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LIPOPOLYSACCHARIDES AND TROPHIC FACTORS REGULATE THE LPS RECEPTOR COMPLEX IN NODOSE AND TRIGEMINAL NEURONS

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Abstract—Binding of bacterial lipopolysaccharides (LPS) to toll-like receptor 4 (TLR4) triggers an innate immunoresponse associated with pain and inflammation. The expression, and to a greater extent the regulation of TLR4 and its auxiliary proteins (myeloid differentiation protein 1 (MD1), myeloid differentiation protein 2 (MD2) and cluster of differentiation 14 (CD14)), are both poorly understood in trigeminal and nodose neurons. We used a combination of Western blotting, semi-quantitative polymerase chain reaction (PCR), pharmacological manipulation and immunohistochemistry. The expression pattern and regulation by LPS and trophic factors of TLR4/MD2/CD14 and radioprotective protein of 105 kDa (RP105)/MD1 were determined in neonatal trigeminal and nodose mice neurons. We found that all these proteins were expressed in both trigeminal and nodose neurons. The trophic factors Artemin and nerve growth factor (NGF) up-regulated MD2 and RP105 mRNA levels in trigeminal neurons. In nodose neurons the trophic factors brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF) up-regulated MD1 and RP105 mRNA levels. Also we observed that in both neuronal types LPS acutely (within 20 min) down-regulated CD14 and MD2 mRNAs. In addition, LPS increased significantly the proportion of trigeminal and nodose neurons expressing nociceptin/orphanin FQ in culture probably acting via TLR4/MD2. Although the exact mechanisms underlying the regulation by trophic factors and LPS require further elucidation, the findings of this study indicate that LPS acts through its archetypical receptor in trigeminal and nodose neurons.

Key words: trigeminal, nodose, nociceptin/orphanin Q, LPS, TLR4, trophic factors.

INTRODUCTION

The immune and nervous systems are functionally related. For example, stimulation by bacterial infection of toll-like receptors (TLR) (Olson and Miller, 2004) present in neuronal and non-neuronal cells (Okun et al., 2011) elicits the release of proinflammatory cytokines leading to inflammation, itch and pain-related behaviors (Liu et al., 2012).

Toll like receptors are a family of 14 evolutionary conserved germ line-encoded pattern recognition receptors that recognize carbohydrates, peptides and nucleic acids expressed in different organisms (Kumar et al., 2011). They initiate the innate response against invading pathogens (Iwasaki and Medzhitov, 2004; Kawai and Akira, 2007). Among the molecules recognized by the innate immune system are cell wall components like lipopolysaccharides (LPS) which are powerful triggers of inflammation and neuropathic pain (Peri and Piazza, 2012). The LPS receptor encompasses several transmembrane proteins: TLR4, the cluster of differentiation 14 (CD14) and the myeloid differentiation protein 2 (MD2). The LPS receptor also includes the radio-protection protein of 105 kDa (RP105) and its auxiliary unit, the myeloid differentiation protein 1 (MD1) (Okun et al., 2011). TLR4 is a transmembrane protein with an extracellular domain involved in the recognition of LPS. MD2 is secreted by the cell into its extracellular media and is necessary for LPS recruitment to the cell membrane and also for its recognition (Nagai et al., 2002a). Although RP105 is similar to TLR4 in the extracellular leucine-rich repeats, it does not have an interleukin 1 (IL-1) receptor-like signaling domain in the cytoplasmic portion (Miyake, 2003). Unlike TLR4, RP105 binds to the MD1 protein, an MD2 homolog (Medzhitov, 2001).

Peripherally induced inflammation induces pathological pain. The increase in TLR4 mRNA levels suggests that it could be involved in the initiation of this pain (Raghavendra et al., 2004; Tanga et al., 2004). TLR4 is also expressed by dorsal root ganglion (DRG)

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Abbreviations: β-ARAC, cytosine β-D-arabinofuranoside; ART, Artemin; BDNF, brain derived neurotrophic factor; BSA, bovine serum albumin; CD14, cluster of differentiation 14; CNTF, ciliary neurotrophic factor; DIV, days *in vitro*; DRG, dorsal root ganglion; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GDNF, glial derived neurotrophic factor; HBSS, Hanks balanced salt solution; IL-1, interleukin 1; LIF, leukemia inhibitory factor; LPS, lipopolysaccharides; MD1, myeloid differentiation protein 1; MD2, myeloid differentiation protein 2; N/OFQ, nociceptin/orphanin-FQ; NGF, nerve growth factor; PBS, phosphate buffered saline; PCR, polymerase chain reaction; RP105, radioprotective protein of 105 kDa; RT, room temperature; RT-PCR, reverse transcription polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TLR, toll-like receptor; TRPM8, transient receptor potential cation channel subfamily M member 8; TRPV1, transient receptor potential vanilloid receptor; WB, Western blot.

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(Acosta et al., 2012) neurons where its activation by LPS leads to up-regulation of the opioid pro-algesic peptide nociceptin/orphanin-FQ (N/OFQ) (Acosta and Davies, 2008).

TLR4 and CD14 expression is present in trigeminal neurons (Wadachi and Hargreaves, 2006) mainly restricted to a subpopulation of capsaicin-sensitive, transient receptor potential vanilloid receptor (TRPV1)-immunoreactive nociceptors (Diogenes et al., 2011). TLR4 is also present in nodose neurons (Hosoi et al., 2005) where its functions are poorly understood. Furthermore, the expression pattern of RP105, MD1 and MD2 in these neuronal types remains unexplored. The study of the LPS receptor in trigeminal neurons is important because these neurons mediate painful sensations from the tooth pulp and the head and are implicated in trigeminal neuralgia (Greenwood and Sessle, 1976; Sessle and Greenwood, 1976; Bossut and Maixner, 1996). The nodose ganglion is a cranial sensory ganglion located in the periphery, whose axons run in the vagus nerve to provide sensory innervations to nearly all the structures within the body cavity including the heart, the lungs, the trachea and the gut (Paintal, 1973). Interestingly, nodose neurons innervating the airways of the lung and trachea express different trophic factor receptors (see for example (Lieu et al., 2011;Lieu and Undem, 2011)). These neurons are thought to mediate LPS activation of the vagus nerve (Huston, 2012).

Another broader question is how the LPS-receptor complex is regulated in neurons and what factors are involved in this regulation. Trophic factors are good candidates because their receptors are expressed in sensory neuron subpopulations and they are important for neuronal development and differentiation (Lewin and Barde, 1996; Chao et al., 2006; Spedding and Gressens, 2008; Allen et al., 2013). Trophic factors such as nerve growth factor (NGF) and glial-derived neurotrophic factor (GDNF) or Artemin (ART) can activate and sensitize nociceptors, suggesting that these molecules contribute to the initiation and maintenance of pain in response to nerve injury and inflammation (Scholz and Woolf, 2007; Jankowski and Koerber, 2010). For example, it has been demonstrated that ART sensitized trigeminal cold nociceptors through modulation of transient receptor potential cation channel subfamily M member 8 (TRPM8) (Lippoldt et al., 2013). Given that trophic factors modulate pain in models of pathological neuropathic and inflammatory pain and that TLR4 is involved in LPS-induced inflammation, we hypothesized that these factors would influence the expression of LPS-receptors in both, trigeminal and nodose afferent neurons.

To test this hypothesis firstly we examined which proteins of the LPS-receptor complex are present in mouse trigeminal and nodose ganglia. Secondly, we explored the influence of selected trophic factors on the mRNA levels for LPS-receptor proteins in trigeminal and nodose neurons *in vitro*. Thirdly, we examined whether activation of the LPS receptor leads to changes in N/OFQ expression in these neurons. We used this opioid peptide as a marker because it is expressed by trigeminal and nodose neurons (Jia et al., 2002; Hou et al., 2003) and it is associated with behavioral changes in models of neuropathic and inflammatory pain (Itoh et al., 2001; Chen et al., 2007).

EXPERIMENTAL PROCEDURES

Animals

Postnatal day 5 (from now on P5) CD-1 mice were used in all experiments. All animals were cared for in accordance with the Guiding Principles in the Care and Use of Animals of the US National Institute of Health. All procedures had been approved by the Institutional Animal Care and Use Committee of the School of Medical Science, Universidad Nacional de Cuyo (protocol approval 21/2014). The number of animals used was minimized and cell cultures used as a model following the recommendations of the 3R's policy.

Cell culture

Trigeminal and nodose ganglia from P5 CD-1 mice were dissected and neurons isolated as follows. Two trigeminal ganglia (one mouse) and six nodose ganglia (three mice) were used for each culture. Briefly, the ganglia were enzymatically dissociated by incubation in Hank's balanced salt solution (HBSS) for 30 min with 0.25% trypsin and 1% collagenase type I at 37 °C (both from Worthington, Lakewood, NJ, USA). Enzymatic activity was stopped by addition of F12 medium containing 10% fetal bovine serum (FBS). The ganglia were then gently triturated, and the resulting cell suspension was pelleted by centrifugation at 2000 rpm for 5 min. To remove most non-neuronal cells the pellet was re-suspended in HBSS, layered on a Percoll gradient (22% for trigeminal and 18% for nodose), and centrifuged at 2000 rpm for 5 min. The supernatant with the non-neuronal cells was discarded, and neuron-enriched pellet was re-suspended in F14-based defined medium. Neurons were then plated in 35-mm tissue culture dishes coated with 2 mg/mm^2 poly-DL-ornithine and 5 ng/mm^2 laminin (Pinon et al., 1997). 5–10 mM β-arabinofuranosylcytosine (β-ARAC) was added 6 h after plating to suppress fibroblast division. The combination of Percoll gradient and β-ARAC treatment resulted in purified cultures of cells that were more than 95% neurons after 1 day *in vitro* (DIV). Plating densities were $\sim 2 \times 10^3$ neurons/ml and 5×10^3 neurons/ml for nodose and trigeminal, respectively. For immunocytochemistry, the cells were plated on poly-DL-ornithine/laminin coated 12-mm-diameter glass coverslips (Bellco Glass, Vineland, NJ, USA). In all experiments, trophic factors and LPS were added to the F14-based defined medium immediately upon plating. Trigeminal neurons were supplemented (depending on the experiment) with no trophic factors (None) or with 10 ng/ml NGF 7S or 20 ng/ml ART or a combination of both. Nodose neurons received one of the following treatments: no trophic factors (None) or 20 ng/ml brain derived neurotrophic factor (BDNF) or 20 ng/ml ART or 10 ng/ml ciliary neurotrophic factor (CNTF) or 20 ng/ml leukemia inhibitory factor (LIF) or combinations of BDNF and ART. LPS (*Escherichia coli*

serotype 055:B5) at 0.5 μg/ml was used throughout. For neuron survival assays or to examine its influence on nociceptin/orphanin Q expression, it was added immediately after plating. For time-course experiments it was added at 1 DIV.

For survival experiments, trigeminal and nodose neurons from three different cultures from different mice were counted. Trophic factors or LPS (as detailed in Section 'Results') were added at the time of plating and again 24 h later. An operator blinded to the treatments performed the counts under bright field optics with an inverted microscope. The initial number of neurons at 6 h was taken as 100%. Subsequent survival after 24 and 48 h in culture was expressed as the percentage of neurons alive relative to this initial number.

Western blotting

We examined the expression of TLR4, MD2, RP105 and MD1 by Western blot (WB) in trigeminal and nodose ganglia from P5 mice ($n = 3$) using standard protocols (Acosta et al., 2012). A total of two trigeminal (from one mouse) and six nodose ganglia (from three mice) were harvested for each WB. Spleen tissue was used as a positive control. Tissue was homogenized at 4 °C in RIPA 1× buffer supplemented with both Complete Protease-Inhibitor Cocktail and Phospo-STOP (Roche Molecular Biochemicals, South Essex, United Kingdom). Samples were then centrifuged at 14,000 rpm for 15 min at 4 °C; the supernatant was recovered, centrifuged again for 1 min, and kept at −20 °C until use. Total proteins were quantified using the Bradford method. 20 μg of protein were run in each lane of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) gels (7.5% gels for TLR4 and RP105 and 12% gels for MD1 or MD2). Proteins were blotted onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P), blocked in 5% semi-skimmed milk 2 h at room temperature (RT) and then incubated overnight at 4 °C with the primary antibody in the same blocking solution. The following dilutions were used: anti-TLR4 (1:500), anti-MD2 (1:500), anti-MD1 (1:500) and anti-RP105 (1:100). A mouse anti-α-tubulin (1:2000) was used as a loading control. Next day, membranes were thoroughly rinsed with TBS/TBST (0.05% Tween 20) and then incubated for 1hr at RT with the appropriate peroxidase-conjugated secondary antibody (1:4000, Vector labs). Reaction products were detected using the ECL Plus staining system (Amersham Biosciences, Amersham, United Kingdom).

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

Relative levels of TLR4, MD2, RP105, MD1 and CD14 mRNAs in cultured trigeminal and nodose neurons were determined by RT-PCR. Total mRNA was obtained using the RNeasy Mini kit (Qiagen, East Sussex, United Kingdom). mRNA was extracted from at least three different cell cultures (from three different mice belonging to the same litter) for each experimental condition. The integrity and concentrations of the mRNA samples were assessed using a spectrophotometer (Nanodrop, Thermo Scientific, Wilmington, DE, USA). To this end, the 28S:18S rRNA ratio was obtained (i.e. calculated from the fluorescence readouts at 260 and 280 nm). The rRNA ratio for our samples fluctuated between 1.7 and 2 showing that they had good to high integrity and did not contain significant RNA lysing impurities (Schoor et al., 2003). Concentrations of RNA were ~200 ng/μl. The samples were then frozen on dry ice and stored at −80 °C until required. cDNA was synthesized from 2 μg total RNA with a mixture of oligo-dT and random primers and SuperScript H⁻ (Invitrogen, Gaithersburg, MD, USA) for 50 min at 37 °C in the presence of RNAguard, a RNAse inhibitor (Amersham Biosciences). cDNA was used as a template for polymerase chain reaction (PCR) amplification in a 20-μl reaction volume containing 1× PCR buffer, 100 nM dNTPs (NEBiolabs),100 nM of each primer and High Fidelity Platinum taq DNA polymerase (Invitrogen). Note that this enzyme offers a very high specificity of reaction and a very low rate of replication errors.

All reactions were performed with an initial denaturation cycle of 94 °C for 1 min. The number of cycles, annealing temperatures and durations were as follows: CD14, 32 cycles, 56 °C for 30 s; TLR4, 30 cycles, 54 °C for 30 s; MD1, 30 cycles, 53 °C for 30 s; MD2, 32 cycles, 52.5 °C for 45 s and RP105, 53 °C for 30 s. All had an extension step at 68 °C for 45 s, followed by a final 10-min 68 °C extension. These PCR conditions were chosen so that (a) none of the RNAs analyzed reached a plateau at the end of the amplification protocol, i.e. they were in the exponential phase of amplification, and (b) that the two sets of primers used in each reaction did not compete with each other. Each set of reactions always included a no-sample negative control. We usually performed a negative control containing RNA instead of cDNA to rule out genomic DNA contamination. Because of the semi-quantitative nature of our approach, it was important to select the appropriate number of cycles so that the amplification product was clearly visible on an agarose gel and could be quantified before the reaction had reached a plateau. When the annealing temperature of the two primer sets was different, annealing temperatures ranging between the optimal temperatures of each primer set were tested.

In all experiments, amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was run simultaneously in the same tube to normalize samples.

Images of the RT-PCR ethidium bromide-stained agarose gels were acquired with a High Performance CCD camera (Hamamatsu Co., Shizuoka, Japan) and quantification of the bands performed by Simple PCI (Digital Pixel, Brighton, UK). Band intensity was expressed as relative density units. The ratio between the sample mRNA to be determined and GAPDH was calculated to normalize for possible initial variations in sample concentration and as a control for reaction efficiency. Unless stated otherwise, all experiments were repeated three times and the data presented as the median of the calculated ratios.

The sequences of the primers used are given in Table 1. All primers were custom designed and checked

Table 1. Primer sequences and gene targets for murine TLR4, MD2, RP105, MD1, CD14 and GAPDH

Gene	Accession	Forward primer	Reverse primer
TLR4	NM_021297.2	TTGAGAAGTCCCTGCTGAGG	TGCCGTTTCTTGTTCTTCC
RP105	D37797.1	GAGCTCGGTACTGGCTGTTT	CTTTGAATGCCTCCGTCTTG
CD14	X13987.1	GCCCTCTCCACCTTAGACCT	TCAGCCCAGTGAAAGACAGA
MD2	NM_016923.2	GACGCTGCTTTCTCCCATA	CTTACGCTTCGGCAACTCTA
MD1	NM_010745.2	CCTATCCCCTTTGTGAGGAG	CTTGGTTATCAGTGGTTCTTGC
GAPDH	NM_008084.2	GGCAAATTCAACGGCACAGT	CAGGGATGATGTTCTGGGCA

for selectivity using Primer-BLAST from NCBI and they were all obtained from Invitrogen.

Immunocytochemistry

Trigeminal and nodose cultures were fixed at 1 DIV with 4% paraformaldehyde plus 4% sucrose in phosphate-buffered saline (PBS) for 20 min at 4 °C and washed three times with PBS. For detection of β-tubulin III and N/OFQ, the cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min (RT), followed by 2 h incubation at RT with 5% bovine serum albumin (BSA) to block nonspecific binding sites. The cultures were incubated with 1:100 goat anti-N/OFQ (C-17, Santa Cruz Biotechnology, Dallas, TX) overnight at 4 °C in 1% BSA. The cultures were then washed three times with PBS, and the cells were incubated for 1 h at RT with 1:500 rabbit anti-goat conjugated with fluorescein isothiocyanate (FITC). Next, the coverslips were incu bated for 3 h at RT with 1:200 mouse anti-β-tubulin III (clone SDL.3D10, Sigma, Buenos Aires, Argentina), washed with PBS and incubated for 1 h at RT with 1:500 rabbit anti-mouse TRITC. The preparations were mounted in FluorSave (Calbiochem, La Jolla, CA, USA), and images were digitally acquired with a LSM510 confocal microscope. Images were analyzed offline using ImageJ.

Only neurons (cells clearly labeled for β-tubulin III a selective marker of neurons) were examined. Neurons were considered positively stained for N/OFQ if their average cytoplasmic pixel intensity was at least 20% above the maximum background level of staining obtained from cultures in which cells were pre-treated with the blocking peptide for C-17 Ab at 37 °C for 6 h.

Antibody preabsorption

To evaluate the selectivity of the goat polyclonal anti-mouse N/OFQ primary antibody we conducted a preabsorption experiment. C-17 Ab (1:100) was preincubated in PBS for 1 h at RT with a specific blocking peptide for this antibody (1:50, sc-9763P). Then immunofluorescence immunocytochemistry (Section 'Immunocytochemistry') was performed on nodose and trigeminal cultured neurons challenged with LPS as described in Section 'Cell culture'. The results are shown in Fig. 2.

Antibodies and reagents

Rat monoclonal IgG2a anti-mouse RP105 (RP14, sc-13592) was from Santa Cruz Biotechnologies (United Kingdom). Affinity-purified rabbit anti-mouse MD1 polyclonal antibody (raised against a KLH-conjugated synthetic peptide corresponding to amino acids 112–125 of human MD1, IMG-357) and the rabbit polyclonal anti-MD2 (IMG-5984A) were both from Imgenex (San Diego, CA, USA). The blocking rat monoclonal anti-TLR4 antibody, clone MTS510 (ab-95562), and the rabbit anti-mouse TLR4 polyclonal antibody (ab-13867) were both from Abcam Ltd. (Cambridge, United Kingdom). All secondary antibodies were from Vector laboratories, Peterborough (United Kingdom). To eliminate sodium azide (which can be cytotoxic), all antibodies used in cell culture experiments were first dialyzed against culture medium. LPS (*E. coli* serotype 055:B5), the monoclonal mouse anti-β-tubulin isotype III (clone SDL.3D10), the mouse anti-α-tubulin (Clone AA13) and all other reagents were obtained from Sigma. NGF 7S (mouse recombinant), ART, BDNF, CNTF and LIF were obtained from Preprotech (London, United Kingdom).

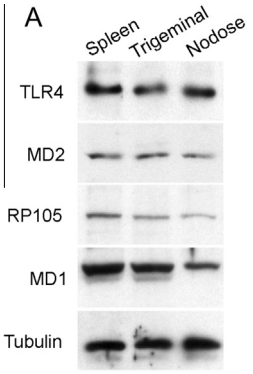
Statistics

In Figs. 2 and 3 statistical comparisons between treatments were non-parametric due to small sample size and because data failed the normality test (D'Agostino–Pearson normality test). Thus Mann–Whitney tests were used for comparison of medians of two groups. Data in Figs. 4 and 5 all passed the normality test. Therefore, comparison between two treatment groups was with paired *t*-tests. All tests were performed with Prism 5 (GraphPad software, San Diego, CA, USA). A level of $p < 0.05$ was considered statistically significant. Significance is indicated on all graphs by $^{*}p < 0.05$, $^{**}p \leqslant 0.01$, $^{***}p \leqslant 0.001$, $^{****}p \leqslant 0.0001$.

RESULTS

Trigeminal and nodose ganglia express the LPS-receptor complex

We first examined whether trigeminal and nodose ganglia expressed the molecular components of the two main LPS-binding receptor complexes: TLR4/MD2 and RP105/MD1. To do this, we performed a WB analysis using whole trigeminal and nodose ganglia from P5 mice (Fig. 1). We used a spleen lysate as positive control. The WB showed that both, trigeminal and nodose ganglia expressed TLR4 (95 kDa) and its auxiliary unit MD2 (18 kDa) and also RP105 (105 kDa) and its auxiliary protein MD1 (19 kDa) (Fig. 1A). Fig. 1B shows a comparison of the expression of these molecules in trigeminal, nodose and DRG neurons. Unexpectedly, and



B

	Trigeminal	Nodose	DRG*1
TLR4	+	+	+
MD2	+	+	+
RP105	+	+	-
MD1	+	+	+

Fig. 1. Trigeminal and nodose ganglia express LPS receptor proteins. (A) Representative Western blots showing the expression of TLR4, MD2, RP105 and MD1 proteins from whole trigeminal and nodose ganglia. A total of two trigeminal and six nodose ganglia from postnatal day 5 CD-1 mice were harvested for each WB. Approximately 20 μg of protein was run in each lane of SDS–PAGE gels (7.5% gels for TLR4 and RP105 and 12% gels for MD1 or MD2). In all experiments, spleen tissue was used as a positive control. Mouse α-tubulin was used as loading control. The WBs showed that trigeminal and nodose ganglia expressed TLR4 (95 kDa) and its auxiliary unit MD2 (18 kDa), RP105 (105 kDa) and MD1 (19 kDa). (B) Comparative table depicting the expression of TLR4, MD2, RP105 and MD1 protein in trigeminal and nodose ganglia from P5 mice and in purified P60 DRG neurons cultured for 24 h. *1data taken from Acosta and Davies (2008). Note that RP105 is not expressed in DRG neurons.

in contrast to what has been reported in DRG neurons (Acosta and Davies, 2008; Tse et al., 2014), both trigeminal and nodose ganglia exhibited RP105 expression. These results showed that trigeminal and nodose neurons possess the necessary receptors to respond to LPS.

Trophic factors regulate the expression of the LPS receptor-complex

The expression of TLR4/MD2 and RP105/MD1 in the whole ganglia (Fig. 1) may reflect proteins present in neurons as well as non-neuronal cells. We therefore examined the expression of the LPS-receptor components in purified neuronal cultures obtained from P5 mice. Nerve cells in culture are necessarily deprived of all electrical signals and all chemical signals transmitted along their axons or dendrites. The last of course is hardly relevant in the case of sensory ganglion cells. Similarly they are deprived of the factors normally present in the extracellular fluid, save for those that the experimenter can modify. Therein lies the power of the methods we have adopted. Nevertheless cultured cells can be useful to assess the effects of selected factors added to the culture media under controlled conditions. Because (a) the phenotype of many neuronal subpopulations is determined during early postnatal life, and (b) this is a process largely mediated by trophic factors (ElShamy and Ernfors, 1997; Luukko et al., 1997a; Baudet et al., 2000) we examined whether some of these factors could play a regulatory role in the mRNA expression of the proteins making up the LPS-receptor complex.

We based our choice of trophic factors on the presence of trophic factor-like receptors in these neurons (Horton et al., 1996; ElShamy and Ernfors, 1997; Luukko et al., 1997b; Ratcliffe et al., 2011). We chose those shown to be involved in the regulation of key properties of the sensory system. Thus, on trigeminal neurons we tested NGF, ART and a combination of both. On nodose neurons we evaluated the effects of adding to the culture medium these factors: BDNF, LIF, CNTF, ART alone or the combination of BDNF and ART.

To exclude possible cytotoxic effects of LPS and also non-specific actions of trophic factors (since this could compromise our interpretation of the effects of these compounds) we first examined neuronal survival in different culture conditions. On cultured trigeminal neurons we tested the effects of NGF, ART and LPS while on cultured nodose neurons we tested BDNF, ART and LPS. We determined the number of neurons alive at 6, 24 and 48 h after adding trophic factor to the

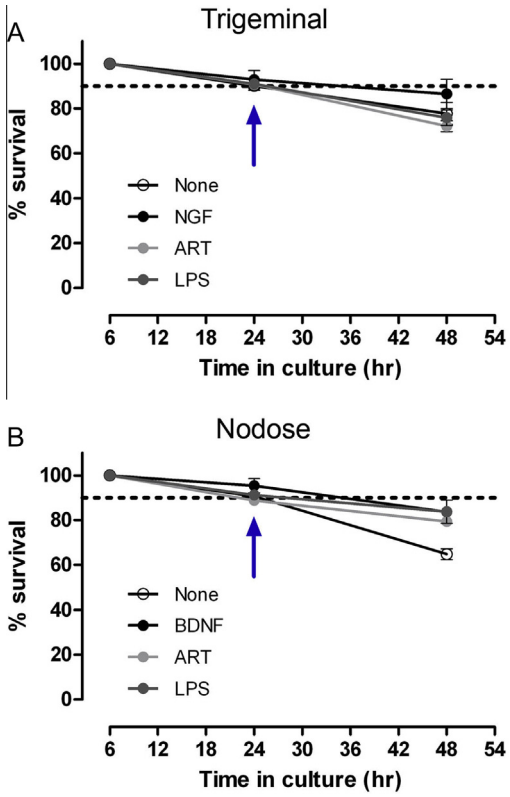


Fig. 2. Survival of trigeminal and nodose neurons cultured with LPS or different trophic factors. Purified cultured trigeminal (A) and nodose (B) neurons from P5 mice were incubated in defined medium alone (None) or medium containing ART (20 ng/ml) or LPS (0.5 μg/ml). Trigeminal neurons were also cultured in the presence of 50 ng/ml NGF while BDNF (20 ng/ml) was used for nodose neurons. The different trophic factors or LPS were added at the time of plating and again 24 h later. The number of cells alive was counted 6, 24 and 48 h after adding trophic factors or LPS to the culture media. Counts were performed by an operator blinded to the treatments using a bright field inverted microscope. The plots show the mean ± SEM percentage survival after 6, 24 and 48 h in culture. Each point represents data obtained from six independent experiments (six cultures from six mice). The arrow indicates the survival rate at 1 DIV; this was the time used for the studies of expression and regulation of the LPS receptor complex because it is the time when cell death was not significant. Note that neither the addition of the trophic factors nor LPS resulted in a significant decline of neuronal survival after 24 h treatment.

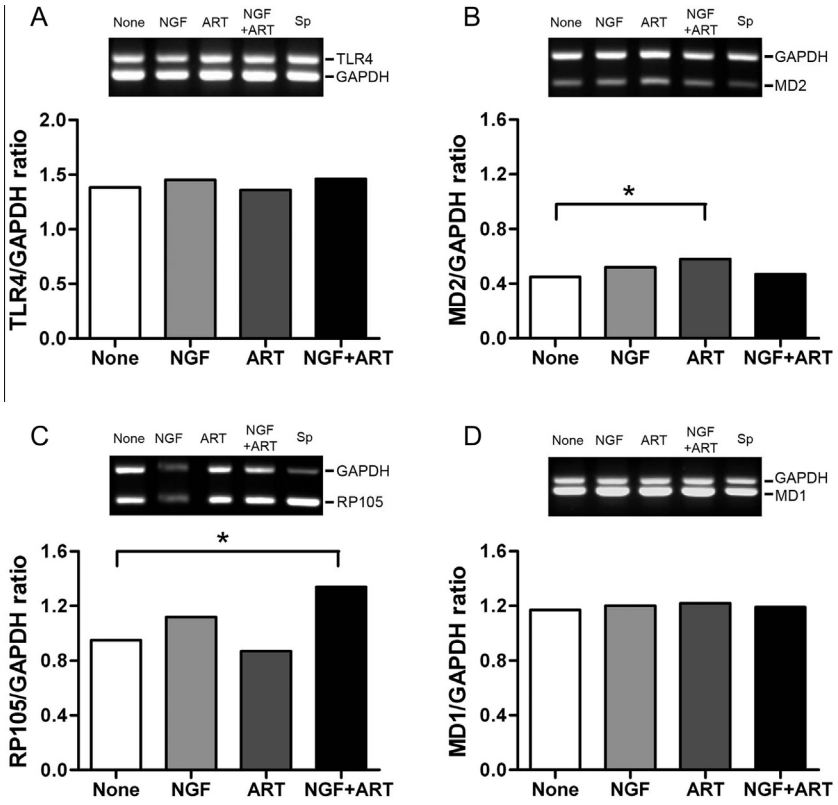


Fig. 3. Trophic factors regulate the expression of LPS receptor mRNAs in cultured trigeminal neurons. Representative gels and corresponding bar graphs of semi quantitative RT-PCR amplification products for TLR4 (A), MD2 (B), RP105 (C) and MD1 (D) mRNAs relative to GAPDH. 2 Trigeminal ganglia (1 mouse) from P5 CD-1 mice were dissected and neurons isolated and cultured for 24 h in defined media. Trigeminal neurons in culture were then supplemented with no trophic factors (None) or with 10 ng/ml NGF 7S or 20 ng/ml Artemin (ART) or a combination of both. Note that ART caused a significant increment in the ratio of MD/GAPDH compared to None (B) and ART + NGF had a similar effect on the ratio of RP105/GAPDH (C). mRNA was extracted from 3 different cell cultures for each experimental condition. Spleen mRNA was used as a positive control. In all experiments, amplification of GAPDH was run in parallel to normalize samples. Quantification of RT-PCR amplification products was performed with Simple PCI as detailed in Section 'Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)'. All experiments were repeated three times and the data presented as the median of the calculated ratios. Statistical significance $^{*}P < 0.05$.

culture media (details in Section 'Cell culture'). Neither the addition of the trophic factors nor LPS resulted in significant decline on neuronal survival after 24 h treatment (Fig 2) suggesting this was a proper time to test the possible effects of trophic factors (Figs. 3 and 4) and LPS (Fig. 5).

Next, we determined the effects of different trophic factors on TLR4, MD2, RP105 and MD1 mRNA expression at 24 h after initial treatment using real-time semi-quantitative PCR. In all cases we compared cultures treated with added trophic factors with cultures produced at the same time that had no added trophic factors (None). We observed no significant changes in TLR4 mRNA expression in trigeminal neurons (Fig. 3A) and only a small (but significant, $P < 0.05$) increment in MD2 mRNA levels with ART (Fig. 3B). Although neither NGF nor ART alone had any significant effect, the combination of both resulted in a significant increment ($P < 0.05$) in the RP105 mRNA levels compared to no trophic factors (Fig. 3C). On the other hand, MD1 levels did not show any significant changes for any of the trophic factors tested (Fig. 3D). These data together suggested (a) that mRNA levels for MD2 and RP105 are subject to trophic factor control in trigeminal neurons; (b) that none of the factors had an effect on TLR4 and MD1 mRNA levels, and (c) that ART regulates differentially the expression of MD2 and RP105.

A different pattern emerged for nodose neurons (Fig. 4). None of the growth factors tested altered TLR4 mRNA levels (Fig. 4A), whereas LIF and BDNF plus ART caused a small but significant reduction of MD2 mRNA levels (Fig. 4B). By contrast, most trophic factors affected RP105 (Fig. 4C) and MD1 (Fig. 4D) mRNA expression to varying degrees. Stimulation of nodose neurons with LIF, CNTF and BDNF plus ART resulted in a significant increment of RP105 mRNA levels compared to no trophic factors (Fig. 4C). With the exception of ART, all the other treatments resulted in significantly elevated MD1 mRNA levels (Fig. 4D).

These findings show that trophic factors are capable of differentially regulating the expression of RP105, its auxiliary unit MD1 and to a lesser extent MD2 with no significant effects on TLR4 expression. Importantly, our data showed regulation of MD2 (but not MD1) by ART in trigeminal cultured neurons while MD1 (but not MD2) was regulated by all tested trophic factors except ART

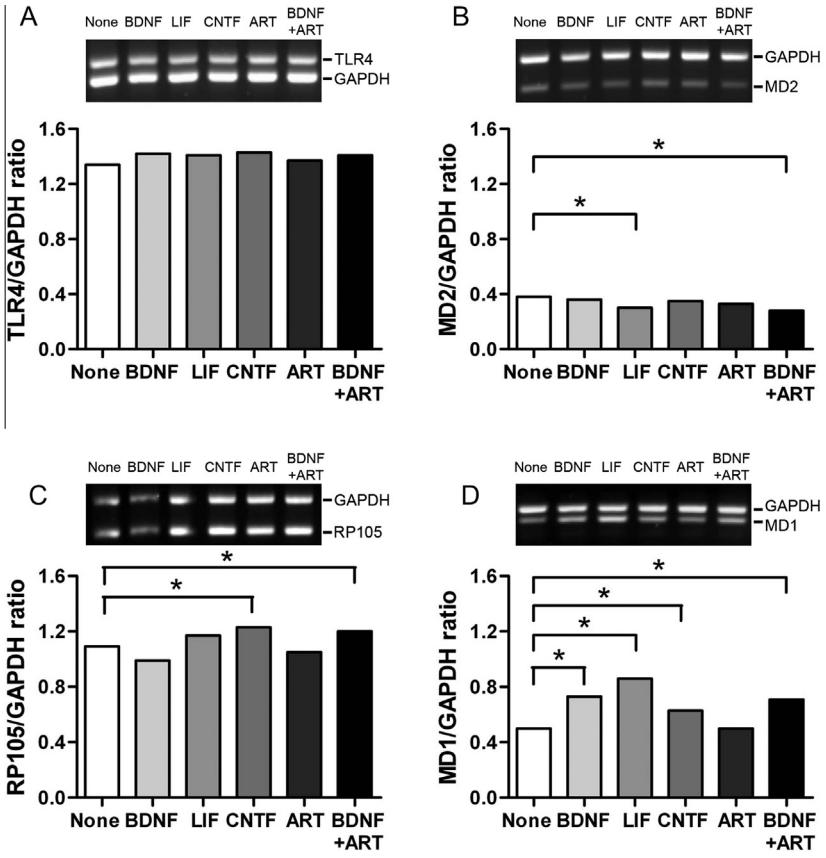


Fig. 4. Trophic factors regulate the expression of LPS receptor mRNAs in cultured nodose neurons. Representative gels and corresponding graph bars of semi quantitative RT-PCR amplification products for TLR4 (A), MD2 (B), RP105 (C) and MD1 (D) mRNAs relative to GAPDH mRNA. Six Nodose ganglia (three mice) from P5 CD-1 mice were dissected and neurons isolated and cultured for 24 h in defined media. Nodose neurons in culture were then supplemented with no trophic factors (None) or with 20 ng/ml BDNF or 20 ng/ml ART or 10 ng/ml CNTF or 20 ng/ml LIF or combinations of BDNF and ART. Interestingly, LIF and BDNF + ART had a detrimental effect on the ratio of MD2/GAPDH (B). BDNF and again BDNF + ART caused a small but significant increment in the ratio of RP105/GAPDH mRNA (C). All tested factors with the exception of ART led to an increment in MD1/GAPDH mRNA ratio compared to None (D). mRNA was extracted from three different cell cultures for each experimental condition. Spleen mRNA was used as a positive control. In all experiments, amplification of GAPDH was run in parallel to normalize samples. Quantification of RT-PCR amplification products (relative to GAPDH) was calculated as explained in Section 'Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)'. Each bar on the graphs represents the median of three independent experiments. Statistical significances are $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

in cultured nodose neurons. Furthermore, in nodose neurons, BDNF plus ART caused up-regulation of the RP105/MD1 complex while at the same time down-regulated MD2.

LPS acutely regulates its own receptor

We next explored whether LPS regulated the proteins of its own receptor complexes. To achieve this we used 24 h-purified cultures of trigeminal and nodose neurons and evaluated the effects of short-term exposure to LPS (within 3 h) on TLR4, MD2, RP105, MD1 and CD14 relative mRNA levels (Fig. 5).

We observed that in trigeminal neurons, LPS caused a significant decrease on CD14 mRNA expression levels after 20 min treatment reaching its lowest level by 60 min. CD14 mRNA expression then recovered to the initial levels after 3 h The other components of the LPS receptor complex exhibited small, non-significant fluctuations over this time interval albeit MD2 mRNA levels tended to decrease by 3 h (Fig. 5A, B).

A similar pattern emerged for nodose neurons: CD14 mRNA levels decreased after 20 min of LPS exposure and recovered to the initial values by 60 min. Interestingly, MD2 mRNA relative levels also dropped significantly after 20 min with LPS, and again returned to its initial level by 3 h (Fig. 5C, D). None of the other mRNAs changed significantly but the RP105/MD1 complex tended to decrease by 3 h.

These findings indicate that LPS probably causes CD14 mRNA to down-regulate acutely in both, trigeminal and nodose neurons, with associated down-regulation of MD2 but only in nodose neurons.

LPS modulates N/OFQ expression in cultured trigeminal and nodose neurons

We have shown that trigeminal and nodose neurons express all the components of the LPS-receptor complex. We therefore asked whether activation of this complex by LPS triggered a change at the cellular level that could be associated with the sensory physiology of

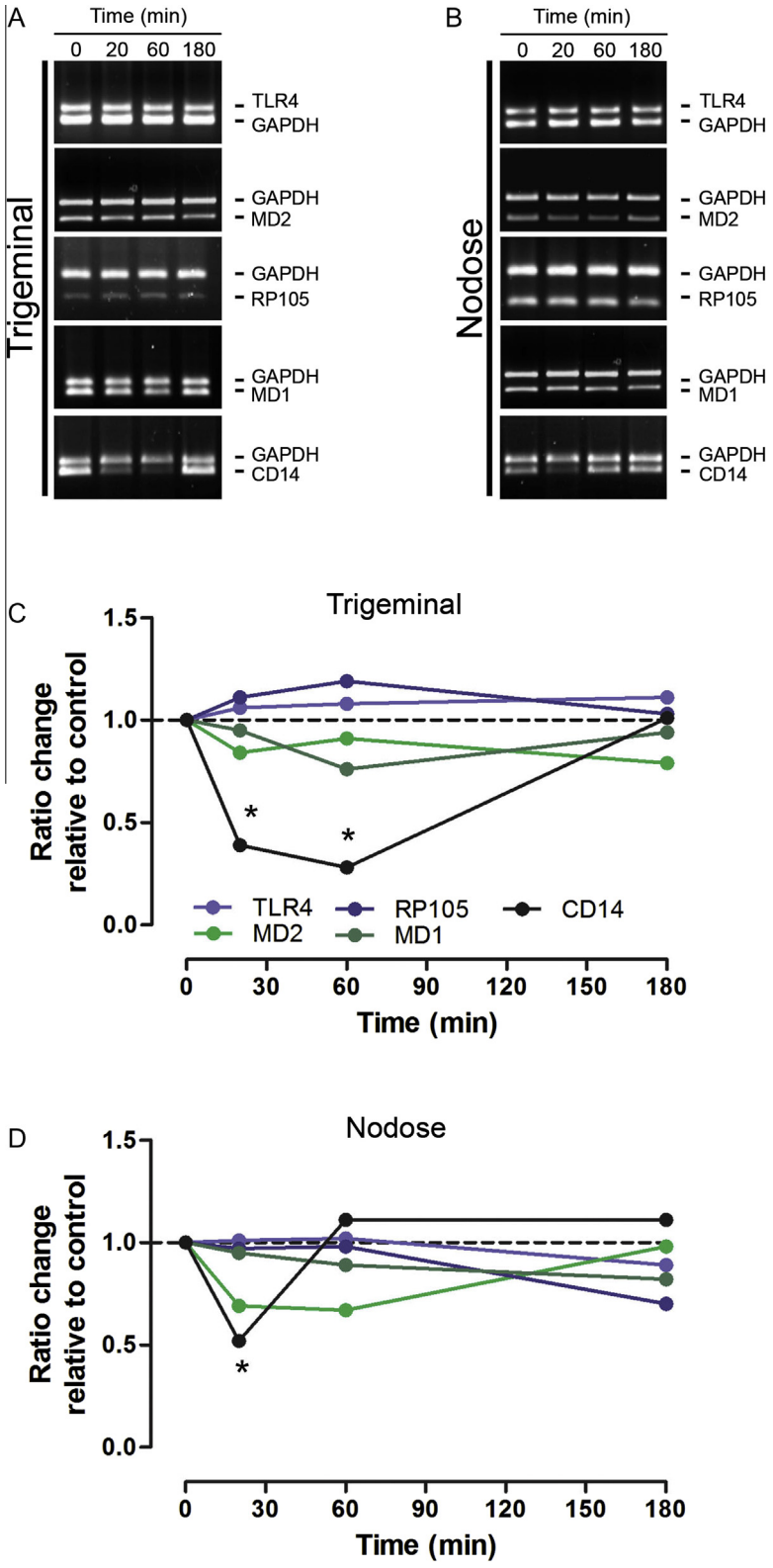


Fig. 5. LPS acutely regulates its own receptor mRNAs. Representative gels of semi quantitative RT-PCR (A and B) and their corresponding bar graphs (C and D) of purified trigeminal (A and C) and nodose (B and D) cultured neurons. Trigeminal and nodose 1 DIV cultured neurons from P5 mice were incubated with 0.5 μg/ml LPS for 0, 20, 60 and 180 min. The neurons were harvested at each time point and the mRNA extracted as detailed in Section 'Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)'. Semi quantitative RT-PCR was performed for TLR4, MD2, RP105, MD1 and CD14 and amplification of GAPDH was run in parallel to normalize samples. The bar graphs (C and D) show the time course of the change in ratio of each mRNA relative to time 0. Each time point represents the mean of three independent experiments. The asterisks show statistically significant ($P < 0.05$) differences relative to time 0. Note that CD14 relative mRNA level decreases in response to exposure to LPS in both, trigeminal and nodose neurons, with associated down-regulation of MD2 only in nodose neurons.

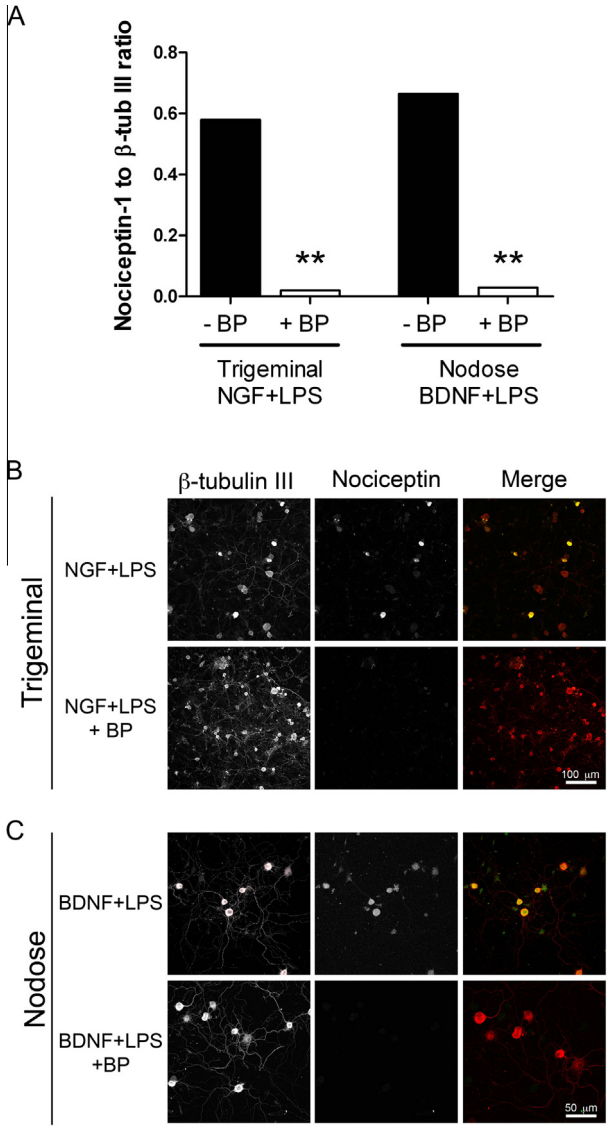


Fig. 6. N/OFQ antibody characterization. Preabsorption experiments were conducted to examine the selectivity of the anti-mouse N/OFQ primary antibody (C-17). Purified cultures of trigeminal and nodose neurons from P5 mice were incubated in defined medium containing NGF (50 ng/ml) and LPS (0.5 μg/ml) for Trigeminal neurons or BDNF (20 ng/ml) and LPS for nodose neurons. The different trophic factors and LPS were added at the time of plating as detailed in Section 'Cell culture'. Three cultures (from three mice) were then fixed with paraformaldehyde and processed for fluorescence immunocytochemistry as described in Section 'Immunocytochemistry'. C-17 Ab (1:100) was preincubated in PBS for 1 h at room temperature with a specific blocking peptide for this antibody (1:50). Images were digitally acquired with a LSM510 confocal microscope and the average cytoplasmic pixel intensity was calculated offline using ImageJ. Only cells clearly labeled for β-tubulin III (a selective marker of neurons) were examined. (A) The plot shows the median ratio of N/OFQ intensity to β-tubulin III intensity for each treatment. The asterisks show statistically significant ($P < 0.05$) differences relative to cultures immunostained with the naked antibody (without the preabsorption step with blocking peptide). Representative images from purified cultured trigeminal (B) and nodose (C) neurons from P5 mice stained for N/OFQ and β-tubulin III immunostained with C-17 antibody preincubated with or without the blocking peptide.

trigeminal neurons (e.g. affect nociception) or nodose neurons (e.g. alter the cough reflex). We chose N/OFQ as the marker because (a) it is upregulated by LPS in sensory neurons (Acosta and Davies, 2008), (b) it has anti-nociceptive activity in the trigeminal ganglia (Wang et al., 1996, 1999) and (c) inhibits acid-induced cough and the release of tachykinins by nodose neurons (Fischer et al., 1998; Jia et al., 2002; Lee et al., 2006).

We established primary purified cultures from trigeminal and nodose ganglia in the continuous presence of NGF or BDNF respectively, to ensure a high rate of neuronal survival (Fig. 2). We treated the cultures for 24 h with LPS and then assessed the proportion of neurons expressing N/OFQ (for details, see Section 'Immunocytochemistry'). Preabsorption of the N/OFQ antibody with its blocking peptide (Section 'Antibody preabsorption') abolished all staining in cultures of trigeminal and nodose neurons, even after immune challenge with LPS (Fig. 6)

As shown in Fig. 7A, C, LPS combined with NGF significantly increased the percentage of neurons expressing N/OFQ (16.3%) compared to either NGF alone (9.3%) or in combination with ART (8.7%). However, co-incubation with a TLR4/MD2 blocking antibody (MTS510) (Qi and Shelhamer, 2005) prevented LPS from incrementing the proportion of trigeminal neurons expressing N/OFQ. This observation strongly suggests that N/OFQ expression was changing in response to LPS acting via TLR4/MD2. Similarly, LPS plus BDNF increased significantly the proportion of N/OFQ immunopositive nodose neurons (16%) compared to BDNF alone (10.9%). Once again, this phenomenon was fully prevented by co-incubation with the TLR4/MD2 blocking antibody (Fig. 7B, C). Interestingly, the combination of BDNF plus ART caused a significant reduction in the number of nodose neurons expressing N/OFQ (from 16% to 3.7%).

These results suggest that LPS is able to increase the percentage of neurons expressing N/OFQ probably via activation of the TLR4/MD2 complex.

DISCUSSION

This study is the first report on the expression of RP105 and the auxiliary units MD1 and MD2 in mouse trigeminal and nodose neurons. We demonstrate that mRNAs for RP105, MD1 and MD2 are regulated by trophic factors at 1 DIV. We also show that LPS acutely (within minutes) down-regulated CD14 relative mRNA levels down in both, trigeminal and nodose cultured neurons. We also show that the TLR4/MD2 receptor is functional and its activation by LPS increases the percentage of trigeminal and nodose neurons expressing the peptide N/OFQ.

Previous studies have shown that trigeminal and nodose neurons could respond to LPS stimulation. Here, we demonstrate that in contrast to what has been observed in DRG neurons, both neuronal types express the five known components of the LPS receptor complex: TLR4/MD2/CD14 and RP105/MD1. In the trigeminal, TLR4 and CD14 are mostly expressed in TRPV1-positive neurons (many, not all, putative nociceptors) (Wadachi and Hargreaves, 2006) and its activation by LPS sensitizes TRPV1 via TLR4 (Ferraz et al., 2011). Here we show that trigeminal neurons also express the auxiliary unit MD2

which is required for membrane expression of TLR4 (Nagai et al., 2002a).

Note that RP105 is mostly restricted to mature B-lymphocytes, is involved in the pathophysiology of autoimmunity (Kimoto et al., 2003) and requires MD1 to reach the cell membrane where it can bind to LPS (Nagai et al., 2002b). Also, stimulation by LPS of RP105/MD1 antagonizes TLR4/MD2 when both receptors are present in the same cells (Miyake, 2004). The fact that this particular LPS receptor seems to be expressed in trigeminal and nodose neurons suggests a) a possible crosstalk between TLR4 and RP105 complexes in response to LPS, and b) that these neurons may respond differentially to LPS depending on whether, or the extent to which, the expression of the two receptors are co-localized. However, we can only speculate about this, because whether they are co-localized or are expressed by different neuronal subpopulations in trigeminal or nodose neurons remains to be established.

Our finding that LPS acutely regulates CD14 in both neuronal types (albeit with different kinetics) suggests this is a key regulatory protein of the LPS receptor. In both cases, CD14 mRNA levels recovered to its original level in the continuous presence of LPS, suggesting that the down-regulation is transient, which may imply a desensitization mechanism. CD14 binds and transfers bacterial LPS to the surface TLR4/MD-2 complex to enable its recognition (Jerala, 2007) and therefore we suggest that the down regulation of CD14 would eventually diminish binding to LPS. This, in turn, may cause an attenuated response to LPS but only after initial exposure to LPS has triggered the downstream signaling pathways leading to a strong initial neuroimmune response (Zanoni and Granucci, 2013). Note that CD14 is necessary for TLR4/MD2 LPS-induced internalization, a process that tends to reduce the surface density of the receptor, especially in the presence of high concentrations of LPS (Shuto et al., 2005). The transient reduction in CD14 mRNA (if it translates to lower soluble CD14) may explain why TLR4 mRNA levels are only slightly (not significantly) elevated after a 3 h LPS treatment. In nodose neurons MD2 mRNA levels also dropped transiently perhaps contributing to a desensitized LPS-receptor that could cause a diminished sensitivity to LPS.

We have shown that LPS acutely alters the mRNA levels for some components of its own receptor. It is also known that longer (> 1 day) exposure to LPS

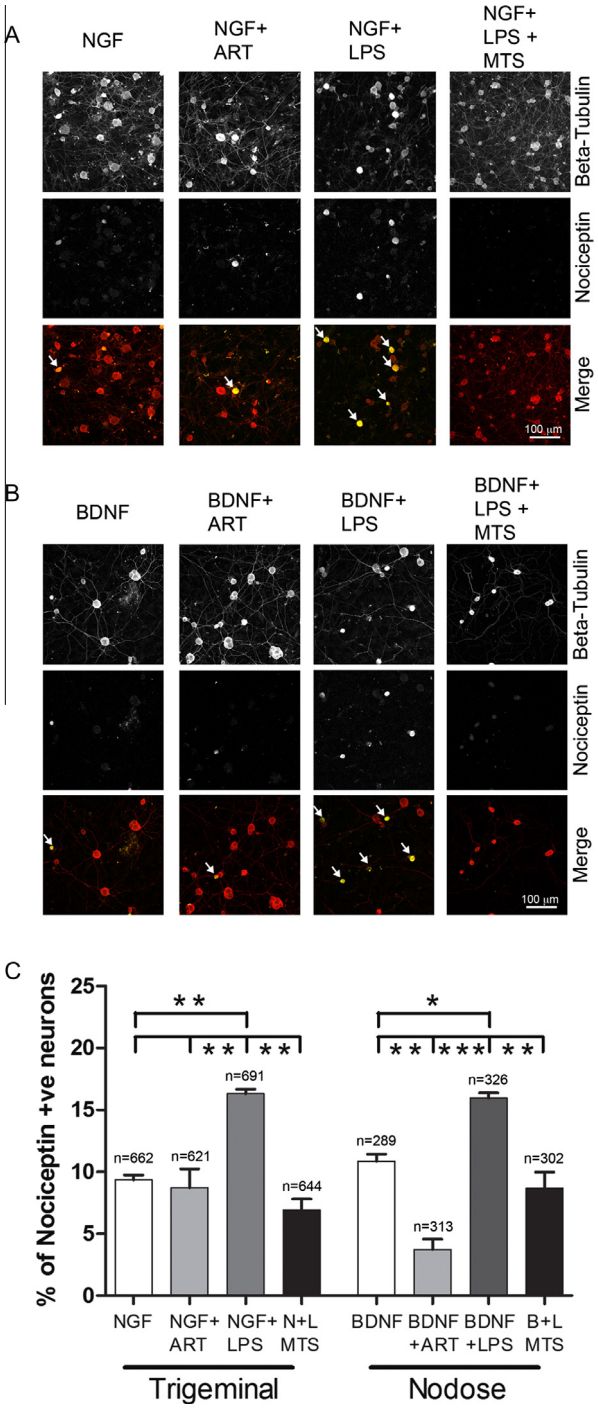


Fig. 7. LPS increases the proportion of N/OFQ immunopositive trigeminal and nodose neurons in culture. Representative microscope images from purified cultured trigeminal (A) and nodose (B) neurons from P5 mice stained for nociceptin-1/Orphanin FQ (N/OFQ) and β-tubulin III (a selective marker of neurons). Merged images are colored to show (in yellow) which neurons were immunopositive for N/OFQ. (A) Trigeminal neurons were incubated in defined medium containing NGF (50 ng/ml) or NGF plus ART (20 ng/ml) or NGF and LPS (0.5 μg/ml) and NGF plus LPS in the continuous presence of the TLR4/MD2 blocking antibody MTS510 (N + L + MTS). (B) Nodose neurons were incubated in defined medium containing BDNF alone (20 ng/ml), BDNF plus ART (20 ng/ml), BDNF plus LPS (0.5 μg/ml) and BDNF plus LPS plus the TLR4/MD2 blocking antibody MTS510 (B + L + MTS). Cultures were fixed and processed for immunolabeling after 24 h treatment as indicated in Section 'Immunocytochemistry'. Arrows indicate typical examples of neurons co-expressing N/OFQ and β-Tubulin III. Scale bar = 100 μm. (C) Shows the mean percentage plus SEM of β-tubulin III-positive cells that were also positively immunostained with the C-17 anti-N/OFQ antibody. n values on top of bars are the number of β-tubulin III-positive neurons measured in each experimental condition. Note that LPS increased the percentage of neurons expressing N/OFQ in both trigeminal and nodose neurons and TLR4/MD2 blocking antibody (MTS510) prevented this effect. Interestingly, the combination of BDNF plus ART caused a significant reduction in the number of nodose neurons expressing N/OFQ. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

induces the *in vitro* synthesis and release of NGF (Arsenijevic et al., 2007) and BDNF (Gomes et al., 2013) among other factors and also increments the expression of their respective receptors (i.e. p75 and TrkA) (Elkabes et al., 1998; Jiang et al., 2007, 2008). *In vivo*, however, there is evidence that levels of NGF, BDNF and NT3 drop in the central nervous system after systemic administration of LPS (Guan and Fang, 2006). Given the central role played by trophic factors in the development and maturation of trigeminal and nodose neurons (Horton et al., 1996; Lewin and Barde, 1996; ElShamy and Ernfors, 1997; Lieu et al., 2011) and that the presence of the LPS-receptor complex confers to neurons the ability to respond to LPS (a key feature of their phenotype), we hypothesized that these factors may regulate the expression of the LPS-receptor complex proteins. Perhaps the differences we see could be traced back to the different embryological origin of DRGs (they are neural crest), while nodose are placodal and trigeminal ganglia are both neural crest and placodal (D'Amico-Martel and Noden, 1983; Hall, 2008; Blentic et al., 2011).

Remarkably, we found that RP105/MD1 were both regulated by trophic factors in trigeminal and nodose neurons, while TLR4/MD2 were not. In the trigeminal, ART increased MD2 mRNA levels and NFG plus ART elevated RP105 mRNA. The latter was not an additive effect but looked synergic suggesting that up-regulation of RP105 requires more than one trophic factor acting simultaneously.

In nodose neurons, RP105 and MD1 mRNAs were both up-regulated by LIF, CNTF and BDNF. This finding agrees with these trophic factors playing a key role in the survival and maturation of nodose neurons (see 'Introduction'). It also suggests that trophic factors may induce the synthesis of the LPS-receptor complexes early during development in trigeminal and nodose neurons. Whether these trophic factors remain active in adulthood (or after neuronal maturation has completed) remains to be studied.

Taking together, our results suggest that some trophic factors influence neuronal plasticity in trigeminal and nodose neurons in terms of their LPS responsiveness and present us with (1) the chance of regulation of the LPS receptor complex and (2) a difference between regulation of the expression of TLR4/MD2 and RP105/MD1.

Up to this point, we have established that nodose and trigeminal neurons express the LPS receptor complex mRNAs that make them potentially responsive to LPS. The physiological importance this expression may have when these neurons face a bacterial infection largely depends on the biological activity of LPS. For instance, LPS-induced inflammation elevates TLR4 and CD14 expression in trigeminal neurons, leading to increased heat and mechanical hyperalgesia probably mediated by neuropeptide release (Kemper et al., 1998; Abd El-Aleem et al., 2004). In nodose neurons, long exposure to LPS stimulates IL-1 release (Lu et al., 2002) and increases tachykinin synthesis (Huang and Lai, 2003;Lai et al., 2003). Furthermore, *in vivo* LPS treatment also elevated endogenous NGF causing higher nodose C-fiber density innervations of the rat airways (Takeda et al., 2011). Therefore LPS may trigger cellular and molecular events leading to a range of physiologically important symptoms that include pain, inflammation, fever, cough, wheeze, headache, toothache and visceral pain. In line with this hypothesis we found that LPS causes an increase in the proportion of N/OFQ expressing nodose and trigeminal neurons. N/OFQ inhibits the release of tachykinins in a subpopulation of nodose neurons that do not synthesize it (Fischer et al., 1998) supporting the notion that the N/OFQ acts on nodose C-fibers preventing acid-induced cough (Lee et al., 2006). However, in the rat vagal paraganglia there is evidence that acute administration of pro-inflammatory cytokines and LPS does not affect the frequency of chemosensory discharges normally or during hypoxia (O'Connor et al., 2012). Furthermore, only very high levels of LPS (equivalent to those found in patients with extreme sepsis) translate into circulating levels of cytokines that could have a physiological impact on the electrical activity of nodose afferents (Goehler et al., 1997;Rummel et al., 2011). This raises the question of why do some nodose neurons express mRNA for all the proteins of the LPS-receptor complex (this study). It is possible that LPS induces physiological changes in vagal afferents only after a prolonged exposure or are restricted to a small number of nodose neurons. Given the contradictory nature of published observations, the role of the LPS-receptor complex in these neurons as a mediator of pro-inflammatory immune responses via changes in electrical activity requires further examination.

In the trigeminal system N/OFQ blocks NMDA-mediated synaptic transmission and inhibits release of substance-P (Wang et al., 1996, 1999; Flores et al., 2001). N/OFQ also blocks high-voltage activated Ca^{2+} channels in a subpopulation of trigeminal neurons that is sensitive to mu opioids (Borgland et al., 2001) thus N/OFQ acts as anti-nociceptive (potentially analgesic). Although LPS is thought to induce acute inflammation-related pain associated with bacterial infection (see Section 'Introduction') it is likely that in the longer term stimulation with N/OFQ could alleviate the severest symptoms of this pain. However, because we do not know whether the N/OFQ acts via an autocrine or paracrine mechanism, it remains unclear which subpopulation of neurons would be more affected by LPS and N/OFQ.

CONCLUSION

The symptoms due to gram-negative bacterial infection affecting the airways of the lung, teeth, tooth pulp or the digestive tract could in part result from activation of afferent neurons by LPS. The whole process is likely to be modulated at the level of LPS-receptor expression by trophic factors and also by LPS itself.

CONFLICT OF INTEREST

The authors declare no competing financial interests.

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