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Western diet induces colonic nitrergic myenteric neuropathy and dysmotility in mice via saturated fatty acid- and lipopolysaccharide-induced TLR4 signalling

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Key points

- A high-fat diet (60% kcal from fat) is associated with motility disorders inducing constipation and loss of nitrergic myenteric neurons in the proximal colon. Gut microbiota dysbiosis, which occurs in response to HFD, contributes to endotoxaemia. High levels of lipopolysaccharide lead to apoptosis in cultured myenteric neurons that express Toll-like receptor 4 (TLR4).
- Consumption of a Western diet (WD) (35% kcal from fat) for 6 weeks leads to gut microbiota dysbiosis associated with altered bacterial metabolites and increased levels of plasma free fatty acids. These disorders precede the nitrergic myenteric cell loss observed in the proximal colon.
- Mice lacking TLR4 did not exhibit WD-induced myenteric cell loss and dysmotility. Lipopolysaccharide-induced *in vitro* enteric neurodegeneration requires the presence of palmitate and may be a result of enhanced NO production.
- The present study highlights the critical role of plasma saturated free fatty acids that are abundant in the WD with respect to driving enteric neuropathy and colonic dysmotility.

Abstract The consumption of a high-fat diet (HFD) is associated with myenteric neurodegeneration, which in turn is associated with delayed colonic transit and constipation. We examined the hypothesis that an inherent increase in plasma free fatty acids (FFA) in the HFD together with an HFD-induced alteration in gut microbiota contributes to the pathophysiology of these disorders. C57BL/6 mice were fed a Western diet (WD) (35% kcal from fat enriched in palmitate) or a purified regular diet (16.9% kcal from fat) for 3, 6, 9 and 12 weeks. Gut microbiota dysbiosis was investigated by fecal lipopolysaccharide (LPS) measurement and metabolomics (linear trap quadrupole-Fourier transform mass spectrometer) analysis. Plasma FFA and LPS levels were assessed, in addition to colonic and ileal nitrergic myenteric neuron quantifications and motility. Compared to regular diet-fed control mice, WD-fed mice gained significantly more weight without blood glucose alteration. Dysbiosis was exhibited after 6 weeks of feeding, as reflected by increased fecal LPS and bacterial metabolites and concomitant higher plasma FFA. The numbers of nitrergic myenteric neurons were reduced in the proximal colon after 9 and 12 weeks of WD and this was also associated with delayed colonic transit. WD-fed Toll-like receptor 4 $(TLR4)^{-/-}$ mice did not exhibit myenteric cell loss or dysmotility. Finally, LPS (0.5–2 ng·ml^{-1}) and palmitate (20 and 30 μM) acted synergistically to induce neuronal cell death *in vitro*, which was prevented by the nitric oxide synthase inhibitor NG-nitro-L-arginine methyl ester. In conclusion, WD-feeding results in increased levels of FFA and microbiota that, even in absence of hyperglycaemia or overt endotoxaemia, synergistically induce TLR4-mediated neurodegeneration and dysmotility.

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Abbreviations BW, body weight; FFA, free fatty acids; GF, germ-free; GE, gastric emptying; GI, gastrointestinal; HEK, human embryonic kidney; HFD, high-fat diet; IFN, interferon; IL, interleukin; KC, keratinocyte-derived chemokine; Lcn2, lipocalin2; L-NAME, N^G-nitro-L-arginine methyl ester; LPS, lipopolysaccharide; NADPH, nicotinamide adenine dinucleotide phosphate; nNOS, neuronal nitric oxide synthase; RD, regular diet; TLR4, Toll-like receptor 4; WD, Western diet; WT, wild-type.

Introduction

Obesity and high-fat diet (HFD) consumption are associated with enteric neuronal dysfunction and gastrointestinal (GI) motility disorders (Taba Taba Vakili et al., 2015; Mushref & Srinivasan, 2013). Myenteric neurons that play a major role in the regulation of motor functions appear to be particularly sensitive to HFD consumption (Stenkamp-Strahm et al., 2013; Reichardt et al., 2013; Rivera et al., 2014; Beraldi et al., 2014; Stenkamp-Strahm et al., 2015; Soares et al., 2015). We previously have shown that HFD-fed mice (60% kcal from fat) exhibited a reduced number of colonic myenteric neurons expressing neuronal nitric oxide synthase (nNOS), which in turn was associated with delayed colonic transit (Nezami *et al.*, 2014). We recently demonstrated the important role of HFD-induced gut microbiota dysbiosis in driving enteric neurodegeneration via Toll-like receptor 4 (TLR4) in mice exhibiting increased plasma lipopolysaccharide (LPS) concentrations (Anitha *et al.*, 2016). High levels of LPS induce proinflammatory cytokines release and apoptosis in cultured myenteric neurons (Arciszewski *et al.*, 2005; Voss & Ekbald, 2014; Coquenlorge *et al.*, 2014). This neurodegenerative process could be also mediated by saturated free fatty acids (FFA) such as palmitate (C16:0) that are abundant in HFD (Voss *et al.*, 2013). Because endotoxaemia and hyperlipidaemia have been described in HFD-fed mice (Sumiyoshi *et al.*, 2006), both LPS and palmitate may simultaneously be present at the level of the myenteric plexus. Both LPS and palmitate can activate TLR4 expressed by enteric neurons (Barajon *et al.*, 2009). However, the relative roles of LPS and palmitate in HFD-induced enteric nitrergic neuronal loss have not yet been defined.

To address this question, we used a Western diet (WD) containing less fat than routinely used in a HFD (35% kcal from fat for WD *vs.* 60% kcal from fat for HFD) that has been designed to represent the typical Western dietary practices in all aspects, including caloric value and macro- and micronutrient composition (Hintze *et al.*, 2012). With this diet, our goal was to assess enteric neuronal damage and determine how LPS and saturated FFA contribute to this disorder. Therefore, the present study aimed to: (i) evaluate the impact of WD consumption on myenteric nitrergic neurons and motility; (ii) characterize the importance of gut microbiota dysbiosis, TLR4 signalling, and plasma LPS and FFA levels in the development of WD-induced neuropathy; and (iii) evaluate the synergic effects of LPS and palmitate *in vitro* on enteric NOS neuronal cell survival.

Methods

Ethical approval

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Emory and Georgia State Universities. Animals were killed by CO_2 inhalation in accordance with IACUC guidelines.

Animals

Six-week-old male C57Bl/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and placed after acclimation on WD (34.5% calories from fat: TD.140304; Harlan Laboratories, Madison, WI, USA) or regular diet (RD) (16.9% calories from fat: TD.140305) for 3, 6, 9 and 12 weeks. Age-matched $TLR4^{-/-}$ mice on a BL/10 background, also purchased from Jackson Laboratories, were fed RD or WD for 12 weeks. Female Germ-Free (GF) wild-type (WT) C57BL/6 mice maintained under GF conditions in the Georgia State University gnobiotic facility using Park Bioservices isolators were fed for 6 weeks. The WD was designed as described by Hintze *et al.* (2012), RD is the control of WD. Diet compositions provided by the manufacturer (Table 1) suggest that the relative palmitate content in WD and RD is ∼4:1. Mice were monitored for body weight (BW) and stool indices throughout the experiments. Blood glucose was measured in 6 h fasted RD and WD mice fed for 12 weeks with a Performa Accu-Check glucose meter (Roche, Basel, Switzerland). Mice were killed after 4 h of fasting.

Plasma measurements

Blood was collected by intracardiac puncture and plasma separated by centrifugation (3000 *g* for 15 min at room temperature). Endotoxin was measured using an LAL assay (QCL-1000; Lonza, Basel, Switzerland). Plasma

Table 1. Diet compositions

Composition ($g\ kg^{-1}$)	RD TD.140305	WD TD.140304
Casein	200	190
L-Cystine	3	2.85
Corn starch	420.086	230
Maltodextrin	132	70
Sucrose	100	260.322
Olive oil	0	28
Soybean oil	70	31.4
Corn oil	0	16.5
Lard	0	28
Beef tallow	0	24.8
Anhydrous milkfat	0	36.3
Cholesterol	0	0.4
Pectin	7.5	8.5
Cellulose	19.9	22.5
Mineral mix	35	35
	AIN-93G-MX (94046)	nTWD (110422)
Sodium chloride	0	4
Vitamin mix	10	10
	AIN93-VX (94047)	nTWS (110423)
Choline bitartrate	2.5	1.4
tert-Butylhydroquinone (TBHQ), anti-oxidant	0.014	0.028

and colonic mucosa FFA ($\geq$ 16 carbons chain) were measured using a free fatty acid Quantification Kit (Abcam, Cambridge, UK). Plasma citrulline, a marker of metabolic disorder development (Sailer *et al.* 2013), was determined by an enzyme-linked immunosorbent assay (BlueGene Biotech, Shanghai, China).

Adipose tissue inflammatory markers

Interferon (IFN)γ, interleukin (IL)-6, keratinocyte-derived chemokine (KC) (or CXCL1) and MCP1 expressions were analysed by quantitative RT-PCR as described previously (Etienne-Mesmin *et al.*, 2016).Total RNA was isolated from mesenteric (fat pads surrounding the proximal colon) and epididymal adipose tissue using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and purified using a RNeasy mini kit (Qiagen, Hilden, Germany). Quantitative RT-PCR was performed in a CFX96 apparatus Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using the iScriptTM One-Step RT-PCR Kit with SYBR Green (Bio-Rad) and gene-specific oligonucleotides primers: IFNγ (forward) 5′-AGCAAGGCGAAAAAGGATGC-3′ and (reverse) 5′-TC ATTGAATGCTTGGCGCTG-3′; IL-6 (forward) 5′-GT GGCTAAGGACCAAGACCA-3′ and (reverse) 5′-GGTT TGCCGAGTAGACCTCA-3′; KC (forward) 5′-TTGTG CGAAAAGAAGTGCAG-3′ and (reverse) 5′-TACAAA CACAGCCTCCCACA-3′; MCP1 (forward) 5′-GCTG GAGCATCCACGTGTT-3′ and (reverse) 5′-TGGGAT CATCTTGCTGGTGAA-3′. Results were normalized to 36B4 (housekeeping gene).

Fecal LPS and flagellin

Fecal LPS and flagellin (TLR5 agonist) levels were quantified using human embryonic kidney (HEK)-Blue-mTLR4 and (HEK)-Blue-mTLR5 cells, respectively (InvivoGen, San Diego, CA, USA), as previously described (Chassaing *et al.*, 2014*a*).

Fecal metabolomics

Feces sampled at 6 weeks were extracted in an acetonitrile/water solution (2:1) containing a mixture of internal standards and analysed as described previously (Uppal *et al.*, 2015). Mass spectrometry was performed using a LTQ-Velos-Orbitrap mass spectrometer (Thermo Fisher, San Diego, CA, USA). Metabolites were characterized according their mass/charge ratio with associated retention time using bioinformatics databases (METLIN: https://metlin.scripps.edu; HMDB: http://www.hmdb.ca). Peaks intensities analyses and heatmap were made using MetaboAnalyst, version 3.0 (www.metaboanalyst.ca).

Fecal lipocalin2

Fecal levels of lipocalin2 (Lcn2) were investigated to evaluate intestinal inflammation using Duoset murine Lcn-2 ELISA kit (R&D Systems, Minneapolis, MN, USA), as described previously (Chassaing *et al.*, 2012).

Enteric neuron staining

Proximal colon and distal ileum (1 cm above the cecum) were freshly dissected to isolate myenteric ganglia and longitudinal muscle layers. Proximal colon mucosa was used to measure FFA content. Myenteric neurons were stained for the neuronal marker peripherin (Millipore, Billerica, MA, USA) or nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase (1 h of incubation with β-NADPH diaphorase, 1 mg ml^{-1}; nitroblue tetrazolium, 0.1 mg ml^{-1}; and 0.3% Triton-X 100 in PBS at 37 °C; all from Sigma-Aldrich, St Louis, MO, USA) that colocalizes with nNOS (Belai *et al.*, 1992). Neuronal quantifications were performed blinded on 10 images per mouse randomly chosen with the same magnification (20×) and expressed by number of neurons/field, as described recently (Carbone *et al.*, 2016). Cryosections from 6-week fed mice colons were stained for cleaved-caspase3 (Cell Signaling, Beverly, MA, USA) and PGP, version 9.5 (Abcam) and five sections per mouse were used to score

the cleaved-caspase3 area within the myenteric tissue using Photoshop (Adobe Systems, San Jose, CA, USA).

GI transit

GI and colonic transit were measured in all mice 3 days and 4 h, respectively, before death, using dye-propagation and bead expulsion techniques, as reported previously (Anitha *et al.*, 2016). Gastric emptying (GE) and small intestinal transit were investigated in 9-week fed mice, as described previously (Anitha *et al.* 2006).

Cell culture

IM-FEN cells were cultured with modified N2 medium (33 °C, 5% CO_2) as described previously (Anitha *et al.*, 2008). Differentiation was induced by changing medium to Neurobasal-A Medium (Gibco, Thermo Fisher, CA, USA) supplemented with 1% FBS, 2 mM L-glutamine, 100 mg ml^{-1} streptomycin (all from Sigma-Aldrich), B27 (0.1x; Gibco) and glial cell-derived neurotrophic factor (GDNF) (10 ng ml^{-1}; Shenandoah Biotechnology Inc., Warwick, PA, USA) for 5 days at 39 °C before treatment. Palmitate (100 mM stock solution in isopropanol), LPS (in ng ml^{-1}, 1 ng $\approx$ 10 EU) and N^G-nitro-L-arginine methyl ester (L-NAME; NOS inhibitor) were provided by Sigma-Aldrich and added to the medium for an additional 24 h. Cell survival was then investigated using the MTS [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay (Promega, Madison, WI, USA) as reported previously (Nezami *et al.*, 2014). Data from five or more experiments were used in the statistical analysis.

Western blotting

Western blot analysis was performed as described previously (Anitha *et al.*, 2016). Lysates obtained from remaining cells after 24 h of treatment were used to probe for nNOS and PGP9.5 sing nNOS (BD Biosciences, San Jose, CA, USA) and PGP9.5 specific antibodies (Abcam). B-actin (Cell Signaling) was used as a loading control. Data from three or more experiments were used for the statistical analysis.

Statistical analysis

Statistical analyses were performed using Student's *t* or Mann–Whitney tests with Prism software (GraphPad Inc, La Jolla, CA, USA) as Pearson's correlations. Data numbers are reported as appropriate. For clarity, only RD and WD animals fed for similar durations were compared together. Results from parametric data are presented as the mean $\pm$ SEM. $P < 0.05$ was considered statistically significant ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

Results

WD increased body-weight and plasma FFA but had no effect on LPS and glucose levels

Our first step was to characterize whether WD consumption results in obesity and diabetes analogous to that previously observed in response to HFD (60% kcal from fat). Therefore, we monitored BW gain and blood glucose in RD and WD mice fed for 3, 6, 9 and 12 weeks. Compared to RD, WD mice gained 21% and 42%, respectively, after 6 and 12 weeks (Fig. 1*A*). Increased mesenteric adiposity contributed to the BW increase at 12 weeks (Fig. 1*B*), although this was not associated with a significant elevation in blood glucose or plasma citrulline (Fig. 1*C* and *D*). Next, we measured levels of LPS and FFA, both of which can potentially activate TLR4. Plasma LPS was not altered in all groups of WD-fed mice (Fig. 1*E*); on the other hand, plasma FFA were found to be increased only in 6-week WD-fed mice (+18%) (Fig. 1*F*). In addition, colonic mucosal/submucosal FFA had a tendency to be increased after 6 weeks (Fig. 1*G*). To investigate whether adipose tissue inflammation is responsible for this FFA increase, we measured proinflammatory gene expression in both mesenteric and epididymal tissues from 6-week fed RD and WD mice. No significant changes were observed in IFNγ, IL-6, KC and MCP1 expression (Fig. 1*H*), suggesting together that 6 weeks of WD feeding induced neither metabolic endotoxaemia, nor adipose tissue inflammation, as observed previously in HFD-fed mice (60% kcal from fat) (Kim *et al.*, 2012; Anitha *et al.*, 2016).

WD induced gut microbiota dysbiosis without intestinal inflammation

Because diet plays a key role in microbial ecology, we assessed the concentration of two bacterial products, LPS and flagellin, which are increasingly being used to reflect microbiota dysbiosis. Fecal LPS, but not flagellin, was significantly increased (+160%) in feces after 6 weeks of WD consumption (Fig. 2*A* and *B*). We next subjected fecal samples of 6-week fed RD and WD mice to linear trap quadrupole-Fourier transform mass spectrometer metabolomics analysis from which 3370 metabolites were quantified. Among the metabolites increased in WD feces compared to RD that we identified, there were compounds related to bacterial wall metabolism (muramic acid and diaminopimelic acid); metabolites common to microbiota and host such as *N*-acetyl glucosamine, tetraethylammonium, glucuronic acid, citrulline, 5-hydroxytryptamine and hypoxanthine; and those resulting from diet consumption (palmitate, triglycerides) (Fig. 2*C*). Taurocholate levels were decreased in WD-fed mice. Finally, no alteration in fecal Lcn2 was observed, suggesting that WD-fed mice did not exhibit intestinal inflammation (Fig. 2*D*).

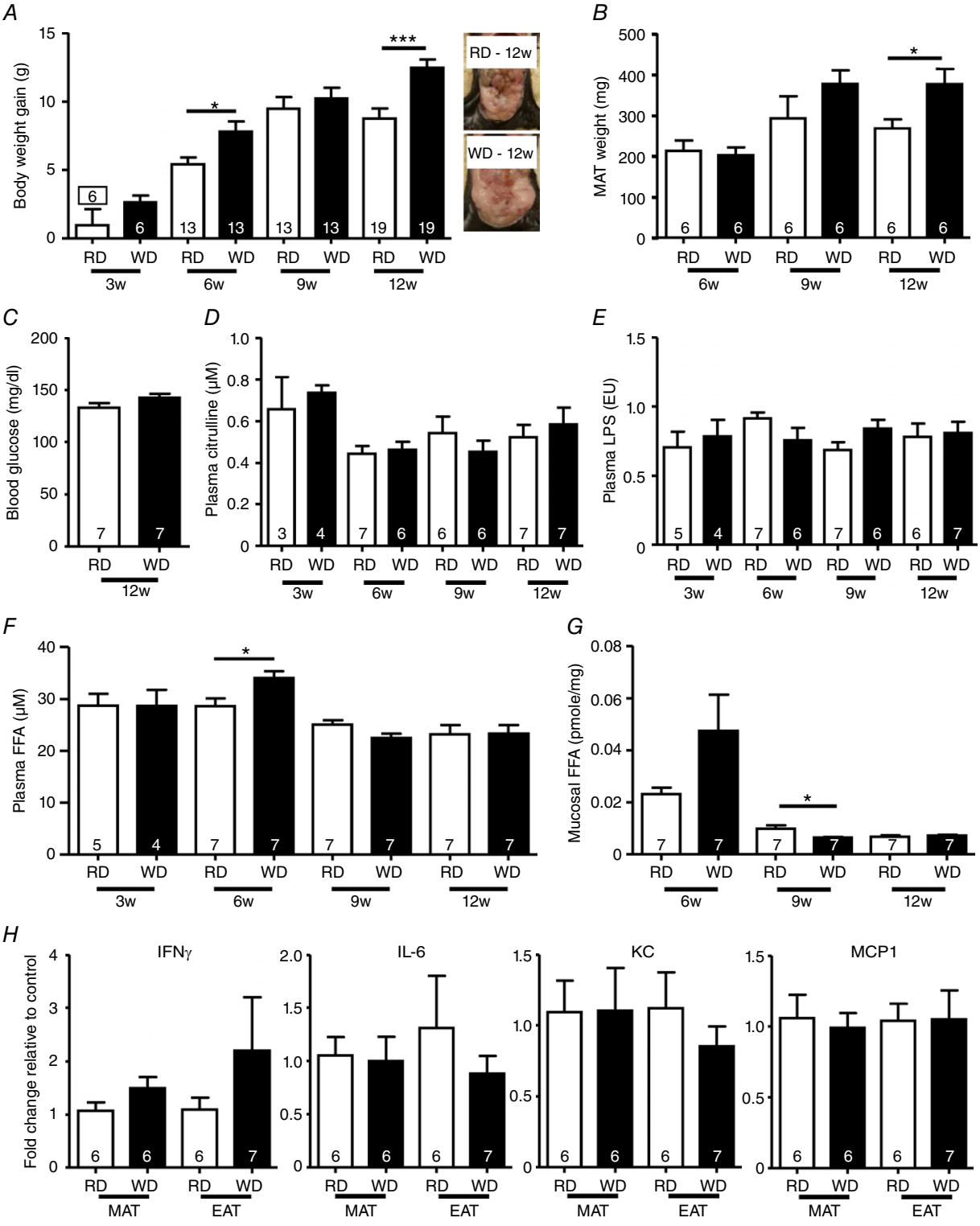

Figure 1. WD increased body-weight gain and induced transitory increase of FFA after 6 weeks of feeding

A, body weight gain of RD or WD mice fed for 3, 6, 9 and 12 weeks and representative pictures of visceral fat from 12-week fed-mice. *B*, mesenteric fat pads from 6, 9 and 12 weeks. *C*, blood glucose from 12-week fed mice. Plasma levels of citrulline (*D*), LPS (*E*) and FFA (*F*) in RD and WD mice fed for 3, 6, 9 and 12 weeks. *G*, FFA levels from proximal colon mucosa/submucosa from RD or WD mice fed for 6, 9 and 12 weeks. *H*, mesenteric (MAT) and epididymal adipose tissue (EAT) mRNAs expression of IFNγ, IL-6, KC and MCP1 from WT mice fed for 6 weeks with a RD or a WD. [Colour figure can be viewed at wileyonlinelibrary.com]

WD increases plasma FFA in a microbiota-dependent manner

We found a positive correlation between fecal LPS and plasma FFA levels in 6-week fed WD-mice (Fig. 3*A*) and used germ-free mice to investigate this phenomenon. In these animals, we observed a slight increase of BW gain in the 6-week WD-fed group (Fig. 3*B*), although no change in FFA was observed (Fig. 3*C*).

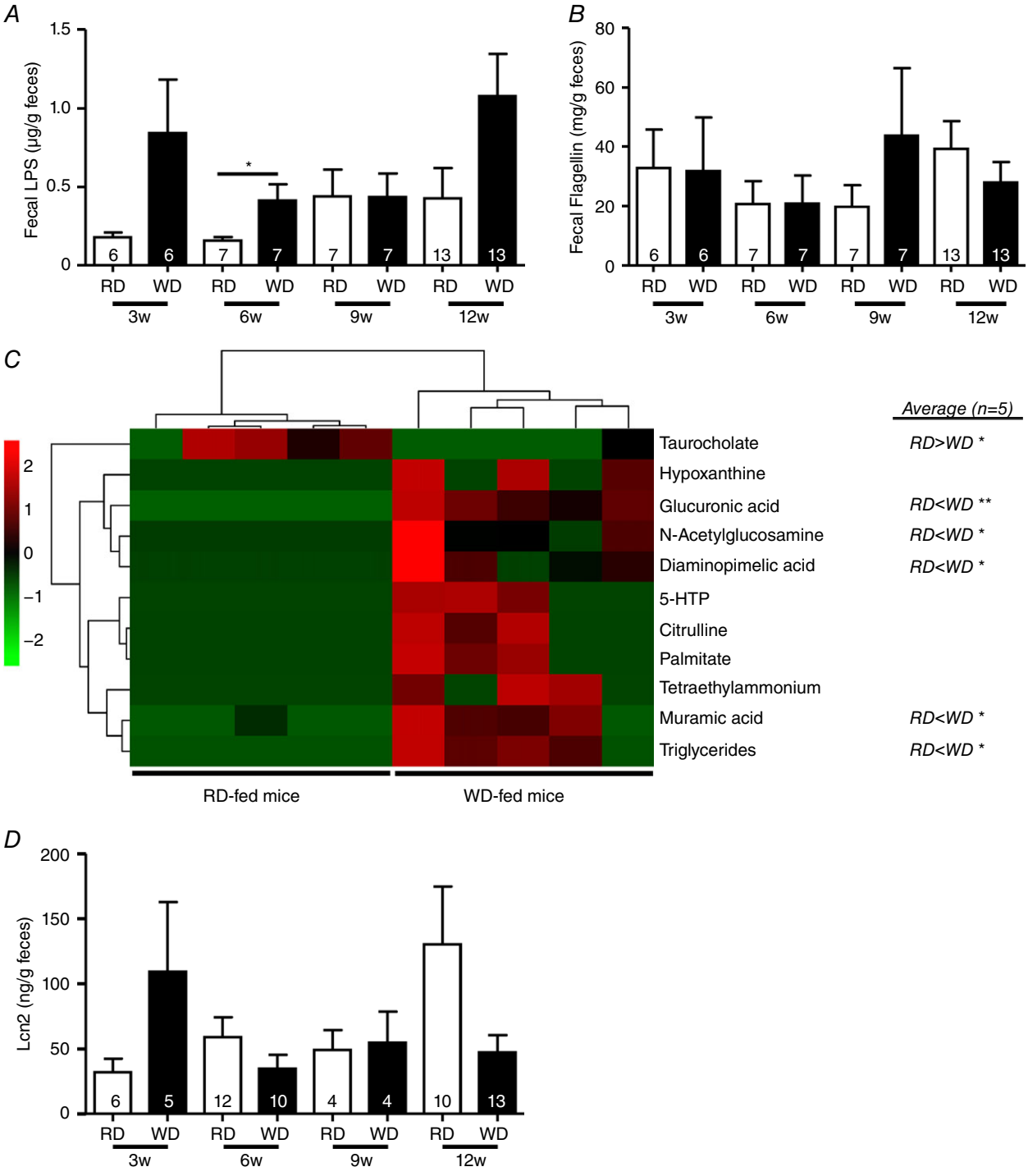

Figure 2. WD for 6 weeks induced gut microbiota dysbiosis
Fecal levels of LPS (*A*) and flagellin (*B*) in RD and WD mice fed for 3, 6, 9 and 12 weeks. *C*, heatmap showing fecal metabolites from RD and WD mice fed for 6 weeks. Statistics after data normalization and averaging ($n = 5$ per group) are shown on the right. *D*, fecal Lcn2 in RD and WD mice fed for 3, 6, 9 and 12 weeks.

WD-fed mice display myenteric neuronal cell loss in the proximal colon but not in distal ileum

Next, we investigated the effects of WD consumption on colonic and ileal myenteric neurons after 6, 9 and 12 weeks. The total number of myenteric neurons in the proximal colon, as shown by peripherin staining, was reduced by 30% and 31%, respectively, after 9 and 12 weeks of WD (Fig. 4*A*). This loss was also observed for NADPH-diaphorase stained neurons that were reduced by 41% and 51%, respectively, after 9 and 12 weeks of WD (Fig. 4*B*), although it was not preceded by increased myenteric cleaved-caspase3 staining at 6 weeks (Fig. 4*C*). Moreover, we observed that the decreased number of nitrergic neurons correlated with a greater BW gain in 12-week fed mice (Fig. 4*D*). By contrast, neither total, nor nitrergic neurons in the ileum were affected by WD (Fig. 4*E* and *F*).

WD-induced colonic neuronal cell loss is associated with dysmotility

We next investigated how intestinal motor functions were impacted by the WD-induced nitrergic neurodegeneration. We observed delayed colonic transit in 12-week WD-fed mice (+ 144%) but not at other time points (Fig. 5*A*). This was not associated with a delay of the whole GI transit at 12 weeks (Fig. 5*B*). At 6 weeks, there was a delay in whole gut transit (+33%) followed by a faster transit at 9 weeks (–31%). In 12-week fed mice, a reduced of number of nitrergic neurons was associated with a longer colonic transit time (Fig. 5*C*). In addition, a delayed colonic transit correlated with an increased BW gain (Fig. 5*D*). Next, we assessed the GE in 9-week fed mice to investigate the faster GI transit; although GE was similar between RD-fed and WD-fed mice (Fig. 5*E*), the dye propagation from the stomach tended to be faster, suggesting upper small intestinal acceleration (Fig. 5*F*). Finally, we observed that WD for 6 weeks did not alter GI transit in GF mice (Fig. 5*G*), indicating that gut microbiota may play a role in the delay observed in WT WD-fed mice.

TLR4$^{-/-}$ mice did not exhibit WD-induced neuronal cell loss and dysmotility

Because we hypothesized that TLR4 is essential in HFD-induced neuronal loss, we assessed the effect of WD feeding on NOS neurons and GI motility in mice lacking this receptor. Compared to WT, 12-week WD-fed TLR4$^{-/-}$ mice did not exhibit any increase in BW gain (Fig. 6*A*). Fecal levels of Lcn2 were not affected by the lack of TLR4 (Fig. 6*B*). However, increased levels of fecal LPS and flagellin were observed in WD-fed TLR4$^{-/-}$ mice compared to RD-fed TLR4$^{-/-}$ and WD-fed WT mice (Fig. 6*C* and *D*). Importantly, no change was seen in NADPH-diaphorase stained myenteric neurons in the proximal colon between RD-fed or WD-fed TLR4$^{-/-}$ mice (Fig. 6*B*), although both exhibited less neurons than control WT mice. The colonic transit was similar between the two groups of TLR4$^{-/-}$ mice (Fig. 6*F*). By contrast, WD-fed TLR4$^{-/-}$ mice exhibited more NADPH-diaphorase stained cells in the distal ileum than their controls (Fig. 6*G*), without an alteration in GI transit (Fig. 6*H*).

Synergistic effects of LPS and palmitate in inducing enteric neuronal loss are TLR4-dependent

Having noted *in vivo* changes in myenteric neurons, we cultured enteric neurons *in vitro* in the presence of palmitate and LPS aiming to clarify the mechanisms leading to neurodegeneration. Incubation for 24 h with palmitate ≥ 30 μM induced enteric neuronal loss (Fig. 7*A*). By contrast, LPS (0.5–2 ng ml^{-1}) alone had no effect on neuronal survival (Fig. 7*B*). When incubated with

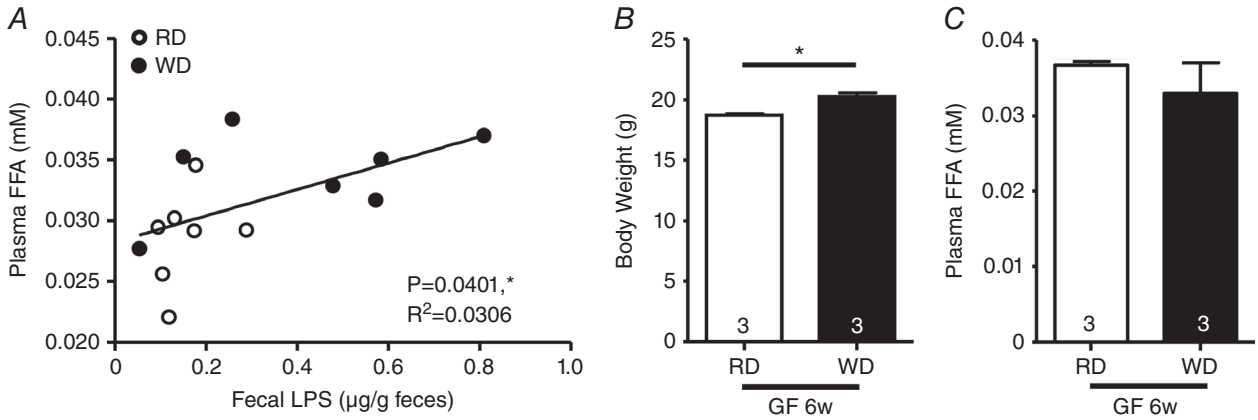

Figure 3. WD-induced plasma FFA increase is microbiota-dependent
A, correlation between fecal LPS levels and plasma FFA concentrations in 6-week fed WT mice (RD, $n = 7$; WD, $n = 7$). *B*, body weight gain and plasma FFA (*C*) in GF mice fed for 6 weeks with a RD or a WD.

palmitate at 20 and 30 μM, LPS 0.5 ng ml^{-1} exacerbated the neuronal loss induced by palmitate alone compared to the vehicle (Fig. 7*C*). This cell loss was substantially mitigated in the presence of the NOS-inhibitor L-NAME (200 μM). Next, we measured nNOS and PGP9.5 expression by Western blotting to estimate nNOS reduction. nNOS expression was not altered by LPS alone 0.5 ng ml^{-1} but significantly reduced after incubation with palmitate 0.02 or 0.03 μM ± LPS (Fig. 7*D*) and this decrease was countered by the addition of L-NAME in the milieu. PGP9.5 expression was slightly reduced after incubation with LPS 0.5 ng ml^{-1} alone and this reduction was even more pronounced in presence of palmitate 20 μM (Fig. 7*E*), Together, these results suggest that LPS and palmitate act synergistically to damage enteric neurons via nNOS activity.

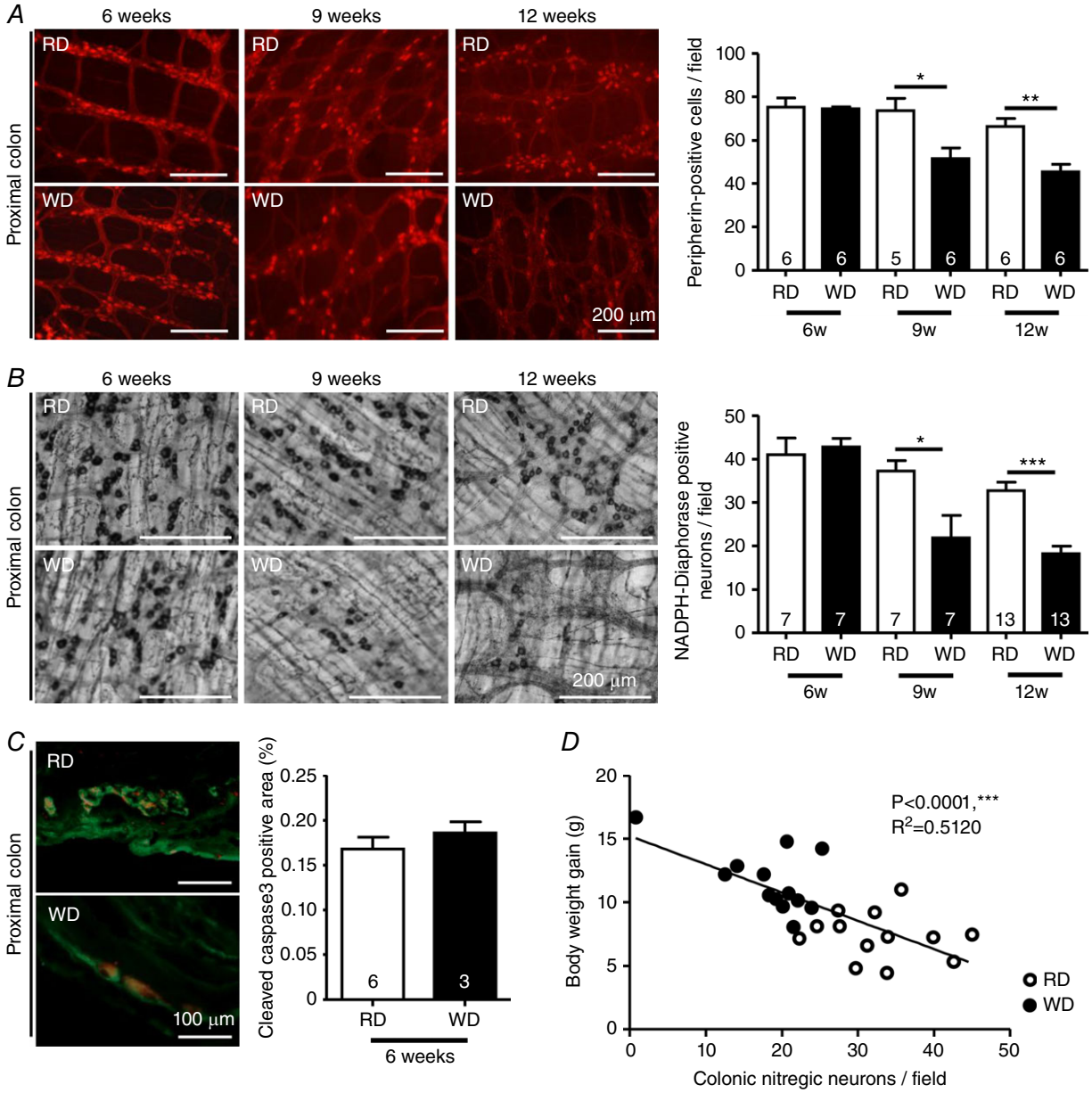

Figure 4. WD reduced the numbers of nitrergic myenteric neurons in the proximal colon
Proximal colon myenteric neurons stained for peripherin (*A*) and NADPH-diaphorase (*B*) in RD and WD mice fed for 6, 9 and 12 weeks. *C*, cleaved-caspase3/PGP9.5 costaining in colonic cryosections from 6-week fed RD or WD mice. *D*, correlation between colonic nitrergic neurons and body weight gain in mice fed for 12 weeks ($n = 13$ for each group). Distal ileum myenteric neurons stained for peripherin (*E*) and NADPH-diaphorase (*F*) in RD and WD mice fed for 6, 9 and 12 weeks.

Discussion

In the present study, we report that, in mice, the consumption of a WD induces nitrergic myenteric neurodegeneration in the proximal colon via TLR4 signalling. This alteration is consequent to gut microbiota dysbiosis and plasma FFA increases after 6 weeks of WD feeding and leads to delayed colonic transit. Finally, cell culture experiments highlight that palmitate potentiates the LPS-neurodegenerative action in enteric neurons via NO production.

One major finding is that, within 9 weeks, WD leads to colonic nitrergic myenteric neuronal loss in the absence of overt hyperglycemia, intestinal inflammation or endotoxaemia, which are three possible causes of the initiation of neuronal loss (Lakhan & Kirchgessner, 2010; Chandrasekharan *et al.*, 2011; Anitha *et al.*, 2016). RD and WD mice fed for 12 weeks exhibited not only similar blood glucose levels, but also no alteration of plasma citrulline, a marker of metabolic syndrome development in HFD-fed mice (Sailer *et al.*, 2013). Enteric cell loss in the absence of glucose intolerance has been recently reported in mice fed a high-fat high-cholesterol diet (21% fat, 2% cholesterol); however, this was after 33 weeks of feeding (Rivera *et al.*, 2014). Intestinal inflammation was investigated through fecal Lcn2 measurement and was similar between RD and WD mice. Finally, we observed no variation of plasma LPS in all the groups of WD mice, although endotoxaemia has been suggested after 3 weeks of HFD containing more fat (45–60% kcal) and reported to be increased in mice exhibiting NOS neurodegeneration after 12 weeks (Hamilton *et al.*, 2015; Anitha *et al.*, 2016).

Fecal LPS, in contrast, was increased in WD-fed mice, although only after 6 weeks, suggesting gut microbial alteration (Chassaing *et al.*, 2014*b*).Moreover, this alteration was associated with a FFA increase in the plasma of those mice. We measured adipose proinflammatory genes to determine adipose tissue expansion or inflammation (Bjorndal *et al.*, 2011; Cullberg *et al.*, 2014). However, mesenteric fat pads were not heavier after 6 weeks of WD and no alteration of genes of interest was observed in mesenteric and epididymal fat pads. In addition, a positive trend in FFA content in proximal colonic mucosa/submucosa was observed after 6 weeks. We found a positive correlation between fecal LPS and plasma FFA and tested the hypothesis that the plasma FFA increase may be related to the gut microbiota dysbiosis. GF mice fed a RD or a WD for 6 weeks did not exhibit hyperlipidaemia despite a slight body weight increase. Future studies will examine the underlying mechanisms that may involve modulation of lipoprotein lipase activity because it is known that microbiota controls the intestinal expression

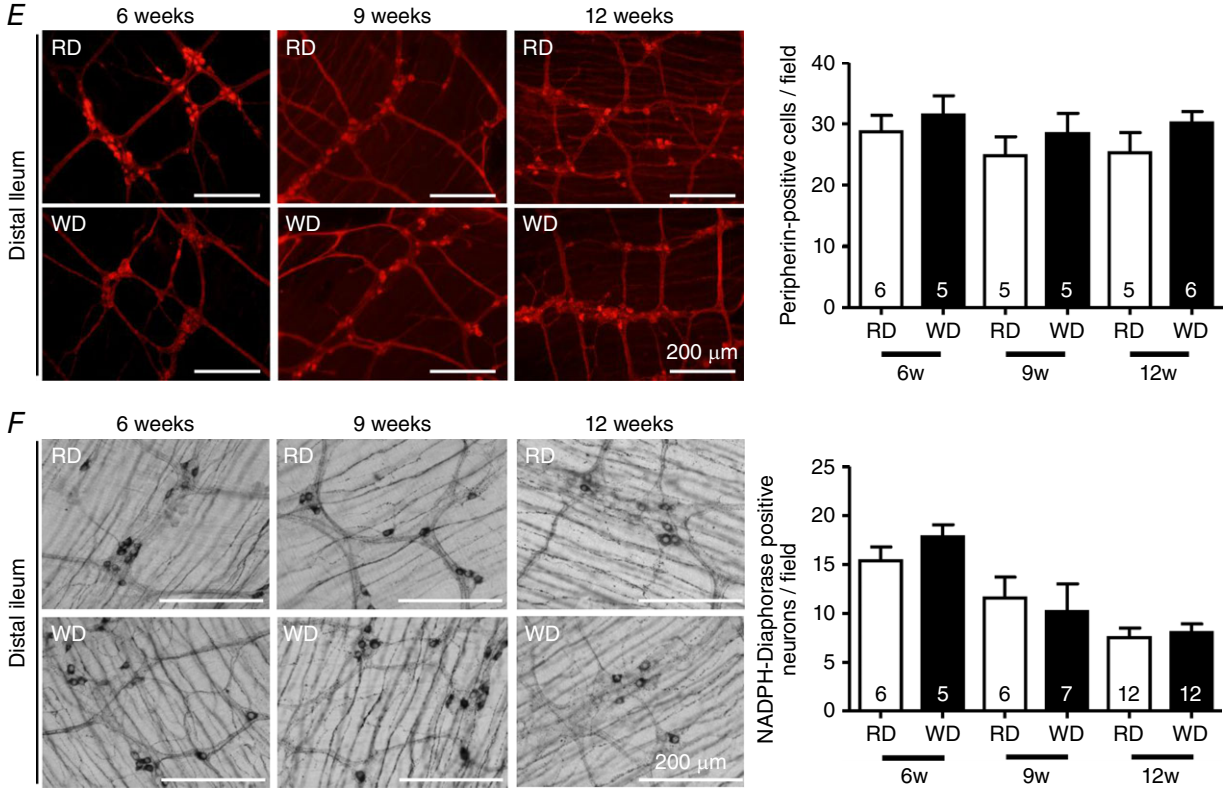

Figure 4. Continued

of a lipoprotein lipase circulating inhibitor (Bäckhed *et al.*, 2004). We will investigate further to what extent the microbial dysbiosis might be related to dyslipidemia in WD-fed mice.

We next analysed fecal metabolites to investigate the gut microbiota alteration suggested by the increased fecal LPS levels in WD-mice fed for 6 weeks. Among those with a bacterial origin, we found increased levels of muramic acid reflecting increased bacterial fermentation and excretion (Sepehr *et al.*, 2003), diaminopimelic acid produced by Gram negative and some Gram-positive bacteria during peptidoglycan synthesis (Gillner *et al.*, 2009) and triamethylammonium associated with gut bacterial ecology disruption (Hong *et al.*, 2010). Moreover, a decreased concentration of the primary bile acid taurocholate in WD-mice feces, compared to control, highlights changes in gut bacterial metabolism. We also found metabolites in WD feces related to the host metabolism: *N*-acetylglucosamine, hypoxanthine produced by intestinal cells during endotoxemia (Schmidt

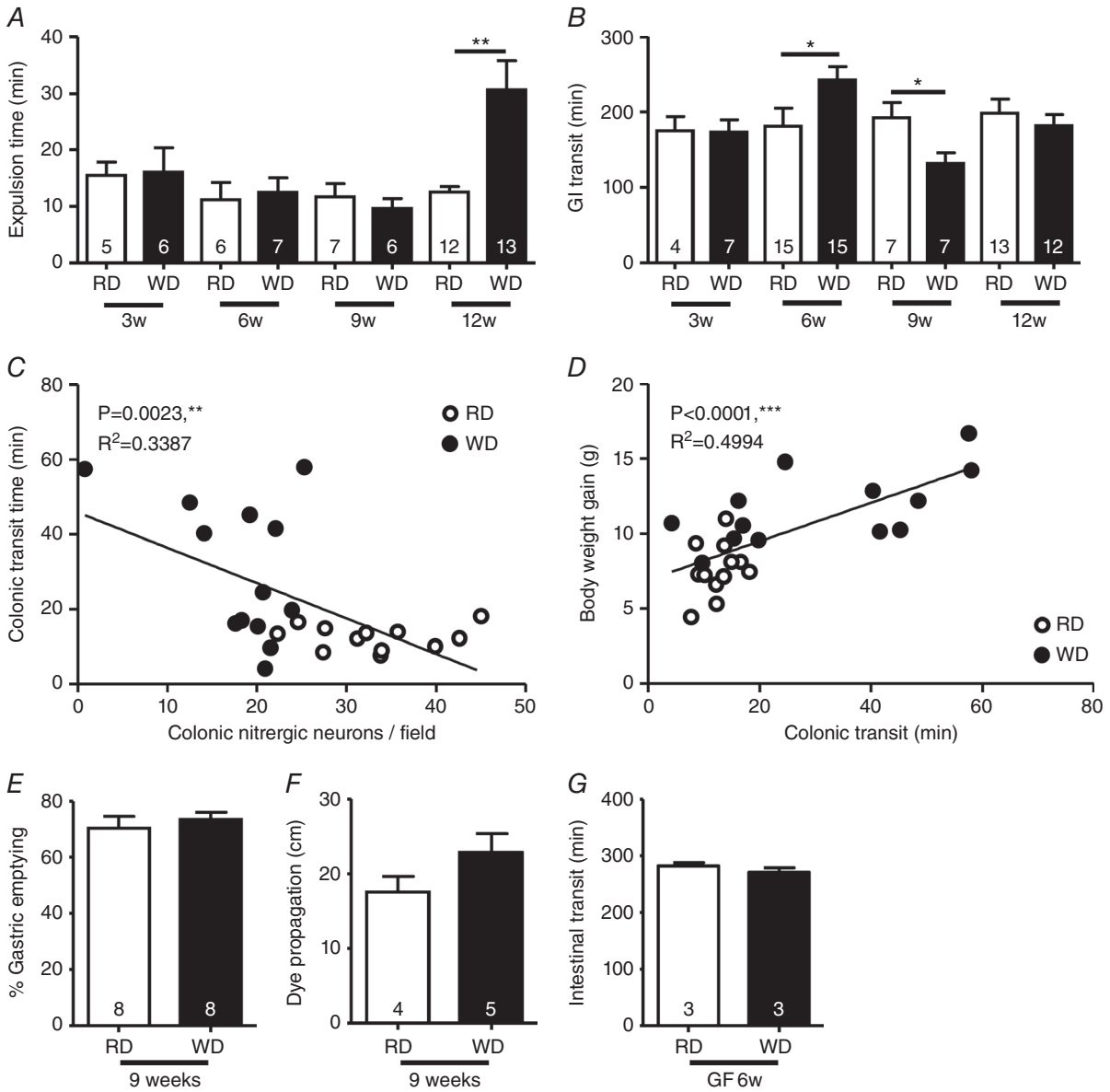

Figure 5. WD altered GI motility
Assessment of GI in mice fed a RD or WD for 3, 6, 9 and 12 weeks, bead expulsion time (*A*) and dye transit (*B*) after oral gavage with Evans blue dye/methyl cellulose solution. Correlations between colonic nitrergic neurons and colonic transit (bead expulsion time) (RD, $n = 12$; WD, $n = 13$) (*C*) and between colonic transit and body weight gain (RD, $n = 13$, WD, $n = 12$) (*D*). GE (*E*) and dye propagation (*F*) in upper small intestine in WD-fed mice for 9 weeks. *G*, GI transit in GF mice fed with a RD or a WD for 6 weeks.

et al., 1997), 5-HTP suggesting higher serotoninergic metabolism as already reported in diet-induce obese mice (Reichardt *et al.*, 2013), citrulline and, finally, glucuronic acid, reflecting an enhanced elimination of excessive metabolites from plasma to feces via the bile (Karanam *et al.*, 2007). Finally, we characterized metabolites related to WD consumption such as palmitate and triglycerides, and similar increases in feces have been already reported not only in HFD-fed WT mice, but also GF mice (Rabot *et al.*, 2010). Certain fecal metabolites, in particular bacterial compounds such as diaminopimelic acid with *N*-acetylglucosamine or muramic acid with triglycerides, were simultaneously increased in a majority of WD-fed mice compared to RD-fed mice.

Focusing next on myenteric neurons, we observed that their total numbers were reduced in WD-fed mice by 30% and 31% in the proximal colon after 9 and 12 weeks, respectively. These losses included NOS neurons that were concomitantly reduced by 41% and 51%. Representing 40% of the total colonic myenteric cells (Murphy *et al.*, 2007), nitrergic neurons are more important in the proximal colon where they are essential to induce greater relaxation for fecal storage and excess fluid absorption (Takahashi & Owyang, 1998). We previously reported that they represent ∼50% in the murine proximal colon (Nezami *et al.*, 2014; Anitha *et al.*, 2016). By contrast, no changes were observed in the distal ileum of WD-fed mice. Neuronal counts have all been reported as counts of nerve cells per microscope field. This method could be affected by changes in intestinal length (e.g. during inflammation); however, we did not observe any evidence of macroscopic inflammation in the intestine.

Colonic nitrergic neurodegeneration is associated with changes in motor function, as illustrated by the correlation

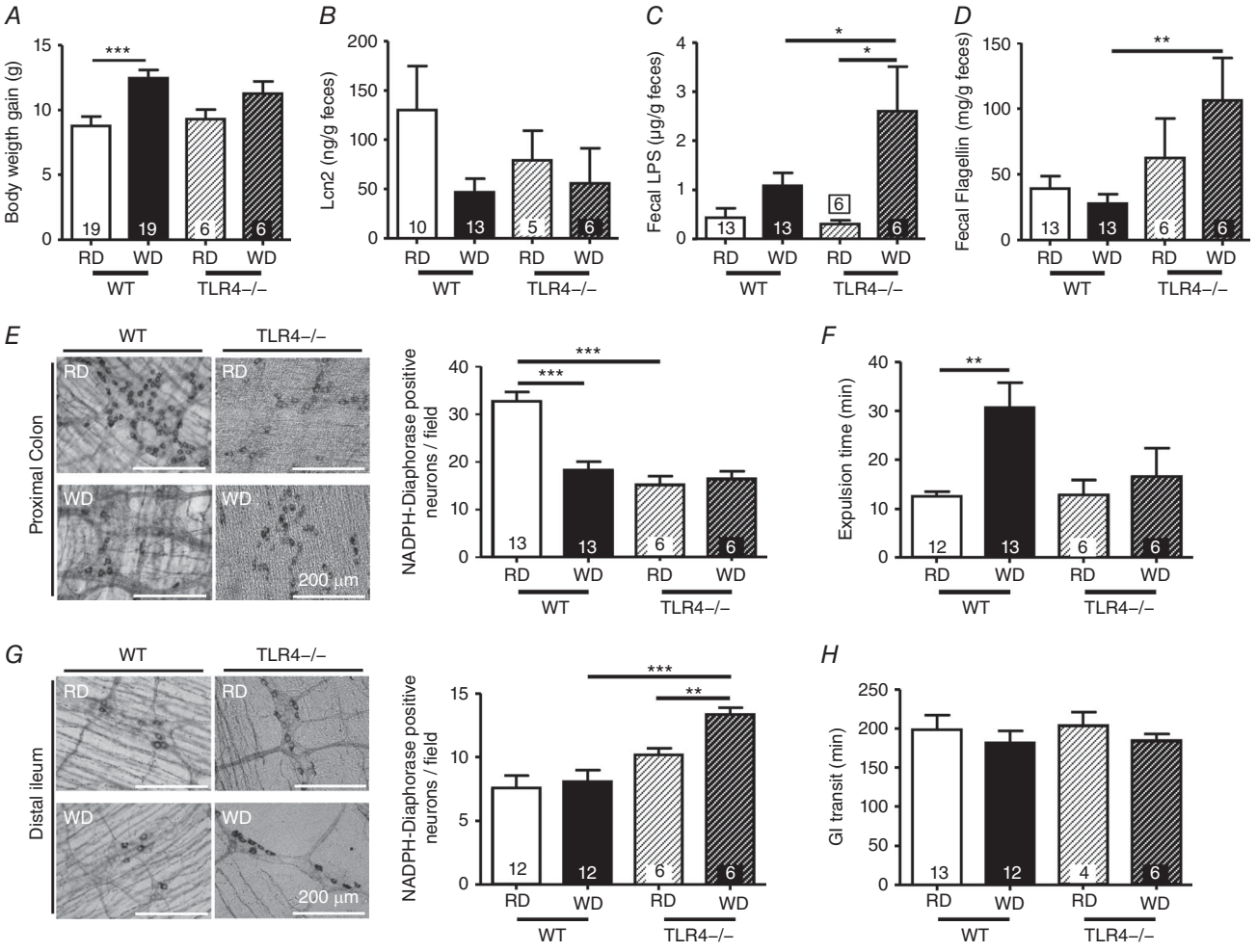

Figure 6. TLR4$^{-/-}$ mice were protected from WD induced neuronal loss and dysmotility
Comparisons of wild type and TLR4$^{-/-}$ fed a WD or RD for 12 weeks. *A*, body weight gain. Fecal levels of Lcn2 (*B*), LPS (*C*) and flagellin (*D*). *E*, proximal colon NADPH-diaphorase staining of WT and TLR4$^{-/-}$ mice myenteric neurons. *F*, colonic transit measured by expulsion time (bead test). *G*, distal ileum NADPH-diaphorase staining of wild-type and TLR4$^{-/-}$ mice myenteric neurons. *H*, GI transit measurement in WT and TLR4$^{-/-}$ fed a RD or WD.

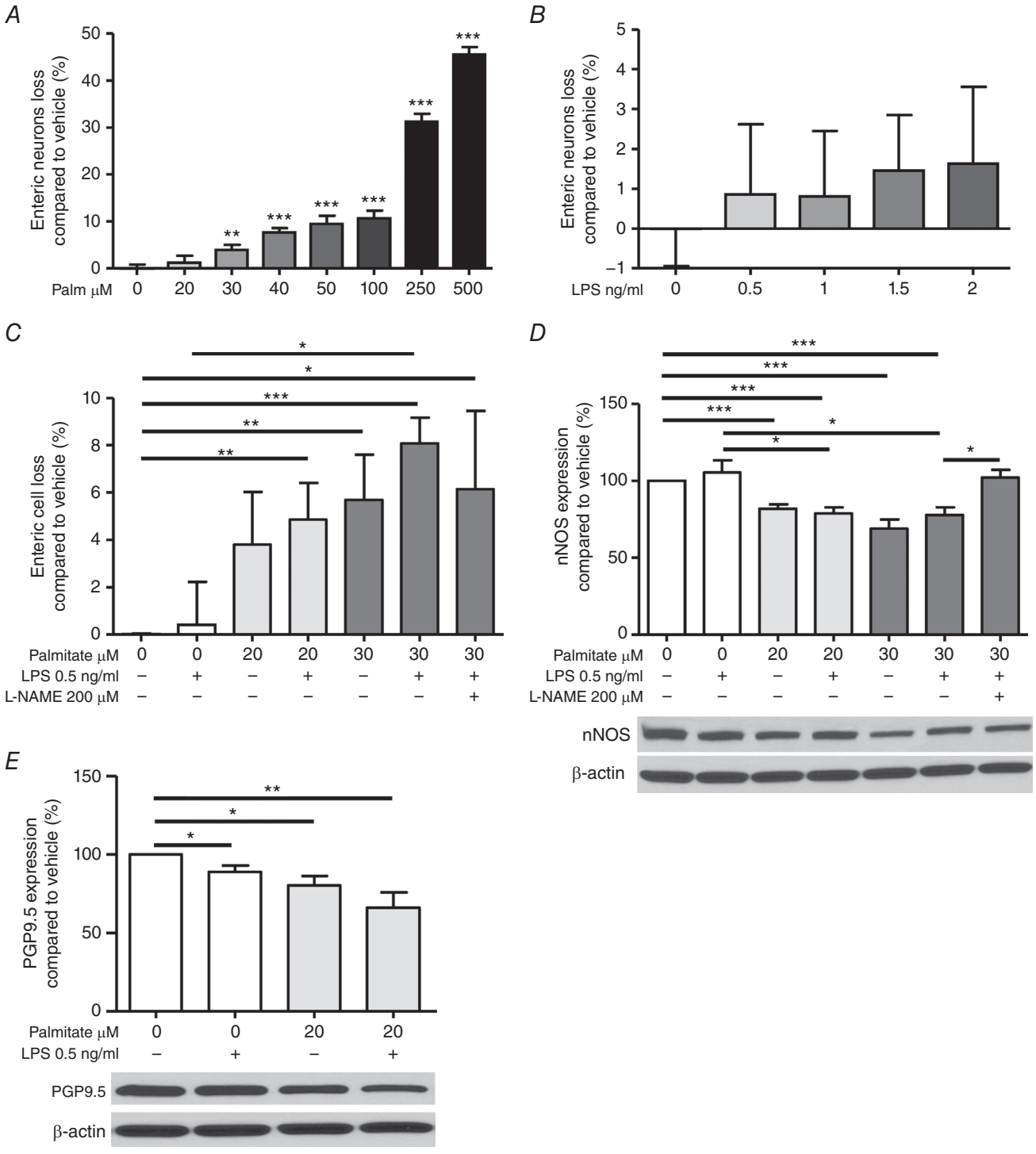

Figure 7. Synergic action of palmitate and LPS on the survival of primary enteric neuronal cells from WT mice

A, incubation for 24 h of an enteric neuronal cell line with palmitate (from 20 to 500 μM) induced cell loss. Results are expressed as the percentage increase in cell viability over vehicle-treated cells. *B*, incubations with LPS (from 0 to 2 ng ml^{-1}). *C*, co-incubations of low LPS (0.5 ng ml^{-1}) with palmitate 20 or 30 μM for 24 h in presence or absence of the NOS inhibitor L-NAME (200 μM). Western blot analyses and representative gels showing nNOS (*D*) and PGP9.5 (*E*) expression after incubation for 24 h with palmitate 20 or 30 μM, +/– LPS 0.5 ng ml^{-1} and + L-NAME (200 μM).

between colonic myenteric NOS neuronal loss after 12 weeks of WD and delayed colonic transit. Moreover, a delayed colonic transit correlates with an increased body weight gain, suggesting that colonic dysmotility could be associated with obesity development. We recently highlighted a relationship between constipation and a high saturated fat diet in humans (Taba Taba Vakili *et al.*, 2015).

By contrast to the colonic transit, the whole GI transit was delayed after 6 weeks and accelerated after 9 weeks, but not altered after 12 weeks. The transit delay after 6 weeks was not observed in WD-fed GF mice, highlighting the interactions among microbiota and GI transit (Kashyap *et al.*, 2013). This suggests that microbiota are critical in the delayed GI transit noted at 6 weeks. This is not neurodegenerative because cleaved-caspase3 staining in colonic myenteric neurons was not increased. Enhanced NOS activity, however, may lead to NO overproduction within cells and oxidative stress, explaining the neuronal loss occurring in WD-fed mice between 6 and 9 weeks of feeding (Rivera *et al.*, 2011). The faster GI transit observed in 9-week WD-fed mice was first considered to be the consequence of an acceleration of the GE because such dysmotility is reported in pre-diabetic HFD-fed mice (Baudry *et al.*, 2012). RD and WD mice fed for 9 weeks exhibited similar GE; however, the proximal intestine transit had a tendency to be accelerated. Enteric neuronal plasticity leading to changes in excitatory neurotransmission may contribute to this accelerated motility phenotype and this needs to be investigated further. Future studies will aim to clarify the impact of HFD on upper intestine motility, in particular the GE that is accelerated in non-diabetic HFD-fed models but delayed during diabetes.

Another major point is that mice lacking TLR4 do not exhibit WD colonic NOS neuron loss and the resulting colonic delay. In the ENS, both neurons and glial cells express TLR4 (Di Liddo *et al.*, 2015). This expression is mainly found on inhibitory nerves and is stronger in the distal large bowel than in the proximal (Arciszewski *et al.*, 2005; Barajon *et al.*, 2009), suggesting why ileal neurons could be less sensitive to WD-induced neurodegeneration. In addition, this receptor is important for NOS neurons development because we showed that TLR4$^{-/-}$ mice possessed reduced numbers of colonic nitrergic neurons (Anitha *et al.*, 2014). This was confirmed in the present study, where NADPH-diaphorase neurons were reduced in TLR4$^{-/-}$. This may indicate that there are two populations of myenteric NOS neurons, expressing or not expressing TLR4, and further experiments will help to characterize this point.

Finally, our *in vitro* experiments highlight that low palmitate, similar to circulating values reported in healthy patients (Normand-Lauziere *et al.*, 2010), potentiates LPS-induced enteric neurodegeneration. We observed first that increasing doses of palmitate induce neuronal loss but not LPS. Next, we saw that a low dose of LPS (0.5 ng·ml^{-1}) amplifies the neurodegeneration cell loss initiated by palmitate (20 or 30 μM). This synergic action was confirmed by the increased reduction of PGP9.5 expression after co-treatment with palmitate (20 μM) and LPS. This suggested that ingested saturated fat, once in the plasma, may enhance nitrergic myenteric neuron

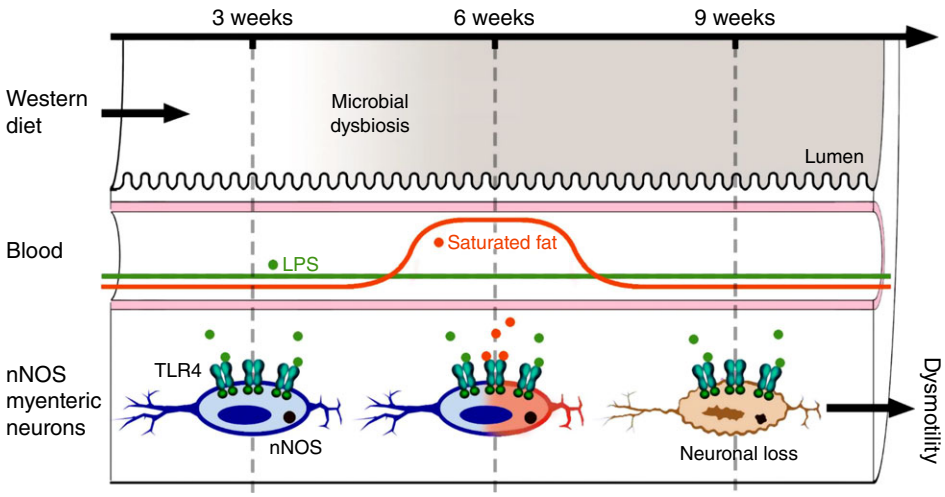

Figure 8. A proposed model where the hyperlipidemia observed after 6 weeks of WD could amplify the TLR4 activation by LPS at the level of the nitrergic myenteric neurons and leads to apoptosis after 9 and 12 weeks of feeding
We propose that WD-induced gut microbiota dysbiosis is accompanied by an increase of plasma saturated fat as observed after 6 weeks of feeding, initiating LPS-induced NOS activity within TLR-4 expressing enteric neurons. This leads to specific nitrergic neurodegeneration, as observed in WD-fed mice for 9 and 12 weeks, and delayed colonic transit.

TLR4 activation by basal LPS. We next confirmed that an enhancement of NOS activity is responsible for cultured neuronal loss because this phenomenon was prevented by the addition of L-NAME (200 μM). nNOS expression was reduced after treatment with palmitate, without further reduction when LPS was also added.

A similar synergic action between LPS and palmitate has been already reported in macrophages where they trigger inflammation (Schilling *et al.*, 2013). Future studies will focus on mechanisms by which palmitate enhances enteric TLR4 signalling. Saturated fatty acids, in addition to being activators, also facilitate TLR4 dimerization (Wong *et al.*, 2009). Variation at a genetic level may be also involved because we showed that TLR4 expression is increased within myenteric ganglia from HFD-fed mice (Anitha *et al.*, 2016). In addition, these new data demonstrate that enteric TLR4 signalling controls nNOS activity. LPS-induced nNOS activation has been already reported in a cultured primary oligodendrocyte precursor (Yao *et al.*, 2010). The question remains as to whether enteric TLR4 induction not only leads to nNOS over activity, but also inducible nitric oxide synthase activation as shown in macrophages, resulting in NO production and oxidative stress (Lee *et al.*, 2005).

Taken together, our data support the idea that WD-induced neuronal loss is initiated by a plasma FFA increase and is dependent on TLR4 signalling. We propose that the transient plasma FFA increase observed after 6 weeks of WD feeding, along with gut microbiota dysbiosis, enhances LPS action and TLR4 signalling within nitrergic myenteric neurons. This initiates nNOS over-activity, leading to neurodegeneration and colonic transit delay, respectively, as observed after 9 and 12 weeks (Fig. 8). Therefore, hyperlipidaemia is essential in HFD-induced neuropathies. The key findings of the present study are the development of a new model representing a typical WD not associated with diabetes or inflammation, but by dysbiosis as illustrated by fecal metabolomics. There was evidence of colonic neuronal loss in this model that is prevented by lack of TLR4 and requires the synergic action of LPS and saturated fatty acids. Further studies from our laboratory will evaluate the proportion of vaso-active intestinal peptide neurons affected by WD feeding and also other neuronal phenotypes such as intrinsic primary afferent neurons, excitatory motor neurons, inter-neurons or interstitial cells of Cajal. Moreover, we plan to investigate liver physiology in WD-fed mice because it was recently suggested by Rivera *et al.* (2014) that enteric neuron damage may contribute to the GI complications of fatty liver disease. In conclusion, the results of the present study suggest that a WD induces colonic dysmotility through myenteric neurodegeneration. Understanding these mechanisms can lead to new strategies to prevent colonic dysmotility in countries where HFDs are widespread.

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Additional information

Conflict of interest

The authors declare that they have no competing interests.

Authors contributions

FR, BC, DJ, ATG and SS designed the experiments, analysed and interpreted the data, and wrote the article. FR, BC, BGN, GL, ST, BL and KU collected and analysed the data. BGN, SM and M-K revised the article critically. All authors contributed to the final version of the manuscript and approved the version submitted for publication.

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Translational perspective

High-fat diet (HFD) consumption is associated with colonic motility disorders inducing constipation and nitrergic myenteric neuronal loss in the proximal colon. The pathophysiology of these disorders may involve the bacterial component lipopolysaccharide (LPS) and the saturated free fatty acids (both increase during HFD) that activate the receptor Toll-like receptor 4 (TLR4) expressed by enteric neurons. To understand the underlying mechanisms of HFD induced neurodegeneration and dysmotility, mice were fed a Western-diet (WD) (35% Cal from fat) containing excessive saturated fat such as palmitate *vs.* a regular diet (16.9% Cal from fat) for 3, 6, 9 and 12 weeks. We observed that WD mice fed for 6 weeks exhibit higher fecal bacterial metabolites such as muramic acid as shown by high-resolution metabolomics suggesting dysbiosis. They also had increased plasma FFA levels but no changes in plasma LPS. After 9 weeks, we found neurodegeneration of nitrergic myenteric neurons in the proximal colon and this was amplified after 12 weeks where it was associated with a delayed colonic transit. We finally highlighted the importance of TLR4 in this process: first by showing that mice lacking this receptor did not exhibit WD-induced disorders, and then by inducing *in vitro* cell death in cultured neurons incubated with LPS and palmitate. Together, these data demonstrate that the WD-induced loss of the nitrergic myenteric neurons is dependent of both gut microbial dysbiosis-associated LPS increase and the plasma FFA increase, and this specific neurodegeneration contributes to the delayed colonic transit. The present study will help to clarify the underlying mechanisms leading to HFD-induced motility disorders.