Cannabinoid CB$_2$ receptors in the enteric nervous system modulate gastrointestinal contractility in lipopolysaccharide-treated rats

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Enhanced intestinal transit due to lipopolysaccharide (LPS) is reversed by cannabinoid (CB$_2$) receptor agonists in vivo, but the site and mechanism of action are unknown. We have tested the hypothesis that CB$_2$ receptors are expressed in the enteric nervous system and are activated in pathophysiological conditions. Tissues from either saline- or LPS-treated (2 h; 65 µg/kg ip) rats were processed for RT-PCR, Western blotting, and immunohistochemistry or were mounted in organ baths where electrical field stimulation was applied in the presence or absence of CB receptor agonists. Whereas the CB$_2$ receptor agonist JWH133 did not affect the electrically evoked twitch response of the ileum under basal conditions, in the LPS-treated tissues JWH133 was able to reduce the enhanced contractile response in a concentration-dependent manner. Rat ileum expressed CB$_2$ receptor mRNA and protein under physiological conditions, and this expression was not affected by LPS treatment. In the myenteric plexus, CB$_2$ receptors were expressed on the majority of neurons, although not on those expressing nitric oxide synthase. LPS did not alter the distribution of CB$_2$ receptor expression in the myenteric plexus. In vivo LPS treatment significantly increased Fos expression in both enteric glia and neurons. This enhanced expression was significantly attenuated by JWH133, whose action was reversed by the CB$_2$ receptor antagonist AM630. Taking these facts together, we conclude that activation of CB$_2$ receptors in the enteric nervous system of the gut, which are much more widely distributed than originally described, which includes the presence of CB$_1$ receptors on non-neural tissues such as liver and adipose tissue and CB$_2$ receptor expression in the brain (7, 15, 20, 45).

Whereas the presence of the CB$_1$ receptor within the ENS has been well characterized (9, 16, 32), there are relatively few studies on CB$_2$ receptor localization or actions in the GI tract (49). Although CB$_2$ receptor mRNA has previously been reported in the stomach and ileum (10, 41), there is little evidence for a functional role for CB$_2$ receptors in the normal GI tract (for review, see Ref. 49). More is known about the actions of CB$_2$ receptors under pathophysiological conditions. In experimental models of colitis (mustard oil and dextran sulfate sodium), the CB$_2$ receptor agonist JWH133 attenuated the severity of colitis, presumably by modulating the CB$_2$ receptor immunoreactive infiltrates that were observed in both these models (18). These data are in partial agreement with a reported upregulation of CB$_2$ receptors on the epithelium and immune cells of colonic tissues from patients with inflammatory bowel disease (IBD) (48). CB$_2$ receptors may also play a role in visceral hypersensitivity associated with IBD, since in a 2,4,6-trinitrobenzenesulfonic model of colitis the CB$_2$ receptor antagonist JWH015 abolished colitis-induced hyperalgesia; however, the site of action of this protective effect is unknown (38).

Our group reported that in a model of lipopolysaccharide (LPS)-enhanced GI transit (22), CB$_2$ receptor activation was able to return the accelerated motility to baseline levels. Although we speculated that this involved the ENS, the presence of the CB$_2$ receptor in the ENS had not been established at that time (22). In the present study, we tested the hypothesis that CB$_2$ receptors are expressed in the ENS and are activated in pathophysiological conditions. Our aim was to determine whether CB$_2$ receptors were expressed in the ENS of the GI tract and could be upregulated by LPS. In addition, we sought to determine whether CB$_2$ receptor activation could modulate neuronal activation and transmission in LPS-treated animals.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (250–350 g; Charles River, Quebec, Canada) and female CB$_2$ receptor gene-deficient (CB$_2^{-/-}$) mice backcrossed onto a C57Bl6/N background and age- and strain-matched controls from the same facility were used for all studies (5).

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Animals were housed in plastic cages with sawdust floors with free access to Purina Laboratory chow and tap water. Animal use for these experiments was approved by the University of Calgary Animal Care Committee, and all protocols were carried out in accordance with the guidelines of the Canadian Council on Animal Care.

**Contractility studies.** Rats were treated with an intraperitoneal injection or either saline or LPS (from *Salmonella typhosa*, Sigma-Aldrich, St. Louis, MO; lot no. 074K4011, 65 μg/kg ip); 2 h later they were euthanized with pentobarbital sodium (≥65 mg/kg ip), and a 10-cm segment of ileum was removed and placed in ice-cold Krebs solution consisting of (in mM) 117 NaCl, 4.8 KCl, 25 NaHCO3, 1.2 NaH2PO4, 1.2 MgCl2, 11 glucose, and 2.5 CaCl2, bubbled with 95% O2-5% CO2. Segments, 1 cm each, were then ligated at each end with 6-0 silk thread, leaving the lumen open. Each segment was mounted longitudinally in a 25-ml tissue bath inside an electrode sleeve and attached to an isometric force transducer. The tissue bath contained Krebs with the nitric oxide synthase inhibitor N3-nitro-l-arginine methyl ester (400 μM to limit relaxations) (4) maintained at a temperature of 37°C and bubbled with 95% O2-5% CO2. Segments were placed under 1-g tension, allowed to equilibrate for 30 min, and rinsed. The mechanical activity of the muscle was detected by using an isometric force transducer (Harvard Apparatus, model 50-7905, Kent, UK), enhanced by a transducer amplifier (Harvard Apparatus, model 50-7970), and relayed to a bioelectric amplifier (Hewlett-Packard, model 8111A, Mississauga, ON, Canada). Data were converted with a CED 1401 PLUS analog-to-digital converter (Cambridge Electronic Design, Cambridge, UK) and recorded on a computer running into Spike 3 strip chart recorder software (Cambridge Electronic Design).

Maximal muscle contractions were obtained by adding 10−4 M bethanechol (Sigma-Aldrich) to the bath. After being rinsed and stabilized, the tissues were subjected to electrical field stimulation (EFS; Grass Instruments, model S88 stimulator, West Warwick, RI) as a continuous stimulus train at 15 Hz, 0.5-ms pulse width at 60 V for 1 s every minute. Tissues were left for 2 h for the EFS-evoked contractile responses to stabilize (1). After this, control responses were determined for each tissue where the mean value was calculated for 10 contractile responses prior to the addition of drugs. Cannabinoid drugs or the equivalent volume of vehicle were then added for 30 min, and rinsed. The mechanical activity of the muscle was determined by using an isometric force transducer (Harvard Apparatus, model 50-7905, Kent, UK), enhanced by a transducer amplifier (Harvard Apparatus, model 50-7970), and relayed to a bioelectric amplifier (Hewlett-Packard, model 8111A, Mississauga, ON, Canada). Data were converted with a CED 1401 PLUS analog-to-digital converter (Cambridge Electronic Design, Cambridge, UK) and recorded on a computer running into Spike 3 strip chart recorder software (Cambridge Electronic Design).

One of the experimental protocol was finished, the tissue response to 10−4 M bethanechol (Sigma-Aldrich) to the bath. After being rinsed and stabilized, the tissues were subjected to electrical field stimulation (EFS; Grass Instruments, model S88 stimulator, West Warwick, RI) as a continuous stimulus train at 15 Hz, 0.5-ms pulse width at 60 V for 1 s every minute. Tissues were left for 2 h for the EFS-evoked contractile responses to stabilize (1). After this, control responses were determined for each tissue where the mean value was calculated for 10 contractile responses prior to the addition of drugs. Cannabinoid drugs or the equivalent volume of vehicle were then added for 30 min, and rinsed. The mechanical activity of the muscle was determined by using an isometric force transducer (Harvard Apparatus, model 50-7905, Kent, UK), enhanced by a transducer amplifier (Harvard Apparatus, model 50-7970), and relayed to a bioelectric amplifier (Hewlett-Packard, model 8111A, Mississauga, ON, Canada). Data were converted with a CED 1401 PLUS analog-to-digital converter (Cambridge Electronic Design, Cambridge, UK) and recorded on a computer running into Spike 3 strip chart recorder software (Cambridge Electronic Design).

Once the experimental protocol was finished, the tissue response to 10−4 M bethanechol was again tested and the average response was calculated. In all cases, tissues responded well at the end of the experiment and no preparations were removed due to reduced viability. At the end of the study, tissues were removed from the bath, the silk was removed, the lumen was opened and mucosa was scraped off. The muscle tissue was weighed to obtain a wet weight, then dried and reweighed to obtain the dry weight.

**CB2 receptor mRNA detection and quantification.** Rats were injected intraperitoneally with either saline or LPS (65 μg/kg). After 2 h the animals were euthanized with pentobarbital sodium (≥65 mg/kg ip) and intracardially perfused with 1 l/kg phosphate-buffered saline (PBS) to remove the blood. The spleen and ileum were then harvested. The ileum was used as full wall thickness or it was further dissected into the ileal mucosa and submucosal layers from the mucosal layer. The ileum was used as full wall thickness or it was further dissected into the ileal mucosa and submucosal layers from the mucosal layer. The ileum was used as full wall thickness or it was further dissected into the ileal mucosa and submucosal layers from the mucosal layer.

**Immunohistochemistry.** Rats treated with either saline or LPS (n = 3 per group; 2 h ip) were euthanized with pentobarbital sodium (≥65 mg/kg ip) and untreated wild-type or CB2−/− mice (n = 3/group) were euthanized with pentobarbital sodium (≥65 mg/kg ip) and intracardially perfused with PBS at 4°C. Full-wall-thickness ileum samples were collected from mice, whereas in rats full-wall-thickness ileal segments, muscle and submucosal layers of the ileum, and the ileal mucosa were collected and homogenized in protease inhibitors, and protein concentrations were determined. Proteins were loaded at 60 μg of the extracted protein of each tissue and separated using SDS-PAGE (12%), using a constant current of 30 mAigel. Molecular weight markers (161-0373, Bio-Rad Laboratories, Hercules, CA) were used in each gel. After separation, the proteins were transferred from the SDS-PAGE gel onto a nitrocellulose membrane as previously described (24). Membranes were then incubated in blocking solution composed of 5% fat-free milk and Tris-buffered saline (TBS-T) for 1 h at room temperature followed by overnight incubation with one of three affinity-purified antibodies directed against the COOH terminus of the CB2 receptor: CB2-A: (1:250, Alpha Diagnostics, San Antonio, TX); KMCB2: (1:10,000, Ken Mackie, Indiana University, Bloomington, IN); PA1-746: (1: 2,000, Affinity Bioreagents, Golden, CO). We have previously established and reported specificity for the Alpha Diagnostics CB2 antibody by preabsorbing with cognate peptide (45). Membranes were washed with TBS-T and incubated in a horseradish peroxidase-conjugated goat anti-rabbit antibody (1:4,000, sc-2004, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. Protein bands were visualized by using a chemiluminescent substrate (ECL kit; Amersham, Little Chalfont, Bucks, UK) and X-Omat film (Eastman Kodak, Rochester, NY). The same membranes were stripped and rebotted with rabbit anti-actin (1:40,000, A-2600, Sigma, Oakville, ON, Canada) and goat anti-rabbit secondary antibody (1:4,000, sc-2004, Santa Cruz Biotechnology). Proteins were semiquantitatively analyzed by densitometric measurement of the bands.
longitudinal muscle-myenteric plexus and submucosal plexus were prepared as previously described (39). Briefly, the mucosa was removed, the submucosal layer was then carefully separated from the layers beneath, and then the circular muscle layer was separated from the longitudinal muscle layer, to which the myenteric plexus remained adherent. Whole-mount preparations were washed in PBS containing 0.1% Triton X-100 (Sigma-Aldrich) and incubated with CB2 receptor antibodies directed against the COOH terminus (1:200-1:6,400) (Alpha Diagnostics CB2, KMCB2, Affinity CB2, Alexis CB2, 210-198-1, Cedarlane Laboratories, Burlington, ON, Canada; Santa Cruz CB2, sc-10079, Santa Cruz Biotechnology) for 48 h at 4°C. Tissues were then washed in PBS three times for 10 min and then incubated in donkey anti-rabbit CY3 secondary antibody (1:100; Jackson, West Grove, PA) for 1 h at room temperature. Tissues were then washed again and mounted on slides with a bicarbonate-buffered glycerol (pH 8.6) and visualized via a Zeiss Axioskan fluorescence microscope and photographed with a digital camera (Sensys, Photometrics, Tucson, AZ).

In some cases, tissues were subsequently incubated with a second primary antibody. Antibodies used were goat anti-calretinin (1:500; cat. no. CGI, Swant, Bellinzona, Switzerland), mouse anti-neuronal nitric oxide synthase (1:200, N-2280, Sigma), mouse anti-neuron-specific enolase (NSE; 1:500, 6720-0704, Biogenesis, Cedarlane Laboratories, Hornby, ON, Canada), mouse anti-S-100B (1:100; mAb079, Chemicon, Temecula, CA), mouse anti-protein gene product 9.5 (PGP 9.5, 1:500, 13C4, Ultraclone, Cedarlane Laboratories), mouse anti-glia fibrillary acidic protein (1:500, RDI-GFAPabm-411, RDI Research Diagnostics, Concord, MA), and mouse anti-synaptotagmin (1:500; SYA-148, Stressgen Biotechnologies, Ann Arbor, MI) to determine colocalization. Secondary antibodies were used as goat anti-rabbit conjugated FITC (1:50; Jackson, 115-095-003), donkey anti-goat conjugated FITC (1:50; Jackson, 705-095-147), and donkey anti-sheep conjugated FITC (1:50; Sigma, F-7634).

Samples were viewed on an Olympus Fluoview FV300 confocal microscope system using krypton-argon and helium-neon lasers. Differential visualization of the fluorophores FITC (excitation 490 nm and emission 520 nm) and CY3 (excitation 552 nm and emission 565 nm) was accomplished through the use of specific filter combinations. Samples were scanned sequentially to avoid any potential for bleedthrough of fluorophores. Images of 1,024 × 1,024 pixels were obtained under identical exposure conditions and were processed identically by using Adobe Photoshop. Confocal micrographs are digital composites of Z-stack scans through 1-μm optical sections.

**Enteric neuron and glial activation.** We modified the in vivo LPS inflammatory model protocol used previously (22) to allow us to establish whether enteric neurons or glia were activated by LPS and to determine whether this could be modulated by a CB2 receptor agonist. Rats were treated with either saline or LPS at 0 min, drugs (vehicle, cannabinoid agonists, and/or antagonists) were administered at 80 min, and at 120 min the animals were euthanized with pentobarbital sodium (>65 mg/kg ip) and a 10-cm segment of ileum was removed. The ileal segment was immediately placed on a Sylgard plate containing PBS with 1 mM nifedipine to prevent muscle contraction; the tissue was then opened along the mesenteric border, pinned flat with the mucosa facing up, and fixed overnight in Zamboni’s fixative and tissue was then opened along the mesenteric border, pinned flat with the mucosa facing up, and fixed overnight in Zamboni’s fixative and processed for Fos immunohistochemistry. Tissues were minimally treated to decrease mechanical stimulation.

**Quantification of Fos-immunoreactive neurons and glia.** To assess the proportion of Fos-immunoreactive cells per ganglia, myenteric plexus preparations of ileum from in vivo-treated animals were double-labeled with antibodies directed toward c-Fos Ab-5 (1:5,000; PC38, Oncogene, Cambridge, MA) and subsequently double labeled for either NSE or the glial marker S-100. Cells positive for c-Fos were counted in at least 20 randomly chosen ganglia throughout the tissue preparation and subsequently expressed as a percentage of total number of neurons per ganglion for enteric neurons and expressed as number of glial cell bodies per ganglia for enteric glia.

**Statistics.** Data were analyzed by a two-way ANOVA followed by Bonferroni-corrected t-tests.

**RESULTS**

**CB receptor agonists and contractility after LPS treatment.** We isolated ileal tissues and determined the effects of the cannabinoid ligands on the cholinergic contractile response elicited by EFS. The contractile responses evoked by EFS were completely blocked by atropine (data not shown), so they were due to acetylcholine release from enteric nerves.

In animals treated with LPS, there was an enhanced contractile response that reached 122.7 ± 5.0% of the contractile response seen in saline-treated rats (n = 9). Thus we could mimic the enhanced motility seen in vivo (22). The CB2 receptor agonist JWH133 had no effect on contractile responses in the saline-treated tissues compared with vehicle control (n = 4; P > 0.05) (Fig. 1). In contrast, in tissues from LPS-treated rats, JWH133 (n = 9) significantly reduced the contractile responses at 3 and 10 μM, where the responses were 78.8 ± 4.9% (P < 0.01) and 75.0 ± 8.1% (P < 0.001) of the vehicle control response, respectively (Fig. 1). The selective CB2 receptor antagonist AM630 (300 nM) blocked the inhibitory effects of JWH133 (data not shown). Thus the JWH133 normalized the enhanced contractility, since the magnitude of the reduction in LPS-treated animals was similar to the degree of enhancement observed in the absence of the drugs (20–25%).

The responses to the CB1 receptor agonists differed from what we observed in vivo (22). The CB1 receptor-selective agonist ACEA was only effective at high concentrations (10 μM) but produced significant reductions in the contractile response (~65% of control responses) in tissues from either saline- or LPS-treated rats (data not shown). We also evaluated the mixed CBα/CB2 agonist WIN55,212-2. This compound caused a concentration-dependent attenuation of responses (Fig. 1) that were significantly attenuated in the presence of the CB1 receptor antagonist AM251 (data not shown) and that was observed in both the tissues from saline- and LPS-treated rats.
animals ($n = 3/4; P > 0.05$). The responses were not significantly different between tissues from saline- and LPS-treated animals at all concentrations tested.

CB$_2$ receptor mRNA is expressed in the rat ileum. CB$_2$ receptor mRNA is expressed in the full-wall thickness ileum, the ileal muscle and submucosal layers that contain the ENS, and the ileal mucosa in saline- and LPS-treated rats (Fig. 2A). No band was detected in the absence of template but, as expected, it was strongly expressed in the spleen used as a positive control. RT-PCR was performed using primers for CB$_2$ receptor or $\beta$-actin, and the expected amplicons were 472 and 277 bp, respectively. RNA from the samples was reverse transcribed or mock treated prior to PCR. No bands were detected in the RNA sample after amplification in the absence of reverse transcription, confirming that the amplified product was not due to genomic DNA in the RNA sample (data not shown). These samples were processed for quantitative real time RT-PCR to determine whether LPS treatment was able to upregulate the CB$_2$ message (Fig. 2B). There was no increase of CB$_2$ receptor message in the spleen or the mucosa. In the full-wall-thickness and muscle and submucosal samples, the LPS-treated tissues sometimes displayed increases in the CB$_2$ receptor mRNA expression, but there was no statistically significant increase in the group data ($n = 5, P > 0.05$).

CB$_2$ receptor protein is expressed in the rat ileum. CB$_2$ receptor protein was detected in full-wall-thickness ileum and was also present in the muscle and submucosal layers and in the mucosa (Fig. 2C). The molecular weight of the detected bands was $\sim 60$ kDa, which is an appropriate size on the basis of previously published work (3, 45). To confirm the specificity of the antibodies used, we performed studies in ileum from wild-type and CB$_2$ knockout mice. In mouse ileal tissue, only the CB$_2$ receptor antibody from Alpha Diagnostics recognized the same molecular weight band observed in rat ileal tissues (Fig. 2C). In the CB$_2^{-/-}$ mice labeling was completely absent, indicating antibody specificity. As before, we compared the tissues from LPS- and saline-treated rats. Analysis by densitometry of CB$_2$-to-$\beta$-actin ratio content for each sample indicated that CB$_2$ receptor expression was not upregulated in the rat ileum by LPS treatment (Fig. 2D).

CB$_2$ receptor is expressed by enteric neurons. We used many different antibodies directed against the COOH terminus of the CB$_2$ receptor to localize CB$_2$ receptor expression in the ENS. Despite robust validated neuronal expression in the central nervous system (CNS) (45) and by Western blot in the GI tract, the Alpha Diagnostics antibody did not produce consistent labeling in the ENS. Similarly, antibodies from Affinity Bioreagents, Alexis, and Santa Cruz either did not label the ENS or produced a clearly nonspecific pattern of immunoreactivity. In contrast, a COOH-terminal affinity-purified antibody that we made (against residues 326–342 of rat CB$_2$ receptor) identified CB$_2$ receptor immunoreactivity in enteric neurons and nerve fibers in both the myenteric (Fig. 3) and submucosal plexus (data not shown) in rat and mouse ileum. This labeling was absent in CB$_2^{-/-}$ mouse ileum (Fig. 3D), which were subsequently double labeled with calretinin to confirm the presence of enteric ganglia. Further control studies in which the primary or secondary antibodies were used alone did not produce any staining (data not shown).

We assessed whether specific classes of enteric neurons expressed the CB$_2$ receptor using double-labeling immunohistochemistry (Fig. 4). CB$_2$ receptor immunoreactivity was found in many cells that expressed the pan-neuronal markers NSE (Fig. 4, top row), PGP 9.5 (data not shown), and calretinin, a marker of longitudinal muscle motor neurons and primary afferent neurons (6, 21) (Fig. 4, center row). However,
only the minority of neurons that express neuronal nitric oxide synthase were found to colocalize with the CB2 receptor (Fig. 4, bottom row). Double labeling of the CB2 receptor with the glial-specific markers S-100 (Fig. 5) or glial fibrillary acidic protein (data not shown) revealed no CB2 receptor expression in these separate cell populations. Double labeling with synaptic vesicles, a marker for synaptic vesicles found in nerve terminals, showed a degree of colocalization (Fig. 5), suggesting the CB2 receptors are present on both nerve terminals and in neurons of the ENS.

Activation of the CB2 receptor reduces Fos expression in the ENS. We used Fos expression to ascertain whether in LPS-treated rats we could observe direct evidence for activation of enteric neurons (27, 30, 44). LPS caused a significant increase in Fos expression in both enteric neurons and enteric glia compared with tissues from saline-treated control rats (Fig. 6; $P < 0.001$). When animals were coinjected with the CB2 receptor agonist JWH133 (1 mg/kg), activation of both enteric neurons and glia were significantly reduced compared with LPS alone ($P < 0.001$). The inhibitory action of JWH133 on LPS-induced Fos expression was reversed by coadministration of the CB2 receptor antagonist AM630 (1 mg/kg). Administration of AM630 alone did not have any effect on activation of enteric neurons and glia ($P > 0.05$).

DISCUSSION

We have made the novel observation that excitatory motor neurons of the rat myenteric plexus, but not enteric glia, express CB2 receptors at both pre- and postsynaptic locations. Functional studies indicate that activation of these receptors had no effect on field-stimulated contractions of ileum in vitro, but that they normalize LPS-induced enhanced contractility of isolated ileal segments and attenuate Fos expression arising from the LPS challenge. This emergent property of CB2 receptor activation after an inflammatory stimulus occurs without altering CB2 receptor mRNA and protein expression in the GI tract. Thus we postulate that CB2 receptor activation interferes with intracellular signaling pathways activated directly or indirectly by LPS.

In our vitro functional approaches allow us to make a number of general observations concerning the possible site of action of cannabinoids in the GI tract. In showing a concentration-dependent reduction in EFS-induced contractile responses by CB1 receptor activation in saline-treated animals, we provide confirmatory evidence in the rat for the observation that CB1 receptor agonists inhibit acetylcholine release and contractility in the guinea pig myenteric plexus (9, 16, 32). We also were able to confirm that CB2 receptor activation had no effect on contractility in saline-treated rats. In the LPS-treated tissues, contractile activity was enhanced; this may account for the enhanced GI transit observed in vivo (22) and so this preparation provides a good model to further explore these findings. The CB2 receptor agonist JWH133, which had no effect under basal conditions, normalized the LPS-enhanced cholinergic contractile responses. Since this is an isolated tissue preparation, it would appear to rule out the central CB2 receptors present in the brain-gut axis (42) and would indicate that the site of action is localized to the gut, and most likely the ENS. Our data are in agreement with other studies that report no actions of CB2 receptor activation in GI motility under physiological conditions (10, 17). However, a recent study reported that CB2 receptor activation inhibits cholinergic neurotransmission in the normal mouse stomach (26), consistent with older observations in which EFS-induced relaxant responses were enhanced in rat gastric fundus in the presence of the CB2 receptor antagonist AM630 (41). In the stomach, it appears that CB2 receptors are functional under normal conditions, whereas in the ileum these receptors appear to be active only after an inflammatory stimulus. These results indicate differences in constitutive cannabinoid receptor activity between stomach and intestine.

The results from the present study do not explain the lack of actions of CB1 receptor activation in the LPS-enhanced transit model in vivo (22). As expected, we saw an inhibition of
contractility in the isolated vehicle-treated ileum by the CB₁ receptor agonists ACEA and WIN55,212-2; however, we also observed a similar inhibition in the LPS-treated tissues. Currently, we do not have an explanation for these observations, except to highlight the many potential sites of action of cannabinoids in the brain-gut axis or immune system (9, 42), which may contribute to the actions of cannabinoids on motility in vivo.

We report that CB₂ receptor message is present throughout the wall of the gut and that this is translated into protein expression, since CB₂ receptors are present under normal conditions in the rat ileum and in the separated muscle and submucosal layers and the intestinal mucosa. Since CB₂ receptor agonists have no actions in vivo or in vitro under normal conditions, it is possible that the actions observed to inhibit LPS-induced enhanced contractility are due to CB₂ receptor upregulation. In support of this possibility, previous studies have reported that CB₂ receptors are present at either nondetectable or at low levels in sensory neurons or the spinal cord under normal conditions but can be induced by nerve damage in models of chronic pain (47, 50). LPS has also been reported to increase CB₂ receptor levels in brain and macrophages (25). In the gut, CB₂ receptors are upregulated in submucosal and mucosal infiltrate in experimental models of colitis and in IBD (18, 48) and in the epithelium after treatment with the probiotic Lactobacillus acidophilus in rats and mice (34). Our data indicate that CB₂ receptors are present in the ileum under normal conditions and expression levels are not affected by LPS treatment, at least within the time course studied in the present experiments. Thus an upregulation of CB₂ receptors is not the mechanism by which the CB₂ receptor agonist JWH133 normalizes LPS-enhanced contractility in vitro or presumably GI transit in vivo (22).

Fig. 4. Confocal fluorescence micrographs of CB₂ receptor immunoreactivity double labeled with enteric neuronal markers in whole-mount preparations of rat ileal myenteric plexus. Single labels for neuron specific enolase (NSE; top row), calretinin (calret; middle row) and neuronal nitric oxide synthase (nNOS; bottom row) are in the first column. CB₂ receptor immunoreactivity is shown in the second column, and the overlay image is shown in the third column. Examples of colocalization are shown by thick arrows. The majority of NSE and calretinin-positive cells colocalize with CB₂ receptor immunoreactivity. There is very little colocalization between neurons expressing nNOS and CB₂ receptor immunoreactivity, shown by thin arrows. Scale bar: 100 μm.
There is a growing body of evidence for the expression of neuronal CB2 receptors. They have now been described in the brain stem (45), isolated vagus nerve (31), sensory nerves (33) and peripheral nerves (10). To determine the localization of the CB2 receptors in the GI tract, we employed immunohistochemical techniques and found that CB2 receptors are expressed in the cell bodies, fibers, and terminals of enteric neurons. These results confirm preliminary observations of neuronal expression of CB2 receptors in the myenteric and submucosal plexus in human ileum (49). Double labeling with the neuronal marker NSE indicates that the majority of enteric neurons express CB2 receptors. The pattern of overlap with NSE and calretinin indicates a cytoplasmic and a likely membrane or submembrane expression of the receptor. In contrast, inhibitory motor neurons expressing neuronal NOS have very little coexpression of CB2 receptors, as is also seen with CB1 receptors (43). The vesicle marker synaptotagmin was used to determine whether CB2 receptors are expressed at presynaptic localizations. The coexpression in terminals of CB2 receptors indicates an action either on postsynaptic excitatory motor neurons or on afferents synapsing onto these cells, which could account for the inhibitory action of CB2 receptor agonists on the enhanced contractility after LPS. In contrast to CB1 receptors (14), CB2 receptors have few reported actions on ion channels (12, 13), so the mechanism of action to modulate neurotransmission is unclear. CB2 receptor activation can inhibit the capsaicin-evoked calcium response in isolated dorsal root ganglia and CGRP release from spinal cord slices (2, 37), but the mechanism has not yet been determined in these systems. Although it is known that CB2 receptors inhibit adenylyl cyclase and regulate of mitogen-activated protein kinase (13), a possible action via these signaling pathways remains to be determined in the myenteric plexus.

We designed an experimental protocol to allow us to evaluate whether LPS can induce myenteric neuronal activity and to determine whether CB2 receptor activation can modulate neuronal activation. Fos expression can be used to examine enteric neuron activation in response to various stimuli (27, 30, 44), and in our hands, LPS induces a robust Fos expression in enteric neurons. The fact that enteric glia were also activated was unexpected. Both enteric neuron and glial Fos expression were attenuated by in vivo activation of CB2 receptors by JWH133. The ability of JWH133 to return the Fos expression to basal levels is in agreement with the contractility studies in which twitch responses were returned to control levels. We speculate that LPS is stimulating the ENS (correlated with increased Fos expression and contractility) either directly or indirectly and CB2 receptors return both of these evoked changes back to the baseline conditions. Presumably, the increased glial Fos expression is secondary to neuronal activation since it can be reduced by CB2 receptor agonists in the absence of CB2 receptors on enteric glia. Although we have not yet fully explored the cellular mechanisms of these observations, we suggest that they involve enhanced transmitter release in the LPS-treated tissues that are brought back to baseline by CB2 receptor activation. Exactly how the CB2 receptors get coupled to a signal transduction system in the stimulated state remains to be determined.
The physiological role of enteric glia is not well understood, but they closely resemble astrocytes in the CNS and so may share some functional properties (36). In the GI tract they surround enteric neurons and are thought to act as a support network, although they may also play a role in physiological and pathophysiological functions (27, 36). We did not observe CB2 receptor expression on enteric glia under normal conditions, which is in agreement with studies on astrocytes (46). Moreover, we did not observe an induction of CB2 receptor expression on enteric glia in the LPS-treated tissues at this time point (data not shown). LPS acts in part through induction of interleukin (IL)-1β (8), and IL-1β can also induce both Fos expression and cytokine release in enteric glia and IL-1β stimulated astrocytes are sensitive to the actions of cannabinoids (35, 40, 44). Our data suggest that CB2 receptor activation can modulate activation of enteric glia by an indirect mechanism, since enteric glia do not appear to express CB2 receptors. These data are in agreement with studies in astrocytes where CB2 receptors are not expressed on the cells but the actions of cannabinoids on LPS-induced cytokine and nitric oxide release are reversed by CB2 receptor antagonism (23, 28). The pathophysiological consequence of inhibiting the activation of enteric glia could be an alteration of GI motility, since we have shown that enteric glia can alter ileal contractility in vitro and GI transit in vivo (27).

In conclusion, we report that CB2 receptors are expressed on enteric neurons. CB2 receptors are present under normal physiological conditions in the gut and the expression pattern or levels are unaffected by LPS treatment. The CB2 receptor does not appear to be functionally active under normal conditions but attenuates the activation of both enteric neurons (directly) and glia (indirectly) in response to a neuroimmune challenge. The functional significance of this activation is decreased neurotransmitter release, thereby reducing muscle contractility, which would account for the “brake” in LPS-enhanced transit observed in vivo (22). These data support a possible role for the CB2 receptor as a therapeutic target in the GI tract for altered motility in states of inflammation and immune activa-

Fig. 6. Fos expression in the rat myenteric plexus after LPS treatment. In vivo LPS-treatment (65 μg/kg ip; 2 h) induces Fos expression in both enteric neurons (A, red nuclei) and glia (D, red nuclei) compared with saline controls. Administration of the CB2 receptor agonist JWH133 in LPS-treated rats attenuates Fos expression in enteric neurons (B, green, labeled with NSE) and glia (E, green, labeled with S-100), which can be reversed by coadministration of the CB2 receptor antagonist AM630 (C, F), which has no effect alone on LPS-induced Fos expression. ***P < 0.001. Scale bar: 100 μm.
tion following infection, since administration of CB2 receptor agonists is not associated with the psychotropic side effects of CB1 receptor agonists and they have minimal actions in normal animals.

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