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Porphyromonas gingivalis lipopolysaccharide rapidly activates trigeminal sensory neurons and may contribute to pulpal pain

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Abstract

Aim To determine whether *Porphyromonas gingivalis* lipopolysaccharide (LPS) can directly activate trigeminal neurons, to identify which receptors are involved, and to establish whether activation leads to secretion of the neuropeptide calcitonin-gene related peptide (CGRP) and/or the translocation of NF-κB.

Methodology Mouse trigeminal ganglion (TG) cells were cultured *in vitro* for 2 days. The effect of *P. gingivalis* LPS (20 μg/mL) on calcium signalling was assessed (by calcium imaging using Cal-520 AM) in comparison to the transient receptor potential channel A1 (TRPA1) agonist cinnamaldehyde (CA; 100 μM), the TRP channel V1 (TRPV1) agonist capsaicin (CAP; 1 μM), and high potassium (60 mM KCl). TG cultures were pre-treated with either 1 μM CLI-095 to block Toll-like receptor 4 (TLR4) signalling or with 3 μM HC-030031 to block TRPA1 signalling. CGRP release was determined using ELISA, and nuclear translocation of NF-κB was investigated using immunocytochemistry. Data were analysed by one-way analysis of variance, followed by Bonferroni's post-hoc test as appropriate.

Results *P. gingivalis* LPS directly exerted a rapid excitatory response on sensory neurons and non-neuronal cells ($p<0.001$ to $p<0.05$). The effects on neurons appear to be mediated via TLR4- and TRPA1-dependent pathways. The responses were accompanied by an increased release of CGRP ($p<0.001$) and by NF-κB nuclear translocation ($p<0.01$).

Conclusions This study reports for the first time that *P. gingivalis* LPS directly activates trigeminal sensory neurons (via TLR4 and TRPA1 receptors) and non-neuronal cells, resulting in CGRP release and NF-κB nuclear translocation. This indicates that *P. gingivalis* can directly influence activity in trigeminal sensory neurons and this may contribute to acute and chronic inflammatory pain.

Introduction

Infection of the dental pulp initiates a variety of pathophysiological changes including the generation of pain. While immune mechanisms are known to indirectly influence nociception, bacteria or their products have also been reported to activate sensory neurons directly. A range of oral bacteria are known to infect dental pulps, many of which are Gram-negative, and amongst which is *Porphyromonas gingivalis* (Rôças *et al.* 2002).

A major component of Gram-negative bacterial surfaces is lipopolysaccharide (LPS) and previous investigators have shown that LPS might directly activate sensory neurons (Diogenes *et al.* 2011, Tse *et al.* 2014). *Escherichia coli* LPS has been shown to increase neuronal excitability of dorsal root ganglia and trigeminal ganglion neurons (Meseguer *et al.* 2014). However, *P. gingivalis* LPS differs from that of *E. coli* and it is not known whether this and other non-enterobacterial LPS that would be found in pulpal infection have the same effects and, if so, which neuronal receptors are involved.

There are two categories of cell surface receptors that are thought to be key participants in a rapid nociceptor response to LPS and the initiation of neurogenic inflammation; Toll-like receptors (TLRs) and transient receptor potential channels (TRPs). TLRs are pattern-recognition receptors highly conserved throughout evolution (Santoni *et al.* 2015), and it is known that they can be activated by some forms of LPS (Diogenes *et al.* 2011, Meseguer *et al.* 2014, Tse *et al.* 2014). Of the 11 TLRs identified in humans so far (Qi *et al.* 2011), TLR4 specifically recognizes LPS of Gram-negative bacteria (Coats *et al.* 2005) and previous workers have shown that TLR4 is expressed by many cell types within the dental pulp, including odontoblasts (Jiang *et al.* 2006), fibroblasts (Hirao *et al.* 2009) and nerve fibres (Wadachi & Hargreaves 2006). TRPs are a family of cation channels that respond to a range of thermal and chemical stimuli including inflammatory stimuli (Geppetti *et al.* 2008, Veldhuis & Bunnett 2013, Santoni *et al.* 2015). *E. coli* LPS is capable of sensitizing TRPV1 via a mechanism involving TLR4 (Diogenes *et al.* 2011). Also, the TRPA1 receptor can be activated by the anionic lipid A of LPS (Meseguer *et al.* 2014). Although TRPV1 and TLR4 co-expression has been demonstrated (Diogenes *et al.* 2011), a complete picture of the crosstalk with other receptors and the actual pathways that these receptors feed into has not been elucidated. Therefore, the aim of this study was to determine whether *P. gingivalis* LPS could induce pulpal pain by directly activating trigeminal neurons and lead to secretion of the neuropeptide CGRP and the translocation of NF-κB.

Materials and methods

Animals

Healthy, 3–9-month-old C57BL/6 wild-type adult mice (30–35 g) were used. All animals were maintained on a 12-hour light/dark cycle in a temperature-controlled environment and given food *ad libitum*. All animal procedures were conducted under the Animal (Scientific Procedures) Act 1986, and approved by the UK Home Office.

Mouse trigeminal ganglia (TG) primary cultures

Mice were sacrificed by an overdose of isoflurane anaesthetic and cervical dislocation, and the TG were removed immediately. TG were placed in ice-cold, calcium- and magnesium-free Hanks' balanced salt solution (HBSS; Invitrogen, Paisley, UK), and then quickly washed twice with HBSS supplemented with 1% penicillin/ streptomycin (PS) (Gibco, Paisley, UK) at 37°C in 5% CO_2. Ganglia were then treated with 1.25% w/v type IV collagenase (Sigma, Gillingham, UK) once for 60 min and then again for 45 min, followed by 1.25% w/v trypsin (Sigma) for 15 min at 37°C. After digestion, the TG were homogenized mechanically by pipetting up and down, and centrifuged at 1500 *g* for 3 min, then re-suspended in supplemented medium (DMEM/F12 media, 1% w/v BSA, 1% N2 supplement, 1% PS; Bottenstein & Sato 1979). The mixtures were transferred to poly-l-lysine and laminin-coated coverslips that were placed at the bottom of 24-well tissue culture trays and cultured for 2–3 days with media change once after 24 h. All experiments on cultured cells were performed on day 2–3.

Intracellular calcium measurements

Cells were loaded for 60 min at 37°C with 5 μM Cal-520 AM (Abcam, Cambridge, UK) in DMEM/F12 medium after which coverslips were transferred into a chamber (Warner Instruments RC-25F; Harvard Apparatus Ltd, Edenbridge, UK) mounted on the stage of a Zeiss Axiovert S100TV microscope (Zeiss, Jena, Germany) and continuously perfused with buffer (142 mM NaCl, 5 mM $NaHCO_3$, 10 mM Hepes, 16 mM Glucose, 2 mM KCl, 2 $CaCl_2$, 1 mM $MgCl_2$, and 0.1% BSA, pH 7.3) at a flow rate of 5 mL/min. Cells were illuminated at 488 nm with a monochromator (TILL Photonics, Kaufbeuren, Germany), and viewed using a band-pass filter (510–540 nm) and 40 × objective (NA1.3; Zeiss). Images were acquired at 6-second intervals using a Photometrics Cascade 512B CCD camera (Photometrics UK Ltd, Marlow, UK) and timelapse sequences recorded using WinFluor® software(V3.8.7; Strathclyde Imaging Software, University of Strathclyde, Glasgow, UK). Following

background subtraction, fluorescence values (measured for every image from regions of interest [ROIs] placed over the soma of cells) were exported and plotted using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). Cells were classified as responsive to a particular stimulus when fluorescence, measured in an ROI, increased by more than five standard deviations over the baseline fluorescence. Baseline fluorescence was measured as the average of the last 10 frames before application of tested products or agonists. Data are presented as the changes in intensity of fluorescence between the baseline and peak of the activated curve. This calculation was used to account for variations in the starting fluorescence of individual cells.

The effect of *P. gingivalis* LPS (20 μg/mL) on calcium signalling was assessed in comparison to the TRPA1 agonist cinnamaldehyde (CA; 100 μM), the TRPV1 agonist capsaicin (CAP; 1 μM), and high potassium (60 mM KCl). To determine whether signalling was occurring via TLR4 or the TRPA1 receptor, we used specific blocking agents applied to the TG cultures prior to exposure to LPS. TG cultures were pre-treated with either 1 μM CLI-095 (Invivogen, UK) for 3 h to block TLR4 or for 1 minute with 3 μM HC-030031 (Tocris Bioscience, Bristol, UK) to block TRPA1 signalling (Kunkler *et al.* 2011, Meseguer *et al.* 2014, Tse *et al.* 2014) and the resultant calcium flux of cells was recorded as described above.

Calcitonin gene-related peptide (CGRP) release assay

Experiments were performed at 37°C in modified HBSS (Invitrogen) (10.9 mM HEPES, 4.2 mM sodium bicarbonate, 10mM dextrose, and 0.1% bovine serum albumin in 1 × HBSS). After two initial washes in HBSS, a 15 min baseline sample was collected. The cells were then exposed to either HBSS alone or HBSS containing *P. gingivalis* LPS (20 μg/mL) for 15 min. Where indicated, inhibitors of TLR4, TRPA1 and TRPV1 were administered at 1 μM CLI-095 for 3 h, 3 μM HC-030031 for 2 min, or 10 μM capsazepine for 2 min prior to the exposure to LPS, as described by previous investigators (Eid *et al.* 2008, Henrich & Buckler 2009, Tse *et al.* 2014). The supernatants were collected and assayed by a competitive enzyme-linked immunosorbent assay (ELISA) for calcitonin gene-related peptide (CGRP). Briefly the ELISA was performed by coating microtitre plate wells with 1:1000 anti-CGRP mouse monoclonal capture antibody (Sigma) in 0.05 M carbonate buffer pH 9.5 overnight at 4°C to immobilize the capture antibody to the wells. Plates were then washed three times with 0.05% PBS-Tween and blocked with 5% BSA in 0.05% PBS-Tween (pH 7.4) for 3 hours at room temperature. After washing, 50 μL of standard CGRP or samples were added to the plate, together with 50 μL of 2 ng/mL biotin-conjugated CGRP (Biorbyt Ltd,

Cambridge, UK), and incubated for 1 hour at 37°C. After washing six times, the plates were incubated with 1:400 streptavidin-horseradish peroxidase conjugate (strep-HRP) for 45 min. Tetramethylbenzidine/hydrogen peroxide substrate solution (TMB; Sigma) was then added until colour developed (10–20 min), and the reaction was stopped by addition of 2M HCl. Resulting colour was read using a spectrophotometer (Infinite 200 PRO; Tecan, Reading, UK) at 450 nm. Unknown CGRP concentrations were calculated by interpolation from a standard curve.

NF-κB translocation

Following application of LPS or PBS for 15 min, cells were washed twice with PBS and then fixed in 4% paraformaldehyde (PFA) for 40 minutes. To block non-specific antibody binding, coverslips were incubated in PBS containing 0.5% Triton X-100 (PBST) and 10% normal donkey serum (NDS) for 1h at room temperature. The coverslips were then incubated in mouse anti-β-tubulin III monoclonal antibody (1:1000, Covance, Harrogate, UK), and rabbit anti-NF-κB polyclonal antibody (1:250, ThermoFisher Scientific, Paisley, UK) diluted in PBST containing 1% NDS, at room temperature for 1h. Cultures were washed twice in PBST for 15 min and incubated with Cy3-conjugated donkey anti-rabbit, and FITC-conjugated donkey anti-mouse secondary antibodies (1:400, Jackson ImmunoResearch, West Grove, PA, USA) diluted in PBST containing 1% NDS, at room temperature for 1h. Finally, the cultures were washed twice in PBS for 15 min and slides were mounted with Vectashield containing DAPI (Vector Laboratories, Burlingame, CA, USA) and visualized by fluorescent microscopy. Immunohistochemistry controls for β-tubulin III and NF-κB were performed by omitting the primary antibodies.

NF-κB nuclear translocation was determined by immunohistochemistry and co-localising with DAPI and β-tubulin III, markers of DNA and neurons, respectively. The translocation of NF-κB could be determined by analysing the proportion of NF-κB fluorescent label in the nuclear and cytoplasmic compartments. Positive nuclear translocations were quantified by measuring the relative fluorescence intensity of NF-κB staining at each intracellular location. DAPI nuclear staining was used to confirm the location of the nuclei. The translocation of NF-κB was counted as positive when the nuc/cyt ratio was >1. Neurons and non-neuronal cells positive for NF-κB nuclear translocation were quantified.

Statistical analysis

Data were analysed by one-way ANOVA, and individual groups were compared by Bonferroni's post-hoc test using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). The statistical significance was set at $p<0.05$.

Results

P. gingivalis LPS can rapidly activate trigeminal ganglion neurons and non-neuronal cells

The responses of trigeminal neurons to *P. gingivalis* LPS, cinnamaldehyde (CA), capsaicin (CAP) and high potassium were assessed using the fluorescent calcium indicator, Cal-520 AM (Figure 1). A total of 90 neurons were assessed from three to four coverslips per experiment and responses were examined from three independent experiments. *P. gingivalis* LPS was found to directly activate approximately 27% of the trigeminal neurons present (24/90). Our study also demonstrated that approximately 13%, and 33% of mouse TG neurons responded to CA and CAP, respectively. Application of high potassium was performed to assess neuron viability and to help distinguish neuronal from non-neuronal cells (Figure 1G, H).

Neuronal cells show a very clear rapid rising and transient response to KCl resulting from depolarization and action potential-evoked calcium signals, the amplitude and duration of calcium transients being determined by the contribution of different ion channels contributing to the action potential (e.g. Mohammed *et al.* 2017); in contrast, non-neuronal cells do not fire action potentials and where calcium signals are observed, these are notably slower, and are likely related to transporters. As shown, neuronal cells also show clear rapid responses to cinnamaldehyde or capsaicin, whereas non-neuronal cells show no or only very weak responses to these compounds. As well as differences in the calcium signalling, the morphology of cells can also be used as a discriminator, as indicated in Figure 1A. Note the non-neuronal cells are much smaller than neurons and lack the extensive thin branching neurites characteristic of neurons.

Approximately 40% of the non-neuronal cells present, such as satellite glial cells and Schwann cells, also responded to *P. gingivalis* LPS (representative data are shown in Figure 1). This suggests that neuronal supporting cells can form part of the responsive population to *P. gingivalis* LPS in the peripheral nervous system (see Figure 1H).

Calcium influx activated by LPS can be abolished by TRPA1 antagonist and modified by TLR4 antagonist

Whether *P. gingivalis* LPS triggered calcium influx *via* TLRs or the TRPs was then investigated. For this, TLR4 and TRPA1 were functionally blocked by pretreating TG neurons with either CLI-095 or HC-030031, respectively. Responses were examined from three independent experiments. A total of 72 neurons in the presence of HC-030031 and

63 neurons in the presence of CLI-095 were assessed from three to four coverslips per experiment. The TRPA1 antagonist (3 μM HC-030031) completely abolished the neuronal response to *P. gingivalis* LPS (0/72), whereas pre-treatment of neurons with a specific TLR-4 signalling inhibitor (1 μM CLI-095) reduced but did not completely inhibit the response. The percentage of neurons responding to LPS in the presence of CLI-095 was reduced to less than 5%, suggesting a contributory role for the TLR4 receptor. Representative, individual traces are shown in Figure 2.

Neuropeptide (CGRP) release by P. gingivalis LPS can be prevented by TLR4, TRPA1, and TRPV1 antagonists

LPS is thought to be a mediator of neurogenic inflammation (Hou *et al.* 2003, Diogenes *et al.* 2011, Ferraz *et al.* 2011) initiated by neuropeptide release. Whether *P. gingivalis* LPS could mediate neuropeptide release from the TG neurons *in vitro* was therefore determined.

TG cultures were either stimulated with vehicle, *P. gingivalis* LPS (20 μg/mL) or capsaicin (1 μM) for 15 minutes at 37°C and the resulting levels of CGRP released into the culture medium determined by ELISA. *P. gingivalis* LPS significantly increased CGRP release from TG neurons (Figure 3, $p<0.001$) as did treatment with the positive control, 1 μM capsaicin (Figure 3).

Since the calcium influx in response to LPS could be abolished by the TRPA1 blocker, and partially removed by TLR4 signalling inhibition, whether TLR4, TRPA1 and TRPV1 inhibitors could prevent CGRP secretion in response to LPS was explored. CGRP release experiments were again performed by exposing TG cultures to LPS following pre-treatment with 3 μM HC-030031, 1 μM CLI-095 or 1 μM capsazepine. CGRP release from TG cultures in the presence of *P. gingivalis* LPS (20 μg/mL) was inhibited by all of three blocking reagents to the level obtained in vehicle-only stimulated samples (Figure 4, $p<0.001$).

P. gingivalis LPS induces NF-κB nuclear translocation in TG neurons and can be modified by TLR4, and TRPA1 antagonists.

P. gingivalis LPS is known to activate nuclear translocation of NF-κB in a range of cell types (Hashimoto *et al.* 2004, Herath *et al.* 2013). Consequently, whether LPS induces NF-κB nuclear translocation in neurons and/or the support cells present in the TG cultures was determined.

Nuclear translocation of NF-κB was considered positive when the nuclear/cytoplasm ratio (nuc/cyt ratio) of NF-κB in neurons was >1, calculated using the methods of Hunot *et al.* (1997) and Kuiken *et al.* (2012). Immunofluorescence revealed that *P. gingivalis* LPS stimulation resulted in positive nuclear translocation of NF-κB in TG neurons and non-neuronal cells over a period of 30 minutes (Figure 5). The percentage of neuronal cells positive for NF-κB translocation was significantly greater in LPS-treated cultures compared with control cultures (62% vs 18%, respectively; $p<0.01$). The percentage of NF-κB translocation-positive non-neuronal cells was also significantly greater following LPS treatment (67% vs 37% in stimulated and control cultures, respectively; $p<0.05$).

Further work showed that both neuronal and non-neuronal nuclear translocation of NF-κB in response to LPS could be inhibited by blocking TLR4 signalling (1 μM CLI-095), but not by blocking TRPA1 (3 μM HC-030031) or TRPV1 signalling (10 μM capsazepine). The percentage of neurons showing nuclear translocation of NF-κB in the presence of 1 μM CLI-095 inhibitor along with LPS decreased significantly from 65% to approximately 30%, and in non-neuronal cells it decreased from 65% to 35% (Figure 6; $p<0.05$).

Discussion

This study reports for the first time that *P. gingivalis* LPS directly activates TLR4 and TRPA1 receptors in trigeminal sensory neurons and non-neuronal support cells. The study also shows that this activation leads to release of the neuropeptide calcitonin-gene related peptide (CGRP) and activation of the NF-κB signalling pathway.

The rationale for the study comes from evidence that some bacterial pathogens can directly stimulate a nociceptor neuronal response (Chiu *et al.* 2013). There is relatively little information, however, about the neuronal response to oral Gram-negative bacteria and since *P. gingivalis* is commonly reported in association with symptomatic pulpitis (Rôças *et al.* 2002, Jacinto *et al.* 2006), whether *P. gingivalis* LPS could directly activate sensory neurons was investigated. For this, an *in vitro* culture model of TG neurons was developed. It was not possible to obtain a culture of neurons free from support cells (glial and Schwann cells) because they are essential for survival of the neurons *in vitro*. However, it was possible to differentiate the responses of neurons from those of non-neuronal cells to various agonists by a combination of calcium imaging and immunofluorescence.

There are a number of receptors that could be involved in the detection and transduction of dental pain following pulpal infection. Some of these involve responses to mediators released as part of the inflammatory response (Igwe 2003, Gonzalez-Rey *et al.* 2010), but there are two categories of cellular receptors that could potentially interact with Gram-negative bacterial LPS. Firstly, TLRs are thought to be primary key participants in inflammatory responses; secondly, TRP receptors have emerged as having critically important roles in peripheral sensitization (Rang *et al.* 1991, Chen *et al.* 2011, Chung *et al.* 2011, Tóth *et al.* 2011). TLRs have been reported to be present on trigeminal and DRG neurons (Wadachi & Hargreaves 2006, Ochoa-Cortes *et al.* 2010, Qi *et al.* 2011) and in the present study ultrapure *P. gingivalis* LPS directly activated more than 20% of TG neurons in the cultures, with a resultant increase in intracellular calcium. This is the first time that *P. gingivalis* LPS has been shown to directly activate TG neurons *in vitro*. Also, the neuronal response could be partially blocked by the specific TLR4 inhibitor, CLI-095, with the proportion of responding cells reducing from around 20% to only 5%.

LPS of Gram-negative oral anaerobic bacteria is known to elicit different host responses from those observed with the classic enterobacterial endotoxin (Darveau & Hancock 1983, Dixon & Darveau 2005, Herath *et al.* 2013). Indeed, *P. gingivalis* LPS has been considered to have

relatively low biological activity because of its low phosphate content, altered length and positions of fatty acids in the lipid A moiety, compared with *E. coli* LPS (Takada & Kotani 1989). Also, Ogawa & Uchida (1996) have described differential cytokine production by mononuclear cells in response to *P. gingivalis* LPS. Notwithstanding these differences, *P. gingivalis* LPS was found to induce inflammatory responses in LPS non-responder mice (TLR4 knockout mice) (Kirikae *et al.* 1999), which suggests a different molecular recognition pathway is involved from that for *E. coli* LPS.

The other category of potential receptors is the transient receptor potential channels (TRPs). Numerous studies have shown that TRP channels are a broad group of ion channels located on nociceptors; TRPA1 and TRPV1 are thought to be involved in responses to bacterial infection (Chung *et al.* 2011, Gibbs *et al.* 2011, Huang *et al.* 2012). TRPV1 is expressed in small and medium diameter nerve fibres throughout the human tooth pulp, which comprise about 20–35% of the TG neurons (Chung *et al.* 2011, Gibbs *et al.* 2011). The data in the present study support this, with 33% of TG neurons functionally responding to the TRPV1 agonist, capsaicin. As well as TRPV1, the receptor TRPA1 could also act as a sensor since it is known to respond to a broad spectrum of endogenous compounds and irritants. However, there has been a report that TRPA1 expression in the trigeminal ganglion is distributed in only a small population of TG neurons (6–10%; Huang *et al.* 2012). The present findings concur with this as it was noted the proportion of neurons in the cultures responding to the TRPA1 agonist CA was less than 15%.

The present study demonstrates that *P. gingivalis* LPS activates trigeminal neurons through TRPA1-dependent pathways, since the response was completely blocked by the TRPA1 inhibitor, HC-030031. This is consistent with the finding of Meseguer *et al.* (2014) that *E. coli* LPS exerted a fast excitatory action on TG neurons via the TRPA1 receptor. However, the neuronal activation by *P. gingivalis* LPS could occur via both TRPA1 and TLR4. This difference could be explained by the different properties of *P. gingivalis* LPS and *E. coli* LPS (Coats *et al.* 2005, Herath *et al.* 2013). It is also possible that activation of TLR4 sensitises TRPA1. As indicated by the present and previous studies (Calvo-Rodríguez *et al.* 2017), TLR4 activation of neurons can increase levels of intracellular calcium; this rise in calcium could then act on TRPA1 to create a positive reinforcement of calcium signalling. Previous studies have reported that a rise in calcium can sensitise TRPA1 (Zurborg *et al.* 2007).

Since TRPA1 has been shown to control vascularity in neurogenic inflammation (Kunkler *et al.* 2011), levels of CGRP release in response to *P. gingivalis* LPS were measured. The

present study demonstrated that a relatively high concentration of *P. gingivalis* LPS (20 μg/mL) could enhance neuronal activation and produce a significant increase in CGRP release ($p<0.001$). In contrast, Ferraz *et al.* (2011) reported that a low concentration (2 μg/mL) of *P. gingivalis* LPS failed to induce CGRP release. However, since only supraphysiological levels of LPS (here 20 μg/mL) triggered a neuronal response, it may indicate a different processing mechanism from that of the classical LPS receptor complex of TLR4 with MD2 and CD14 co-factors. With supraphysiological LPS concentrations, LPS signalling could occur via CD14-independent mechanisms (Ulevitch & Tobias 1995) and so the mechanisms revealed in this study may not completely represent the canonical pathway of TLR4–LPS interaction. Moreover, the effect of LPS on the release of CGRP could be reversed by not only TRPA1 or TLR4 signalling blockers, but also by a TRPV1 blocker. This finding may support the notion that *P. gingivalis* LPS somehow induces a TRPV1-dependent release of CGRP, which would be consistent with the findings of Diogenes *et al.* (2011). These workers showed that TLR4 stimulation could enhance TRPV1 activation, leading to the release of CGRP. Even though there has been very limited evidence to show how LPS modulates peripheral sensitization, it has been thought that Ca^{2+} permeation through TRPV1 leads to exocytosis, which underlies CGRP and SP release (Henrich & Buckler 2009) and which can account for a rapid neuronal response. However, it is also possible that CGRP release may also be influenced by the triggering of PKC/PLC pathways (Vellani *et al.* 2010), as a latent consequence. Taken together, the present data cannot rule out the possibility that LPS sensitivity of TG neurons requires TRPV1-mediated release of CGRP.

In order to have more prolonged effects on neurons, LPS would be expected to result in activation of the NF-κB pathway because this is the major transcription factor responsible for modulating gene expression in response to LPS (Arias-Salvatierra *et al.* 2011). Also, both TRPA1 and TLR4 mediate the production of inflammatory mediators through the NF-κB pathway and this pathway is thought to be related to voltage-gated channel over-expression (e.g. Ca^{2+}, Na^{+} channels) and possibly inducing allodynia, thermal hyperplasia, or chronic pain (Huang *et al.* 2006). Thus, the observed secretion of CGRP may relate to involvement of both these receptors (TRPA1 and TLR4), though CGRP secretion itself appears to involve the TRPV1-dependent pathway. These data lead us to propose a possible model for LPS stimulation of TG cells as shown in Figure 7.

Several previous reports have suggested that satellite glial cells could be a possible therapeutic target for modulation of pain (Watkins & Maier 2002, Takeda *et al.* 2009). This is

because neuronal excitability could be developed and maintained through the sensory neuron along with the surrounding satellite glial cells acting as a functional unit (Takeda *et al.* 2009, Villa *et al.* 2010, Kushnir *et al.* 2011, Poulsen *et al.* 2014, Costa & Moreira Neto 2015, Hanani 2015). In the present study, TG neuronal supporting cells were directly activated by *P. gingivalis* LPS (e.g. using calcium influx and NF-κB nuclear translocation measurements). This is consistent with the finding of Yoon *et al.* (2012) who reported that *E. coli* LPS could increase expression of satellite cell markers in DRG. Taken together, the activation of neuronal supporting cells, such as satellite glial cells and/or Schwann cells, may be a crucial element in generation and maintenance of orofacial pain, however studies are currently being carried out to further characterize their contribution.

Conclusion

This study sheds light on the mechanisms involved in the pain pathways that can be directly activated by bacterial infection of the pulp. Contact between oral Gram-negative bacteria, such as *P. gingivalis*, and TG neuronal and non-neuronal cells can involve their direct activation through a combination of TRPA1, TLR4 and TRPV1 receptors. This results in NF-κB nuclear translocation and release of the neuropeptide CGRP.

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Figure legends

Figure 1. Analysis of intracellular calcium in primary trigeminal cell culture. (A) Phase-contrast and (B–F) time-lapse calcium imaging in trigeminal ganglion (TG) neurons (white arrow) and non-neuronal cells (white arrowhead) loaded with Cal-520 AM, during application of LPS, cinnamaldehyde (CA), capsaicin (CAP), and high potassium chloride (KCl). The high potassium application is to assess neuron viability and to help distinguish neuronal from non-neuronal cells. (G,H) Graphs show traces of the transient change in calcium in individual cells over time after application of LPS and the agonists in neurons (G), and non-neuronal cells (H); data plotted are in arbitrary fluorescent units.

Figure 2. The effects of *P. gingivalis* LPS after TRPA1 and TLR4 blocking. Representative samples of responses to ultrapure *P. gingivalis* LPS in TG neurons following pre-incubation with (A) no block, (B) 1 μM CLI-095 [TLR4 block], and (C) 3 μM HC-030031 [TRPA1 block]; data plotted are in arbitrary fluorescent units. Note that panel A is identical to the image in Figure 1G, and is included here for ease of comparison with panels B and C. Figure 2D shows the percentage of LPS-responding neurons in three groups; no block, TLR4 block, and TRPA1 block.

Figure 3. CGRP levels in LPS-, and capsaicin-stimulated TG neurons. The secreted CGRP response to *P. gingivalis* LPS or capsaicin was determined by the incubation of TG cells for 15 minutes with ultrapure *P. gingivalis* LPS (20 μg/mL) or capsaicin (1 μM) at 37°C in 5% CO_2. Measurement of CGRP levels was performed using enzyme-linked immunosorbent assay. Data are the mean ± SD from three independent experiments using cells cultured from six ganglia collected from three different animals; each independent experiment assessed responses from four coverslips. Error bars are ± SD; *$p<0.001$.

Figure 4. CGRP levels in TG neuron cultures in response to LPS in the presence of specific agonist inhibitors. The secreted CGRP response to *P. gingivalis* LPS or LPS in the presence of TLR4, TRPA1, and TRPV1 blockers was determined by incubation of TG cells for 15 min with ultrapure *P. gingivalis* LPS (20 μg/mL), or 20 μg/mL ultrapure *P. gingivalis* LPS with 1 μM CLI-095 [TLR4 block], 3 μM HC-030031 [TRPA1 block], or 1 μM capsazepine [TRPV1 block] at 37°C in 5% CO_2. Measurement of CGRP levels was performed using enzyme-linked immunosorbent assay. Data presented are the means from two independent

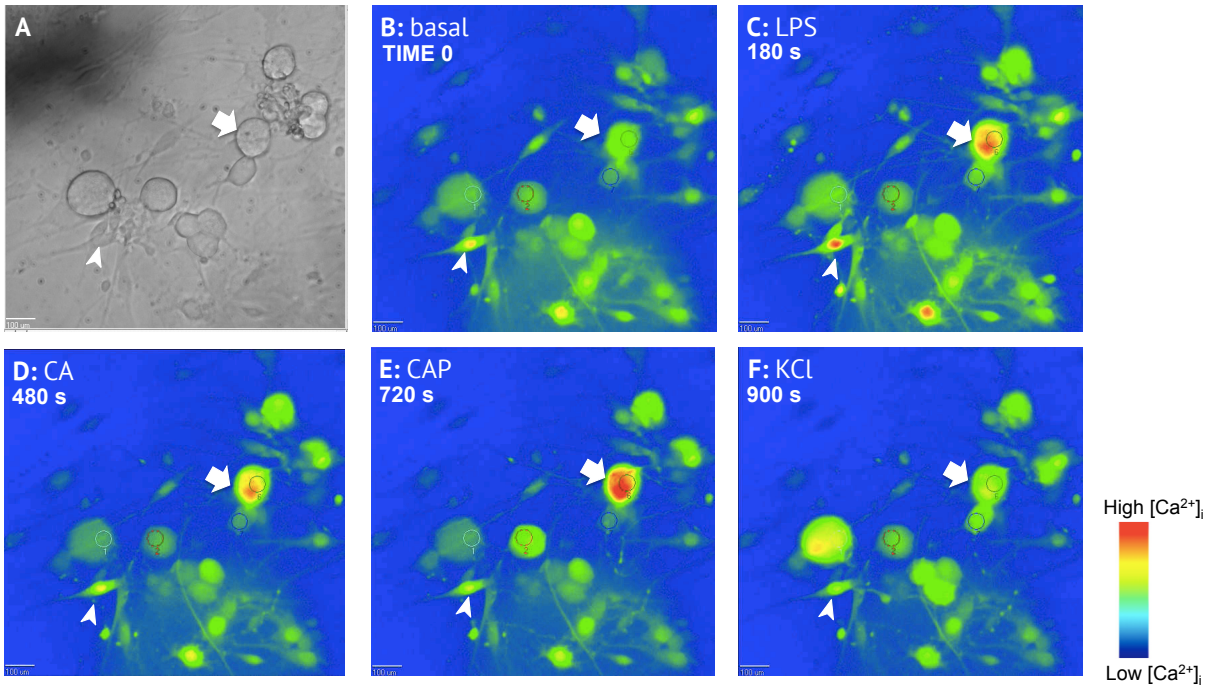
experiments using cells cultured from four ganglia collected from two different animals; each independent experiment assessed responses from four coverslips. Error bars are ± SD; ***p<0.001.

Figure 5. Photomicrographs of p65 NF-κB nuclear translocation in *P. gingivalis* LPS-treated TG neurons and non-neuronal cells. The TG cultures were (A) left untreated, or (B) stimulated with *P. gingivalis* LPS for 30 minutes. Cells were permeabilized with 0.1% Triton X-100 and subsequently stained with primary antibodies against anti-p65 NF-κB and appropriate secondary antibodies. The β-tubulin III, and p65 NF-κB control samples showed no positive labelling (C and D, respectively). The β-tubulin III is shown in green (A1,B1) and p65 NF-κB appears in red (A2,B2), nuclear staining with DAPI appears blue (A3,B3). The merged images (A4,B4) show the combined p65 NF-κB, β-tubulin III and DAPI staining. Scale bars = 70 μm. Localization of p65 NF-κB is indicated in neurons by yellow arrows and non-neuronal cells by white arrows. The translocation of NF-κB was counted as positive when nuc/cyt ratio was >1. Quantitative analysis of neuronal nuclear translocation stimulated by *P. gingivalis* LPS is shown in figure E. Neurons and non-neuronal cells positive for NF-κB nuclear translocation were quantified. Data are the mean ± SD from three independent experiments. Error bars are ± SD;*p< 0.01, **p<0.05.

Figure 6. Percentages of p65 NF-κB nuclear translocation in (A) *P. gingivalis* LPS-treated TG neurons and (B) non-neuronal cells in the presence of TLR4, TRPA1, and TRPV1 signalling inhibitors. Positive nuclear translocations were quantified by measuring the fluorescence intensity at each intracellular location. The translocation of NF-κB was counted as positive when nuc/cyt ratio was >1. Neurons and non-neuronal cells positive for NF-κB nuclear translocation were quantified. Data are the mean from two independent experiments. Error bars are ± SD; *p<0.05.

Figure 7. Proposed model for LPS stimulation of TG neurons. LPS can activate TG neurons via TRPA1 and TLR4; this activation can be blocked by their specific antagonists HC-030031 and CLI-095, respectively. The neuronal activation can result in both rapid responses (e.g. CGRP release) and delayed responses (e.g. NF-κB nuclear translocation).

Fig. 1


A
B: basal
TIME 0
C: LPS
180 s
D: CA
480 s
E: CAP
720 s
F: KCl
900 s
High [Ca2+]i
Low [Ca2+]i

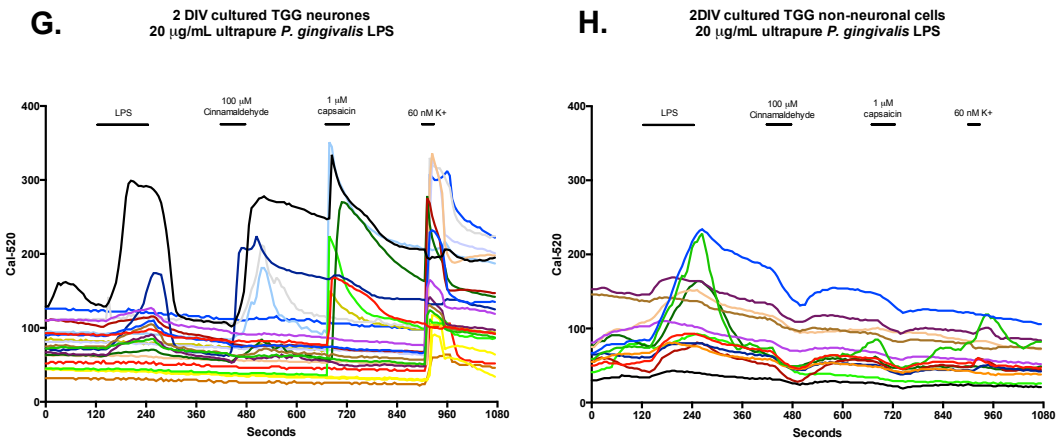

G.
2 DIV cultured TGG neurones
20 µg/mL ultrapure P. gingivalis LPS
H.
2DIV cultured TGG non-neuronal cells
20 µg/mL ultrapure P. gingivalis LPS
LPS
100 µM Cinnamaldehyde
1 µM capsaicin
60 nM K+
Cal-520
Seconds

Fig. 2

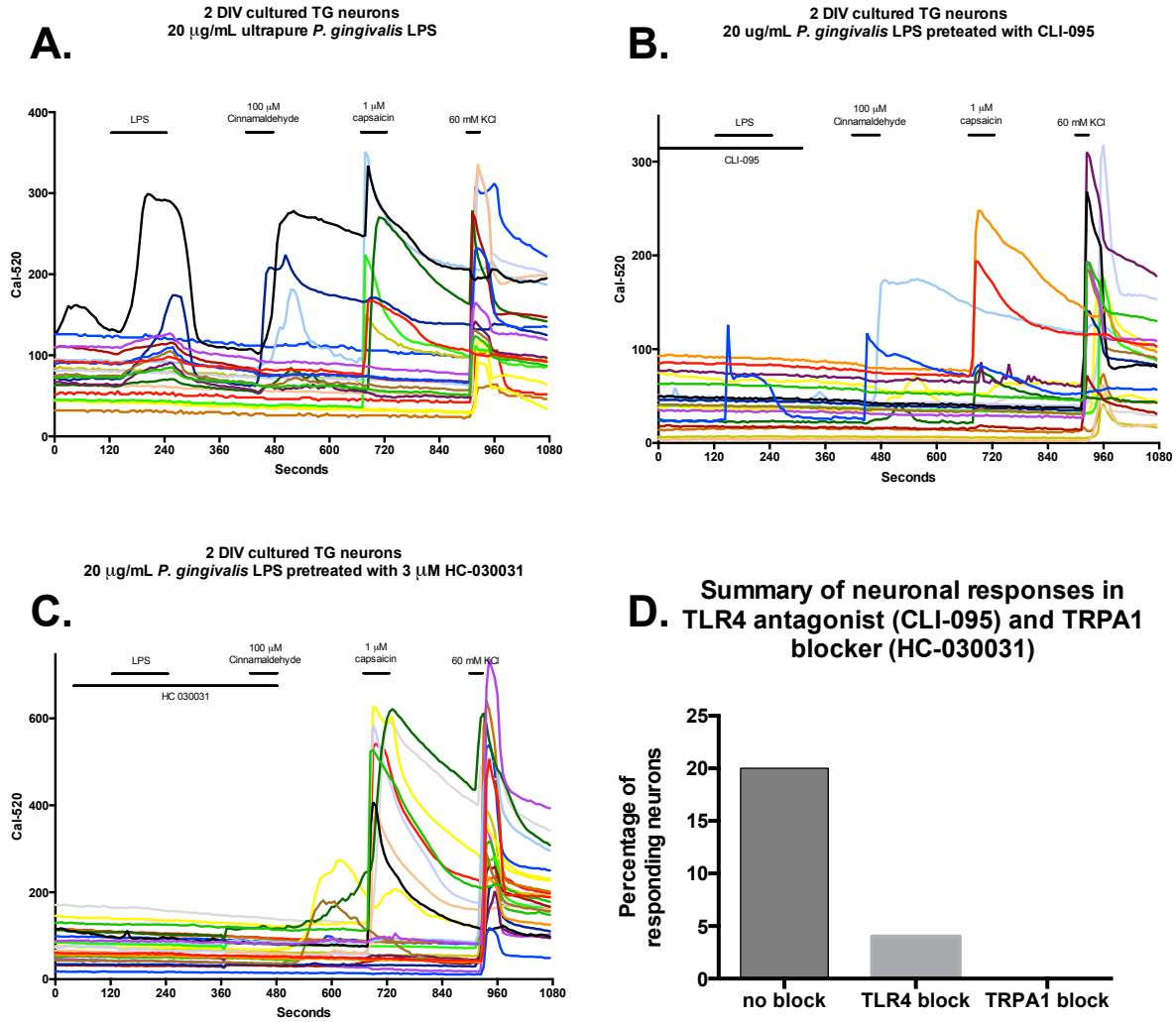

A.
2 DIV cultured TG neurons
20 μg/mL ultrapure P. gingivalis LPS
LPS
100 μM Cinnamaldehyde
1 μM capsaicin
60 mM KCl
Cal-520
Seconds
B.
2 DIV cultured TG neurons
20 ug/mL P. gingivalis LPS preteated with CLI-095
CLI-095
C.
2 DIV cultured TG neurons
20 μg/mL P. gingivalis LPS pretreated with 3 μM HC-030031
HC 030031
D.
Summary of neuronal responses in TLR4 antagonist (CLI-095) and TRPA1 blocker (HC-030031)
Percentage of responding neurons
no block
TLR4 block
TRPA1 block

Fig. 3

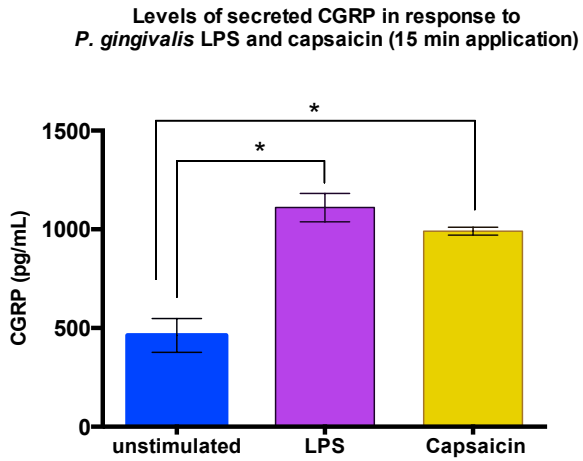

Levels of secreted CGRP in response to
P. gingivalis LPS and capsaicin (15 min application)
CGRP (pg/mL)
1500
1000
500
0
*
*
unstimulated
LPS
Capsaicin

Fig. 4

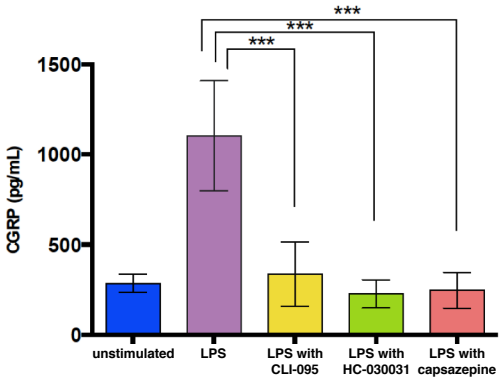

***
***
***
1500
1000
500
0
CGRP (pg/mL)
unstimulated
LPS
LPS with CLI-095
LPS with HC-030031
LPS with capsazepine

Fig. 5

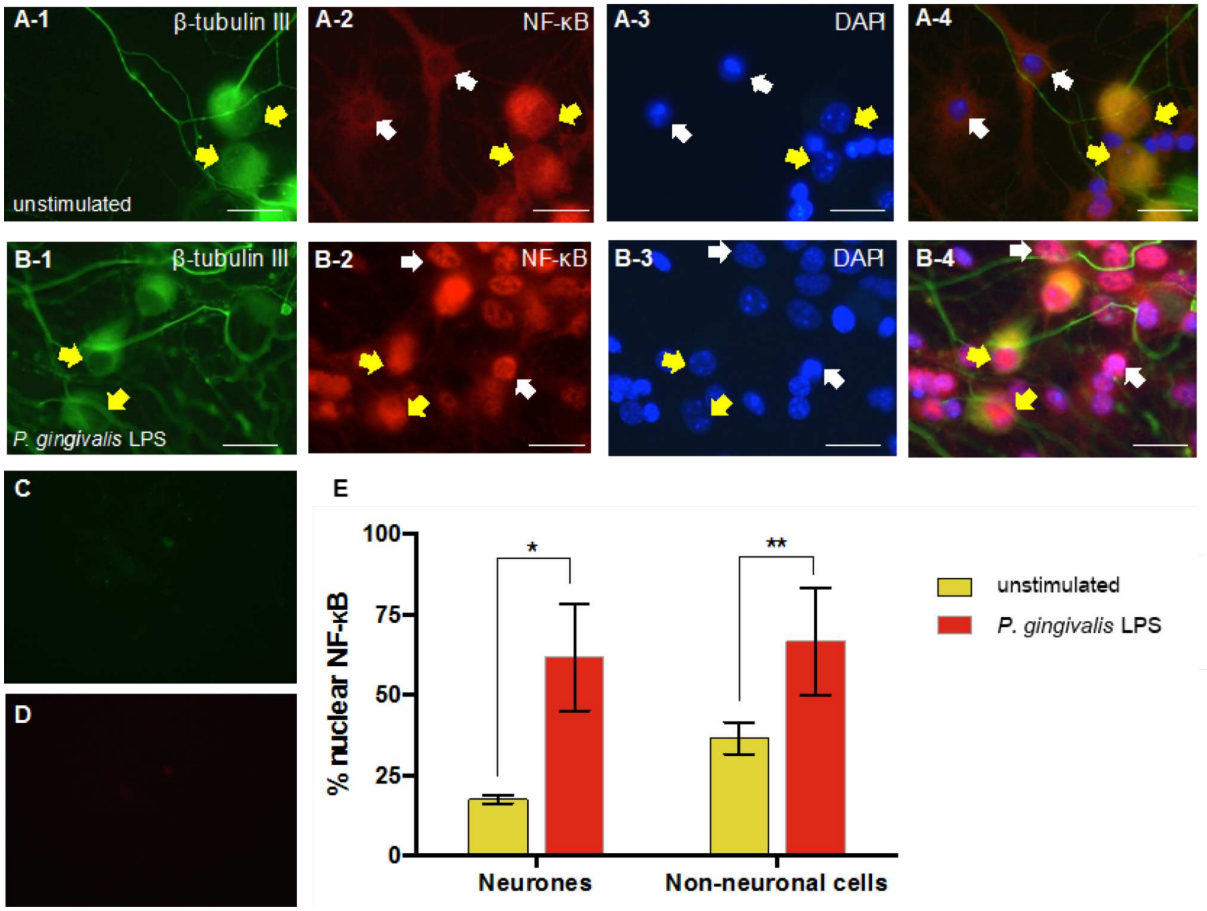

A-1
β-tubulin III
unstimulated
A-2
NF-κB
A-3
DAPI
A-4
B-1
β-tubulin III
P. gingivalis LPS
B-2
NF-κB
B-3
DAPI
B-4
C
D
E
% nuclear NF-κB
100
75
50
25
0
*
**
Neurones
Non-neuronal cells
unstimulated
P. gingivalis LPS

Fig. 6

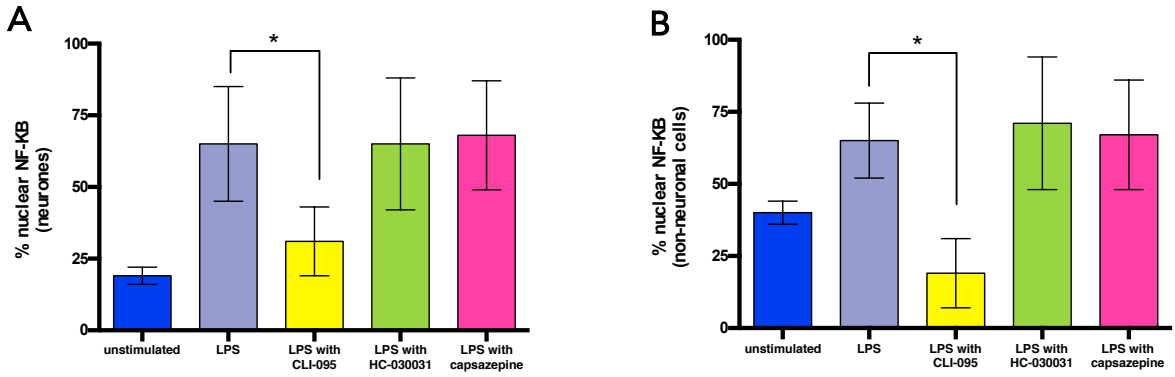
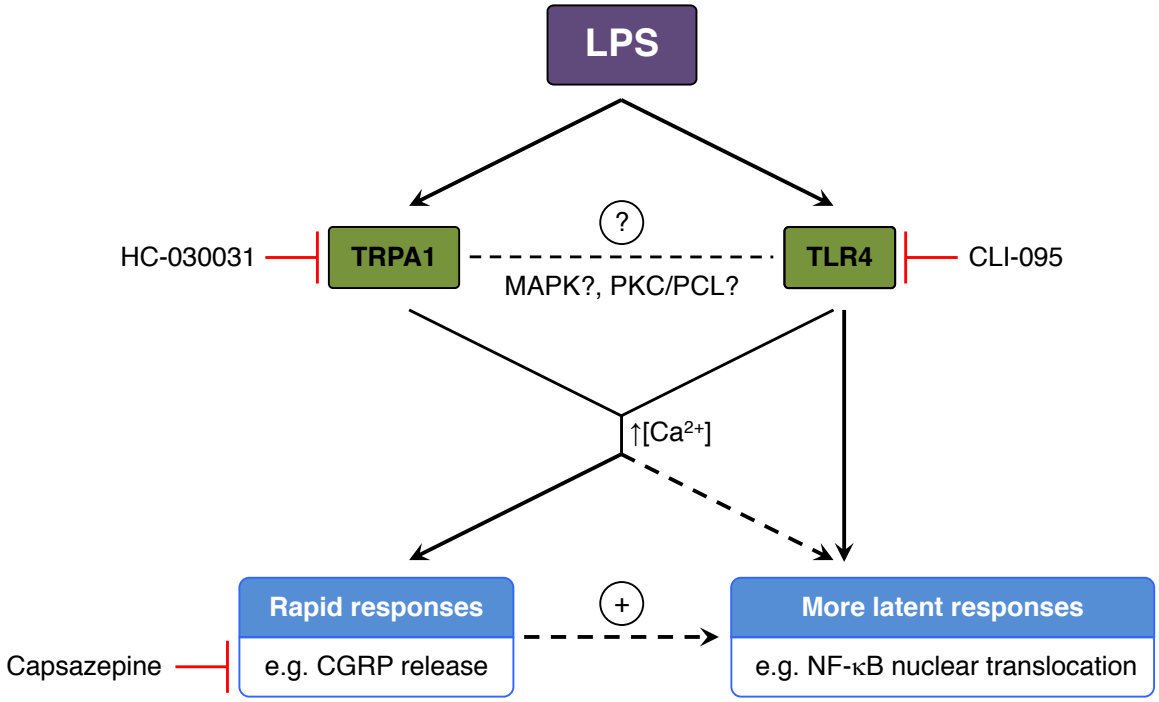

A
% nuclear NF-KB (neurones)
100
75
50
25
0
*
unstimulated
LPS
LPS with CLI-095
LPS with HC-030031
LPS with capsazepine
B
% nuclear NF-KB (non-neuronal cells)
100
75
50
25
0
*
unstimulated
LPS
LPS with CLI-095
LPS with HC-030031
LPS with capsazepine

Fig. 7


LPS
HC-030031
TRPA1
?
MAPK?, PKC/PCL?
TLR4
CLI-095
↑[Ca2+]
Capsazepine
Rapid responses
e.g. CGRP release
+
More latent responses
e.g. NF-κB nuclear translocation