Cellular/Molecular

Lipopolysaccharide-Induced TRPA1 Upregulation in Trigeminal Neurons is Dependent on TLR4 and Vesicular Exocytosis

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Pain from bacterial infection was believed to be the consequence of inflammation induced by bacterial products. However recent studies have shown that bacterial products can directly activate sensory neurons and induce pain. The mechanisms by which bacteria induce pain are poorly understood, but toll-like receptor (TLR)4 and transient receptor potential A1 (TRPA1) receptors are likely important integrators of pain signaling induced by bacteria. Using male and female mice we show that sensory neuron activation by bacterial lipopolysaccharides (LPS) is mediated by both TRPA1 and TLR4 and involves the mobilization of extracellular and intracellular calcium. We also show that LPS induces neuronal sensitization in a process dependent on TLR4 receptors. Moreover, we show that TLR4 and TRPA1 are both involved in sensory neurons response to LPS stimulation. Activation of TLR4 in a subset of sensory neurons induces TRPA1 upregulation at the cell membrane through vesicular exocytosis, contributing to the initiation of neuronal sensitization and pain. Collectively these data highlight the importance of sensory neurons to pathogen detection, and their activation by bacterial products like LPS as potentially important to early immune and nociceptive responses.

Key words: **lipopolysaccharide; pain; sensory neuron; TLR4; trigeminal system; TRPA1**

Significance Statement

Bacterial infections are often painful and the recent discovery that bacteria can directly stimulate sensory neurons leading to pain sensation and modulation of immune system have highlighted the importance of nervous system in the response to bacterial infection. Here, we showed that lipopolysaccharide, a major bacterial by-product, requires both toll-like receptor (TLR)4 and transient receptor potential A1 (TRPA1) receptors for neuronal activation and acute spontaneous pain, but only TLR4 mediates sensory neurons sensitization. Moreover, we showed for the first time that TLR4 sensitize sensory neurons through a rapid upregulation of TRPA1 via vesicular exocytosis. Our data highlight the importance of sensory neurons to pathogen detection and suggests that TLR4 would be a potential therapeutic target to modulate early stage of bacteria-induced pain and immune response.

Introduction

Although bacterial infections are frequently quite painful, the mechanisms by which bacteria produce pain remain poorly understood. Bacteria, through the production of pathogen-associated molecular patterns (PAMPs) molecules such as lipopolysaccharide (LPS), lipoteichoic acid, or flagellin are detected by pattern recognition receptors including receptors from the toll-like receptor (TLR) family (Fitzgerald and Kagan, 2020). These receptors, highly expressed in innate immune cells, mediate the development of inflammation and the release of proinflammatory mediators. These mediators contribute to immune cell recruitment to control the infection, while also indirectly producing pain by activating and sensitizing sensory afferent fibers (Schäfers et al., 2003; Neeb et al., 2011; Cook et al., 2018; Fitzgerald and Kagan, 2020). However, PAMPs can also directly activate and sensitize sensory neurons, driving pain responses as well as the release of neurotransmitters that, in turn, potently

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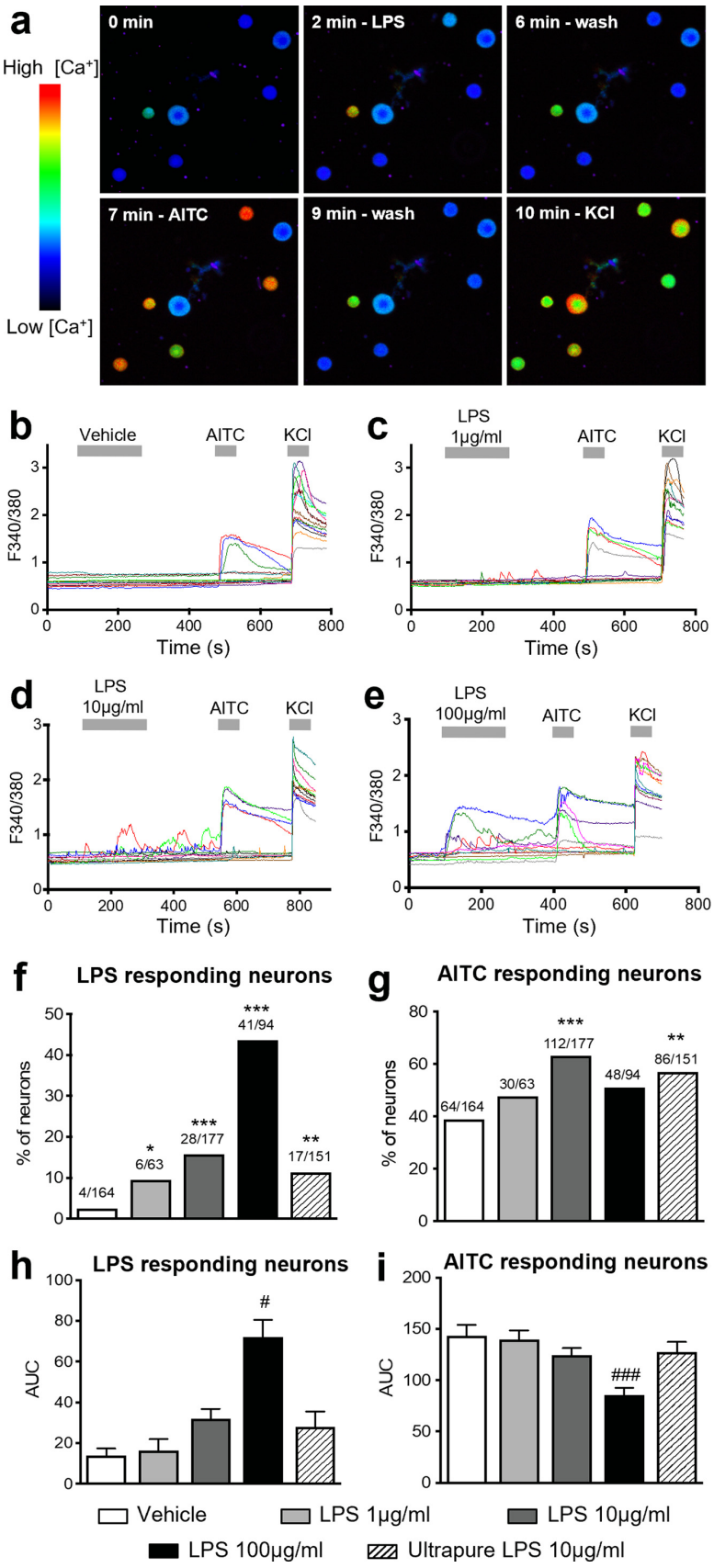


Figure 1. Effects of LPS on trigeminal ganglion neuron activation and sensitization. Trigeminal neurons from female mice were cultured for 2–4 h before performing live cell calcium imaging. Neurons were treated for 3 min with LPS or vehicle, washed for 3 min and stimulated with AITC 250 µM for 1 min. At the end of the experiments, neurons were stimulated with KCl 75 µM to identify live and healthy cells. Only neurons responding to KCl were included in the quantitative analyses. ***a***, Representative images of calcium levels in neurons stimulated with LPS (10 µg/ml) at different time during calcium imaging experiment. ***b–e***, Modification of calcium levels in trigeminal neurons treated with different concentration of LPS (1–100 µg/ml). Each trace represents the calcium level in one

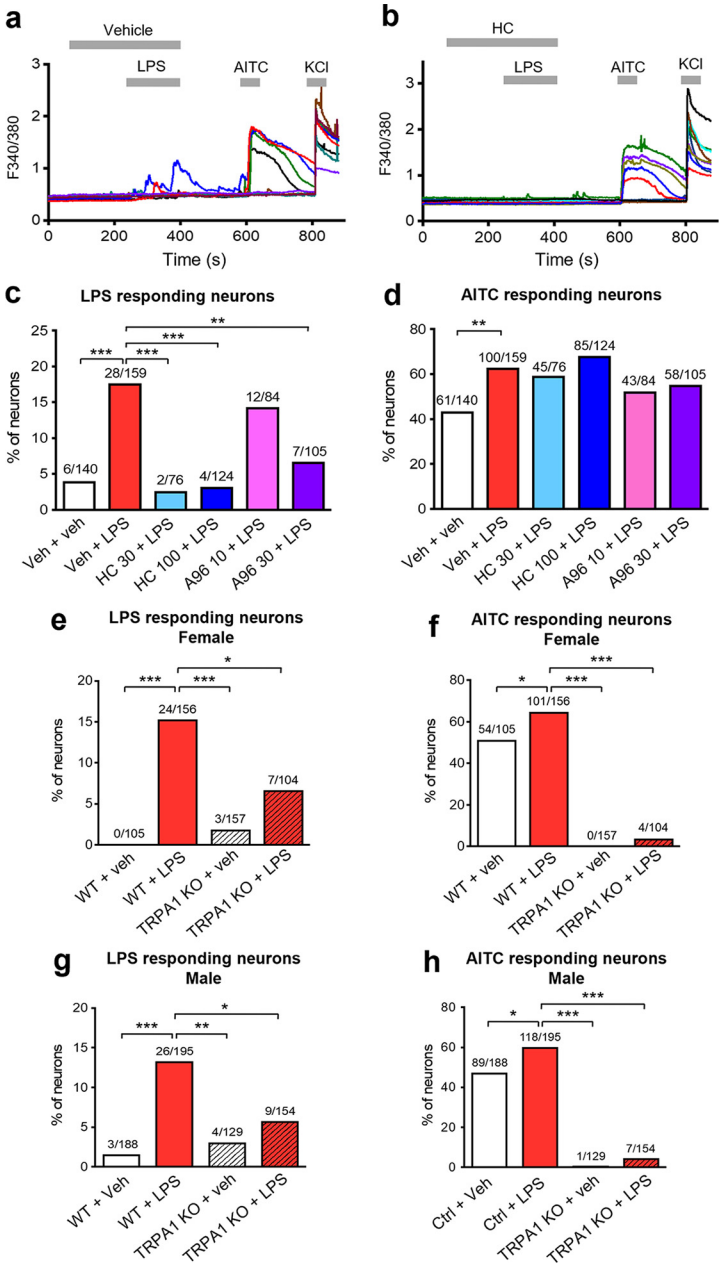


Figure 2. Involvement of TRPA1 in LPS-induced trigeminal neuron activation. ***a***, ***b***, Modification of calcium levels in female mouse TG neurons treated with vehicle or the TRPA1 antagonist HC030031 (100 μM). Each trace represents the calcium level in one TG neuron. Horizontal gray bars indicate the duration of each stimulation. ***c***, ***d***, Live cell calcium imaging was performed in TG neurons successively treated with a TRPA1 antagonist (30–100 μM HC030031 or 10–30 μM A967079) or vehicle (veh) for 3 min, with TRPA1 antagonist/veh + LPS (10 μg/ml) for 3 min, washed for 3 min and stimulated with AITC (250 μM) for 1 min. ***e–h,*** Live cell calcium imaging was performed in TG neurons from male and female TRPA1 KO mice or WT mice. Neurons were cultured for 2–4 h before performing live cell calcium imaging. Neurons were treated with LPS (10 μg/ml), washed for 3 min, stimulated with AITC (250 μM) for 1 min. ***c***, ***e***, ***g***, Proportion of neurons responding to LPS treatment and (***d***, ***f***, ***h***) proportion of AITC responding neurons; the number of activated neurons/total number of neurons is indicated above the bar-graphs. ***c–f***, Experiments performed in female and (***g***, ***h***) in male mice. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, Fisher's exact test, $n = 76–195$ neurons from three to five independent cell cultures.

←

trigeminal neuron. Horizontal gray bars indicate the duration of each stimulation. ***f***, Proportion of neurons responding to LPS treatment; the number of LPS responding neurons/total number of neurons is indicated above the bar-graphs. ***g***, Proportion of AITC responding neurons pretreated with different concentration of LPS; the number of AITC responding neurons/total number of neurons is indicated above the bar-graphs. ***h***, Each bar is the average ± SEM of the area under the curve (AUC) of LPS response traces. ***i***, Each bar is the average ± SEM of the AUC of AITC response traces. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, Fisher's exact test. $\#p < 0.05$, $\#\#\#p < 0.001$, Tukey's test, $n = 63–177$ neurons from three to four independent cell cultures. KCl = potassium chloride; SEM = standard error of the mean.

modulate the immune response and the progression of pathogen-mediated pathology (Pinho-Ribeiro et al., 2018). LPS form an intrinsic component found in the cell wall of Gram-negative bacterial pathogens. LPS is known to cause significant inflammation and pain *in vivo* when injected into mice. LPS was shown to directly activate sensory neurons through the transient receptor potential A1 receptor (TRPA1; Meseguer et al., 2014).

TRPA1 is an ion channel receptor expressed in sensory neurons and is activated by irritant molecules (acrolein, mustard oil) as well as mechanical and noxious cold stimuli (Kwan et al.,

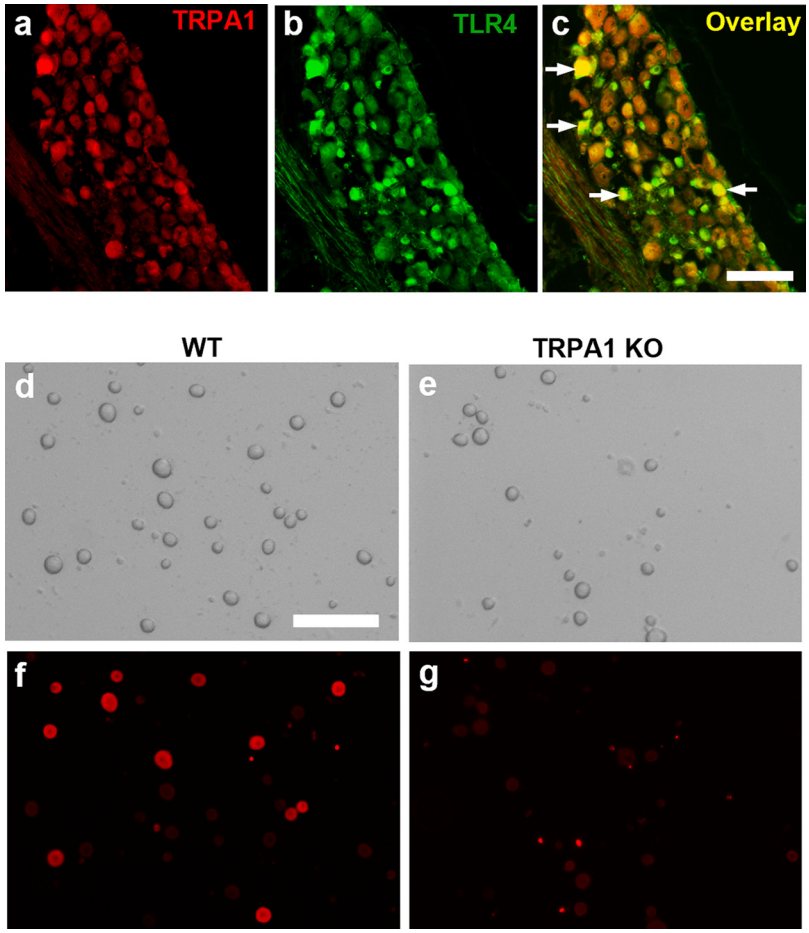


Figure 3. Immunostaining of TRPA1 and TLR4. Trigeminal ganglia from C57Bl6 mice were immunolabeled for TRPA1 (***a***) and TLR4 (***b***). Arrows show neurons stained for both TRPA1 and TLR4 (***c***). TG neurons culture from WT (***d***, ***f***) and TRPA1 KO (***e***, ***g***) mice were immunolabelled with an antibody binding to an extracellular domain of TRPA1. No immunostaining was observed in TRPA1 KO TG neurons. Scale bars = 100 μM.

2006). TRPA1 expression and activity are upregulated in sensory neurons in multiple pain conditions that encompass both neuropathic and inflammatory pain (Obata et al., 2005; Chen and Hackos, 2015). Moreover, the pharmacological or genetic blockade of TRPA1 reduces pain behaviors in preclinical models (Obata et al., 2005; Chen et al., 2011). TRPA1 has a central role in pain hypersensitivity, neuronal activation and the subsequent secretion of neuropeptides and the development of neurogenic inflammation (Pedersen-Bjergaard et al., 1991; Gebhardt et al., 2020). Collectively, there is good evidence that TRPA1 is an important molecular integrator of pain signaling and inflammation including bacterial activation of sensory neurons via LPS. This appears to be a highly conserved mechanism, as fruit flies also require TRPA1 to detect endotoxin contaminated food (Soldano et al., 2016).

However, it would be unusual for LPS-signaling sensory neurons to function completely independently of the TLR4 receptor. Notably, TLR4 is the most well characterized receptor for LPS and is indeed the most highly expressed TLR receptor in peptidergic sensory neurons (Wadachi and Hargreaves, 2006; Wu et al., 2019; Donnelly et al., 2020). The TLR4 receptor is necessary for rapid LPS-induced increases in neuropeptide release (Diogenes et al., 2011), suggesting that neuronal TLR4 receptors could mediate pain sensitization. The TLR4 receptor plays an important role in mediating pain signaling in a range of pain models and is an important integrator of pain physiopathology. The inhibition of TLR4 both prevents and reversed allodynia and hyperalgesia in animal models of inflammatory and neuropathic pain (Woller et al., 2016; Li et al., 2017). As such, with TLR4 being found on sensory neurons and an important integrator of pain signaling, it is also a strong candidate for mediating bacterial activation of sensory neurons via LPS.

In the present study we evaluated the contribution of both TRPA1 and TLR4 to the sensitization and activation of trigeminal sensory neurons by LPS. We found that both TRPA1 and TLR4 contributes to LPS-induced neuronal activation, and that LPS-induced neuronal sensitization does not involve calcium mobilization but a new mechanism by which TLR4 mediates TRPA1 upregulation at the cell membrane through vesicular exocytosis. This finding highlights the importance of interactions between TLR4 and TRPA1 in sensory neurons for the detection and early response to bacterial infection.

Materials and Methods

Animals

Experiments were conducted on 8- to 12-week-old male and female C57Bl6, TRPA1 knock-out (KO; B6;129P-Trpa1$^{tm1Kykw/J}$), C3H/HeJ (TLR4*mut*; carrying nonfunctional TLR4 receptor) and C3H/HeOuJ (control for TLR4*mut* mice) mice, purchased from Jackson Laboratories. Mice were housed two to five per cage in an environment with 12/12 h dark/light cycle and had free access to food and water. All animal experiments were approved by the Institutional Animal Care and Use Committee at New York University and Harvard University and followed the guidelines provided by the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

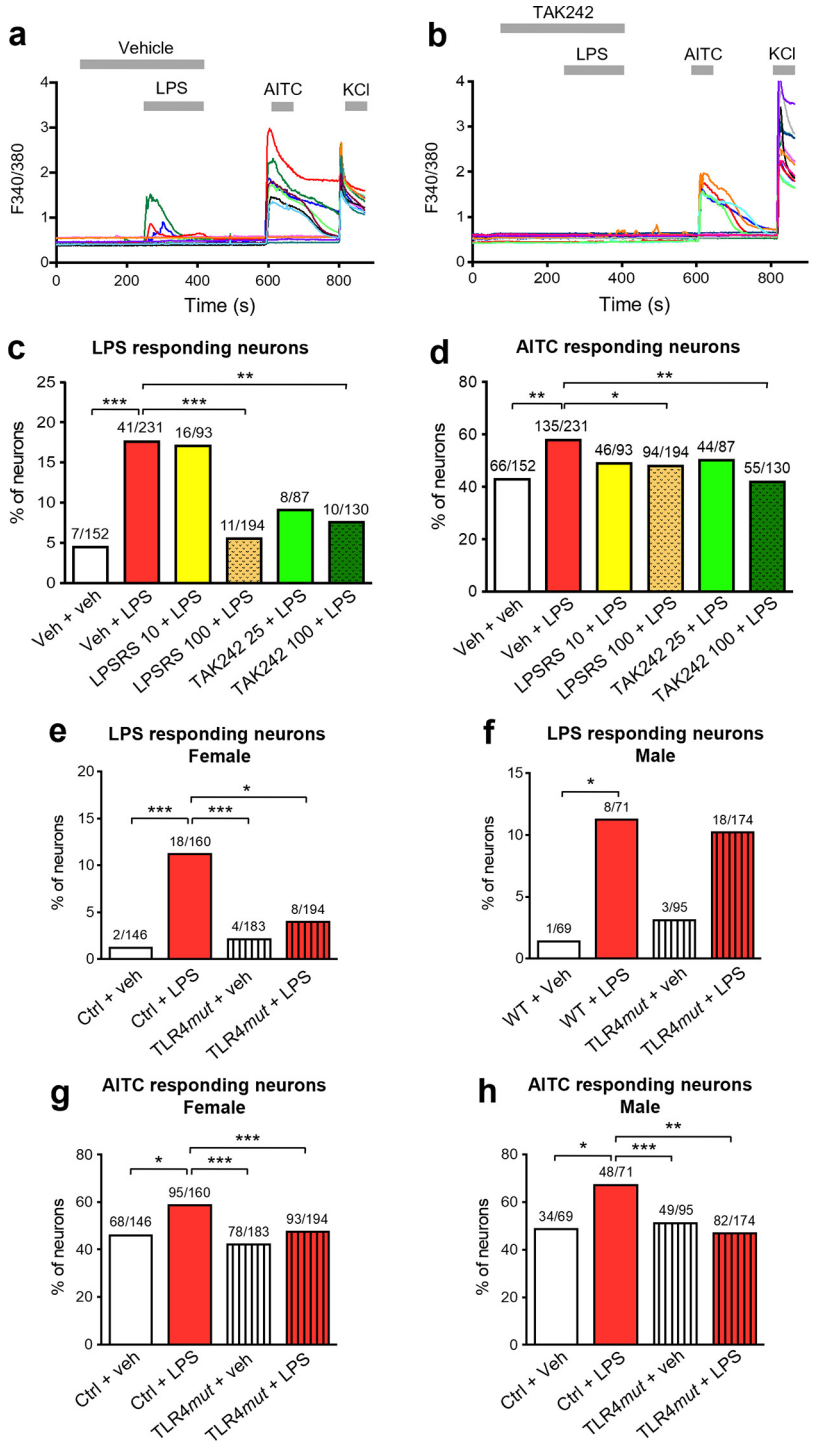


Figure 4. Involvement of TLR4 in LPS-induced trigeminal neuron activation and sensitization. ***a***, ***b***, Modification of calcium levels in female mouse TG neurons treated with vehicle or the TRPA1 antagonist TAK242 (100 μM). Each trace represents the calcium level in one TG neuron. Horizontal gray bars indicate the duration of each stimulation. ***c***, ***d***, Live cell calcium imaging was performed in TG neurons successively treated with a TLR4 antagonist (10–100 μM LPS-RS or 25–100 μM TAK242) or vehicle (veh) for 3 min, with TLR4 antagonist/veh + LPS (10 μg/ml) for 3 min, washed for 3 min, and stimulated with AITC (250 μM) for 1 min. ***e–h***, Live cell calcium imaging was performed in TG neurons from male and female mice expressing a nonfunctional TLR4 receptor (TLR4*mut*) or control (Ctrl) mice. Neurons were treated with LPS (10 μg/ml) or veh for 3 min, washed for 3 min, stimulated with AITC (250 μM) for 1 min. ***c***, ***e***, ***f***, Proportion of neurons responding to LPS treatment and (***d***, ***g***, ***h***) proportion of AITC responding neurons; the number of activated neurons/total number of neurons is indicated above the bar-graphs. ***c–e***, ***g***, Experiments performed in female and (***f***, ***h***) in male mice. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, Fisher's exact test, $n = 69–231$ neurons from three to four independent cell cultures.

Trigeminal ganglion (TG) neuron culture

TG from C57Bl6, TRPA1-KO, C3H/HeJ, C3H/HeOuJ mice were collected in F12 culture media (Sigma-Aldrich) supplemented with 5% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (2.5 μg/ml). Trigeminal neurons were isolated as described by Malin et al. (2007). TGs were collected and sectioned into 10 pieces each and incubated for 20 min with papain 40 units/ml in HBSS without calcium. After 2 min of centrifugation at 180 × *g*, the supernatant was removed, and TGs incubated for 20 min in collagenase-dispase solution (3.33–4.66 mg/ml in HBSS without calcium). Cells were centrifuged for 4 min at 400 × *g* the supernatant was removed. Neurons were resuspended in F12 culture media and centrifuged for 6 min at

450 × *g* to remove the excess of collagenase-dispase solution. Then, neurons were suspended in F12 media supplemented with 5% fetal bovine serum, mechanically dissociated with a P200 pipet and seeded into poly-D-Lysine-coated 25-mm round glass coverslips. After a 2- to 4-h incubation at 37°C in a humidified atmosphere containing 5% CO_2, neuronal culture was processed for subsequent experiments.

Single-cell calcium imaging

TG neurons were washed for 10 min in HBSS and loaded in the dark with the fluorescent calcium indicator fura-2 acetoxy-methyl ester (fura-2 AM; 5 μM) for 45 min at room temperature. Coverslips were washed for 10 min in HBSS and mounted in a microscope chamber. Loaded cells were excited successively (2 Hz) for fura-2 AM at 340 nm and 380 nm for 200 ms and emitted fluorescence was monitored at 510 nm using a charged device sensor camera coupled to an inverted Nikon Eclipse Ti microscope. Fluorescence intensities from single cells excited at the two wavelengths were recorded separately, corrected for the background and the fluorescence ratio (F340/F380) was calculated using the software NIS Elements-AR version 4.0. All neurons were treated for 3 min with antagonist/vehicle, followed by antagonist/vehicle + LPS (1, 10, or 100 μg/ml), washed for 3 min with HBSS, stimulated for 1 min with Allyl isothiocyanate (AITC; 250 μM), washed for 3 min with HBSS and stimulated for 1 min with KCl (75 mM). Only healthy neurons, which responded to KCl stimulation, were used for the quantitative analyses. A positive neuronal response was defined as a minimum of 20% increase of the ratio F340/F380 relative to the baseline F340/F380 value. The proportion of AITC responding neurons was calculated as the ratio of the number LPS or AITC responsive neurons/number of KCl responding neurons. The intensity of the AITC response was evaluated by calculating the area under the curve (AUC) during the period from 10 s before AITC stimulation to 180 s after the beginning of the stimulation using GraphPad Prism 5 Software (GraphPad Software).

Drugs and neuron culture treatments

After isolation and loading with fura-2 AM, neurons were treated with LPS (1–100 μg/ml in HBSS; L3129; Sigma-Aldrich) or ultrapure LPS (10 μg/ml; L5024; Sigma-Aldrich) for 3 min. AITC 250 μM (dissolved in HBSS + 0.025% ethanol; Sigma-Aldrich) for 1 min.

HC030031 (TRPA1 antagonist, 30 and 100 μM in DMSO 0.1%; Sigma-Aldrich), A967079 (TRPA1 antagonist, 10–30 μM in DMSO 0.03%; Tocris), LPS-RS (TLR4 antagonist, 10–100 μM in HBSS; tlrl-prslps; Invivogen), TAK242 (TLR4 antagonist, 25–100 μM in DMSO 0.1%; Cayman), 2-APB (IP3 receptor antagonist, 100 μM in ethanol 0.2%; Tocris), or EGTA (2 mM in calcium free HBSS; Sigma-Aldrich) were added for 3 min before and during LPS treatment.

BAPTA-AM (intracellular calcium chelator, 10 μM in DMSO 0.05%; Abcam) treatment was performed immediately after neuron loading with fura-2 AM. Neurons were incubated with BAPTA-AM for 15 min at 37°C/5% CO_2 in the dark before processing for calcium imaging.

Tetanus toxin (Tetx 30 nM in HBSS; Sigma-Aldrich) treatment was performed before neuron loading with fura-2 AM; neurons were incubated with Tetx for 3 h in an incubator at 37°C/5% CO_2.

Pain behavior

Female and male mice were placed individually in small transparent plastic testing box (15 × 15 × 17 cm) with ventilation holes and of sufficient size to allow them to move around freely and stand on hindlimbs. A video camera was positioned in front of the mice that the image of the mouse head can be observed. A mirror was placed on the back allowing for a two-sided view of the mouse. Mice were habituated in the cage before initiating data collection, by placing them in the box for 15 min for 2 consecutive days. On test days, the mice were acclimated for 15 min, and then 10 μl of LPS (1 μg/μl, s.c.) was injected into the vibrissal pad using insulin syringe with needle (31 G). Pretreatment with TAK242 (3 mg/kg, i.p.) was performed 30 min before LPS injection. The mouse was put back into the box immediately after the injection, and then recorded for 10 min. Video recordings were scored by a blinded observer for facial wipes and bouts of grooming.

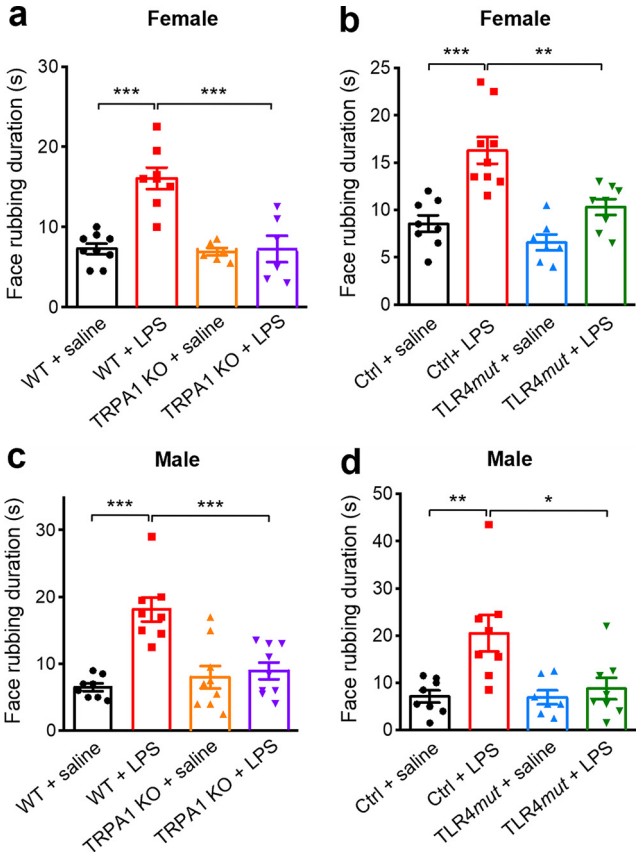


Figure 5. Involvement of TRPA1 and TLR4 on LPS-induced pain behavior in female and male mice. ***a***, ***c***, LPS (10 μg/10 μl) was injected in the vibrissal territory of TRPA1 KO and WT mice and face rubbing was quantified for 10 min after injection. ***b***, ***d***, LPS (10 μg/10 μl) was injected in the vibrissal territory of TLR4*mut* and control mice and face rubbing was quantified for 10 min after injection. ***a***, ***b***, Experiments performed in female and (***c***, ***d***) in male mice. Each bar is the average ± SEM of the rubbing duration. $^{**}p < 0.01$, $^{***}p < 0.001$, Tukey's test, $n = 7–9$ animals per group.

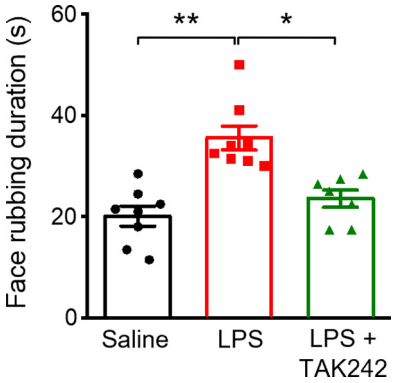


Figure 6. Effects of TAK242 on LPS-induced pain behavior in female mice. C57Bl6 mice were pretreated with TAK242 (3 mg/kg, i.p.) or vehicle 30 min before LPS (10 μg in 10 μl, s.c.) or saline injection in the vibrissal territory. Face rubbing was quantified for 10 min after LPS injection. Each bar is the average ± SEM of the rubbing duration. $^{*}p < 0.05$, $^{**}p < 0.01$, Tukey's test, $n = 7–8$ animals per group.

Immunofluorescence

Mice were euthanized and perfused intracardially with 10 ml of PBS (1×) with heparin (10 U/ml) followed by 50 ml of 10% formalin in PBS. TGs were removed, postfixed overnight in 10% formalin and cryoprotected for 24 h in PBS with 30% sucrose. TGs were sectioned (16-μm-thick sections) with a Cryostat (−20°C) and collected on superfrost glass slides. Sections were washed for 30 min in PBS, followed by 1-h

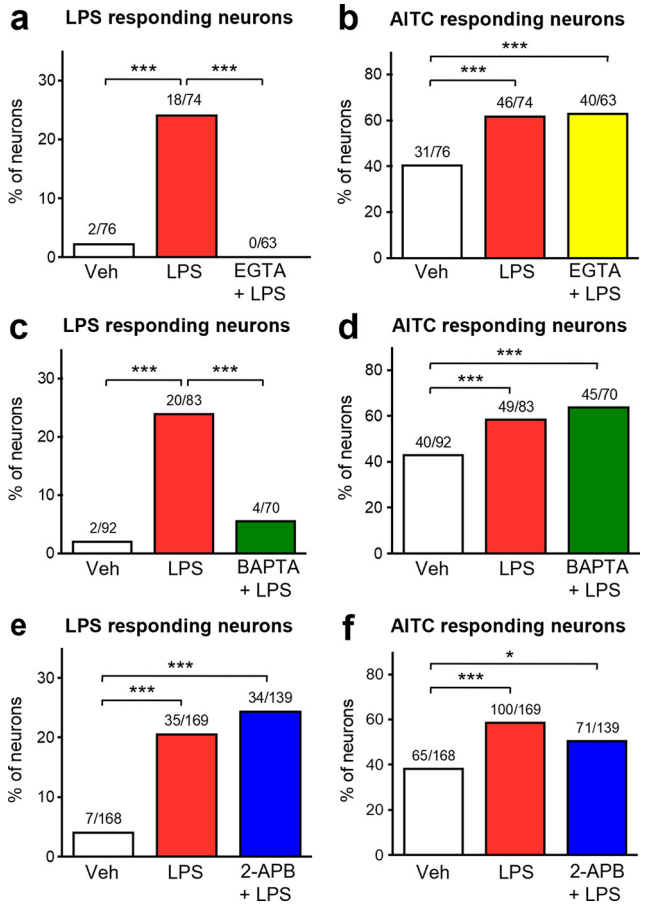


Figure 7. Involvement of intracellular and extracellular calcium stores in LPS-induced trigeminal neuron activation and sensitization. Live cell calcium imaging was performed in female mouse TG neurons treated with EGTA (2 mM; ***a***, ***b***), BAPTA-AM (10 μM; ***c***, ***d***), or 2-APB (100 μM; ***e***, ***f***) to evaluate the involvement of extracellular calcium, intracellular calcium and IP3 receptor, respectively, in LPS-induced neuronal activation and sensitization. Then, neurons were treated with LPS (10 μg/ml) or vehicle (veh) for 3 min, washed for 3 min, and stimulated with AITC (250 μM) for 1 min. ***a***, ***c***, ***e***, Proportion of neurons responding to LPS treatment and (***b***, ***d***, ***f***) proportion of AITC responding neurons; the number of activated neurons/total number of neurons is indicated above the bar-graphs. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, Fisher's exact test, $n = 63–169$ neurons from three to four independent cell cultures.

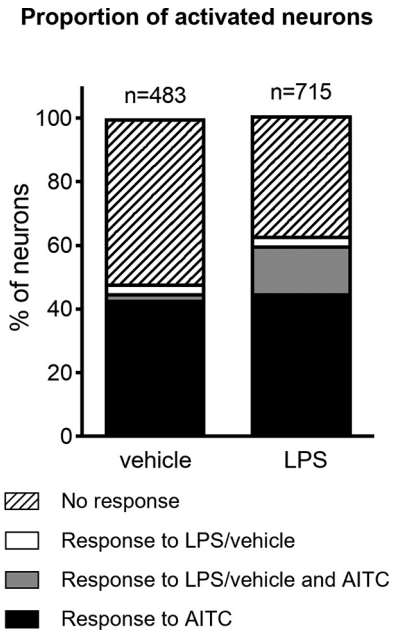


Figure 8. Identification of neuronal subpopulations activated by LPS and/or AITC. Live cell calcium imaging was performed in TG neurons successively treated with vehicle or LPS (10 μg/ml) for 3 min, washed for 3 min and stimulated with AITC (250 μM) for 1 min. Neurons were characterized as insensitive to LPS and AITC (no response), activated by LPS only (response to LPS), activated by AITC only (response to AITC) or activated by both LPS and AITC (response to LPS and AITC). $n = 483–715$ from 12 independent cell cultures

incubation in a blocking solution (1% Triton X-100 and 5% normal goat serum in PBS). Then, sections were incubated overnight at 4°C with rabbit anti-TRPA1 antibody (1:1000; AB58844; Abcam), and mouse anti-TLR4 antibody (1:100; sc-52962; Santa Cruz). After a 30-min wash in the blocking solution, samples were incubated for 2 h with the secondary antibody Alexa Fluor 488-conjugated goat anti-rabbit (1:500; Life Technology) or Alexa Fluor 546-conjugated goat anti-mouse (1:500; Life Technology). After a final 30-min wash, sections were coverslipped. Photo-micrographs of TGs were collected with a fluorescent inverted microscope (Nikon Eclipse Ti).

Immunocytochemistry

Cultured TG neurons were washed PBS and fixed with 10% formalin for 15 min. Cell membrane staining was performed before neuronal fixation using CellBrite NIR750 cell membrane dye (30-min incubation; Biotium). Fixed cells were washed for 15 min with PBS and incubated with a blocking solution #1 (1% BSA in PBS without Triton X-100) to prevent cell membrane permeabilization and allow anti-TRPA1 antibody to bind only TRPA1 present at surface of the cell membrane. Sections were incubated overnight with rabbit anti-TRPA1 (extracellular) primary antibody (1:1000; ACC-037; Alomone). After 15-min wash with PBS, cells were successively incubated for 1 h with a blocking solution #2 (1% BSA +1% Triton X-100) and overnight with mouse anti-TLR4 primary antibody (1:1000; sc-52962; Santa Cruz) or guinea pig anti-TRPV1 antibody (1:200; ACC-030-GP; Alomone). After a 15-min wash in the blocking solution, samples were incubated for 2 h with the secondary antibody Alexa Fluor 488-conjugated goat anti-rabbit (1:500; Life Technology), Alexa Fluor 546-conjugated goat anti-mouse (1:500; Life Technology) and Dylight 550-conjugated goat anti guinea pig (1:500; Life Technology). After a final 15-min wash, sections were coverslipped. Photo-micrographs of TG neurons were collected with a fluorescent inverted microscope (Nikon Eclipse Ti). High-magnification images are acquired on an inverted epifluorescent microscope (Leica DMI8) with a 40× plan-apo objective and processed by the THUNDER image processing algorithms.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 Software. Data are expressed as mean + SEM or as percentage for the proportion of LPS or AITC responding neurons. The proportion of LPS/TRPA1 responsive neurons was analyzed with a Fischer's exact test. AUC of calcium response and behavioral data were analyzed with a one-way ANOVA followed by Tukey's test. Significance was set at the $p < 0.05$ level.

Results

LPS activates and sensitizes trigeminal sensory neurons

First, we measured calcium signaling in primary cultures of neurons to study *Escherichia coli* derived LPS activation of sensory neurons. Like others, we found that LPS induces a rapid, dose dependent increase in the activation of TG neurons, evidenced by the increase of intracellular calcium concentration. LPS increased both the proportion of responding neurons and the intensity of the calcium response (Fig. 1*a–e*). LPS at 100 μg/ml induced the highest neuronal response, 43.6% of responding neurons and the intensity was increased from an area under the curve (AUC) of 14.0 ± 3.3 in control group to 72.1 ± 8.5 in LPS at 100 μg/ml group (Fig. 1*f*,*h*).

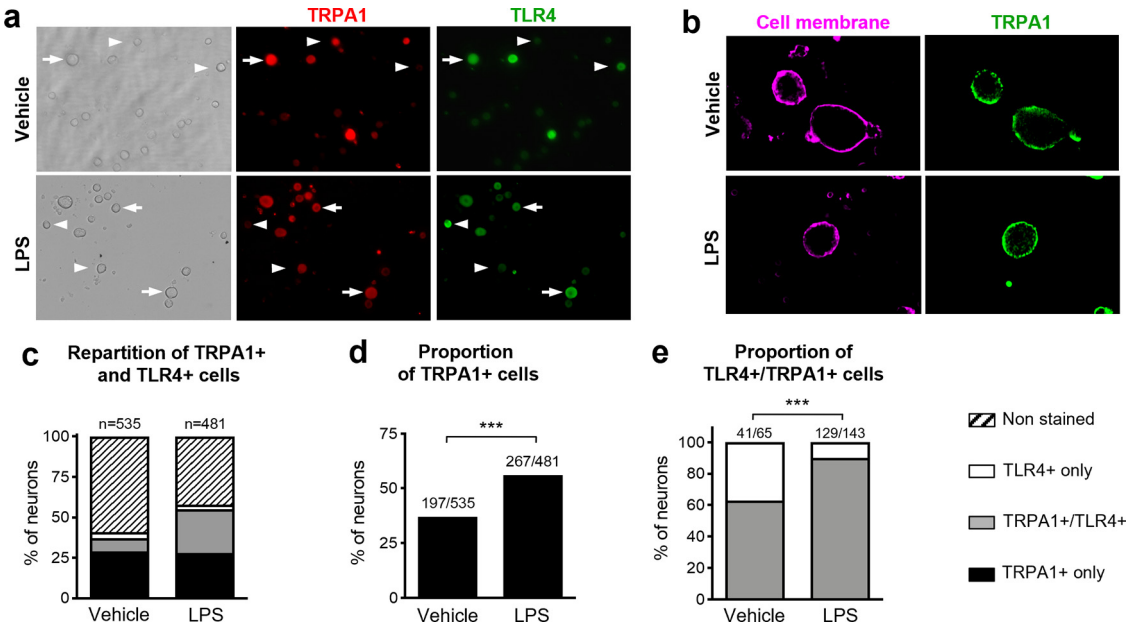


Figure 9. TRPA1 and TLR4 expression in response to LPS treatment. Co-immunostaining of TRPA1 and TLR4 was performed on female mouse TG neuron culture treated for 3 min with LPS (10 μg/ml) or vehicle. ***a***, Representative images of immunostaining of TRPA1-extracellular domain (red) and TLR4 (green). Arrows show neurons co-expressing TRPA1 and TLR4. Arrowheads show neurons expressing either TRPA1 or TLR4. ***b***, High-magnification images showing the localization of TRPA1 at the cell membrane (green). Cell membrane was stained with CellBrite membrane dye (magenta). ***c***, Quantification of the proportion of neurons expressing TRPA1, TLR4 or co-expressing TRPA1 and TLR4 in response to LPS. ***d***, Proportion of neurons expressing TRPA1 after LPS treatment. ***e***, Proportion of TLR4 positive neurons which express or not TRPA1 after LPS treatment. ***$p < 0.001$, Fisher's exact test. $n = 481–535$ neurons from three independent cell cultures.

This brief treatment with LPS (3 min) also increases the proportion of neurons responding to a subsequent stimulation with the TRPA1 agonist allyl-isothiocyanate (AITC) from 39.0% in the control group to 63.2% in the groups treated with LPS 10 μg/ml (Fig. 1*g*), indicating a rapid sensitization of the neurons. Interestingly, the highest dose of LPS (100 μg/ml) does not further increase the proportion of AITC responding neurons, inducing an increase of AITC responding neurons similar to the LPS 1 μg/ml (51.1% and 47.6%, respectively; Fig. 1*g*), indicating a decrease in the specificity of LPS effects or a desensitization of TRPA1 receptors. Contrary to the effects of LPS on the proportion of AITC responding neurons, the intensity of the AITC-induced calcium response was not increased (Fig. 1*i*).

To evaluate whether potential impurities in LPS could affect neuronal activation and sensitization we tested the effects of ultra-pure LPS and show a similar increase in the proportion of activated neurons and the proportion of AITC responding neurons in the groups treated with 10 μg/ml of ultrapure LPS and regular LPS (Fig. 1*g*). For the following experiments we used LPS 10 μg/ml, the dose showing the highest effects on TRPA1 sensitization.

TRPA1 receptors mediate LPS-induced neuronal activation but not neuronal sensitization

To further investigate the contribution of TLR4 and TRPA1 to LPS activation and sensitization of sensory neurons we used pharmacologic and genetic approaches. First, we evaluated the effects of the inhibition of TRPA1. The TRPA1 antagonist HC30031 (30 and 100 μM) completely blocked the activation of sensory neurons by LPS whereas the antagonist A967079 (30 μM) partially blocked LPS effects (Fig. 2*c*). Similarly, TRPA1 knock-out (KO) mice have a lower proportion of LPS responding neurons than wild-type (WT; 6.7% and 15.4%, respectively; Fig. 2*e*,*g*), indicating that TRPA1 is essential for LPS-induced neuronal activation. However, TRPA1 is not involved in LPS-mediated neuronal sensitization as we show that in both male and female mice, LPS-mediated sensitization of TRPA1 responses remains intact, despite TRPA1 receptor blockade during the LPS stimulation (Fig. 2*d*,*f*,*h*).

TLR4 receptors mediate LPS-induced neuronal activation and sensitization

As both TLR4 and TRPA1 are expressed in trigeminal sensory neurons (Wadachi and Hargreaves, 2006; Diogenes et al., 2011; Michot et al., 2018) and TLR4 is the most well established receptor for LPS, we wanted to determine whether TLR4 also mediates LPS activation and/or sensitization of sensory neurons. We also found that TLR4 and TRPA1 are colocalized in a subset of TG neurons (Fig. 3*c*) confirming data from RNA sequencing showing a co-expression of TLR4 and TRPA1 in peptidergic sensory neurons (Zheng et al., 2019). Two TLR4 antagonists, LPS-RS (100 μM) and TAK242 (25 and 100 μM), both prevented LPS-induced neuronal activation (Fig. 4*c*). A similar decrease in the proportion of neurons activated by LPS in mice carrying non-functional TLR4 receptors (11.3% vs 4.1% in control and TLR4*mut* female mice; Fig. 4*e*) confirmed that TLR4 contributes to LPS induced neuronal activation. Thus, both TRPA1 and TLR4 are responsible for activation of sensory neurons by LPS.

Interestingly, whereas LPS did not induce neuronal activation in female TLR4*mut* mice (Fig. 4*e*), it induced a similar increase in the proportion of activated neurons in control and TLR4*mut* male mice (Fig. 4*f*) showing that TLR4 contributes to LPS-induced neuronal activation in female but not in male mice.

We next evaluated the involvement of TLR4 on LPS-induced neuronal sensitization and show that pretreatment with the highest doses of the TLR4 antagonists LPS-RS or TAK242 both significantly blocked LPS effects (Fig. 4*d*). This role of TLR4 in LPS sensitization of TRPA1 was confirmed using trigeminal neurons

from male and female TLR4*mut* mice (Fig. 4*g*,*h*), showing that the LPS-induced TRPA1 sensitization is indeed mediated by TLR4 activation. In addition, we showed that TLR4 and TRPA1 are colocalized in a subset of TG neurons (Fig. 3). The presence of both receptors in the same neurons suggests the possibility of their direct interaction.

Both TRPA1 and TLR4 receptors contribute to LPS-induced pain

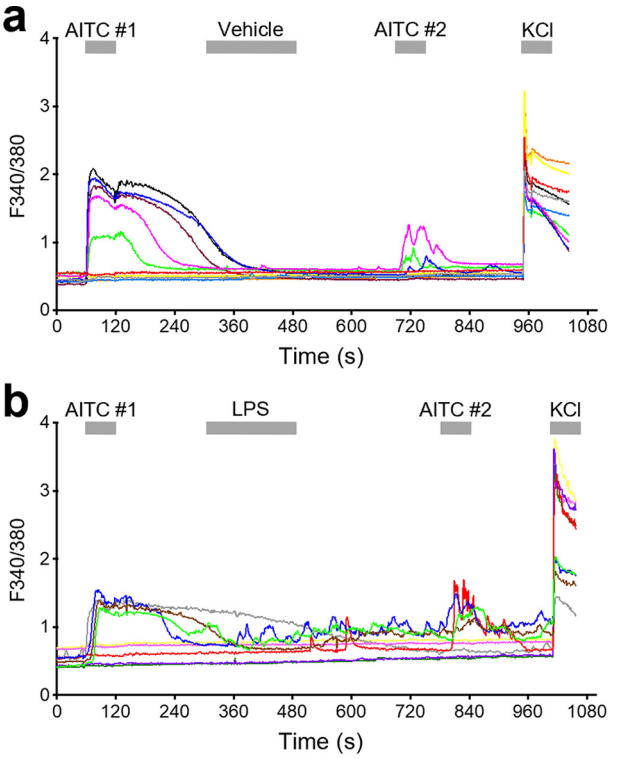
As LPS-induced sensory neuron activation/sensitization contributes to spontaneous pain, we used a mouse model of LPS induced spontaneous nocifensive behavior to further assess the contributions of TRPA1 and TLR4. LPS was injected subcutaneously in the vibrissal territory and the time spent in spontaneous face rubbing after the injection was quantified. LPS injection in female control mice induced pain behavior evidenced by the increase of face rubbing in the injected area (7.2 ± 0.6 vs 16.1 ± 1.3 s in saline vs LPS-injected mice; Fig. 5*a*). This increase in pain behavior induced by LPS was prevented in both TRPA1 KO and TLR4*mut* mice (Fig. 5*a*,*b*). Moreover, pretreatment with the TLR4 antagonist TAK242 also prevented LPS induced acute pain behavior (Fig. 6). Similar results were found in TRPA1 KO and TLR4*mut* male mice, showing no clear sex differences related to the role of TRPA1 or TLR4 in LPS-induced spontaneous pain behavior (Fig. 5*c*,*d*). Collectively, it appears both TRPA1 and TLR4 are important for acute pain-like behaviors induced by LPS.

Effects of calcium signaling modulators on LPS-induced neuronal activation and sensitization

As calcium is one of the most important modulators of TRPA1 activity (Hu et al., 2021), we investigated the role of calcium signaling in LPS activation and sensitization of sensory neurons. EGTA in calcium free media was used to evaluate the contribution of extracellular calcium in LPS effects. Treatment with EGTA abolished LPS induced TG neuron activation (Fig. 7*a*) but did not affect LPS-induced TRPA1 sensitization (Fig. 7*b*). Similarly, blocking intracellular calcium with BAPTA-AM prevented LPS-induced neuronal activation (Fig. 7*c*) but not LPS-induced TRPA1 sensitization (Fig. 7*d*). We also evaluated whether the IP3 receptor, a major contributor of intracellular calcium mobilization, is involved and found that LPS-induced neuronal activation and sensitization are independent of the IP3 receptor pathway (Fig. 7*e*,*f*). These data suggest that LPS-induced neuronal activation might be mediated by both extracellular calcium mobilization through TRPA1 and intracellular calcium mobilization through TLR4.

LPS sensitizes neurons via upregulation of TRPA1 at the cell membrane

LPS treatment rapidly increases the proportion of AITC responsive neurons in a TLR4 dependent manner (Fig. 4). Interestingly, further analysis shows that after LPS treatment ~45% of neurons responded to AITC only (a similar proportion to AITC responding neurons in the control group, 43%) and ~15% responded to both AITC and LPS (Fig. 8) Thus, a subpopulation of TG neurons becomes responsive to TRPA1 agonists after a brief exposure to LPS. We next evaluated whether LPS changes the expression of the TRPA1 receptor at the cell surface membrane. Using an antibody that binds to the first extracellular loop of TRPA1 receptor (Figs. 3*d*–*g*, 9*b*), we showed that LPS increases the proportion of neurons expressing TRPA1 at the cell surface membrane (Fig. 9*c*,*d*). Interestingly, the number of TRPV1 immunoreactive neurons is not modified in response to LPS, suggesting that TRPA1 and TRPV1 are differently upregulated in bacterial-mediated inflammatory conditions.



c

Treatment	Proportion of neurons responding to AITC: AITC #1 and AITC #2	Proportion of neurons responding to AITC: AITC #2 only
Vehicle	17.4% (8/46)	0% (0/46)
LPS	17.9% (16/89)	5.6% (5/89)

Figure 10. Effects of LPS on repeated AITC stimulations. Live cell calcium imaging was performed in TG neurons successively stimulated with AITC (AITC #1; 250 μM) for 1 min, LPS (10 μg/ml) for 3 min, AITC (AITC #2; 250 μM) for 1 min and KCl (75 μM). Only alive and healthy neurons which responded to KCl were included in the quantitative analyses. ***a***, ***b***, Modification of calcium levels in TG neurons treated with vehicle or LPS. Each trace represents the calcium level in one trigeminal neuron. Horizontal gray bars indicate the duration of each stimulation. ***c***, Quantitative analysis of the proportion of neurons responding to either AITC stimulation #1 and #2 or AITC stimulation #2 only in groups treated with vehicle or LPS. $n = 46$–89 neurons from two independent cell cultures.

Moreover, we found that the increase in the proportion of TRPA1 immunoreactive neurons is dependent on TLR4 receptor expression. Whereas the proportion of neurons expressing TRPA1, and not TLR4 is similar in control and LPS-treated groups, the proportion of neurons that co-express both TRPA1, at the cell membrane, and TLR4 increases more than threefold after LPS treatment (7.7% and 26.8% in control and LPS-treated groups, respectively; Fig. 9*c*).

In order to clarify whether LPS treatment causes neurons to become functionally responsive to TRPA1 agonists, we evaluated whether a subpopulation of sensory neurons become newly responsive to AITC subsequent to LPS treatment. Using single cell calcium imaging, we stimulated TG neurons first with AITC to

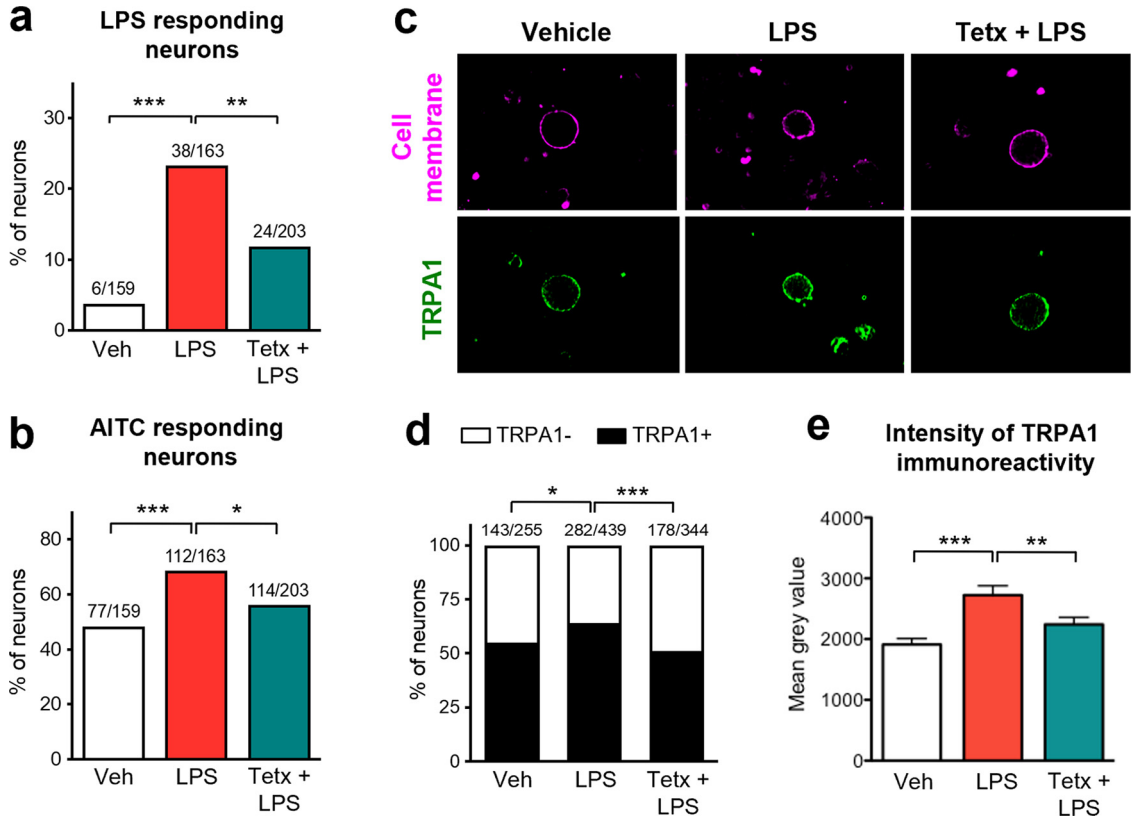


Figure 11. Effects of tetanus toxin on LPS-induced trigeminal neuron activation and sensitization. ***a***, ***b***, Live cell calcium imaging was performed in female mouse TG neurons treated with tetanus toxin (Tetx, 30 nM) to evaluate the effects of exocytosis inhibition on LPS-induced neuronal activation and sensitization. Then, neurons were stimulated with LPS (10 µg/ml) or vehicle (veh) for 3 min, washed for 3 min, and stimulated with AITC (250 µM) for 1 min. ***a***, Proportion of neurons responding to LPS treatment and (***b***) proportion of AITC responding neurons. ***c–e***, Immunostaining of TRPA1-extracellular domain in sensory neurons treated with vehicle, LPS or LPS+Tetx. ***c***, Representative images of TRPA1 immunostaining at the cell membrane (green). Cell membrane was stained with CellBrite membrane dye (magenta). ***d***, Proportion of TRPA1 positive neurons after LPS or LPS+Tetx treatment. ***e***, Quantification of the intensity of TRPA1 immunostaining at the cell membrane in neurons treated with vehicle, LPS or LPS+Tetx. The number of activated or immunoreactive neurons/total number of neurons is indicated above the bar-graphs. Each bar is the average ± SEM of the mean grey value of fluorescence. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, Fisher's exact test, $n = 159–439$ neurons from three to four independent cell cultures. $\#\#p < 0.01$, $\#\#\#p < 0.001$, Tukey's test, $n = 52–61$ neurons from two independent cell cultures.

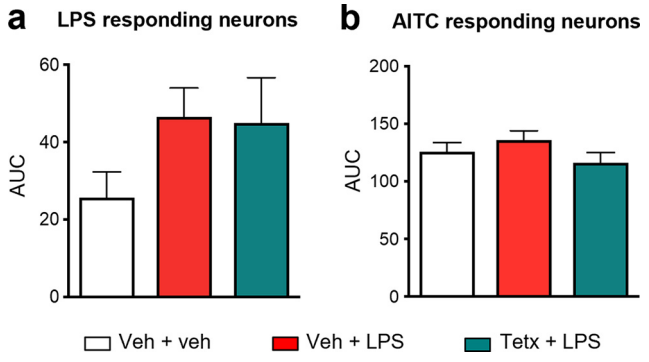


Figure 12. Effects of tetanus toxin on the intensity of TG neuron response to LPS. Live cell calcium imaging was performed in female mouse TG neurons treated with tetanus toxin (Tetx, 30 nM) and then stimulated with LPS (10 µg/ml) or vehicle (veh) for 3 min, washed for 3 min, and stimulated with AITC (250 µM) for 1 min. Each bar is the average ± SEM of the area under the curve (AUC). ***a***, Intensity of neuronal response to LPS treatment and (***b***) intensity of neuronal response to AITC. $n = 6–114$ neurons from four independent cell cultures.

identify AITC-sensitive neurons and then treated these neurons with LPS before a second stimulation with AITC. Indeed, we observed a population of neurons which were insensitive to the first AITC stimulation, show a novel response after the second AITC stimulation in the LPS-treated group (Fig. 10). Altogether these data show that LPS sensitizes a TLR4+ TG neuron subpopulation by increasing the expression of TRPA1 at the cell membrane.

Vesicular exocytosis is involved in LPS-induced TRPA1 sensitization

To understand the mechanisms underlying LPS-induced increases in TRPA1 expression at the cell membrane we hypothesized that a subpopulation of TLR4 expressing TG neurons express TRPA1 intracellularly, in storage vesicles. These vesicles would be released at the cell membrane, via exocytosis, in response to LPS stimulation, allowing these neurons to become newly responsive to subsequent AITC stimulation. Using the exocytosis inhibitor tetanus toxin (Tetx) on LPS-induced TRPA1 sensitization, we found that Tetx partially prevented LPS-induced neuronal activation (Fig. 11*a*), and decreased the proportion of AITC responding neurons compared with control (56.1% and 68.7%, respectively; Fig. 11*b*) but did not change the intensity of LPS or AITC response (Fig. 12). We also evaluated TRPA1 expression specifically at the cell membrane and found that Tetx reduces the number of TRPA1 immunoreactive neurons in Tetx-treated group compared with LPS-treated group (Fig. 11*d*) indicating that vesicular exocytosis mediates LPS-induced TRPA1 upregulation. Similarly, the presence of TRPA1 at the cell membrane is increased by LPS and prevented by Tetx (Fig. 11*e*)

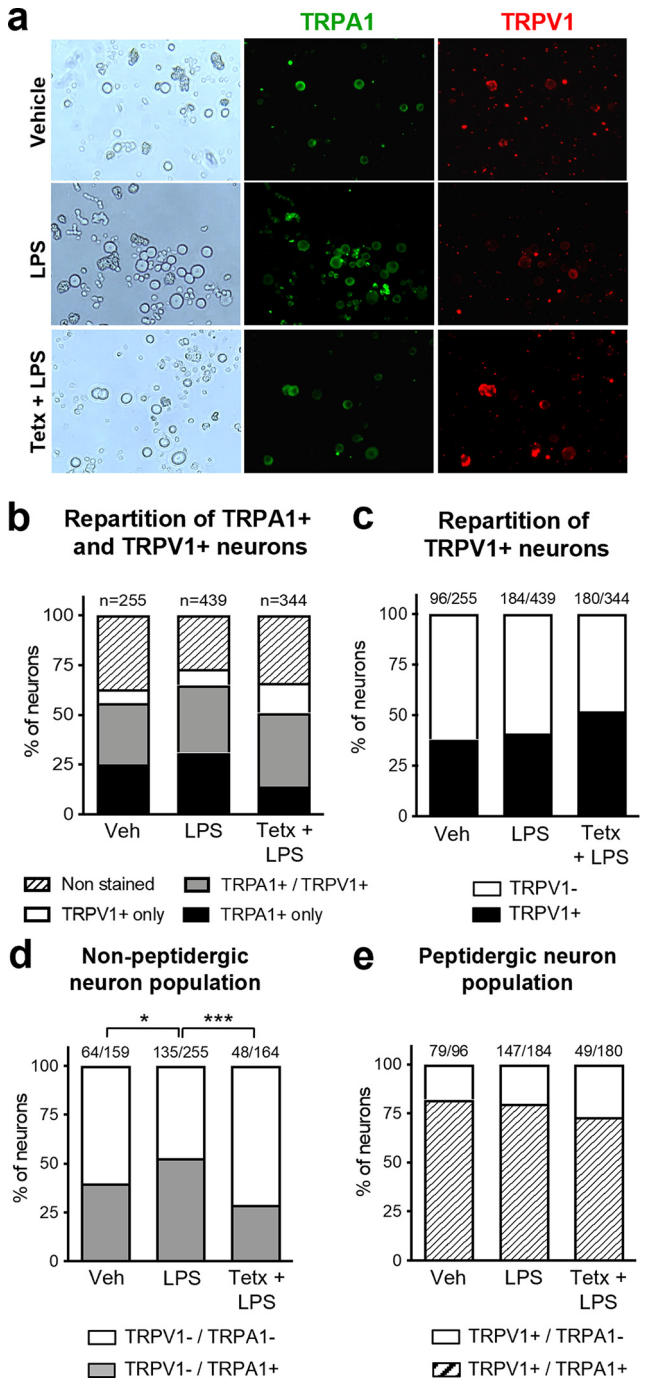


Figure 13. TRPA1 and TRPV1 expression in response to LPS treatment. Co-immunostaining of TRPA1 and TRPV1 was performed on female mouse TG neuron culture pretreated with vehicle or tetanus toxin (Tetx, 30 nM) and then, stimulated with LPS (10 μg/ml) or vehicle for 3 min. ***a***, Representative images of immunostaining of TRPA1 (green) and TRPV1 (red). Arrows show neurons co-expressing TRPA1 and TRPV1. Arrowheads show neurons expressing either TRPA1 or TRPV1. ***b***, Quantification of the proportion of neurons expressing TRPA1, TRPV1 or co-expressing TRPA1 and TRPV1. ***c***, Quantification of the proportion of neurons TRPV1 immunoreactive. ***d***, Proportion of TRPA1 positive neurons in nonpeptidergic neuronal population identified as TRPV1 negative neurons. ***e***, Proportion of TRPA1 positive neurons in peptidergic neuronal population identified as TRPV1 positive neurons. The number of immunoreactive neurons/total number of neurons is indicated above the bar-graphs. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, Fisher's exact test, $n = 159$–439 neurons from three independent cell cultures.

As TRPA1 is expressed in peptidergic and nonpeptidergic neurons (Barabas et al., 2012), we measured the changes of TRPA1 expression in a subpopulation of these neurons identified by their immunoreactivity to TRPV1 (Woodbury et al., 2004; Cavanaugh et al., 2011). LPS treatment increases the proportion of TRPA1 immunoreactive neurons in the TRPV1− neuronal population (40.2% and 52.9% in control and LPS-treated group, respectively; Fig. 13*d*) but do not change the proportion of TRPA1 immunoreactive neurons in the TRPV1+ neuronal subpopulation (Fig. 13*e*). This increase in the proportion of TRPV1− neurons expressing TRPA1 was prevented by Tetx (Fig. 13*d*). This finding supports that LPS-induced TRPA1 sensitization is dependent on vesicular exocytosis and mainly involves nonpeptidergic sensory neurons.

Discussion

In this study, we investigated the mechanisms by which the bacterial toxin LPS is involved in trigeminal sensory neuron activation, sensitization, and pain. We showed that LPS induced neuronal activation and pain is mediated by both TLR4 and TRPA1 receptors and that LPS-induced neuronal sensitization is mediated by TLR4 and involves vesicular exocytosis (Fig. 14).

Although, the effects of LPS on sensory neuron activation were previously studied, questions remain. Meseguer and colleagues initially concluded that LPS-activation of sensory neurons was mediated by TRPA1 but not TLR4 receptors (Meseguer et al., 2014). A more recent study shows LPS induces sensory neuron activation through TLR4 and the activation of NFkb signaling pathways (Kaewpitak et al., 2020). Using both antagonists and KO mice our results also support that activation of TLR4 or TRPA1 receptors by LPS can produce neuronal activation, providing additional evidence of an important role of neuronal TLR4 in mediating LPS effects. It is of note that we used sensory neurons acutely cultured for 2–4 h in our *in vitro* experiments to avoid the physiological TRPA1 upregulation that occurs at later stages of neuron culture, which could have complicated interpretation of the effects of LPS treatment (Barabas et al., 2012). However, our results using early-stage neuron culture might not be fully extended to more mature or native nociceptors and additional investigation is needed to confirm the mechanisms we describe are present in other experimental models.

The differences in the involvement of TLR4 in LPS-induced neuronal activation from previous studies and ours could be explained by the bacterial origin of the LPS used, *Porphyromonas gingivalis* or *E. coli*, as suggested by Kaewpitak et al. (2020) or perhaps by the type of neurons used in the Meseguer study, as nodose ganglia and TG were used interchangeably. However, our data support that sexual dimorphism may also be involved in these differences. We observed that LPS-induced neuronal activation remained intact in male TLR4 KO mice as previously reported (Meseguer et al., 2014). Intriguingly, our study shows that LPS-induced neuronal activation was abolished in female TLR4 KO mice. To our knowledge, previous studies did not evaluate female mice.

These data give additional mechanistic information about important sexual dimorphisms regulating TLR4 function. Multiple studies have shown that involvement of TLR4 in pain mechanisms is sex dependent. Spinal TLR4 receptors contribute to the development of pain behavior induced by intrathecal injection of LPS in male but not female mice (Sorge et al., 2011; Woller et al., 2016). Moreover, TLR4 expressed in peripheral sensory neurons mediates pain in females but not males in a nerve injury model

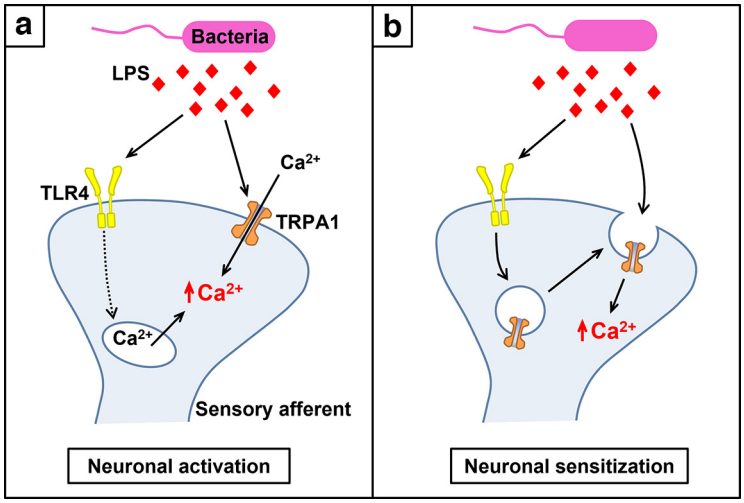


Figure 14. Schematic representation of contribution of TLR4 and TRPA1 in LPS induced sensory neuron activation and sensitization. During bacterial infection, LPS, a major PAMP produced by gram-negative bacteria, can directly stimulate sensory afferents via the activation of TLR4 and TRPA1 receptor contributing to the initiation of LPS-induced pain symptoms (***a***). This neuronal activation is mediated by the mobilization of both intra and extracellular calcium. TLR4 would mediate intracellular calcium mobilization and TRPA1 mediate extracellular calcium influx in the cell. LPS also induce a rapid neuronal sensitization by increasing the proportion of neurons expressing TRPA1 (***b***). A subset of sensory neurons, mainly nonpeptidergic neurons, express TRPA1 receptor that stays located intracellularly, in storage vesicles, and serves as spared receptors that are mobilized in response LPS detection. LPS activates TLR4 in this neuronal subpopulation, inducing the trafficking of TRPA1 at the cell membrane. The recruitment of this additional population of TRPA1 expressing neurons enhances response to subsequent TRPA1 activation and pain behavior.

(Szabo-Pardi et al., 2021). Although various inflammatory and neuropathic pain models at the spinal and peripheral levels were used, collectively the sex dependent contribution of TLR4 in pain mechanisms is well established.

Interestingly, contrary to the sex dependent effect of LPS-induced neuronal activation, we did not show any sex difference in LPS induced pain behavior. LPS injection in the orofacial area was prevented in both male and female TLR4mut mice suggesting that, despite different cellular mechanisms in male and female mice, the overall effects of LPS depends on TLR4 in this acute pain model.

To our knowledge, this study evaluated for the first time the involvement of TLR4 in LPS induced pain in the orofacial area. Previous studies investigated the effects of LPS and the involvement of TLR4 in pain affecting the spinal system (Sorge et al., 2011; Meseguer et al., 2014; Woller et al., 2016; Szabo-Pardi et al., 2021). This could explain the difference of the involvement of TLR4 in male mice in the present work versus others studies as it was shown that orofacial pain and pain affecting the spinal system have different physio-pharmacological characteristics (Ambalavanar et al., 2005; Giniatullin et al., 2008; Latrémolière et al., 2008; Michot et al., 2012; Michot et al., 2014).

LPS-induced neuronal activation was described to be dependent on the activation of TRPA1 which activation increases cation influx into the cell and neuronal depolarization (Meseguer et al., 2014; Kaewpitak et al., 2020). Our work also shows that TLR4 is involved in trigeminal sensory neuron activation; both pharmacological and genetic inhibition of TLR4 prevents LPS induced increase of intracellular calcium and neuronal activation. However, TLR4 may not be directly linked to signaling pathways involving calcium. TLR4 can activate different signaling pathways including MyD88, TRIF, NF-kB, and MAPK (Stokes et al., 2013; Li et al., 2015; Kaewpitak et al., 2020) but whether these signaling messengers leading to calcium increase in the neurons in not clear. We showed that both intracellular and extracellular calcium are involved in LPS induced neuronal activation suggesting that extracellular calcium mobilization would be mediated by TRPA1 and intracellular calcium mobilization could be mediated by TLR4. However, we cannot exclude that in our model TLR4 could trigger extracellular calcium mobilization through activation of the voltage dependent calcium channel Cav 3.2 as previously reported (Li et al., 2017).

In the present study we investigated how LPS could contribute to the initiation of spontaneous pain-like behavior via neuronal sensitization. We show that LPS, through TLR4 activation, induces neuronal sensitization by upregulation of TRPA1. Specifically, the proportion of neurons responding to TRPA1 agonist was increased after LPS stimulation and prevented by TLR4 inhibition. In addition, we show that some neurons that are basally insensitive to AITC become responsive to TRPA1 agonists after LPS stimulation, suggesting that a subpopulation of neurons is sensitized by LPS and upregulate TRPA1 at the cell membrane.

TRPA1 is highly sensitive to pro-inflammatory mediators and its expression and activity are rapidly upregulated in inflammatory or neuropathic pain conditions (Obata et al., 2005; Frederick et al., 2007; Chen and Hackos, 2015). Interestingly, we show that LPS induces TRPA1 sensitization via the increase in the number of neurons sensitive to AITC but we did not observe an increase in the intensity of AITC response (Fig. 1). It would be possible that TRPA1 desensitization occurs in some neurons (Raisinghani et al., 2011) but would be compensated by the recruitment of the neuronal population newly responsive to AITC after LPS treatment. Different mechanisms are involved in TRPA1 upregulation, bradykinin activate PLC and PKA signaling pathway in neurons leading to TRPA1 phosphorylation, enhancing its response to AITC activation (Wang et al., 2008a; Meents et al., 2017). Proinflammatory mediators as well as nociceptive stimuli increase trafficking of TRPA1 to the cell membrane, leading to neuronal sensitization (Schmidt et al., 2009; Meng et al., 2016). In addition, it was shown that low intracellular calcium level can directly activate TRPA1 and enhance its response to selective agonists (Zurborg et al., 2007; Wang et al., 2008b; Hasan and Zhang, 2018). On the contrary, high intracellular calcium levels inactivate TRPA1 receptor (Wang et al., 2008b), highlighting the dual effects of calcium on TRPA1.

Among these mechanisms, changes in gene expression are not involved as we show the TRPA1 upregulation occurs within a few minutes after LPS stimulation. The LPS-induced TRPA1 sensitization is not mediated by calcium signaling, as inhibition of both extracellular and intracellular calcium mobilization did not prevent LPS-induced TRPA1 sensitization. On the contrary, we showed that both TLR4 inhibition and vesicular exocytosis inhibition prevented LPS-induced TRPA1 upregulation indicating that LPS directly sensitizes trigeminal neuron through TLR4 activation and TRPA1 trafficking at the cell membrane. These results are in line with previous data showing that TLR4 activation induces vesicular trafficking to the cell membrane and mediator release in macrophages (Ren et al., 2014) and that proinflammatory cytokines increase TRPA1 expression at the cell membrane through vesicular exocytosis (Schmidt et al., 2009; Meng et al., 2016).

Finally, we identified a trigeminal sensory neuron subpopulation that co-expresses TLR4 and TRPA1 but mobilize intracellular TRPA1 to the cell membrane, becoming functionally responsive to TRPA1 agonist only after LPS stimulation. These results suggest that this neuronal subpopulation express TRPA1 in storage vesicles which are mobilized in response to proinflammatory stimulation. This pool of TRPA1 receptor would allow a rapid increase in sensory neuronal activation in response to bacterial infection, contributing to the initiation of neuronal sensitization, pain and neurogenic inflammation that would precede the development of immune cell-mediated inflammation. Collectively this highlights the importance of sensory neurons to pathogen detection, and their activation by toxins such as LPS as being a potentially important mechanism in the early immune response.

References

Ambalavanar R, Moritani M, Dessem D (2005) Trigeminal P2X3 receptor expression differs from dorsal root ganglion and is modulated by deep tissue inflammation. Pain 117:280–291.

Barabas ME, Kossyreva EA, Stucky CL (2012) TRPA1 is functionally expressed primarily by IB4-binding, non-peptidergic mouse and rat sensory neurons. PLoS One 7:e47988.

Cavanaugh DJ, Chesler AT, Bráz JM, Shah NM, Julius D, Basbaum AI (2011) Restriction of transient receptor potential vanilloid-1 to the peptidergic subset of primary afferent neurons follows its developmental downregulation in nonpeptidergic neurons. J Neurosci 31:10119–10127.

Chen J, Hackos DH (2015) TRPA1 as a drug target–promise and challenges. Naunyn Schmiedebergs Arch Pharmacol 388:451–463.

Chen J, et al. (2011) Selective blockade of TRPA1 channel attenuates pathological pain without altering noxious cold sensation or body temperature regulation. Pain 152:1165–1172.

Cook AD, Christensen AD, Tewari D, McMahon SB, Hamilton JA (2018) Immune cytokines and their receptors in inflammatory pain. Trends Immunol 39:240–255.

Diogenes A, Ferraz CC, Akopian AN, Henry MA, Hargreaves KM (2011) LPS sensitizes TRPV1 via activation of TLR4 in trigeminal sensory neurons. J Dent Res 90:759–764.

Donnelly CR, Chen O, Ji RR (2020) How do sensory neurons sense danger signals? Trends Neurosci 43:822–838.

Fitzgerald KA, Kagan JC (2020) Toll-like receptors and the control of immunity. Cell 180:1044–1066.

Frederick J, Buck ME, Matson DJ, Cortright DN (2007) Increased TRPA1, TRPM8, and TRPV2 expression in dorsal root ganglia by nerve injury. Biochem Biophys Res Commun 358:1058–1064.

Gebhardt LA, Kichko TI, Fischer MJM, Reeh PW (2020) TRPA1-dependent calcium transients and CGRP release in DRG neurons require extracellular calcium. J Cell Biol 219:e201702151.

Giniatullin R, Nistri A, Fabbretti E (2008) Molecular mechanisms of sensitization of pain-transducing P2X3 receptors by the migraine mediators CGRP and NGF. Mol Neurobiol 37:83–90.

Hasan R, Zhang X (2018) Ca2+ regulation of TRP ion channels. Int J Mol Sci 19:1256.

Hu F, Song X, Long D (2021) Transient receptor potential ankyrin 1 and calcium: interactions and association with disease (review). Exp Ther Med 22:1462.

Kaewpitak A, Bauer CS, Seward EP, Boissonade FM, Douglas CWI (2020) Porphyromonas gingivalis lipopolysaccharide rapidly activates trigeminal sensory neurons and may contribute to pulpal pain. Int Endod J 53:846–858.

Kwan KY, Allchorne AJ, Vollrath MA, Christensen AP, Zhang DS, Woolf CJ, Corey DP (2006) TRPA1 contributes to cold, mechanical, and chemical nociception but is not essential for hair-cell transduction. Neuron 50:277–289.

Latrémolière A, Mauborgne A, Masson J, Bourgoin S, Kayser V, Hamon M, Pohl M (2008) Differential implication of proinflammatory cytokine interleukin-6 in the development of cephalic versus extracephalic neuropathic pain in rats. J Neurosci 28:8489–8501.

Li Y, Tatsui CE, Rhines LD, North RY, Harrison DS, Cassidy RM, Johansson CA, Kosturakis AK, Edwards DD, Zhang H, Dougherty PM (2017) Dorsal root ganglion neurons become hyperexcitable and increase expression of voltage-gated T-type calcium channels (Cav3.2) in paclitaxel-induced peripheral neuropathy. Pain 158:417–429.

Li Y, Zhang H, Kosturakis AK, Cassidy RM, Zhang H, Kennamer-Chapman RM, Jawad AB, Colomand CM, Harrison DS, Dougherty PM (2015) MAPK signaling downstream to TLR4 contributes to paclitaxel-induced peripheral neuropathy. Brain Behav Immun 49:255–266.

Malin SA, Davis BM, Molliver DC (2007) Production of dissociated sensory neuron cultures and considerations for their use in studying neuronal function and plasticity. Nat Protoc 2:152–160.

Meents JE, Fischer MJ, McNaughton PA (2017) Sensitization of TRPA1 by protein kinase A. PLoS One 12:e0170097.

Meng J, Wang J, Steinhoff M, Dolly JO (2016) TNFα induces co-trafficking of TRPV1/TRPA1 in VAMP1-containing vesicles to the plasmalemma via Munc18-1/syntaxin1/SNAP-25 mediated fusion. Sci Rep 6:21226.

Meseguer V, Alpizar YA, Luis E, Tajada S, Denlinger B, Fajardo O, Manenschijn JA, Fernández-Peña C, Talavera A, Kichko T, Navia B, Sánchez A, Señarís R, Reeh P, Pérez-García MT, López-López JR, Voets T, Belmonte C, Talavera K, Viana F (2014) TRPA1 channels mediate acute neurogenic inflammation and pain produced by bacterial endotoxins. Nat Commun 5:3125.

Michot B, Bourgoin S, Viguier F, Hamon M, Kayser V (2012) Differential effects of calcitonin gene-related peptide receptor blockade by olcegepant on mechanical allodynia induced by ligation of the infraorbital nerve vs the sciatic nerve in the rat. Pain 153:1939–1948.

Michot B, Kayser V, Bastian G, Bourgoin S, Hamon M (2014) Differential pharmacological alleviation of oxaliplatin-induced hyperalgesia/allodynia at cephalic versus extra-cephalic level in rodents. Neuropharmacology 79:432–443.

Michot B, Lee CS, Gibbs JL (2018) TRPM8 and TRPA1 do not contribute to dental pulp sensitivity to cold. Sci Rep 8:13198.

Neeb L, Hellen P, Boehnke C, Hoffmann J, Schuh-Hofer S, Dirnagl U, Reuter U (2011) IL-1β stimulates COX-2 dependent PGE_2 synthesis and CGRP release in rat trigeminal ganglia cells. PLoS One 6:e17360.

Obata K, Katsura H, Mizushima T, Yamanaka H, Kobayashi K, Dai Y, Fukuoka T, Tokunaga A, Tominaga M, Noguchi K (2005) TRPA1 induced in sensory neurons contributes to cold hyperalgesia after inflammation and nerve injury. J Clin Invest 115:2393–2401.

Pedersen-Bjergaard U, Nielsen LB, Jensen K, Edvinsson L, Jansen I, Olesen J (1991) Calcitonin gene-related peptide, neurokinin A and substance P: effects on nociception and neurogenic inflammation in human skin and temporal muscle. Peptides 12:333–337.

Pinho-Ribeiro FA, Baddal B, Haarsma R, O'Seaghdha M, Yang NJ, Blake KJ, Portley M, Verri WA, Dale JB, Wessels MR, Chiu IM (2018) Blocking neuronal signaling to immune cells treats streptococcal invasive infection. Cell 173:1083–1097.e22.

Raisinghani M, Zhong L, Jeffry JA, Bishnoi M, Pabbidi RM, Pimentel F, Cao DS, Evans MS, Premkumar LS (2011) Activation characteristics of transient receptor potential ankyrin 1 and its role in nociception. Am J Physiol Cell Physiol 301:C587–C600.

Ren H, Teng Y, Tan B, Zhang X, Jiang W, Liu M, Jiang W, Du B, Qian M (2014) Toll Toll-like receptor-triggered calcium mobilization protects

mice against bacterial infection through extracellular ATP release. Infect Immun 82:5076–5085.

Schäfers M, Svensson CI, Sommer C, Sorkin LS (2003) Tumor necrosis factor-alpha induces mechanical allodynia after spinal nerve ligation by activation of p38 MAPK in primary sensory neurons. J Neurosci 23:2517–2521.

Schmidt M, Dubin AE, Petrus MJ, Earley TJ, Patapoutian A (2009) Nociceptive signals induce trafficking of TRPA1 to the plasma membrane. Neuron 64:498–509.

Soldano A, Alpizar YA, Boonen B, Franco L, López-Requena A, Liu G, Mora N, Yaksi E, Voets T, Vennekens R, Hassan BA, Talavera K (2016) Gustatory-mediated avoidance of bacterial lipopolysaccharides via TRPA1 activation in *Drosophila*. Elife 5:e13133.

Sorge RE, LaCroix-Fralish ML, Tuttle AH, Sotocinal SG, Austin JS, Ritchie J, Chanda ML, Graham AC, Topham L, Beggs S, Salter MW, Mogil JS (2011) Spinal cord Toll-like receptor 4 mediates inflammatory and neuropathic hypersensitivity in male but not female mice. J Neurosci 31:15450–15454.

Stokes JA, Cheung J, Eddinger K, Corr M, Yaksh TL (2013) Toll-like receptor signaling adapter proteins govern spread of neuropathic pain and recovery following nerve injury in male mice. J Neuroinflammation 10:148.

Szabo-Pardi TA, Barron LR, Lenert ME, Burton MD (2021) Sensory neuron TLR4 mediates the development of nerve-injury induced mechanical hypersensitivity in female mice. Brain Behav Immun 97:42–60.

Wadachi R, Hargreaves KM (2006) Trigeminal nociceptors express TLR-4 and CD14: a mechanism for pain due to infection. J Dent Res 85:49–53.

Wang S, Dai Y, Fukuoka T, Yamanaka H, Kobayashi K, Obata K, Cui X, Tominaga M, Noguchi K (2008a) Phospholipase C and protein kinase A mediate bradykinin sensitization of TRPA1: a molecular mechanism of inflammatory pain. Brain 131:1241–1251.

Wang YY, Chang RB, Waters HN, McKemy DD, Liman ER (2008b) The nociceptor ion channel TRPA1 is potentiated and inactivated by permeating calcium ions. J Biol Chem 283:32691–32703.

Woller SA, Ravula SB, Tucci FC, Beaton G, Corr M, Isseroff RR, Soulika AM, Chigbrow M, Eddinger KA, Yaksh TL (2016) Systemic TAK-242 prevents intrathecal LPS evoked hyperalgesia in male, but not female mice and prevents delayed allodynia following intraplantar formalin in both male and female mice: The role of TLR4 in the evolution of a persistent pain state [Erratum in Brain Behav Immun 69:632]. Brain Behav Immun 56:271–280.

Woodbury CJ, Zwick M, Wang S, Lawson JJ, Caterina MJ, Koltzenburg M, Albers KM, Koerber HR, Davis BM (2004) Nociceptors lacking TRPV1 and TRPV2 have normal heat responses. J Neurosci 24:6410–6415.

Wu Y, Wang Y, Wang J, Fan Q, Zhu J, Yang L, Rong W (2019) TLR4 mediates upregulation and sensitization of TRPV1 in primary afferent neurons in 2,4,6-trinitrobenzene sulfate-induced colitis. Mol Pain 15:1744806919830018.

Zheng Y, Liu P, Bai L, Trimmer JS, Bean BP, Ginty DD (2019) Deep sequencing of somatosensory neurons reveals molecular determinants of intrinsic physiological properties. Neuron 103:598–616.e7.

Zurborg S, Yurgionas B, Jira JA, Caspani O, Heppenstall PA (2007) Direct activation of the ion channel TRPA1 by Ca2+. Nat Neurosci 10:277–279.