent of TRPV1 Protein Complexes

Next, utilizing the Strep or Flag moiety of the tagged receptor, we affinity purified protein complexes extracted from DRG of transgenic animals expressing both the tagged and the native form of the receptor. Wild-type animals devoid of the tagged receptor served as control.

Mass spectrometry revealed the presence of TRPV2—a TRP family member previously shown to interact with TRPV1 (Rutter et al., 2005)—in purified protein complexes, further validating our approach. The $GABA_{B1}$ receptor subunit was identified as one of the most abundant components of native TRPV1 protein complexes, regardless of Tag-TRPV1 expression level (Figures 2A and S2A and Table S1).

Interestingly, $GABA_B$ receptors in the CNS have long been known to mediate analgesia (Sokal and Chapman, 2003). However, $GABA_B$ mechanisms specifically targeting the peripheral nociceptive pathway have not been identified. Surprisingly, $GABA_B$ receptors are robustly expressed in peripheral nociceptive neurons (Charles et al., 2001; Towers et al., 2000), suggesting an unrecognized role for $GABA_B$ in the pain pathway. We were able to confirm these results and found that most, if not all, TRPV1-positive sensory neurons express the $GABA_{B1}$ subunit (Figure 2B).

We validated the presence of $GABA_{B1}$ in TRPV1 protein complexes by immunoprecipitation (IP) of Tag-TRPV1 or $GABA_{B1}$ (Figures 2C and 2D).

To further confirm the close proximity of the two transmembrane receptors in their native environment, we carried out proximity ligation assays (PLA). In this assay, a fluorescent signal is generated when two proteins of interest either physically interact directly or coexist within close molecular distance (Söderberg et al., 2006). We therefore cultured DRG neurons of *Tag-Trpv1*

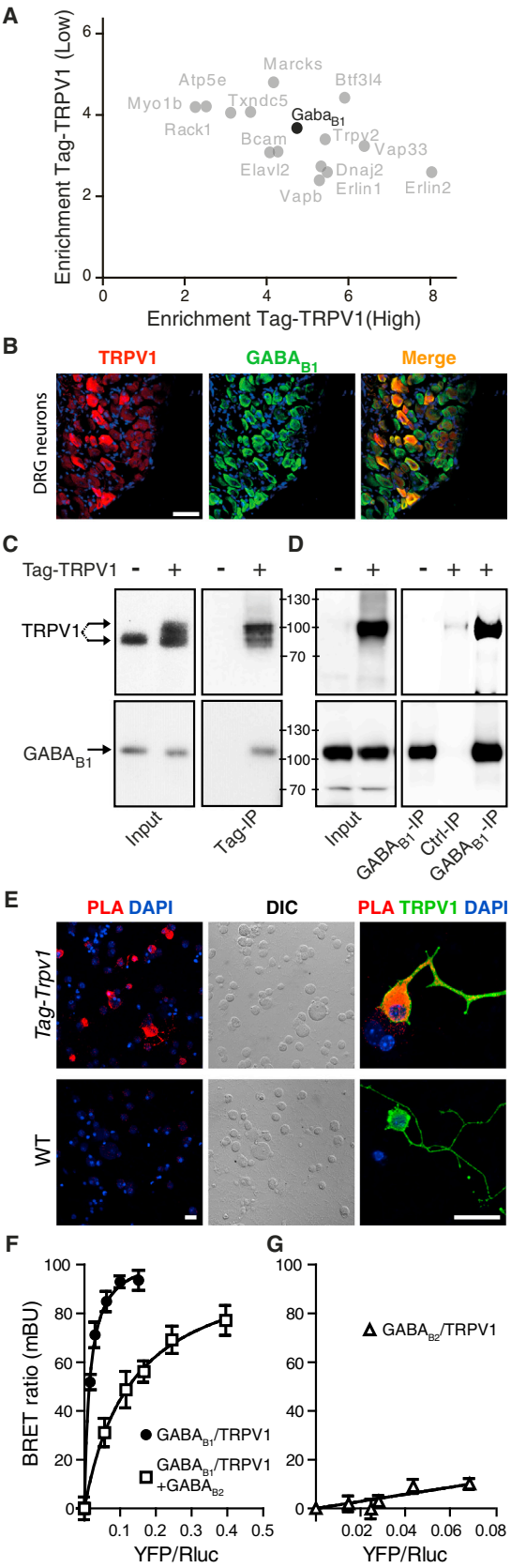

Figure 2. $GABA_{B1}$ and TRPV1 Reside in Common Protein Complexes

(A) Mass spectrometric analysis and quantification of Flag-affinity-purified Tag-TRPV1 protein complexes derived from DRG of mice expressing either high or low levels of Tag-TRPV1. Dot plot shows significantly enriched proteins as $\log_2$ fold ratio (Tag-TRPV1/WT). The analysis reveals specific and robust enrichment for $GABA_{B1a}$ protein.

(B) Immunostaining of DRG of WT mice with anti-TRPV1 (red) and anti-$GABA_{B1}$ (green) antibodies shows co-expression of both receptors in nociceptive neurons. Scale bar, 50 μm.

(C and D) Immunoprecipitations of solubilized protein extracts derived from DRG of *Tag-Trpv1* or WT control animals using immobilized anti-Flag antisera (C), anti-$GABA_{B1}$, or control antisera (D). Western blots are probed with anti-$GABA_{B1}$ (bottom) or anti-TRPV1 (top panels in C) or anti-Flag antisera (top panels in D). $GABA_{B1}$ only co-elutes from affinity resin in the presence, but not in the absence, of TRPV1 (bottom right panel in C). Note that DRG neurons predominantly express the $GABA_{B1a}$ isoform and little or no $GABA_{B1b}$. Similarly, TRPV1 is specifically enriched in isolated $GABA_{B1}$ protein complexes (top right panel in D). Note that endogenous (untagged) TRPV1 protein is precipitated together with Tag-TRPV1, confirming the biochemical isolation of native tetrameric TRPV1 protein complexes (top right panel in C).

(E) PLA of dissociated DRG neurons of *Tag-Trpv1* and WT animals. Antibodies against Flag and $GABA_{B1}$ were used to detect proximity of the two receptors. PLA signal (red) is only present in cell bodies and neurites derived from *Tag-Trpv1* animals. Scale bar, 25 μm.

(F) BRET assay using Rluc-TRPV1 and $GABA_{B1}$-YFP fusion proteins in the presence or absence of $GABA_{B2}$, showing that TRPV1 and $GABA_{B1}$ strongly interact and that $GABA_{B2}$ competes with TRPV1 for $GABA_{B1}$ binding. Error bars represent SEM.

(G) No interaction was detected between Rluc-TRPV1 and $GABA_{B2}$-YFP. Error bars represent SEM.

See also Figure S2 and Table S1.

mice and wild-type control animals and performed PLA using anti-Flag and anti-$GABA_{B1}$ antibodies. A robust PLA signal was detected in *Tag-Trpv1* neurons, but not in WT controls, demonstrating that a TRPV1-$GABA_{B1}$ complex is present in intact sensory neurons and their projecting neurites (Figure 2E).

Intriguingly, we were not able to detect the $GABA_{B2}$ subunit either by mass spectrometry or by western blotting of TRPV1 protein complexes (Figures S2B and S2C). Similarly, KCTD proteins—found to constitutively interact with the $GABA_{B2}$ receptor subunit (Schwenk et al., 2010)—were absent, and only $GABA_{B1}$ selectively co-purified with TRPV1 complexes.

Additionally, when co-expressing TRPV1, $GABA_{B1}$, and $GABA_{B2}$, lower amounts of $GABA_{B1}$ protein were detected in TRPV1 IPs when compared to samples containing TRPV1 and $GABA_{B1}$ alone (Figures S2D and S2E). These findings suggested that TRPV1 and $GABA_{B2}$ might compete for binding to $GABA_{B1}$. To further examine this possibility, we carried out bioluminescence resonance energy transfer (BRET) assays (Ayoub and Pfleger, 2010) and expressed *Renilla* Luciferase BRET donor (Rluc-TRPV1) and YFP BRET acceptor ($GABA_{B1}$-YFP, $GABA_{B2}$-YFP, or YFP-TRPV1) fusion proteins in a heterologous cell expression system. While $GABA_B$ BRET probes have been characterized previously (Adelfinger et al., 2014), we first confirmed the functionality of the TRPV1 BRET probes (Figures S2F and S2G).

The BRET assay confirmed close association of TRPV1 and $GABA_{B1}$ (Figure 2F). Again, no interaction was detectable between TRPV1 and $GABA_{B2}$ (Figure 2G). Moreover, similar to the co-IP experiments (Figures S2D and S2E), co-expression

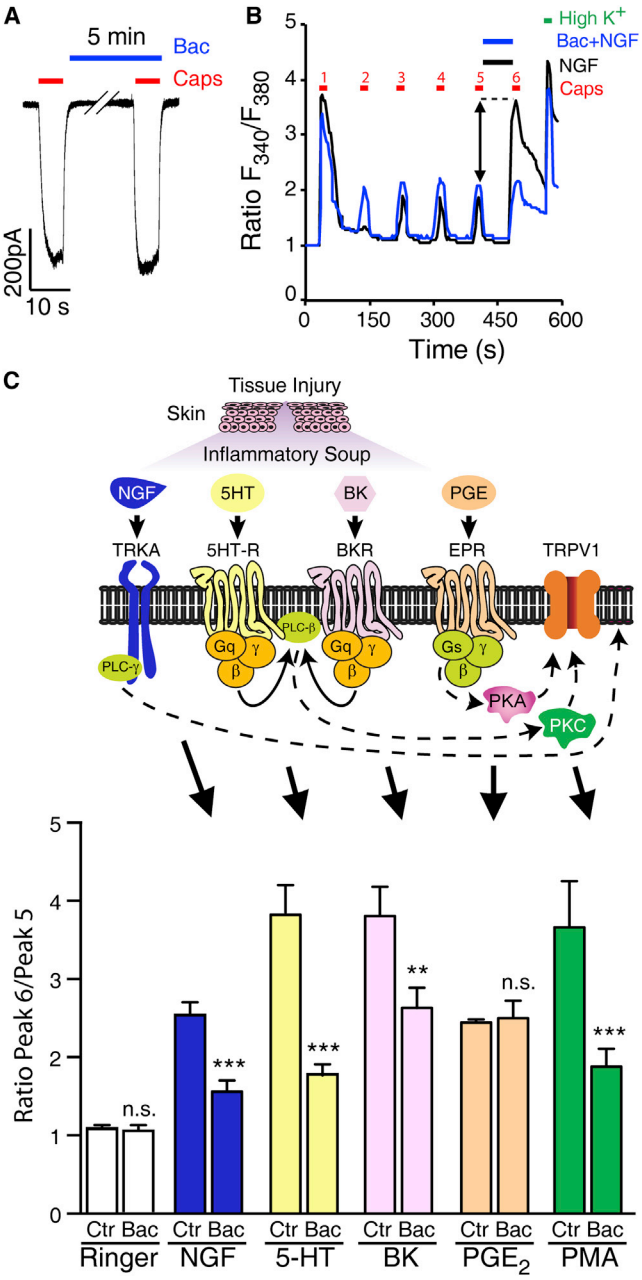

Figure 3. Effect of Baclofen on Native TRPV1 Receptors in Cultured Sensory Neurons

(A) Electrophysiological recordings of cultured DRG neurons. TRPV1 currents elicited by capsaicin (500 nM) were not affected by application of 100 μM baclofen. Rise time (10%–90%, in s): control, 6.5 ± 1.8; Baclofen, 5.8 ± 1.3. Decay (tau, s): control, 7.6 ± 1.5; Baclofen, 6.8 ± 1.8.

(B) Ca^{2+} responses (measured as normalized fluorescence ratios) of DRG neurons stimulated repetitively with capsaicin (100 nM). NGF (100 ng/ml) sensitized TRPV1 activity resulting in a larger response magnitude. Co-application of baclofen (100 μM) with NGF resulted in inhibition of TRPV1 sensitization.

(C) Top: simplified cartoon showing the release of inflammatory mediators during tissue injury. Components of the "inflammatory soup" activate diverse receptor types, which signal via PKC, PKA, and lipid modifiers, such as PLC, to mediate TRPV1 sensitization. Bottom: quantification of averaged fluorescence ratios as shown in (B). Bar graphs represent the ratios between peaks 6 and 5 (after and before application of inflammatory agents) in the presence or absence of baclofen (100 μM). Baclofen effectively attenuated TRPV1 sensitization induced by NGF (100 ngl/ml), serotonin (100 μM), bradykinin (10 nM), and PMA (1 μM), but not by PGE_2 (1 μM). Cells treated with vehicle or baclofen served as a negative control. Error bars represent SEM.

See also Figure S3.

of $GABA_{B2}$ interfered with the interaction of Rluc-TRPV1 and $GABA_{B1}$-YFP (Figure 2F), shown by the increased amount of BRET acceptor ($GABA_{B1}$-YFP) required to reach 50% of the maximal BRET signal (Figures 2F, S2H, and S2I).

In summary, $GABA_{B1}$ and TRPV1 form a protein complex that appears to lack the G protein signaling subunit $GABA_{B2}$.

$GABA_B$ Signaling Counteracts Sensitization of TRPV1

Given $GABA_B$'s established role in modulating KIR3 and Ca_V channels (Padgett and Slesinger, 2010), it is conceivable that $GABA_B$'s known analgesic property could stem in part from inhibiting TRPV1 activity.

Different to its effect on KIR3 channels (Figure S3A), neither calcium imaging nor electrophysiological recordings revealed any influence of the $GABA_B$ agonist baclofen on TRPV1 currents elicited by capsaicin (Figures 3A and S3B). Tachyphylaxis, a form of rapid TRPV1 desensitization, was also not affected by baclofen application (Figure S3C).

Under inflammatory conditions, multiple pathways sensitize TRPV1. Paramount among the different inflammatory sensitizers is NGF, activating parallel TRKA receptor signaling cascades that converge on the capsaicin receptor to enhance its sensitivity.

We thus tested whether NGF-mediated TRPV1 sensitization is modulated by $GABA_B$ activation. To this end, we monitored nociceptive neuron populations for NGF-enhanced TRPV1 activity using calcium imaging (Bonnington and McNaughton, 2003). Indeed, pre-incubation of sensory neurons with baclofen (but not saline) robustly blocked TRPV1 sensitization (Figures 3B, 3C, and S3D).

Apart from the receptor tyrosine kinase TRKA, several G-protein-coupled receptors (GPCRs) mediate TRPV1 sensitization (Figure 3C). Notorious in this regard are bradykinin- and serotonin-triggered GPCR cascades (Chuang et al., 2001; Huang et al., 2006). Strikingly, baclofen was equally effective in inhibiting TRPV1 sensitization induced by both inflammatory mediators (Figures 3C, S3E, and S3F).

Given that the initial signaling events controlled by the two GPCRs are quite different to that of TRKA, these results suggest that $GABA_B$ exerts its effect at a converging point downstream of the different pathways, potentially at TRPV1 itself. In agreement with this hypothesis, bypassing upstream inflammatory receptor signaling by direct pharmacological PKC activation also resulted in baclofen-reversible TRPV1 sensitization (Figure 3C).

The inflammatory prostaglandin PGE_2 mediates TRPV1 sensitization largely via a G_s-coupled/PKA pathway (Gu et al., 2003; Lopshire and Nicol, 1998; Moriyama et al., 2005). Intriguingly, baclofen did not inhibit PGE_2-mediated TRPV1 sensitization (Figure 3C). Canonical $GABA_B$-$G_{i/o}$ coupling would be expected to effectively counteract G_s-mediated sensitization. In agreement

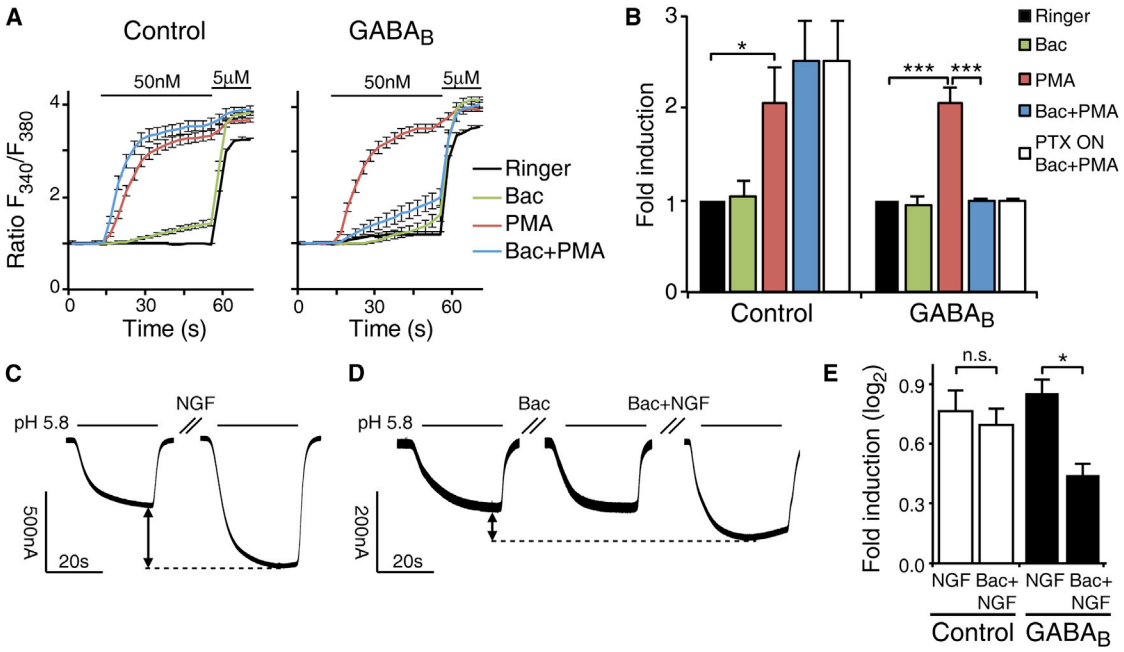

Figure 4. Reversal of TRPV1 Sensitization by GABA$_B$ Is Recapitulated in Cellular Expression Systems In Vitro

(A) Calcium responses elicited by 50 nM capsaicin pulses were assessed in TRPV1-HEK293 cells with or without GABA$_B$ after incubation with either baclofen (100 μM), PMA (1 μM), or the combination of PMA+baclofen as indicated. Depicted are representative ratiometric traces from one experiment (n = 50 cells). PMA promotes PKC-induced TRPV1 sensitization, and baclofen inhibits sensitization only in the presence of GABA$_B$. Error bars represent SEM.

(B) Quantification of experiments shown in (A). PTX (500 ng/ml) did not abrogate the baclofen effect, indicating that $G_{i/o}$ proteins are not involved in counteracting TRPV1 sensitization by GABA$_B$.

(C and D) Electrophysiology recordings of oocytes injected with *Trpv1*, *TrkA*, and *Gaba$_B$*, following TRPV1 activation with pH 5.8, before and after treatment with NGF (100 ng/ml) (C) and NGF + baclofen (D).

(E) Quantification of the experiments shown in (C) and (D); sensitization was measured as log_2 ratio of capsaicin-elicited steady-state currents before and after drug treatment. TRPV1 sensitization mediated by NGF is attenuated by baclofen only in the presence of GABA$_B$. Error bars represent SEM.

See also Figure S4.

with the apparent absence of the GABA$_{B2}$ subunit from TRPV1-GABA$_{B1}$ complexes, this result further pointed at a potentially unrecognized GABA$_B$ mechanism modulating TRPV1 sensitization independent of $G_{i/o}$ signaling.

Non-Canonical GABA$_B$ Signaling Counteracts TRPV1 Sensitization

To test directly whether $G_{i/o}$ protein is involved in mediating the observed GABA$_B$ effect, we pre-incubated sensory neurons with pertussis toxin (PTX). This selective and potent blocker of $G_{i/o}$ proteins did not attenuate baclofen's effect on TRPV1 sensitization (Figures S4A and S4B), whereas another $G_{i/o}$-signaling cascade present in DRG neurons was effectively inhibited by PTX (Figures S4C and S4D), thereby serving as a positive control.

The positive allosteric modulator CPG7930 potentiates GABA$_B$ receptor signaling by binding to the GABA$_{B2}$ subunit (Binet et al., 2004). However, inhibition of TRPV1 sensitization was not significantly enhanced by CPG7930 (Figure S4E) at concentrations that robustly potentiated GABA$_B$-induced KIR3 currents (Figure S4F), again pointing toward a non-canonical GABA$_B$ pathway.

Next, we tested GABA$_B$'s modulatory effect on TRPV1 sensitization in two heterologous expression systems.

We first recapitulated PKC-induced TRPV1 sensitization in a TRPV1-HEK293 cell line (Siemens et al., 2006). Pre-incubating the cells with baclofen in the absence of GABA$_B$ had no effect and did not abrogate PKC-mediated TRPV1 sensitization. However, co-expressing GABA$_B$ completely reverted TRPV1 hyperactivity upon baclofen incubation (Figures 4A and 4B). Similar to our results in native sensory neurons, PTX did not prevent baclofen to exert inhibition of TRPV1 sensitization (Figure 4B).

Additionally, we reconstituted the NGF sensitization pathway and expressed the TRKA receptor together with TRPV1 and GABA$_B$ in *Xenopus* oocytes. Again, we found that baclofen was able to significantly reduce NGF sensitization in a GABA$_B$-dependent manner (Figures 4C–4E).

In agreement with the observed close molecular proximity of the two receptors, these results demonstrate a direct inhibitory effect of GABA$_B$ receptors on TRPV1 sensitization, independent of classical $G_{i/o}$ signaling downstream of GABA$_{B2}$.

GABA$_{B1}$ Reverts TRPV1 Sensitization Independent of GABA$_{B2}$ Signaling

In order to examine the role of the two GABA$_B$ receptor subunits in more detail, we next employed GABA$_B$ receptor knockout mice. A conditional "floxed" GABA$_{B1}$ mouse line (*Gaba$_{B1}^{f/f}$*; Haller et al., 2004) was crossed to *Trpv1*-Cre mice (Cavanaugh et al.,

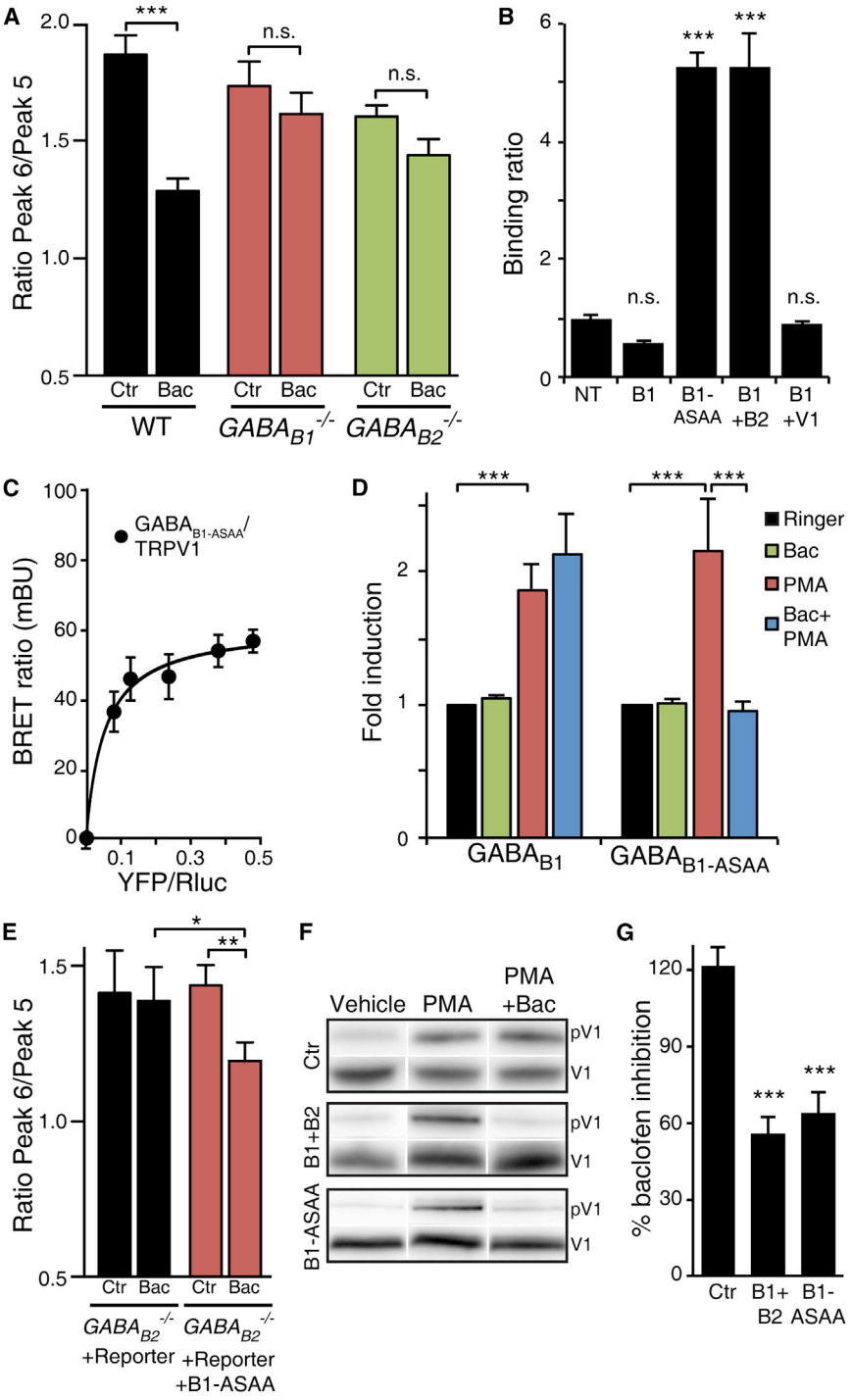

Figure 5. Surface Expression of $GABA_{B1}$ in the Absence of $GABA_{B2}$ Is Sufficient to Mediate Inhibition of TRPV1 Sensitization

(A) Quantification of capsaicin responses in DRG neurons from *Trpv1-Cre;Gaba$_{B1}^{f/f}$* (*Gaba$_{B1}^{-/-}$*), *Gaba$_{B2}^{-/-}$*, and WT control mice in the presence or absence of baclofen (100 μM) following incubation with serotonin (100 μM). Error bars represent SEM.

(B) Cell surface expression of $GABA_{B1}$ and $GABA_{B1\text{-}ASAA}$ in the presence or absence of $GABA_{B2}$ or TRPV1 was assessed by measuring the binding of [^{3}H]CGP54626 in intact CHO cells. Unspecific binding to non-transfected (NT) cells is set to 1. Error bars represent SEM.

(C) BRET experiment demonstrating that RLuc-TRPV1 and surface-localized $GABA_{B1\text{-}ASAA}$-YFP fusion proteins interact. Error bars represent SEM.

(D) Calcium responses in TRPV1-HEK293 cells transfected with $GABA_{B1}$ or $GABA_{B1\text{-}ASAA}$ after incubation with either baclofen (100 μM), PMA (1 μM), or the combination of PMA+baclofen. Error bars represent SEM.

(E) Quantification of serotonin-sensitized capsaicin responses of cultured *Gaba$_{B2}^{-/-}$* DRG neurons transfected with a fluorescent reporter (tomato) in the presence or absence of $GABA_{B1\text{-}ASAA}$ plasmid, incubated with baclofen (100 μM). Error bars represent SEM.

(F) Representative western blot showing phosphorylated (pV1) and total TRPV1 protein (V1) of HEK cells transfected with control plasmid, $GABA_{B1+B2}$, or $GABA_{B1\text{-}ASAA}$ alone after treatment with PMA, PMA+baclofen, or vehicle. Three biological replicates are loaded onto the gel, but only one representative lane for each condition is shown.

(G) Quantification of (F). Phosphorylated TRPV1 intensity values were normalized to total TRPV1 intensities and expressed as percent of baclofen-mediated inhibition (PMA+baclofen/PMA). Error bars represent SEM.

See also Figure S5.

2011) to specifically ablate the $GABA_{B1}$ subunit in TRPV1-positive sensory neurons (Figure S5A). As expected, inhibition of TRPV1 sensitization by baclofen was absent in sensory neurons derived from conditional *Trpv1-Cre;Gaba$_{B1}^{f/f}$* mice, attesting to baclofen's high specificity and selectivity for the $GABA_{B1}$ subunit (Figure 5A). In DRG neurons derived from *Gaba$_{B2}^{-/-}$* mice (Gassmann et al., 2004), the baclofen effect was also abrogated (Figure 5A), demonstrating that the $GABA_{B2}$ subunit is required for mediating TRPV1 modulation, despite its apparent absence from TRPV1 protein complexes and the dispensability of $G_{i/o}$ signaling downstream of the $GABA_{B2}$ subunit.

It is well established that $GABA_{B1}$ does not translocate to the cell surface autonomously, but the $GABA_{B2}$ subunit mediates trafficking of the heteromeric receptor complex by masking an ER retention signal present in the $GABA_{B1}$ protein (Margeta-Mitrovic et al., 2000; Pagano et al., 2001). We therefore asked whether TRPV1 promotes cell surface translocation of $GABA_{B1}$ independent of the $GABA_{B2}$ subunit. Analyzing cell surface binding of a $GABA_{B1}$-specific radioligand [^{3}H]CGP54626 (Galvez et al., 2001), we find that TRPV1, different to $GABA_{B2}$, does not promote cell surface translocation of $GABA_{B1}$ (Figure 5B).

Mutating the ER-retention signal encoded by the peptide sequence "RSRR" to "ASAA" allows surface translocation of $GABA_{B1}$ independent of $GABA_{B2}$ without changing $GABA_{B1}$'s signaling capability (Margeta-Mitrovic et al., 2000; Pagano et al., 2001). Interestingly, this constitutively surface-localized $GABA_{B1}$ variant, $GABA_{B1\text{-}ASAA}$ (Figure 5B), interacts with TRPV1 similar to its wild-type counterpart (Figure 5C), suggesting that the $GABA_{B1}$-TRPV1 protein complex can form at the cell surface.

Expectedly, ER-retained native $GABA_{B1}$ protein was not in the position to mediate inhibition of TRPV1 sensitization when expressed in the absence of the $GABA_{B2}$ subunit (Figure 5D). Strikingly, surface-localized $GABA_{B1\text{-}ASAA}$ alone robustly inhibited TRPV1 sensitization in HEK293 cells (Figure 5D). Moreover, $GABA_{B1\text{-}ASAA}$ reinstalled baclofen-mediated inhibition of TRPV1 sensitization in $GABA_{B2}$-deficient sensory neurons (Figures 5E, S5B, and S5C), demonstrating the ability of $GABA_{B1}$ to signal to TRPV1 directly and independently of the $GABA_{B2}$ subunit.

PKC phosphorylation of TRPV1 sensitizes the receptor (Mandadi et al., 2006) and is a convergence point downstream of NGF, bradykinin, and serotonin signaling. We therefore examined whether PMA-induced PKC phosphorylation of TRPV1 is attenuated by $GABA_{B1}$ signaling. Indeed, we find that $GABA_{B1\text{-}ASAA}$ alone (similar to the native $GABA_{B1+B2}$ receptor pair) effectively inhibits PKC phosphorylation of TRPV1 (Figures 5F and 5G).

$GABA_B$ Reverts TRPV1 Hypersensitivity in Models of Inflammatory and Neuropathic Pain

Given the robust cellular effects of $GABA_{B1}$ on TRPV1 sensitization, we next tested whether these results translate into $GABA_{B1}$-mediated inhibition of pain hypersensitivity in animal models. One nocifensive assay that allows robust measurement of TRPV1-dependent heat hyperalgesia is the Hargreaves assay. In this assay, inflammatory stimuli, such as NGF and bradykinin, decrease the paw withdrawal latency in a TRPV1-dependent manner (Chuang et al., 2001). Indeed, we observed significant inhibition of sensitization in baclofen-injected mice compared to mice that solely received NGF. No effects were detected in *Trpv1*$^{-/-}$ animals (Figure S6A). To rule out any potential central effects of baclofen (Sokal and Chapman, 2003), we slightly modified the testing paradigm and induced inflammatory hyperalgesia in both hind paws of the same animal (Figure S6B). We found robust and dose-dependent inhibition of sensitization only in the ipsilateral paw that had received baclofen, but not in the contralateral paw (Figures 6A and S6C). Moreover, baclofen's inhibitory effect was completely blunted in conditional *Trpv1-Cre;Gaba*$_{B1}^{f/f}$ mice (Figure 6B). These findings are in agreement with our cellular data (Figure 5A) and demonstrate that $GABA_{B1}$'s beneficial effect on heat hyperalgesia is mediated in peripheral TRPV1-positive nociceptors in a cell-autonomous fashion, rather than in higher-order pain-processing CNS areas.

Similarly, $GABA_{B1}$ receptor activation strongly attenuated serotonin- and bradykinin-triggered heat hyperalgesia but spared PGE_2-triggered heat hyperalgesia (Figures 6A and S6D), an effect that was not further enhanced by the positive allosteric modulator CPG7930 that stimulates $GABA_{B2}$ (Figure S6E).

For therapeutic purposes, it would be beneficial to identify an intervention that is able to revert pre-existing pain hypersensitivity. Applying baclofen at a time point when NGF had already sensitized nociceptors reduced heat withdrawal thresholds (Figure S6F), establishing that $GABA_{B1}$ activation has the capacity for both prevention and reversal of capsaicin receptor sensitization.

Given the ability of the $GABA_{B1}$ subunit to inhibit sensitization triggered by multiple different inflammatory pathways, we asked whether it is also effective in attenuating hyperalgesia as a consequence of intraplantar CFA (complete Freund's adjuvant) injection, a widely used model of persistent inflammatory pain. We found that intraplantar application of baclofen 2 days after inducing CFA-mediated inflammation significantly reduced heat hyperalgesia compared to vehicle-treated controls (Figure 6C). The beneficial effect of a single dose lasted for a minimum of 3 hr and had completely ceased 24 hr after application.

Similar to the cellular assays, our behavior experiments confirm that $GABA_{B1}$ activation specifically attenuates hypersensitivity of TRPV1 but did not impinge upon acute responsiveness of the receptor to either a heat stimulus, as tested in the Hargreaves assay (Figure S6G), or assessed by measuring acute capsaicin-induced nocifensive responses (Figure S6H).

$GABA_{B1}$ and TRPV1 Co-Localize at Peripheral Nerve Endings

Our data suggest that $GABA_{B1}$ receptors mediate their anti-hyperalgesic effect in the periphery. Consequently, $GABA_{B1}$ would be expected to reside in free nerve endings in close proximity to TRPV1. To elucidate whether the two receptors indeed co-localize at nociceptor endings, we utilized transgenic *Gaba*$_{B1}$*-GFP* reporter mice (Casanova et al., 2009). Similar to skin, the cornea is densely innervated by TRPV1-positive sensory fibers, attesting to strong reactions of the eye to inflammatory insults. We analyzed both skin and cornea tissue for co-expression of the two receptors in peripheral nerve endings that project from DRG and trigeminal (TG) sensory neurons, respectively. Not only did we find $GABA_{B1}$ to be present in peripheral nociceptive fibers, but we also observed substantial co-localization with TRPV1 in nerve terminals (Figures 6D–6F and S6I–S6K and Movie S1). In agreement with the close association of TRPV1 and $GABA_{B1}$ found in Tag-TRPV1-positive DRG neurons (Figure 2E), 89% of TRPV1-positive corneal fibers co-localize the two receptors at their terminals (Figure S6L).

GABA Is Released from Peripheral Nociceptors and Modulates Pain Hypersensitivity

Interestingly, we found that local application of the competitive $GABA_{B1}$ receptor antagonist CPG52432 enhanced NGF-mediated TRPV1 sensitization and induced a small but significant increase in mouse pain behavior compared to NGF alone (Figure 7A). This result suggested that GABA, the native agonist of $GABA_A$ and $GABA_B$ receptors, is endogenously present at peripheral nerve endings to produce a basal $GABA_B$ receptor tone that regulates TRPV1 sensitivity. Indeed, in blister fluid obtained from human skin and extracted mouse corneal fluid, we find GABA at concentrations (161 ± 42 nM and 335 ± 54 nM, respectively) sufficient to inhibit TRPV1 sensitization in cultured DRG neurons in a $GABA_{B1}$-dependent manner (Figure 7B).

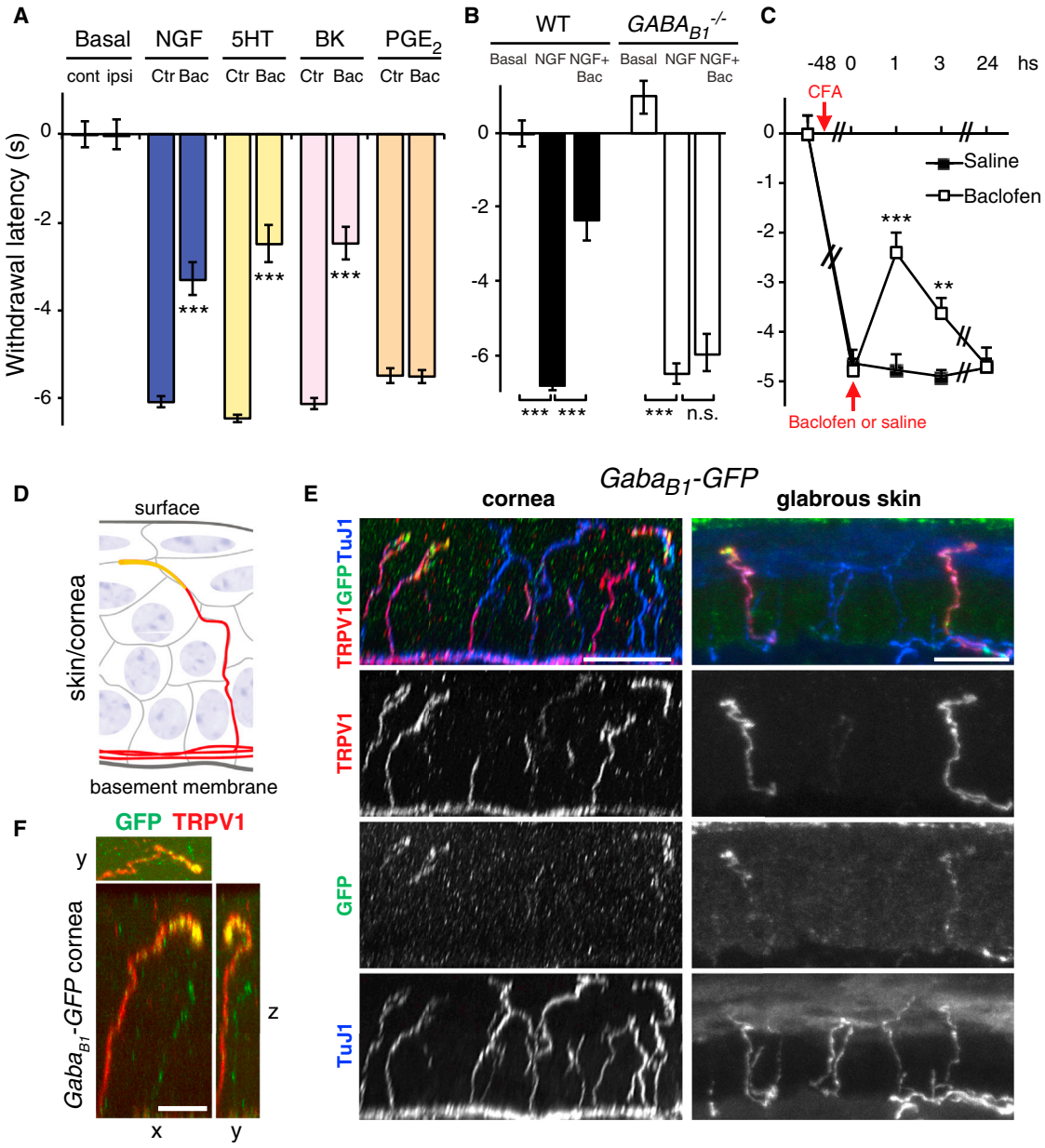

Figure 6. $GABA_{B1}$ Activation in Peripheral Nociceptive Terminals Decreases Thermal Hyperalgesia

(A) Thermal responses were measured using the Hargreaves test. NGF (2 μg/paw), serotonin (5HT, 100 nmol/paw), bradykinin (10 nmol/paw), or PGE_2 (1 nmol/paw) were injected into both mouse hind paws 30 min after unilateral administration of baclofen (Bac, 3 μg/paw) into the ipsilateral paw and vehicle (Ctr) into the contralateral paw. Bar graph shows the differences in mean paw withdrawal latency upon radiant heat stimulation relative to the basal (non-stimulated) withdrawal latency. Baclofen decreased thermal hyperalgesia for all inflammatory mediators, except PGE_2. Basal withdrawal latencies were measured 24 hr before the experiment. Error bars represent SEM.

(B) Baclofen does not attenuate NGF-induced thermal hyperalgesia in conditional *Trpv1-Cre;*$Gaba_{B1}^{f/f}$ mice ($Gaba_{B1}^{-/-}$). Error bars represent SEM.

(C) Heat hyperalgesia 2 days after CFA induction is attenuated by baclofen (3 μg/paw) for a minimum of 3 hr when compared to mice that received vehicle only (saline). Error bars represent SEM.

(D) Cartoon depicting skin or cornea innervation shown in (E) and (F).

(E) Immunostaining of skin and cornea sections obtained from $Gaba_{B1}$*-GFP* transgenic mice and labeled with antisera for TRPV1 (red), GFP (green), and Tuj1 (blue). Scale bars, 20 μm.

(F) Higher magnification of a single corneal fiber. $GABA_{B1}$-GFP localizes in TRPV1-positive fibers and is concentrated at the terminal close to the epithelial surface (demonstrated by orthogonal maximal projections in the x, y, and z dimensions). Scale bar, 10 μm.

See also Figure S6 and Movie S1.

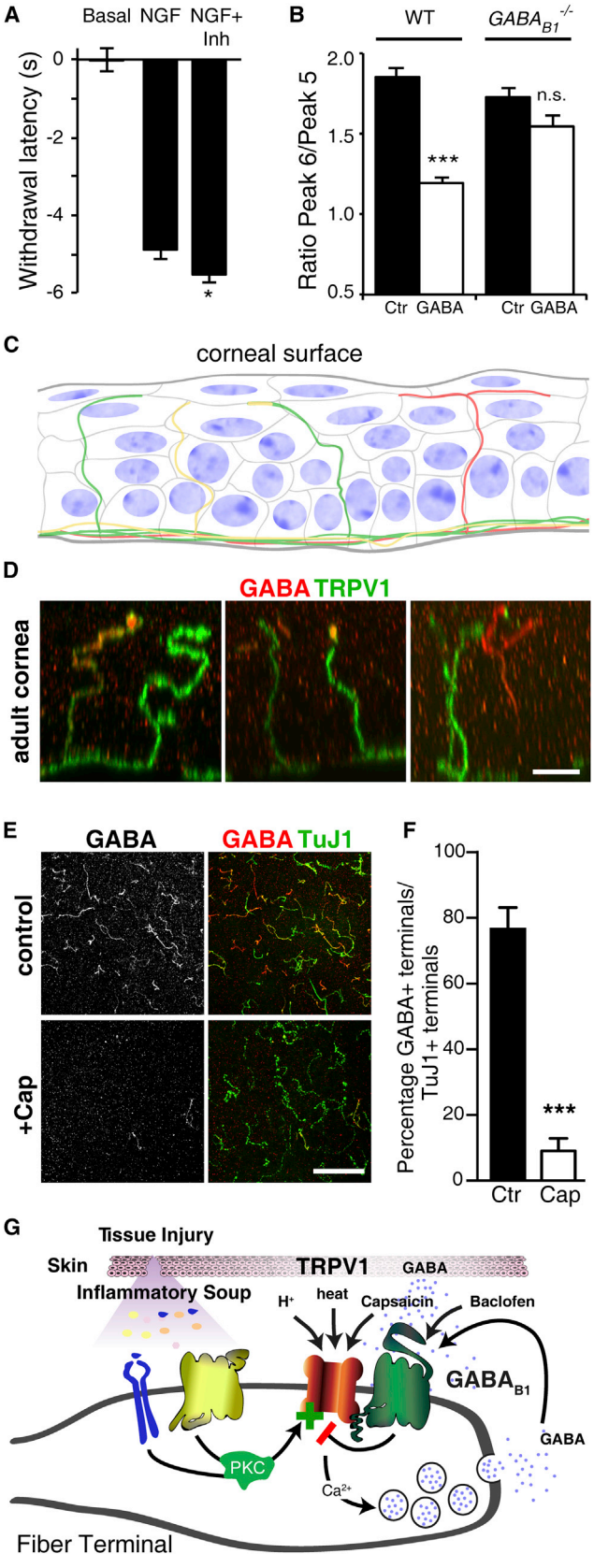

Figure 7. Peripheral Effects and Localization of GABA and Its Release from Peripheral Fibers

(A) Withdrawal latencies in mice sensitized with NGF are significantly reduced by the application of the $GABA_{B1}$ inhibitor CPG52432 (17 nmol/paw) compared to mice that received vehicle. Error bars represent SEM.

(B) 200 nM GABA is sufficient to attenuate serotonin sensitization of TRPV1 in WT but not in *Trpv1-Cre;Gaba*$_{B1}^{f/f}$ (*Gaba*$_{B1}^{-/-}$) sensory neurons. Error bars represent SEM.

(C) Cartoon depicting cornea innervation shown in (D); nerve fibers emerge from the sub-basal nerve plexus and branch out toward the corneal surface.

(D) Sensory fibers innervating the mouse cornea were immune labeled with antisera decorating GABA (red) and TRPV1 (green). GABA is either distributed throughout the length of the fibers (left) or concentrated at the terminals close to the corneal surface (middle) of TRPV1-positive fibers. Although most fibers contain GABA and TRPV1 conjointly, some fibers harbor GABA in the absence of TRPV1 (right). Scale bar, 10 μm.

(E) Corneas of WT mice either treated with capsaicin (10 μM) or vehicle (10 min, 32°C) and subsequently prepared for immunostainings using antisera recognizing GABA and TuJ1. GABA staining was strongly reduced in capsaicin-treated corneas compared to control, indicating that TRPV1 activation stimulates GABA release. Scale bar, 50 μm.

(F) Quantification of (E). Error bars represent SEM.

(G) Model illustrating inhibition of TRPV1 sensitization by $GABA_{B1}$ receptor signaling. TRPV1 is sensitized by a multitude of signaling cascades that are initiated in the context of inflammation. Several cascades converge on PKC, a kinase shown to robustly mediate TRPV1 sensitization via direct receptor phosphorylation. Sensitization augments TRPV1 channel activity, which leads to increased calcium influx and concomitant GABA release to attenuate TRPV1 sensitization via $GABA_{B1}$. Activation of this autocrine loop dampens (or resolves) hyperactivity of TRPV1.

See also Figure S7.

Although GABA is well known for its presence in inhibitory CNS neurons, much less is known about its localization in the periphery. Using a GABA-specific antibody, we found GABA localized to the terminal endings of corneal nociceptors, many of which also express TRPV1 (Figures 7C, 7D, and S7A). Given that primary afferent nociceptors are glutamatergic, it was unexpected to find GABA at their peripheral endings. Presumably, in these fibers, GABA is stored in vesicles as many of the terminals also reacted with antibodies for vGAT, the vesicular GABA transporter (Figure S7B). Additionally, DRG and TG sensory neurons exhibit low Gad2 transcript levels (Figure S7C). Consistently, we find GABA to be present in DRG and TG neuronal cell bodies (Figure S7D), albeit the GABA labeling was less pronounced compared to that observed in TRPV1-positive sensory terminals (Figure 7D), likely reflecting efficient vesicular transport to distal terminals.

Given the juxtaposition of GABA vesicles and TRPV1 at peripheral nerve terminals, we tested whether TRPV1 activation and subsequent calcium influx would constitute an adequate stimulus to release GABA. Stimulating isolated corneas with capsaicin reduces GABA content at the terminals compared to corneas kept without capsaicin, strongly suggesting that GABA release has taken place (Figures 7E and 7F). The capsaicin-mediated release of GABA is specific to TRPV1 activation, as it was absent from capsaicin-stimulated corneas of *Trpv1*$^{-/-}$ mice (Figure S7E).

Taken together, these results support a model by which peripheral GABA limits (and resolves) TRPV1 sensitization via $GABA_{B1}$ receptors, directly at the site where painful stimuli are first encountered (Figure 7G).

DISCUSSION

Genetic-Proteomic Dissection of TRPV1 Modulation

Counteracting sensitization of TRPV1—without blocking acute channel activity—is a promising approach for developing analgesics. Using infusible peptides mimicking intracellular TRPV1 domains, it has been demonstrated that interference with TRPV1 sensitization has beneficial effects in models of inflammatory pain (Fischer et al., 2013). However, in contrast to a multitude of pathways sensitizing TRPV1, endogenous pathways inhibiting sensitization have remained elusive.

Combining a genetic-biochemical approach with quantitative mass spectrometry to probe the molecular environment of native TRPV1 receptors, we report here the identification of the $GABA_{B1}$ receptor as a modulator of TRPV1 sensitization.

Non-Canonical $GABA_B$ Signaling Aborts TRPV1 Sensitization

In CNS neurons, most, if not all, $GABA_B$ receptor effects are mediated by coupling to inhibitory $G_{i/o}$-type G proteins (Padgett and Slesinger, 2010). Activating $G_{i/o}$-coupled signaling in sensory neurons is able to promote TRPV1 sensitization (Forster et al., 2009; Loo et al., 2012). Hence, it is difficult to reconcile the observed inhibitory $GABA_B$ effect on TRPV1 sensitization with a classical $G_{i/o}$-coupled signaling cascade.

Multiple lines of evidence suggest that a $GABA_{B1}$ mechanism that is independent of $GABA_{B2}$ signaling targets TRPV1 hypersensitivity: (1) $GABA_{B2}$ appears to be absent from $GABA_{B1}$-TRPV1 complexes; (2) neither PTX nor CPG7930—inhibiting $G_{i/o}$ activation or enhancing G protein coupling of $GABA_{B2}$, respectively—had any effect on $GABA_B$'s potential to attenuate TRPV1 sensitization; and (3) plasma-membrane-localized $GABA_{B1}$ can inhibit TRPV1 sensitization in the absence of $GABA_{B2}$.

Our data suggest that a ternary complex encompassing TRPV1 and both $GABA_B$ subunits is not favored; rather, $GABA_{B2}$ competes with TRPV1 for $GABA_{B1}$ interaction. Interestingly, it has been shown that TRPV1 activation by capsaicin can promote dissociation of the two $GABA_B$ subunits (Laffray et al., 2007). Thus, it is tempting to speculate that TRPV1 activation promotes the formation of a surface-localized TRPV1-$GABA_{B1}$ liaison at the expense of a heteromeric $GABA_{B1}$-$GABA_{B2}$ receptor complex, thereby priming the nociceptor to guard against sensitization.

$GABA_B$ Inhibits Convergent PKC-Mediated TRPV1 Sensitization

How does the $GABA_{B1}$ receptor mediate its effect on TRPV1? Given the close proximity of the two proteins, it is possible that baclofen-induced conformational changes are directly transmitted onto the TRP ion channel to control and regulate its sensitization status. This would be reminiscent of dopamine receptors, which directly modulate $GABA_A$-type ion channels, independent of a downstream signaling cascade (Liu et al., 2000).

Alternatively, a putative signaling effector downstream of $GABA_{B1}$ may inhibit TRPV1 sensitization. Signaling cascades downstream of $GABA_{B1}$ (and independent of $GABA_{B2}$) have remained elusive. However, β-arrestin2 has been implicated to act downstream of $GABA_B$ receptors, independent of $G_{i/o}$ protein signaling (Lu et al., 2012). β-arrestin2 has also been proposed to modulate tachyphylactic TRPV1 responses (Por et al., 2012), suggesting that this protein could, in principle, couple $GABA_B$ receptors to TRPV1. However, we did not detect β-arrestin2 in TRPV1-$GABA_{B1}$ protein complexes, nor did we find tachyphylactic TRPV1 responses to be modulated by $GABA_B$ receptor activation (Figure S3).

Different to the important functions for β-arrestins in other GPCR signaling cascades, their role in $GABA_B$-mediated pathways is less clear (Perroy et al., 2003; Sudo et al., 2012), suggesting that β-arrestin2 is an unlikely candidate to mediate the modulatory action of $GABA_{B1}$ on TRPV1.

Different than the inflammatory pathways initiated by NGF, serotonin, or bradykinin, we find that PGE_2-triggered sensitization of TRPV1 was not inhibited by the $GABA_{B1}$ receptor. The differential susceptibility to $GABA_{B1}$ receptor modulation coincides with different points of signal convergence on TRPV1; a common denominator of NGF, serotonin, and bradykinin signaling is the activation of PLC/PKC pathways that result in sensitization of the capsaicin receptor (Huang et al., 2006). By triggering PKC activation directly, we find that this branch of TRPV1 sensitization can be inhibited by $GABA_{B1}$ receptor activity, resulting in reduced TRPV1 phosphorylation.

In contrast, PGE_2 signaling leads to PKA-dependent phosphorylation of TRPV1 (Gu et al., 2003; Lopshire and Nicol, 1998; Moriyama et al., 2005), and its sensitizing effect was not attenuated by $GABA_{B1}$. Phosphorylating TRPV1 at different sites may have different functional consequences despite a similar net effect on sensitization—recent studies demonstrate unique structural features of TRPV1 with an upper and lower gate that are independently engaged by different pro-algesic agents to open and modulate ion channel conductance (Cao et al., 2013). Thus, it is conceivable that differential phosphorylation by PKC and PKA mediates different types of TRPV1 gating, the first of which is susceptible to $GABA_{B1}$ receptor-mediated inhibition, whereas the latter is not.

Given that PKC is not the sole mediator of TRPV1 sensitization utilized by the inflammatory pathways susceptible to $GABA_{B1}$ inhibition, it is very well possible that other mechanisms are also targeted and that $GABA_B$ provides a broader protection (or "shielding") against TRPV1 sensitization.

$GABA_B$ receptors trigger a surprising range of different cellular responses. Heteromeric receptor assembly, as well as the recent discovery of auxiliary $GABA_B$ subunits (Schwenk et al., 2010), has been proposed to explain contextual signaling diversity and challenge the classical view of GPCR organization and function. Our study further corroborates $GABA_B$ receptor signaling complexity and highlights a $GABA_{B2}$-independent pathway that mitigates TRPV1-mediated pain hypersensitivity.

Peripheral GABA Regulates Nociceptor Sensitization

It came as a surprise to find not only $GABA_B$ receptors but also their cognate agonist, GABA, localized at peripheral nerve terminals. Classically, vesicular GABA is a hallmark of inhibitory synapses of CNS neurons. Although it is known that the excitatory transmitter glutamate is present in peripheral nerves, where it may promote excitability and neurogenic inflammation (Miller et al., 2011), to our knowledge nothing similar has been reported for inhibitory transmitters.

Here, we provide evidence that, under physiological conditions, peripheral GABA limits TRPV1-mediated hyperalgesia. It will be interesting to ascertain whether the peripheral GABA-$GABA_{B1}$ feedback on TRPV1 is altered under pathological pain conditions. Exploiting this endogenous feedback system (e.g., by promoting peripheral GABA release or rendering a pharmacological $GABA_{B1}$ agonist non-permeable to the blood-brain barrier) may be a valuable route for anti-pain therapy, circumventing severe adverse effects associated with baclofen's dominant CNS activity.

Processing and modulation of painful signals have mainly been attributed to higher-order brain centers such as the dorsal spinal cord and beyond. Our model may—after all—not only inspire new approaches for developing TRPV1-centric pain therapeutics, but it also offers a fresh look at modulation of sensory input directly at the site of sensory transduction in nerve terminals, a paradigm that may also be relevant for other somatosensory modalities.

EXPERIMENTAL PROCEDURES

Generation of *Tag-Trpv1* BAC Transgenic Mice

The SF (Strep-Flag) tag was seamlessly integrated into a mouse BAC clone (RP23-390G23) encoding the *Trvp1* genomic locus, and transgenic mice were obtained by pronuclear injection.

Behavioral Studies

All animal experiments were in accordance with the local governing bodies. Thermal pain was assessed by measuring the response latency to a radiant heat stimulus focused onto the plantar surface of the paw (Hargreaves assay).

Biochemical Protein Complex Purification and Mass Spectrometry Analysis

Following plasma membrane fractionation of DRG, protein complexes were affinity isolated using Anti-Flag magnetic beads. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was performed with in-solution digested affinity purified protein samples on a Q Exactive mass spectrometer (Thermo Scientific). Label-free quantitation (LFQ) was performed using MaxQuant Analysis Software.

BRET and PLA Assays

Luminescence and fluorescence signals of COS-1 cells transiently transfected with plasmids encoding Rluc BRET donor and YFP BRET acceptor fusion proteins were analyzed using an Infinite F500 microplate reader (Tecan).

PLA was performed using Duolink reagents (Sigma) per manufacturer's instructions.

Calcium Imaging and Electrophysiological Recordings

For calcium imaging experiments, primary sensory neurons or HEK293 cells were loaded with the calcium indicators Fura-2 or Cal-520 AM. Electrophysiological recordings were performed using a 700B amplifier and 1440A Analog Digital Converter (Molecular Devices) in whole-cell voltage clamp configuration.

GABA Measurements

GABA concentrations were determined by LC-MS/MS at Brainsonline (Groningen).

Statistical Analyses

Data are presented as mean ± SEM. Statistical significance was evaluated employing Student's t test for paired comparisons unless indicated otherwise. p values < 0.05 were considered statistically significant, with $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Additional Information is available in the Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, one table, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2015.01.022.

AUTHOR CONTRIBUTIONS

J.S. conceived the project and designed the experiments together with C.H., M.M., W.C.L., and H.W., who performed and analyzed experiments. M.K., L.A., K.S.-S., A.T.-T., C.W., H.K., M.G., and D.R. conducted and analyzed experiments. B.B., M.G., G.R.L., and M.S. contributed to the study design and experiments. J.S. wrote the paper. All authors commented on and approved the paper.

ACKNOWLEDGMENTS

We thank David Julius, Rohini Kuner, Thomas Müller, Björn Schröder, Christoph Stein, Gerd Zolles, and Bernd Fakler for critical tools and reagents. We also thank Jana Rossius and Christina Steinmeyer-Stannek for expert technical support and Christopher Bohlen for helpful criticism and reading of the manuscript. This work was supported by the European Research Council (ERC-2011-StG-280565 to J.S. and ERC-2011-AdG-294678 to G.R.L.) and the Alexander von Humboldt Foundation/BMBF to M.M. and J.S. Additional support came from the National Center for Competences in Research "Synapsy, Synaptic Bases of Mental Health Disease" Swiss National Science Foundation (3100A0-117816) to B.B. Dr. Roggenkamp is an employee of Beiersdorf AG.

Received: April 22, 2014
Revised: November 16, 2014
Accepted: January 6, 2015
Published: February 12, 2015

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