There has been a long-standing debate regarding the role of peripheral afferents in mediating rapid-onset anorexia among other responses elicited by peripheral inflammatory insults. Thus, the current study assessed the sufficiency of peripheral afferents expressing toll-like receptor 4 (TLR4) to the initiation of the anorexia caused by peripheral bacterial lipopolysaccharide (LPS). We generated a Tlr4 null (Tlr4\textsuperscript{LoxB}) mouse in which Tlr4 expression is globally disrupted by a loxP-flanked transcription blocking (TB) cassette. This novel mouse model allowed us to restore the endogenous TLR4 expression in specific cell types. Using Zp3-Cre and Na\textsubscript{v}1.8-Cre
mice, we produced mice that express TLR4 in all cells (Tlr4<sup>LoxTB</sup> X Zp3-Cre) and in peripheral afferents (Tlr4<sup>LoxTB</sup> X Na<sub>1.8</sub>-Cre), respectively. We validated the Tlr4<sup>LoxTB</sup> mice, which were phenotypically identical to previously reported global TLR4 knock-out mice. Contrary to our expectations, the administration of LPS did not cause rapid-onset anorexia in mice with Na<sub>1.8</sub>-restricted TLR4. The later result prompted us to identify Tlr4-expressing vagal afferents using in situ hybridization (ISH). In vivo, we found that Tlr4 mRNA was primarily enriched in vagal Na<sub>1.8</sub> afferents located in the jugular ganglion that co-expressed calcitonin gene-related peptide (CGRP). In vitro, the application of LPS to cultured Na<sub>1.8</sub>-restricted TLR4 afferents was sufficient to stimulate the release of CGRP. In summary, we demonstrated using a new mouse model that vagally-expressed TLR4 is selectively involved in stimulating the release of CGRP but not in causing anorexia.

**Key words:** Cre-loxP; innate immunity; neuropeptide; vagus nerve

**Introduction**

Toll-like receptor 4 (TLR4) is the main membrane receptor for lipopolysaccharides (LPS), which are endotoxins derived from the outer membrane of Gram-negative bacteria (Poltorak et al., 1998a,b; Chow et al., 1999). Hence, the immunologic effects of LPS are blunted in TLR4-deficient mice (Hoshino et al., 1999). That said, other membrane proteins, including the myeloid differentiation factor 2 (Park et al., 2009) and TRP channels (Meseguer et al., 2014; Alpizar et al., 2017) also contribute to LPS signaling. Upon exposure to LPS, anorexia ensues (Kent et al., 1992; Porter et al., 1998a) as the direct result of the actions of proinflammatory molecules on the CNS (Layé et al., 1994; Grossberg et al., 2010; Daniel et al., 2016; Essner et al., 2017). While the anorexic effects of LPS occur within minutes (Liu et al., 2016), LPS does not cross the blood-brain barrier (Banks and Robinson, 2010), suggesting that a peripheral neural mechanism involving vagal afferents may contribute to LPS-induced anorexia. This is because the peripheral endings of vagal afferents are outside the blood-brain barrier and directly accessible to bacterial products located in vagally-innerated tissues. Experiments conducted in deafferented animals are in agreement with this view (Blüthé et al., 1994; Bret-Dibat et al., 1995; Layé et al., 1995; Konstantinou et al., 2000). However, it was never clear whether LPS could directly act on peripheral neurons until TLR4 was found to be expressed in peripheral afferents, including those in the vagus nerve (Hua et al., 1996; Hosoi et al., 2005; Ochoa-Cortes et al., 2010; de Lartigue et al., 2011; Diogenes et al., 2011; Due et al., 2012; Kunda et al., 2014; Helley et al., 2015; Li et al., 2015; Boonen et al., 2018).

Moreover, the ability of isolated sensory neurons to directly and rapidly respond to LPS has been reported in different species using electrophysiology and calcium signaling (Riley et al., 2013; Meseguer et al., 2014). The in vivo administration of LPS has also been reported to produce changes in the neuropeptide expression and firing rate of vagal afferents (Huang and Lai, 2003; Liu et al., 2007). Thus, it is plausible that this mechanism allows peripheral sensory neurons to detect infections in a rapid manner (Clatworthy and Grose, 1999; Chiu et al., 2013; Soldano et al., 2016). Finally, it must be noted that within the brain, TLR4 is expressed in endothelial and glial cells, but not in neurons (Laflamme and Rivest, 2001; Chakravarty and Herkenham, 2005; Kigerl et al., 2007). The latter observation suggests that peripheral afferents are likely the only neurons in the body able to sense changing levels of TLR4 activators in a direct manner.

Other findings contradict the view that TLR4 is present and functional in vagal afferents. Another RNA sequencing study of vagal afferents failed to detect any vagal afferents with significant Tlr4 mRNA expression (Wang et al., 2017). This is without mentioning that several...
laboratories that interrupting vagal afferents does not prevent the host of physiological responses to peripheral LPS, including anorexia (Bret-Dibat et al., 1997; Schwartz et al., 1997; Hansen et al., 2000; Martin et al., 2000; Wieczorek et al., 2005). Considering that vagal afferents are exquisitely sensitive to a wide range of proinflammatory molecules (Ek et al., 1998; Yu et al., 2005; Feldman-Goriachnik et al., 2015), it remains possible that the aforementioned cellular and physiological effects of LPS are not directly mediated by TLR4 signaling in vagal afferents themselves. Given the above uncertainties in the field, the current study aimed to reassess the sufficiency of vagal afferents TLR4 to select LPS-induced responses using a newly generated transgenic mouse model.

Materials and Methods

Mice

Mice were all housed in a barrier facility in a light-controlled and temperature-controlled environment [zeitgeber (ZT)0 = 6 A.M.] with ad libitum access to standard chow, unless specified otherwise (Harlan Teklad TD.2016 Global). Experimental mice were males, whereas females were only used for breeding purposes. Single housing was required during the course of food intake studies. Otherwise, animals were group housed at all time. All the procedures in this study were approved by our Institutional Animal Care and Use Committees and in accordance with the Society’s Policies on the Use of Animals and Humans in Neuroscience Research.

Wild-type (WT) C57Bl/6 mice

A total of six male mice, between six and eight weeks of age, on a pure C57Bl/6 background were used for chromogenic in situ hybridization (ISH) experiments. These mice were obtained from our Animal Resource Center.

Na1.8-CreChR2-YFP reporter mice

We also generated and genotyped Na1.8-Cre-Chr2-YFP mice carrying one Cre allele and one Chr2-YFP allele. A total of four males between six and eight weeks of age, were used for chromogenic ISH combined with GFP immunostaining experiments.

Generation of novel Cre-reactivated TLR4-null mice (Tlr4LoxTB)

To generate Tlr4LoxTB mice, BAC clones containing the murine Tlr4 gene derived from the 129/Sv strain were obtained from the Sanger Institute. The BAC DNA clone number bMQ58F22 was electroporated into EL350 bacteria. First, the loxP sequences present in the BAC backbone were removed by homologous recombination as previously described (Lee et al., 2001). To identify the recombinant clones, genomic DNA was extracted from ES cells as previously described (Berglund et al., 2012) and used for long PCR assays to discriminate between ES clones hosting WT and loxP-modified Tlr4 allele. Correct targeting was further confirmed by a custom multiplex TaqMan quantitative PCR (qPCR) assay. Briefly, extracted DNA was used as template (5–100 ng). Melanocortin 4 receptor (MC4R) was used as an endogenous reference (ID Mm00457483_s1, FAM dye-labeled probe; Applied Biosystems). Custom primers (5’-TCTTACAACAACTGGAGAAACATGGTAC-3’ and 5’-AGGAATCCAGCTATGAGGACCTGAGAAAT-3’) from Biosearch Technologies were multiplexed with the MC4R assay, DNA-based qPCRs were conducted on an Applied Biosystems PRISM 7900HT sequence detection system. Correctly targeted recombinant ES cells were injected into blastocysts of C57Bl/6 mice. Chimeric male mice (F0) were crossed to C57Bl/6 female mice and their pups (F1) were screened for germ line transmission by PCR using the same strategy as for genotyping. F1 pups bearing the Cre-reactivable Tlr4-null allele (Tlr4LoxTB) were then crossed with zona pellucida 3 (Zp3)-Cre transgenic mice (stock #003651 C57BL/6-Tg(Zp3-cre)93Knw/J) to restore endogenous TLR4 expression in all tissues (Tlr4LoxTB X Zp3-Cre). Mice
carring a WT and/or Cre-mediated recombination allele (Tlr4<sup>LoxTB</sup> X Zp3-Cre) were genotyped by primer pairs P1 (5'-CTGACTGGTGAGTGAAGTGGA-3') and P3 (5'-GCTGATGATGCTAGCAGATAA-3'). Primers P2 (5'-CTGACAAACATGGCTGGAA-3') and P3 were used to screen the Tlr4<sup>LoxTB</sup> allele. Globally reactivated mice were used for validation studies and feeding tests. The group size is indicated in figure legends.

**Tlr4<sup>LoxTB</sup> X Na<sub>1.8</sub>-Cre mice**

F1 pups bearing the Cre-reactivated Tlr4<sup>LoxTB</sup> allele were then crossed with Na<sub>1.8</sub>-Cre mice to produce mice with endogenous TLR4 reactivation only in Na<sub>1.8</sub> neurons (Tlr4<sup>LoxTB</sup> X Na<sub>1.8</sub>-Cre). These mice are referred to as Na<sub>1.8</sub>-restricted Tlr4 mice. For all studies, breeding was set up to obtain the following groups: control, Tlr4<sup>LoxTB</sup>, and Na<sub>1.8</sub>-restricted Tlr4 mice. Controls consisted of mice expressing one copy of the Na<sub>1.8</sub>-Cre allele. Tlr4<sup>LoxTB</sup> mice expressed two copies of the Tlr4<sup>LoxTB</sup> allele without Cre. Na<sub>1.8</sub>-restricted Tlr4 mice expressed two copies of the Tlr4<sup>LoxTB</sup> allele and one Cre allele. To validate our ISH probe, we also compared the ganglia from two Tlr4<sup>LoxTB</sup> mice to that of two globally reactivated mice (Tlr4<sup>LoxTB</sup> X Zp3-Cre). Mice were maintained on a mixed (C57Bl/6 and 129) genetic background. Male mice entered the experiments at 8–16 weeks of age and were used for feeding and calcitonin gene-related peptide (CGRP) studies. The group size is indicated in figure legends.

**LPS preparation and dosage**

For animal and culture experiments, we kept 1 mg/ml aliquots of LPS solution (Sigma L2880 *Escherichia coli* 055:B5) in sterile pyrogen-free 0.9% saline (Sigma) at −20°C. When needed, LPS was diluted 100 times in sterile saline for animal studies or in sterile media for culture studies. In all our feeding studies, LPS was administered at a single dosage of 2 mg/kg, intraperitoneally. One hour postinjection, mice received an overdose of chloral hydrate (500 mg/kg, i.p.) and their nodose-jugular ganglia were rapidly removed and frozen in liquid nitrogen. Total RNAs were extracted using RNA Stat60 (Teltest) according to the manufacturer’s instructions. RNA concentration and quality were determined by NanoDrop 1000 Spectrophotometer (Thermo Scientific). Complementary DNA was synthesized using the High Capacity cDNA kit and was performed at 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min (Applied Biosystems). Primers for Tlr4 (ID: Mm0445274_m1), Nfkbia (ID: Mm00477798_m1), and 18s (ID: Hs99999901_s1) were purchased from Applied Biosystems. The mRNA contents were normalized to 18s RNA levels. qPCR was performed in an ABI Prism 7900HT sequence detection system (Applied Biosystems) using TaqMan Master Mix (Applied Biosystems) and 10 ng of synthesized cDNA. Samples were run in triplicates. The relative amounts of all mRNAs were calculated using the ΔΔCT assay.

**ISH, microscopy, and digital images analysis**

Mice received an overdose of chloral hydrate (500 mg/kg, i.p.), before being transcardiacally perfused with 10% formalin (Sigma). The nodose-jugular ganglionic mass was rapidly dissected and kept in formalin for an additional 24 h at 4°C. Fixed ganglia were next incubated with 20% sucrose in PBS (pH 7.4) at 4°C before being frozen on a bed of dry ice. Series of 14-μm sections were collected on SuperFrost slides using a cryostat. Tissue sections from Nav1.8-CreChR2-YFP mice were labeled for Tlr4 alone or Tlr4 combined with Calca (CGRP preparatory gene) were counterstained with a hematoxylin solution (GHS132 Sigma). Tissue sections from Na<sub>1.8</sub>-Cre<sup>ChR2-YFP</sup> mice were not counterstained, but further processed for GFP immunohistochemistry. Briefly, after several PBS washes, the sections were incubated overnight at room temperature in a chicken anti-GFP primary antiserum (Aves Laboratory; GFP-
Table 1: List of reagents used for ISH

| Gene style name(s) | Accession number | Probe region | Catalog number channel | Chromogenic labels | Pretreatment | Kits |
|--------------------|------------------|--------------|------------------------|--------------------|--------------|------|
| Tlr4-C1            | NM_021297.2      | 3006–3775    | 316801-C1              | Fast-Red           | RNAscope Hydrogen Peroxide | RNAscope 2.5 HD Reagent Kit-RED assay |
| Calca-C1           | NM_007587.2      | 44–995       | 417961-C1              | HRP-based Green    | RNAscope Hydrogen Peroxide | RNAscope2.0 2-plex |
| Tlr4-C2            | NM_021297.2      | 2404–3775    | 316801-C2              | AP-based Fast Red  | RNAscope Target Retrieval | RNAscope Target Retrieval |

10.120; RRID:AB_10000240; 1:1000) in 3% normal donkey serum with 0.25% Triton X-100 in PBS. On the following day, sections were washed and incubated for 1 h in a biotinylated anti-chicken secondary antibody (catalog #703065155; Jackson ImmunoResearch; 1:1000), followed by Streptavidin Alexa Fluor 488 (Invitrogen, #S11223; 1:1000). All our slides were coverslipped with EcowMount mounting medium (Biocare Medical, LLC EM897L).

Bright-field images were captured using a Zeiss microscope (Imager ZI) attached to a digital camera (Axiocam). A camera lucida attached to the microscope was also used to draw tissue sections. Drawings were digitalized and exported to Adobe Illustrator Artwork 15.1. The Zeiss microscope (Imager ZI) was also used for manual counts of the ISH signals for cell profiles positive for Tlr4/H&E as well as Tlr4/Calca. Cell profiles were counted on digital images by an experimenter unaware of our experimental design. Fluorescent digital images were all acquired with the 20× objective of a Zeiss microscope (Imager ZI) or the 63× objective of a Leica TCS SP5 confocal microscope (The University of Texas Southwestern Live Cell Imaging Core). Scanning parameters were adjusted appropriately to improve the signal/background. We collected Z-stacks separated by ~0.35–0.45 µm in a 512 × 512-pixel format. NIH ImageJ software was used to generate our final TIFF images with combined Z stacks. Adobe Photoshop CS2 software was used to combine digital images into plates with annotations. The contrast and brightness of digital images were uniformly adjusted. Neuronal versus non-neuronal profiles were usually easily identifiable by the size, shape and intensity of the counterstaining of their nuclei. Our counts should be considered estimates rather than absolute counts. Cells that were considered doubly labeled included profiles with accumulation of Tlr4 mRNA (at least one red dot per profile) within the boundaries of one Calca-positive profile. In addition, the circumference of each identified cell profile was assessed using the measurement tools in ImageJ. Images acquired by confocal microscopy were further evaluated for doubly labeled cells using plot of fluorescence intensities. Briefly, we manually traced a line across identified cell profiles using the ImageJ software (NIH, Fiji version). Plot profiles of gray values were generated for each color channel (overlapping red and green). Representative plot profiles were included in our figures. Cells with colocalized fluorescence displayed overlapping red and green plots of fluorescence across the line profile. Cells without staining or minimal colocalization displayed a flat plot profile in at least one channel. Colocalization data were expressed as the mean percentage of identified cell profiles ± SEM, and the absolute numbers of counted profiles were included in the figures.

Organotypic nodose ganglion and CGRP assays

Our protocol was based on previous literature (Gavini et al., 2018). In particular, we chose to culture ganglia for 5–7 d to give the samples time to rebound from the injury caused by the acute dissection. Male mice (five to eight weeks) were deeply anesthetized with isoflurane before being decapitated and the nodose/jugular ganglia were quickly removed and stored in chilled HBSS (Invitrogen) on ice. The isolated ganglia were placed on a 30 mm, 0.4 µm pore size, hydrophilic Millicell culture insert (Millipore Sigma; catalog #PICM03050) and maintained in the insert on DMEM F-12 GlutaMax media (Invitrogen) supplemented with 20% heat-inactivated horse serum (Invitrogen, Life Technologies), and 1 x penicillin streptomycin (Invitrogen). Cultures were maintained for 5–7 d with media changes every other day. After an overnight incubation in low serum (2.0%), cultures were stimulated with 500 ng/ml LPS or vehicle for 24 h before supernatant was collected. Supernatants were centrifuged to remove debris and loaded to a CGRP ELISA kit (Cayman Chemical; catalog #589001) and the protocol ran according to manufacturer’s instructions.

Statistical analysis

All of the quantitative data are expressed as the mean value ± SEM. The numbers of mice per group are indicated in each figure. Statistical analysis was performed using GraphPad Prism software, version 6.07. As indicated in the legends, the data were analyzed using Student’s unpaired t test, one-way ANOVA or two-way ANOVA, followed by the post hoc test recommended by the software; p < 0.05 was considered statistically significant. Lower case letters were used to indicate groups found to be significantly different by the post hoc analysis. We also followed the journal’s recommendations in terms of data representations and estimation statistics (Ho et al., 2018).
Results

Validation of a novel reactivable TLR4 mouse model

We designed and generated a novel Tlr4 null (Tlr4LoxTB) mouse whose Tlr4 expression is globally disrupted by a loxP-flanked TB cassette (LoxTB) that was inserted into the coding region of the Tlr4 gene (Fig. 1A). Crossing Tlr4LoxTB mice with Zp3-Cre mice produced mice in which expression of endogenous Tlr4 was globally reactivated in all cells (Fig. 1B). As a result, the LPS-induced inflammatory response in these mice was identical to WT mice (Fig. 1C, D). Plasma TNFα levels were measured in WT, Tlr4LoxTB and Tlr4LoxTB X Zp3-Cre mice (n=3–5) 1.5 h after intraperitoneally injection of LPS with the concentration of either 1 mg/kg BW (C) or 0.5 mg/kg BW (D). In C, data were analyzed using one-way ANOVA for genotype (p=0.0013), followed by Dunnett’s test (WT vs Tlr4LoxTB p=0.0017). In D, data were analyzed using one-way ANOVA for genotype (p=0.0004), followed by Dunnett’s test (WT vs Tlr4LoxTB p=0.0003). For cytokines, letters denote significant differences between columns with p<0.05, compared with corresponding Tlr4LoxTB mice. E, Graphs of 1-h cumulative food intake in response to saline or LPS (100 μg/kg, i.p.) in control ZP3Cre (white), Tlr4LoxTB (black), and Zp3-Cre-reactivated mice (gray). Groups consisted of n=5. Data are expressed as mean ± SEM and were analyzed using two-way ANOVA for genotype (p=0.0116), treatment (p=0.0002), and interaction (p=0.001), followed by Sidak’s post hoc test. For each genotype, letters denote significant differences between columns with p<0.05, compared with corresponding saline-treated mice. Estimation statistics was calculated in LPS-treated mice. Estimation statistics are included in Extended Data Figure 1-1.

Tlr4-expressing vagal afferents do not initiate LPS-induced anorexia

To test for functional TLR4 signaling in vagal afferents in vivo, we used the previously described Na1,8-Cre line (Stirling et al., 2005) to generate cohorts of mice that endogenously express Tlr4 only in Na1,8 neurons. By qPCR, we show that Tlr4 mRNA expression in the nodose-jugal ganglion was 15-fold higher in control mice than in Tlr4LoxTB mice (without Cre; Fig. 2A). In Na1,8-restricted Tlr4 animals, Tlr4 expression in the nodose-jugal ganglion was ~8-fold higher than in Tlr4LoxTB mice (Fig. 2A). Tlr4 was selectively re-expressed in Na1,8 neurons, but not other Tlr4-expressing cell types. For example, Tlr4 was not reactivated in the liver, a tissue with high endogenous expression of Tlr4, but without Na1,8-Cre cells (Fig. 2B). Lastly, the administration of LPS (2 mg/kg, i.p.) was fully restored in mice with Tlr4 reactivated in all cells that normally express it (Fig. 1E). This is because the expression of Tlr4 remains driven by its endogenous promoter. These studies demonstrate the validity of our reactivable model.
i.p.) stimulated the expression of Nfkbia, a well-known TLR4 target gene (Quan et al., 1997), in the nodose-jugular ganglion of control animals (Fig. 2C). As anticipated, LPS-treated mice (2 mg/kg, i.p.) stimulated the expression of Nfkbia, a well-known TLR4 target gene (Quan et al., 1997), in the nodose-jugular ganglion (Fig. 2C). LPS also failed to significantly increase Nfkbia expression in the ganglia of Na1,8-restricted Tlr4 mice (Fig. 2C). This result is consistent with the view that TLR ligands and cytokines are generally poor inducers of the NF-κB pathway in neurons compared with other cell types (Listwak et al., 2013). Overall, our data established the successful re-expression of Tlr4 in vagal afferents.

Early LPS-induced anorexia was investigated in control Na1,8-Cre mice with intact Tlr4 expression. As anticipated, they showed rapid anorexia, starting ~15 min following LPS injection (Fig. 3A). The total amount of food that LPS-treated mice ate over 1 h was also significantly reduced (Fig. 3B). Weight gain was significantly reduced in LPS-treated mice (Fig. 3C). In contrast, LPS did not elicit anorexia or weight loss in mice that completely lacked TLR4 expression (Fig. 3D–F). In mice expressing TLR4 only in Na1,8 cells, LPS similarly failed to trigger anorexia or weight loss (Fig. 3G, H). We concluded that TLR4-bearing vagal Na1,8 neurons do not contribute to the anorectic and cachectic actions of LPS at this particular dose and time point.

**TLR4 is selectively enriched in CGRP-positive afferents**

Although Tlr4 mRNA has previously been described in the nodose ganglion (Hosoi et al., 2005), little information is available regarding its exact cellular distribution. Chromogenic ISH allowed us to detect the signal for the Tlr4 transcript as a red precipitate (FastRed) visible under brightfield and fluorescent illuminations. The majority of the cellular profiles in the nodose and jugular ganglia was Tlr4-negative or contained very few Tlr4 signals (Fig. 4A–D). Diffuse and weak Tlr4 signals were seen in both neurons and non-neuronal cells in the nodose-jugular ganglia and the vagal trunk itself. However, a subset of cells resembling neurons contained moderate to high levels of Tlr4 signals (Fig. 4A–D). Notably, neurons with high expression levels were not uniformly distributed but were concentrated in the jugular ganglion and the rostral pole of the nodose ganglion (Fig. 4A–D). The caudal nodose ganglion itself, which contains a large population of vagal neurons including gastrointestinal afferents, was largely devoid of Tlr4-positive neurons. Based on our estimates, <12% of vagal afferents located in the rostral nodose ganglion proper contained moderate to high levels of Tlr4 signals (Fig. 4D). Considering that the boundary between the nodose and jugular-petrosal ganglia is ambiguous, many Tlr4-positive neurons observed in the nodose ganglion may have been part of the jugular ganglion. Indeed, over 50% (n = 3) of vagal afferents in the jugular ganglion were Tlr4-positive and close to 25% (n = 3) of them contained moderate to high levels of Tlr4.

**Figure 2.** A, Tlr4 mRNA expression in the nodose-jugular ganglionic mass of control, Tlr4\(^{loxTB}\), and Tlr4\(^{loxTB}\) crossed with Na1,8-Cre mice (Na1,8-restricted TLR4). Our qPCR demonstrated the re-expression of Tlr4 in vagal afferents of reactivated animals. Groups consisted of \(n = 8\)–15 mice. B, Restoration of Tlr4 expression was not observed in the liver of reactivated mice, demonstrating the specificity of the Cre-reactivated allele. Groups consisted of \(n = 3\)–7 mice. C, Expression of Nfkbia mRNA in saline-treated and LPS-treated mice (2 mg/kg, i.p.) of each respective genotype. Groups consisted of \(n = 4\)–7 mice. Data were all obtained by qPCR, and 18S was used as a control gene. Data were analyzed using two-way ANOVA (treatment and genotype factors) followed by Sidak’s post hoc test. Letters denote significant differences between columns with \(p < 0.05\). NG-JG: nodose-jugular ganglia; Nfkbia, NFκB inhibitor α. Estimation statistics are included in Extended Data Figure 2-1.
40% \( (n = 4) \) of Tlr4-expressing neurons also being Calca-positive (Fig. 6E). In summary, our anatomic data established that Tlr4 was predominantly, but not exclusively, expressed by a subset of vagal afferents producing Calca, which are known to be mainly airways and facial somatosensory and nociceptive afferents (Helke and Niederer, 1990; Mazzone and Undem, 2016).

CGRP is an immunomodulatory peptide released from peripheral afferents during bacterial infections (Lai et al., 2017). Thus, we tested whether TLR4 signaling is sufficient and/or required for the release of CGRP from cultured vagal afferents. As anticipated, the application of LPS robustly raised CGRP release (Fig. 7A,B). In Tlr4^{LoxTB} mice, no significant changes in CGRP release were noticed following LPS. In the ganglia of Na\(_v\)1.8-restricted Tlr4 mice, the application of LPS raised CGRP release to the same degree as in the ganglia of control mice (Fig. 7A,B). These data demonstrated that TLR4 signaling in vagal afferents directly mediates CGRP release on LPS exposure. Of note, the application of LPS did not alter the

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**Figure 3. A, D, G.** Graphs of the cumulative food intake in response to saline (white) or LPS (black; 100 \( \mu \)g/kg, i.p.) in control, Tlr4^{LoxTB}, and Na\(_v\)1.8-restricted TLR4 littermates. Groups consisted of \( n = 5-8 \) mice. Data were analyzed using two-way ANOVA (time and treatment) separately for each genotype, followed by Sidak’s post hoc test. The small letter b indicates points that are different from the saline-treated group. **B, E, H,** Graph representing food intake or body weight change (C, F, I) over the hour following LPS or saline treatment. Data were analyzed using Student’s unpaired t test. Small letters indicate significant differences between columns. Data are all expressed as the mean values ± SEM over a period of 60 min. Estimation statistics are included in Extended Data Figure 3-1.
transcription of Tlr4 and Calca in sensory ganglia cultured for 5 d (see Extended Data Figure 7-1).

**Discussion**

**Novel tools for the study of TLR4**

LPS is a potent inducer of proinflammatory molecules, a number of which share the same cellular targets and intracellular signaling pathways as TLR4 (Miyamoto and Verma, 1995; Muta et al., 2002; Skelly et al., 2013). Combined with the redundancy of cells expressing TLR4 throughout the body (Poltorak et al., 1998a; Lin et al., 2000; Laflamme and Rivest, 2001; Liu et al., 2002), this has made it particularly difficult to tease apart the cellular and molecular determinants of TLR4 actions. Here, we developed and validated a novel mouse model to manipulate TLR4 expression in a cell type-specific manner within the context of intact animals. In the current study, we used this model to investigate the role played by peripheral afferents in LPS-induced anorexia. In theory, this approach can be used to test TLR4 signaling in any genetically-targeted cells provided that a Cre line is available. It has also been difficult to study TLR4 actions because of the lack of validated anti-TLR4 antibodies. While radioisotopic ISH has been used to detect Tlr4-expressing cells in the CNS (Laflamme and Rivest, 2001; Chakravarty

**Figure 4.** A–D. Chromogenic ISH for Tlr4 mRNA (red) in the nodose and jugular ganglia of C57Bl6/J mice. Using fluorescent illumination, scattered positive cells resembling neurons are observed mostly in the jugular portion of the ganglionic complex (white arrows). Under bright-field illumination, representative neuronal profiles were labeled as expressing Tlr4 signals at high (H), medium (M), or low levels (L). Cells with no signals were also apparent (N). Black arrows indicate putative non-neuronal cells surrounding neuronal profiles. Tissues were counterstained with hematoxylin (purple) to facilitate the identification of cellular profiles. The scale bars in B applies to C, D. Graph showing the percentage of neuronal profiles with different ISH signal strengths for Tlr4. Data are expressed as the mean percentage ± maximal values of Tlr4-expressing neurons in the nodose and jugular ganglia. The total number of counted profiles is indicated above each bar graph. The Extended Data Figure 4-1 includes a control of specificity for the Tlr4 probe. c, caudal; H, high intensity signal; HS, hematoxylin stain; M, medium intensity signal; N, no visible signal; cNG, caudal nodose ganglion; rNG, rostral nodose ganglion; JG, jugular ganglion; L, low intensity signal; r, rostral.
and Herkenham, 2005), the present study has refined these techniques to specifically analyze Tlr4 gene expression in vagal afferents. In particular, we used a highly sensitive ISH procedure called RNAscope (Advanced Cell Diagnostic; Wang et al., 2014; Grabinski et al., 2015). The latter technique allowed us to detect Tlr4 mRNA with virtually no background. It also allowed us to easily detect Tlr4 in combination with other transcripts or GFP.

Role of vagal afferents during inflammatory anorexia

It is likely that peripheral afferents can rapidly convey information to the brain relevant to a potentially lethal threat, such as a Gram-negative bacterial infection. This phenomenon might possibly exist throughout the animal kingdom because sensory neurons in Aplysia sea slugs have been reported to be sensitive to bacterial LPS (Clatworthy and Grose, 1999). In rodents, numerous studies have suggested the ability of both vagal and spinal sensory neurons to respond to a broad range of inflammatory stimuli (Goehler et al., 1997; Ek et al., 1998; Ross et al., 2003; Eijkelkamp et al., 2010; La and Gebhart, 2011; Chiu et al., 2013). Prior studies have established that rodents with bilateral subdiaphragmatic surgical vagotomy showed diminished anorexia in response to LPS (Bret-Dibat et al., 1995; Sergeev and Akmaev, 2000). However, despite all of the evidence implicating peripheral afferents in sensing LPS, the requirement of vagal afferents in mediating LPS-induced anorexia has been difficult to demonstrate. For instance, other studies have
reported identical degrees of LPS-induced anorexia in animals with capsaicin-mediated or surgery-mediated deafferentation (Schwartz et al., 1997; Porter et al., 1998b; Wieczorek et al., 2005). To our knowledge, prior in vivo studies have rarely examined LPS-induced anorexia before the 1-h postinjection time point (Kent et al., 1992; Liu et al., 2016). However, our data reinforced the view that anorexia is initiated rapidly after intraperitoneal LPS exposure. This observation led us to hypothesize that first, LPS may directly act on vagal afferents to suppress feeding; and second, that prior studies may have missed the early anorectic response to LPS. Contrary to expectation, our

Figure 6. A. Camera lucida drawing of one vagal ganglionic complex processed for double chromogenic ISH for Tlr4 and Calca mRNAs. Each symbol represents one individual neuronal profile identified as positive for Tlr4 (red dots), Calca (open circles), or both signals (circles filled with red). Note that both Tlr4-positive and Calca-positive cells preferentially accumulated in the rostral pole of the ganglionic mass. B–D. Representative bright-field images of doubly labeled neurons for Tlr4 (red) and Calca (blue) in the ganglionic mass of C57Bl6/J mice. Overall, these data indicate that Tlr4 and Calca were frequently co-expressed in the same neurons. The numbers are meant to indicate four examples of profiles co-expressing Calca and Tlr4 mRNAs. Ganglia were counterstained with hematoxylin. E. Quantification of Tlr4 signals (dots) in cell profiles negative (left) or positive (right) for Calca. Individual cell profiles are displayed as black circles. A total of 923 cell counterstained profiles were counted, n = 4 mice. Using ImageJ, the circumference of each profile was determined and further used to categorize cell profiles into small or large/medium cells. Absolute numbers of tlr4-positive profiles is also indicated above each graph. Scale bar in C applies to D. cNG, caudal nodose ganglion; rNG, rostral nodose ganglion; JG, jugular ganglion; X, vagus nerve trunk.
findings indicate that TLR4 signaling in peripheral afferents alone is not sufficient to initiate anorexia. As explained below, we believe these findings can be explained by the fact that TLR4 signaling occurs mostly in rostral nodose and jugular vagal afferents supplying the airways, rather than those involved in feeding. Instead, it is likely that LPS-induced anorexia is initiated by the de novo production of cytokines and prostaglandins within the brain (Yao et al., 1999; Layé et al., 2000; Sachot et al., 2004; Chakravarty and Herkenham, 2005; Ching et al., 2007; Leow-Dyke et al., 2012; Calvo-Rodríguez et al., 2017). A recent single-cell sequencing study determined that the molecular make-up of jugular afferents is reminiscent of that of spinal nociceptors rather than vagal afferents of the nodose ganglion (Kupari et al., 2019). Thus, the difference of expression of Tlr4 mRNA in the nodose versus jugular ganglion is not completely surprising. Considering that the jugular and nodose ganglia are fused in rodents, one prior report of Tlr4 expression in the whole nodose ganglion may have been because of a jugular contamination (Hosoi et al., 2005).

**TLR4 signaling and vagal CGRP**

In the CNS, TLR4 is enriched in non-neuronal cells, most notably including microglial cells (Lacroix et al., 1998; Laflamme and Rivest, 2001; Lehnardt et al., 2002; Olson and Miller, 2004; Chakravarty and Herkenham, 2005; Kigerl et al., 2007; Madore et al., 2013; Larochelle et al., 2015). Several laboratories also reported TLR4 expression in neurons (Rolls et al., 2007; Yoo et al., 2011; Leow-Dyke et al., 2012; Calvo-Rodríguez et al., 2017). However, the latter studies relied on *in vitro* data and/or unvalidated antibodies against TLR4. In contrast, high resolution ISH mapping studies have established that TLR4 expression was restricted to non-neuronal cells in normal, inflamed, and injured CNS (Laflamme and Rivest, 2001; Chakravarty and Herkenham, 2005; Kigerl et al., 2007). Hence, a general agreement is that CNS neurons do not express TLR4, or they do at very low levels. In contrast, TLR4 expression was found to be relatively high in a small subset of vagal afferents. Notably, we found that LPS did not significantly increase NfκB expression in Tlr4-expressing Na1.8 neurons. This result is in agreement with the fact that LPS is a poor inducer of the NF-κB pathway in neurons compared with other cell types (Quan et al., 1997; Stern et al., 2000; Laflamme and Rivest, 2001; Chakravarty and Herkenham, 2005; Kigerl et al., 2007; Listwak et al., 2013). In addition, NfκB-independent signaling mechanisms may account for LPS actions on sensory neurons (Li et al., 2014; Meseguer et al., 2014; Allette et al., 2017; Boonen et al., 2018). This putative non-genomic effect of TLR4 on peripheral afferents differs from its canonical NF-κB-dependent actions on immune, endocrine, and glial cells. Thus, additional studies are warranted in our Na1.8-restricted TLR4 mice to elucidate LPS sensing mechanisms in vagal afferents. Considering the large amount of LPS that is constitutively contained within the gut lumen (Hersoug et al., 2016), we anticipated that TLR4 would be expressed by vagal afferents supplying the intestines. Based on our data, we cannot rule out entirely that certain vagal afferents innervating the gut expressed TLR4. In fact, a small subset of CGRP-positive vagal afferents was reported to supply the rodent stomach (Bai et al., 2019). Nonetheless, it must be stressed that Tlr4 mRNA was rarely seen in neurons of the caudal nodose ganglion, where reside vagal afferents innervating the gut. Thus, the common proposition that LPS signaling in vagal afferents sense gut microbial communities is not supported by our findings. Instead, we found abundant expression of TLR4 in CGRP-producing vagal afferents, a subgroup of vagal afferents supplying the upper airways, meninges, and facial skin (Mazzone and Undem, 2016). Moreover, our *in vitro* data indicate that TLR4 signaling is
required and sufficient to mediate LPS-induced CGRP release. Interestingly, bacterial infections in the respiratory tract are common and can lead to sepsis more often than in any other primary sites of infection. CGRP is a well-known immuno-modulatory peptide released from vagal and spinal C-fiber endings (Myers et al., 1996; Abbadie et al., 2002; Abrahamsen et al., 2008; Chiu et al., 2013). Vagal afferents play a key role in airways defenses and coughing (Prescott et al., 2020; Ruhl et al., 2020). Therefore, it is tempting to speculate that the stimulation of TL4 in vagal airway afferents plays an important immuno-modulatory role in the airways and lungs. For instance, a recent study demonstrated that the release of CGRP from respiratory vagal afferents is critical to bacterial clearance in lungs (Baral et al., 2018). Therefore, further studies in TL4 reactivated mice are warranted to verify their susceptibility to respiratory infections. Our prediction is that the stimulation of TL4-bearing vagal (and/or spinal) neurons supplying the airways during a Gram-negative bacterial infection would trigger an effener response, probably involving the release of CGRP, aimed at containing inflammation and favoring bacterial clearance. It is without saying that molecules other than TL4 may also be involved in mediating CGRP release during a bacterial infection, as suggested by several studies (Diogenes et al., 2011; Meseguer et al., 2014; Boonen et al., 2018). In particular, Meseguer and colleagues showed that the release of CGRP from isolated tracheas is a TRPA1-dependent phenomenon. However, based on data obtained with a Trpa1 global knock-out, it cannot be certain that neuronal TRPA1 was involved in the observed effects. Indeed, CGRP is also released from immune cells (Baliu-Piqué et al., 2014) and TRPA1 itself has been reported in immune and epithelial cells (Khalil et al., 2018). Moreover, the high dose of LPS used in this prior TRPA1-related study is believed to cause TL4-independent effects because of LPS interacting with the membrane itself (Redeker and Briscoe, 2019; Yin et al., 2020). Using a lower dose of LPS, other authors have shown TRPA1 to be dispensable for LPS calcium imaging response in sensory neurons (Diogenes et al., 2011; Boonen et al., 2018).

Once again, the advantage of our model is that we manipulated TL4 only in Na,1.8 neurons of the nodose ganglion. In other words, it is likely that TL4 and TRP channels are synergistically involved in the release of CGRP from neuronal endings, but TRP channels do not mediate this response alone. Lastly, it must be noted that the transcriptional profile of jugular vagal afferents resembles that of spinal and trigeminal nociceptors (Kupari et al., 2019). It is therefore likely that our findings may be extrapolated to other Trl4-expressing peripheral nerves including spinal and trigeminal CGRP-positive afferents.

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