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# LPS Sensitizes TRPV1 *via* Activation of TLR4 in Trigeminal Sensory Neurons

## ABSTRACT

Recent studies have demonstrated that the lipopolysaccharide (LPS) receptor (TLR4) is expressed in TRPV1 containing trigeminal sensory neurons. In this study, we evaluated whether LPS activates trigeminal neurons, and sensitizes TRPV1 responses *via* TLR4. To test this novel hypothesis, we first demonstrated that LPS binds to receptors in trigeminal neurons using competitive binding. Second, we demonstrated that LPS evoked a concentration-dependent increase in intracellular calcium accumulation $(Ca^{2+})_i$ and inward currents. Third, LPS significantly sensitized TRPV1 to capsaicin measured by $(Ca^{2+})_i$, release of calcitonin gene-related peptide, and inward currents. Importantly, a selective TLR4 antagonist blocked these effects. Analysis of these data, collectively, demonstrates that LPS is capable of directly activating trigeminal neurons, and sensitizing TRPV1 *via* a TLR4-mediated mechanism. These findings are consistent with the hypothesis that trigeminal neurons are capable of detecting pathogenic bacterial components leading to sensitization of TRPV1, possibly contributing to the inflammatory pain often observed in bacterial infections.

**KEY WORDS:** neuropeptides/transmitters, neuroscience/neurobiology, pain, endotoxin, pharmacology.

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## INTRODUCTION

The innate immune response represents the first line of defense against invading micro-organisms. Its activation relies on "pattern recognition" receptors (PRRs) that detect specific molecular motifs that are highly conserved throughout evolution in different species (Roach *et al*., 2005). Eleven Toll-like receptors (TLRs) have been identified in humans (Chuang and Ulevitch, 2001; Tabeta *et al*., 2004). These transmembrane receptors bind to distinct components of various pathogens (Barton and Medzhitov, 2002). For example, Gram-negative bacteria are recognized by the Toll-like receptor 4 (TLR4) binding bacterial-derived lipopolysaccharides (LPS) expressed in the cell walls of these organisms (Xu *et al*., 2000).

The traditional hypothesis for pain associated with bacterial infections includes sensitization and activation of nociceptors by inflammatory mediators released from immune cells in response to the presence of bacteria or their constituents and toxins (Cunha *et al*., 1992; Julius and Basbaum, 2001). However, recent studies have demonstrated that TRPV1-containing trigeminal nociceptors express the receptors for LPS, namely, TLR4 and CD14 (Wadachi and Hargreaves, 2005). These findings suggested the hypothesis that nociceptors may directly detect Gram-negative bacteria *via* the recognition of LPS by TLR4, but the functional consequence of this recognition, if any, is unknown. In the present study, we tested the hypothesis that LPS directly activates TLR4 in trigeminal sensory neurons and whether this response produces neuronal activation and sensitization of TRPV1-mediated responses.

## MATERIALS & METHODS

### Animals

Adult male Sprague-Dawley rats (200-250 g, Charles River, Wilmington, MA, USA) were used in this study. The Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio approved all animal study protocols. Animals were housed for 1 wk prior to the experiments, with food and water available *ad libitum*.

### Chemicals

For the cultures of primary trigeminal neurons, nerve growth factor (NGF; Harlan, Indianapolis, IN, USA) was added to the culture media. For the immunofluorescence experiments, the following antibodies were used: rabbit polyclonal TLR4 (1:250; Santa Cruz Biotechnologies, Santa Cruz, CA,

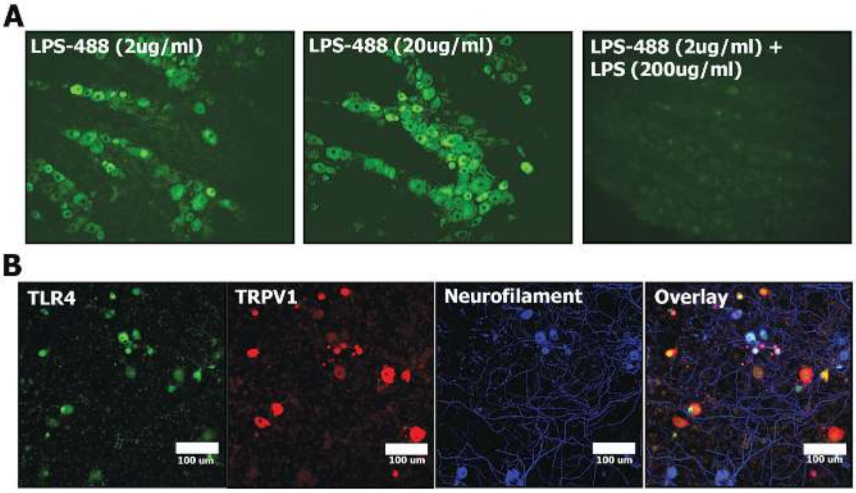

**Figure 1.** Trigeminal neurons express TLR4 in a TRPV1-containing subpopulation of neurons. **(A)** LPS selectively binds to a receptor in the plasma membrane of trigeminal (TG) neurons. The binding of the fluorescently labeled LPS (LPS-488) was competitively displaced with the co-incubation with an unlabeled LPS. **(B)** Representative confocal micrograph of trigeminal neurons cultured in coverslips for 5 days. TLR4 is visualized in green, TRPV1 in red, and neurofilament heavy in blue. The majority of immunoreactive TLR4 is found co-expressed with TRPV1.

USA), guinea-pig polyclonal TRPV1 (1:3000; Neuromics, Bloomington, MN, USA), and chicken polyclonal 200 kDa neurofilament heavy (1:2000; Abcam, Cambridge, MA, USA). For LPS competitive binding, lipopolysaccharide isolated either from *Escherichia coli* serotype 055:B5 conjugated with Alexa Fluor® 488 (LPS-488; Molecular Probes, Eugene, OR, USA) or 200 µg/mL of wild-type LPS from *E. coli* (Wt-LPS; Sigma, St. Louis, MO, USA) were used. For single-cell calcium imaging, immunoreactive calcitonin gene-related peptide (iCGRP) release and electrophysiology experiments, LPS from *E. coli* (InvivoGen, San Diego, CA, USA), a LPS antagonist derived from a mutated strain of *E. coli* lacking the myristoyl fatty acid moiety of the lipid A (*E. coli* K12 *msbB* strain; InvivoGen), and capsaicin (Sigma) were used.

## Rat Trigeminal Ganglia (TG) Primary Cultures

Rat TG were quickly removed after decapitation and neuronal cultures prepared as previously described (Diogenes *et al.*, 2006). Cells were plated on 24-well Poly-D-Lysine-coated plates (for iCGRP experiments) or Poly-D-Lysine-/laminin-coated coverslips (for single-cell calcium imaging, immunohistochemistry, and patch-clamp electrophysiology). The TG cultures were maintained at 37°C, 5% $CO_2$ for 5 days in the presence of 100 ng/mL NGF (Harlan).

## Immunofluorescence and LPS Binding Assay

For the immunofluorescence experiments, TG neurons cultured on coverslips were processed as described previously (Jeske *et al.*, 2009). Cells were then incubated overnight with antibodies anti-TLR4 (1:250), anti-TRPV1 (1:3000), and anti-200-kDa neurofilament heavy (1:2000), and immunoreactivity was visualized with species-specific Alexa Fluors 488, 568, and 633 IgG secondary antibodies (1:200; Molecular Probes). TG neurons were evaluated with a Nikon C1si laser scanning confocal microscope (Nikon Instruments, Melville, NY, USA). EZ-C1 v3.20 (Nikon) was used for acquisition of all images. Controls consisted of evaluation of cells that were stained as described above but that lacked primary antibodies. These control preparations lacked specific immunofluorescence.

For the LPS binding assay, freshly harvested rat TG were frozen in Neg-50 (Richard-Allen Scientific, Kalamazoo, MI, USA) and sectioned at 200 µm by means of a cryostat (Leica Microsystems, Bannockburn, IL, USA). The cryosections were immersed in phosphate-buffered saline (PBS) containing 2 µg/mL of lipopolysaccharide isolated either from *E. coli* serotype 055:B5 conjugated with Alexa Fluor 488 (LPS-488; Molecular Probes), or 200 µg/mL of wild-type LPS from *E. coli* (Wt-LPS; Sigma), or just vehicle, for 5 min. Sections were washed in cold PBS for 5 min, fixed with 4% formaldehyde, mounted onto slides, and visualized by confocal microscopy.

## Single-cell Calcium Imaging

The $Ca^{2+}$ imaging experiments and ratiometric data conversion were performed as previously described (Akopian *et al.*, 2007). We calculated the net changes in $Ca^{+2}$ influx by subtracting the basal $[Ca^{+2}]_i$ (mean value collected for 60 sec prior to addition of the first compound) from the peak $[Ca^{+2}]_i$ value achieved after exposure to the drugs. $Ca^{2+}$ influxes above 50 nM were considered positive. This minimal threshold criterion was established by application of 0.1% DMSO as a vehicle.

## Immunoreactive Calcitonin Gene-related Peptide (iCGRP) Release Assay

Experiments were performed on 5-day TG cultures at 37°C with modified Hanks (Gibco, Grand Island, NY, USA) buffer (10.9 mM HEPES, 4.2 mM sodium bicarbonate, 10 mM dextrose, and 0.1% bovine serum albumin were added to 1x Hanks). After 2 initial washes, a 15-minute baseline sample was collected. The cells were then exposed to either vehicle or LPS derived from *E. coli* strain 0111:B4 for 15 min and then stimulated with capsaicin (50 nM) for 15 min. In the antagonism experiments, cells were pre-treated with vehicle or a LPS antagonist derived from *E. coli* K12 *msbB*-LPS (InvivoGen) at 200 µg/mL (Somerville *et al.*, 1999), followed by exposure to LPS at 0.2 µg/mL for 15 min, and a subsequent application of capsaicin (50 nM) for 15 min. All treatments were collected for analysis of iCGRP content by radioimmunoassay (RIA), and experiments were repeated 3 times with n = 6 for each group.

### iCGRP RIA

A previously used primary antibody against CGRP (final dilution 1:1,000,000, kindly donated by Dr. Iadarola, NIH) was used as described previously (Diogenes *et al*., 2006).

### Patch-clamp Electrophysiology

Recordings were made in whole-cell perforated patch voltage clamp [holding potential ($V_h$) of –60 mV] configuration at 22-24°C from the somata of cultured TG neurons (15-40 pF). Data were acquired and analyzed by means of an Axopatch 200B amplifier and pCLAMP9.0 software (Molecular Devices). Recording data were filtered at 0.5 kHz and sampled at 2 kHz. Access resistance ($R_s$) was compensated for (40-80%) when appropriate up to the value of 13-18 MΩ. Data were rejected when $R_s$ changed > 20% during recording, leak currents were > 50 pA, or input resistance was < 200 MΩ. Currents were considered positive when their amplitudes were 5-fold bigger than displayed noise (in root mean square).

Standard external solution (SES) contained (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 D-glucose, and 10 HEPES, pH 7.4. The pipette solution consisted of (in mM): 140 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 EGTA, and 10 HEPES, pH 7.3. Drugs were applied in a fast, pressure-driven, and computer-controlled 8-channel system (ValveLink8; AutoMate Scientific, San Francisco, CA, USA).

### Statistics

All experiments were conducted in triplicate, with n = 24-126 cells *per* group for the calcium imaging experiments, n = 6 wells *per* group for iCGRP release experiments, and n = 12-24-14 *per* group for the patch-clamp electrophysiology. Data were analyzed with Prism software version 5 (GraphPad Software, San Diego, CA, USA). The results were analyzed by one-way ANOVA, and individual groups were compared by Bonferroni's *post hoc* test. The statistical significance was set at $p < 0.05$.

## RESULTS

### LPS Binds Selectively to Receptors in Trigeminal Ganglia Neurons

Prominent labeling of trigeminal neuronal cell bodies was observed by confocal microscopy in the samples exposed to fluorescently labeled LPS (LPS-488) at 2 µg/mL and 20 µg/mL concentrations for 5 min (Fig. 1A). This specific labeling was mostly eliminated by competitive binding following cell exposure to both fluorescently labeled LPS-488 (2 µg/mL) and unlabeled *E. coli*-derived LPS (Wt-LPS) at a concentration 100x greater (200 µg/mL) (Fig. 1A).

### TLR4 Co-localizes with TRPV1 in TG Sensory Neurons

We next evaluated whether the cultured TG neurons expressed TLR4. We used confocal microscopy to examine 5-day cultures, since this is the same time-point evaluated in single-cell calcium imaging, iCGRP release, and electrophysiology patch-clamp experiments. This examination showed the expression of TLR4 (LPS receptor) within a subset of sensory neurons. Co-localization studies showed the prominent co-expression of TLR4 within a subpopulation of small-diameter TRPV1-positive neurons. It can also be noted that TLR4 is present in some non-TRPV1 neurons (Fig. 1B).

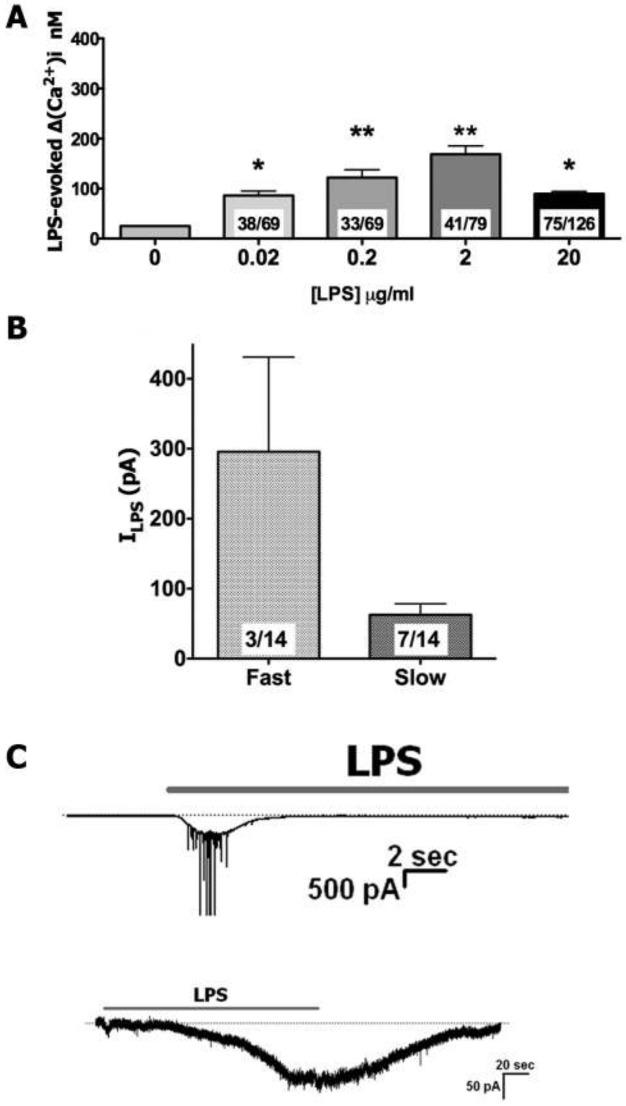

**Figure 2.** LPS activates TG sensory neurons *in vitro*. **(A)** Treatment of trigeminal neurons *in vitro* with *E. coli*-derived LPS evoked a concentration-dependent $(Ca^{2+})_i$ accumulation. Data are presented as mean ± SEM, $^*p < 0.05$, and $^{**}p < 0.01$. **(B)** Treatment of trigeminal neurons *in vitro* with LPS (2 µg/mL) evoked inward currents in ~ 71% of the neurons evaluated. These currents were classified as either slow (30%) or fast (70%). **(C)** Representative current traces from patch-clamp experiments illustrating both types of currents (slow and fast).

### LPS Activates TG Sensory Neurons

We next examined whether LPS is able to activate TG sensory neurons. Administration of LPS to trigeminal neurons for 5 min

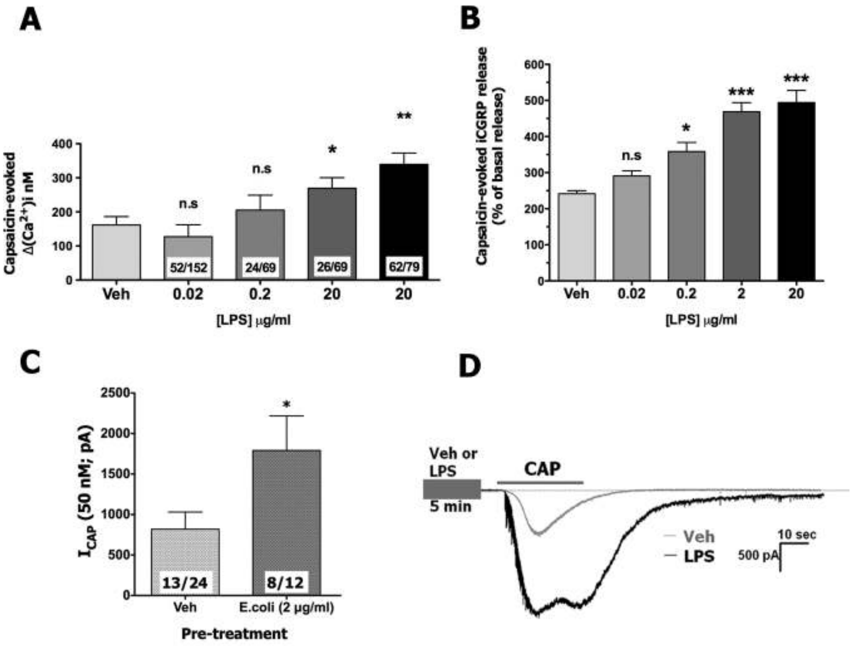

**Figure 3.** LPS sensitizes TRPV1 responses in a concentration-dependent manner. **(A)** Pre-treatment of trigeminal neurons with LPS evoked a concentration-dependent sensitization of a subsequent capsaicin-evoked accumulation of intracellular calcium. Data are presented as mean ± SEM, Veh = vehicle, n.s. = not significant, $*p < 0.05$, $**p < 0.01$. **(B)** Pre-treatment of trigeminal neurons with LPS evoked a concentration-dependent sensitization of capsaicin-evoked iCGRP release. Data are presented as mean ± SEM, Veh = vehicle, $*p < 0.05$, $***p < 0.001$. **(C)** Pre-treatment of trigeminal neurons with LPS (2 μg/mL) led to the sensitization of a subsequent capsaicin-evoked inward current. Data are presented as mean ± SEM, Veh = vehicle, $*p < 0.05$. **(D)** Representative traces of LPS sensitization of capsaicin-evoked currents.

increased $[Ca^{2+}]_i$ levels in a concentration-dependent manner in approximately 48% of the cells, with levels typically returning to baseline values after a two-minute wash (Fig. 2A). The LPS concentration-dependent increase in $[Ca^{2+}]_i$ showed an $EC_{50}$ (concentration required to elicit 50% of maximum response) of ~ 0.06 μg/mL and an $E_{MAX}$ (concentration required to elicit maximum response) of ~ 2 μg/mL (Fig. 2A). Importantly, there was a significant decrease of response at the concentration of 20 μg/mL when compared with $E_{MAX}$ (2 μg/mL), suggestive of pharmacological desensitization of the TLR4 receptor complex.

To confirm activation of TG by LPS, we performed whole-cell voltage clamp recordings from 5-day-cultured TG neurons treated by LPS (2 μg/mL). LPS-evoked inward currents ($I_{LPS}$) were recorded from approximately 71% of the small-to-medium neurons (25-37 pF or 22-36 μm) (Fig. 2B). Two types of $I_{LPS}$ were registered: slow activation kinetic current with 24.28 ± 4.3 sec, and fast activation with 4.5 ± 1.65 sec of 5-95% rise time to peak (Figs. 2B, 2C).

## LPS Sensitizes Capsaicin-evoked Responses in TG Sensory Neurons

Since LPS can generate hyperalgesia, we next evaluated whether LPS modulates TRPV1 channel function by LPS. Pre-treatment of TG neurons with LPS led to a significant, concentration-dependent sensitization of capsaicin (30 nM)-evoked $[Ca^{2+}]_i$ accumulation in approximately 61% of the capsaicin-responsive cells (Fig. 3A). Thus, a 3-minute pre-treatment with LPS 20 μg/mL produced nearly a two-fold increase in capsaicin-evoked $(Ca^{2+})_i$ accumulation (339.5 ± 33.28 nM, *vs.* 161.7 ± 24.6 nM; one-way ANOVA, $p < 0.001$).

Analysis of the iCGRP-release assay and patch-clamp electrophysiology data supports $Ca^{2+}$-imaging findings. Indeed, LPS significantly increased the capsaicin-evoked (50 nM) iCGRP release in a concentration-dependent manner (Fig. 3B). In addition, LPS treatment for 5 min led to a > 2-fold increase in capsaicin-evoked inwards currents ($I_{cap}$) (819.2 ± 211.5 pA, *vs.* 1791 ± 426.4 pA; $p < 0.05$) (Figs. 3C, 3D). In summary, LPS both activates TG neurons and sensitizes TRPV1-mediated capsaicin responses in sensory neurons.

## LPS Sensitizes TRPV1 Function *via* the TLR4 Pathway in TG Sensory Neurons

To test the hypothesis that sensory neuron activation and the TRPV1 sensitization by LPS are mediated *via* TLR4 pathways, we used a pharmacological approach. A previously validated TLR4 antagonist (Somerville *et al.*, 1999) at a concentration of 200 μg/mL was used. Pre-treatment with this TLR4 antagonist significantly blocked the direct LPS (2 μg/mL)-evoked $(Ca^{2+})_i$ accumulation (Fig. 4A). As described in the previous experiment, pre-treatment with LPS at 2 μg/mL significantly increased the capsaicin-evoked increase of $(Ca^{2+})_i$. This increase in response was significantly inhibited by pre-treatment with a specific TLR4 antagonist (Fig. 4A). Further, the antagonist significantly blocked LPS (2 μg/mL)-induced potentiation of capsaicin-triggered iCGRP (Fig. 4B). Finally, administration of the antagonist alone did not evoke any significant $(Ca^{2+})_i$ (Fig. 4A) or iCGRP responses (Fig. 4B).

## LPS Increases the Release of Immunoreactive Calcitonin Gene-related Peptide (iCGRP) Mediated by TRPV1 in a Concentration-dependent Manner *via* TLR4

Administration of LPS at concentrations ranging from 0.02 to 20 μg/mL did not evoke a significant release of iCGRP (data not shown). However, it significantly increased the iCGRP release evoked by capsaicin (50 nM) in a concentration-dependent manner (Fig. 3B). Importantly, a selective TLR4 antagonist significantly blocked the maximum effect observed with 2 μg/mL, whereas the antagonist by itself had no effect on the basal iCGRP release (Fig. 4B).

## DISCUSSION

In this study, we demonstrated that LPS can directly activate sensory neurons and sensitize TRPV1-mediated capsaicin responses in trigeminal sensory neurons *in vitro* via TLR4 pathways. This is the first demonstration that LPS displays distinct direct effects on neuronal activation as well as selective sensitization of TRPV1-mediated activities in trigeminal sensory neurons.

In the first set of experiments, we demonstrated that immunoreactive TLR4 receptors in trigeminal neurons selectively bound fluorescently labeled LPS. Importantly, the binding of the labeled LPS was displaced with competitive binding of unlabeled LPS, demonstrating that competitive receptor binding interactions occur in the plasma membrane of neuronal cell bodies. Further, the expression of TLR4 was verified by immunofluorescence in trigeminal neurons cultured under the same conditions as those used in the functional assays.

TRPV1 is an ionotropic channel that, within the sensory ganglia, is expressed exclusively in nociceptors, and is required for the development of inflammatory hyperalgesia (Caterina *et al*., 2000). There is a substantial body of evidence demonstrating that TRPV1 integrates the signaling of several inflammatory mediators (Bhave *et al*., 2003; Diogenes *et al*., 2006). In this study, in addition to directly activating TG neurons, we investigated whether LPS could directly sensitize TRPV1 activation by capsaicin. We found that LPS sensitizes capsaicin-evoked intracellular calcium accumulations, iCGRP release, and inward currents. The increase of capsaicin-evoked responses is not an additive effect observed of the previous LPS effect (pre-treatment), since for the intracellular calcium accumulation and electrophysiology experiments, those responses returned to baseline prior to the administration of the capsaicin stimulus, and for the iCGRP release experiments, LPS by itself had no effect. Therefore, the effect of LPS in trigeminal neurons is not a global effect of increased neuronal activity, but rather a selective mechanism leading to the sensitization of the TRPV1 channel.

Besides TLR4, LPS has other co-receptors (LBP, CD14, and MD-2) that appear to have an amplifying effect but are not sufficient to mediate LPS signaling (Schumann *et al*., 1990). In this study, we used LPS from a mutated strain of *E. coli* (*E. coli* K12 *msbB* strain) to block the effects of LPS activation of TLR4 in trigeminal neurons. This mutated strain of LPS is known to be a competitive antagonist of LPS binding to TLR4 (Somerville *et al*., 1999). Analysis of these data demonstrated that, despite possible complex interaction with other receptors, TLR4 mediates and is essential to the LPS effects observed in TG neurons *in vitro*.

LPS represents one of the primary initiators of the innate immune response in the dental pulp as caries progresses and the microbial flora becomes composed of predominantly facultative and obligate Gram-negative anaerobes (Drucker *et al*., 1992; Gomes *et al*., 2004). Additionally, at the concentrations tested in this study, LPS has been found in infected root canal systems and correlated with pain (Jacinto *et al*., 2005). In this study, we have demonstrated that trigeminal sensory neurons are activated and have TRPV1 responses sensitized by LPS. A subpopulation of these nociceptors densely innervates the dental pulp, with some primary afferents extending their free-nerve endings into the dentinal tubules (Byers, 1980). A peptidergic subpopulation of these nociceptors releases vasoactive neuropeptides such as CGRP and substance P, causing vasodilatation and plasma extravasation, namely, neurogenic inflammation (Kilo *et al*., 1997). Therefore, trigeminal nociceptors may initially recognize LPS reaching the dental pulp through the dentinal tubules, leading to pain and inflammation (*i.e.*, pulpitis), a warning sign of the recognition of invading Gram-negative anaerobic bacteria. Thus, neuronal activation and sensitization by LPS can potentially amplify the inflammatory reaction in the dental pulp in conjunction with cells of the innate immune response and ancillary cells such as odontoblasts and fibroblasts, known to express both TLR4 and TRPV1 (Botero *et al*., 2003; Miyamoto *et al*., 2005; Jiang *et al*., 2006; Staquet *et al*., 2008; El Karim *et al*., in press).

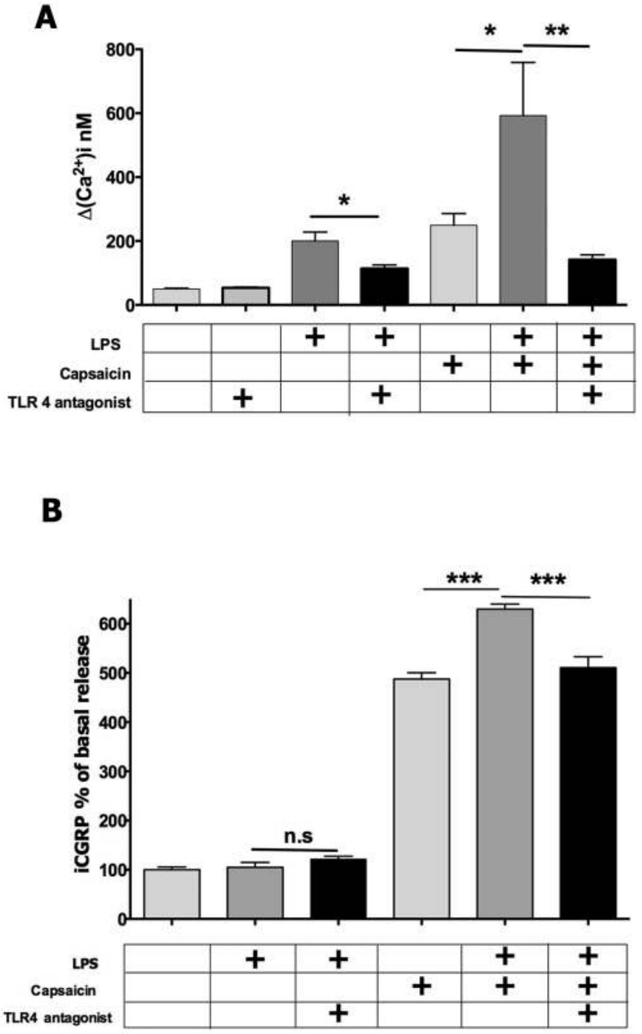

**Figure 4.** LPS activates sensory neurons and sensitizes TRPV1 *via* activation of TLR4. **(A)** Pre-treatment of trigeminal neurons with a TLR4 antagonist significantly blocked both LPS-evoked $(Ca^{2+})_i$ accumulation and the sensitization of TRPV1 responses. Data are presented as mean ± SEM, $^*p < 0.05$, $^{**}p < 0.01$. **(B)** Pre-treatment of trigeminal neurons with a TLR4 antagonist significantly blocked LPS-mediated sensitization of capsaicin-evoked iCGRP release. Data are presented as mean ± SEM, n.s. = not significant, $^{***}p < 0.001$.

Analysis of these data, collectively, demonstrates that TLR4 receptors expressed in trigeminal sensory neurons are capable of binding to LPS, leading to rapid neuronal activation and sensitization of TRPV1 in a concentration-dependent manner. This study expands our knowledge of the mechanisms of peripheral nociceptive sensitization, and sheds light on the clinical challenge of managing pain associated with bacterial infections.

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