SI APPENDIX

Morphine activates neuroinflammation in a manner parallel to endotoxin

Xiaohui Wang[a], Lisa C. Loram[b,c], Khara Ramos[b,c], Armando J. de Jesus[a], Jacob Thomas[d], Kui Cheng[a], Anireddy Reddy[b,c], Andrew A. Somogyi[d], Mark R. Hutchinson[e], Linda R. Watkins[b,c], Hang Yin[a,c,f,1]

[a]Department of Chemistry and Biochemistry, [b]Department of Psychology and Neuroscience, [c]Center for Neuroscience, and [f]Biofrontiers Institute, University of Colorado at Boulder, Boulder, CO 80309, USA; [d]Discipline of Pharmacology and [e]Discipline of Physiology, School of Medical Sciences, University of Adelaide, Adelaide, South Australia 5005, Australia

[1]To whom correspondence should be addressed. Email:Hubert.Yin@colorado.edu.

SI Appendix

SI Materials and Methods

Reagents

Lipopolysaccharide (LPS), biotin-LPS, LPS-RS, TLR4 antagonist CIL-095, IκB-α inhibitor BAY 11-7082 and HEK Blue hTLR4 cells were obtained from Invivogen (San Diego, CA, USA). Natural morphine ((-) configuration) was provided by Mallinckrodt, Inc. (St. Louis, MO, USA). (+)-Morphine was provided by National Institute on Drug Abuse (NIDA). Morphine-6-glucuronide (M6G) was purchased from Cerilliant (Round Rock, TX, USA). Validated TLR4 and MD-2 SMARTpool siRNAs and mock siRNA were purchased from Dharmacon (Lafayette, CO, USA). Transfection vehicle Lipofectamine LTX reagent was purchased from Invitrogen (Grand Island, NY, USA). Mammalian Protein Extraction Reagent, protease inhibitor cocktails, BCA protein assay kit, streptavidin coated plates, goat anti-mouse IgG-horseradish peroxidase (HRP) conjugate, immunoprecipitation (IP) kit and Super-Signal West Pico Chemiluminescent Substrate were purchased from Pierce (Rockford, IL, USA). Cell Proliferation Reagent WST-1 was purchased from Roche Diagnostics (GmbH, Mannheim, Germany). Phospha-Light™ SEAP Reporter Gene Assay System was obtained from Applied Biosystems (Foster, CA, USA). Cignal Lenti NF-κB Reporter kit, RNeasy Mini Kit, RT² Easy First Strand cDNA Synthesis Kit and RT^2 SYBR Green PCR Master Mix were purchased from SABioscience (Frederick, MD, USA). Dual-Glo luciferase assay system was purchased from Promega (Madison, MI, USA). IL-1β and TNF-α ELISA kits, TMB substrate reagent set, bright linearized baculovirus DNA and SF-9 insect cell were purchased from BD Bioscience (San Jose, CA, USA). Curcumin, roxithromycin, protein A, rabbit anti-GFP antibody, rabbit anti-Flag antibody and mouse IgG-HRP conjugate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse anti-MD-2 antibody (9B4), which specifically recognizes free MD-2, but not LPS bound MD-2 (1), was purchased from eBiosceince (San Diego, CA, USA). Rabbit anti-MD-2 antibody, rabbit ant-TLR4 antibody, rabbit anti-IκB-α antibody, rabbit anti-NF-κB p65 antibody, morphine antibody and goat anti-rabbit IgG-HRP were purchased from Abcam (Cambridge, MA**,** USA). p-p38 MAPK (Thr180/Tyr182) and p-p44/42 MAPK (Erk1/2) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Odyssey blocking buffer and IRDye 800CW conjugates of goat-anti-rabbit-IgG and goat-anti-mouse-IgG were purchased from LICOR Bioscience (Lincoln, NE, USA) and DRAQ staining was obtained from Biostatus Limited (Leicestershire, UK). IgG sepharose 6 fast flow, benzamidine sepharose 4 fast flow, protein A sepharose 4 fast flow and thrombin were obtained from GE Lifescience (Piscataway, NJ, USA). The murine microglial BV-2 cell line was provided by Dr Rona Giffard (Stanford University). Ba/F3 cells, which over-express human TLR4-Flag, human TLR4-GFP, human CD14, and human MD-2, were provided by Dr Kensuke Miyake (Osaka University, Japan) (2). Insect expression human MD-2-pAcGP67A vector was provided by Dr Jie-Oh Lee (KAIST, Korea) (3). High 5 insect cell was provided by Dr Xuedong Liu (University of Colorado, Boulder). HEK293 cells stably secreting the human TLR4 (1-632)-Fc were provided by Dr Douglas T. Golenbock (Massachusetts Medical School) (4).

MD-2 and TLR4 expression and purification

MD-2 baculovirus was prepared by co-transfection of SF-9 insect cells with MD-2-pAcGP67A vector and bright linearized baculovirus DNA as described by the manufacturer's protocol (BD Bioscience, San Diego, CA, USA). After 2-3 rounds of amplification, the MD-2 baculovirus suspension reached a titer of ~10^8/mL virus particles and was used to transfect high 5 insect cells to express MD-2. MD-2 was secreted into the medium. After 3-4 day transfection, the medium was harvested and subjected to IgG sepharose affinity purification. When necessary, protein A tag was removed using thrombin. MD-2 was further purified by IgG sepharose purification and benzamidine sepharose purification according manufacture's instructions to remove protein A tag and thrombin. SDS-PAGE analysis showed that the purity of the prepared protein was >95% (Fig.S1A). Circular dichroism (CD) experiments showed the MD-2 protein was well folded (Fig.S1A) and thermal stable (T_m, around 80 ºC) (Fig. S1B). To further demonstrate the prepared MD-2 was active, we tested the binding of MD-2 with a well known MD-2 ligand, curcumin (5, 6) A K_d of 0.55 ± 0.36 μM was obtained (Fig. S1C), which is agreement with that of previous reported value (0.37 ± 0.12 μM) (5).

HEK293 cells stably secreting the human TLR4 were cultured in CD 293 medium supplemented penicillin (50 unit/mL), streptomycin (50 μg/mL) and G418 (250 μg/mL). When reaching confluence,

medium was harvested and subjected to protein A affinity purification according to manufacture's instruction.

Chemical Synthesis

All reactions were performed in oven-dried or flame-dried glassware under a dry nitrogen or argon atmosphere. Flash chromatography was performed using 32-64 μm silica gel. ^{1}H NMR spectra were recorded at 500 MHz in $CDCl_3$ using residual $CHCl_3$ (7.26 ppm) as the internal standard. ^{13}C NMR spectra were recorded at 75 MHz in $CDCl_3$ using residual $CHCl_3$ (77.23 ppm) as an internal reference. Exact mass was determined using electrospray ionization.

Biotin-morphine was synthesized according to previously described method (7). The synthesis scheme was shown in Fig.S2.

Compound **1** and **3** was synthesized according to the method reported elsewhere (8). The scheme was shown in Fig. S20.

Synthesis of Compound **2**: 1-Adamantanecarbonyl chloride, S7 (238 mg, 1.20 mmol) was added to a mixture of 1,4-diphenylenediamine (108 mg, 1.00 mmol), triethylamine (202 mg, 2.00 mmol), DMAP (6 mg, 0.05 mmol) and 1,4-dioxane (4.0 mL) at room temperature. After stirring for 12 h, half of the reaction solvent was evaporated and the subsequent solution was subjected to column chromatography with ethyl acetate as the eluent. The intermediate S8 was obtained as a colorless powder; yield 113 mg, 40%. Isovaleryl chloride (14 mg, 0.10 mmol) was added to a solution of the intermediate (27 mg, 0.10 mmol), triethylamine (20 mg, 0.20 mmol), *N*, *N*'-dimethylaminopyridine (1.2 mg, 0.010 mmol) and 1, 4-dioxane (1.0 mL) at room temperature. After stirring for 12 h, the reaction mixture was subject to column chromatography (1:1 ethyl acetate-hexanes). The compound **2** was obtained as colorless powder; yield 30 mg, 85 %. The scheme was shown in Fig. S21.

ELISA assays

Four different ELISA assays were performed to investigate the morphine and MD-2 interaction.

ELISA 1: Biotin-morphine was immobilized on streptavidin coated plates in phosphate buffer solution (PBS, 137 mM NaCl, 2.7 mM KCl, 8.1 mM, 1.76 mM KH_2PO_4, pH 7.4) at room temperature for 2 h as the capturing probe. The wells were washed 3 times with PBST buffer (PBS supplemented with 0.05% Tween-20) and then blocked with 5% BSA solution at room temperature for 1 h. After washing with PBST 3 times, indicated concentration of MD-2-protein A or MD-2-protein A and LPS reaction mix was added and incubated for 1 h at room temperature. After washing with PBST 5 times, mouse IgG-HRP conjugate was diluted at the ratio of 1:4000 and added into the wells and incubated at room temperature for 1 h. After washing with PBST 7 times, 100 μL of TMB reagents were added to each well and incubated at room temperature for 10-30 min. 50 μL of 1 M H_3PO_4 was subsequently added into each well to stop the color reaction and the absorbance at 450 nm was measured on a Beckman-Coulter DTX 880 micro-plate reader and 620 nm was chosen as the reference wavelength.

ELISA 2: The 96-well ELISA microplate (BD Bioscience, San Jose, CA, USA) was coated with 10 μg/mL MD-2-Protein A or BSA in 0.1 M acetate buffer (pH 5.0). The wells were blocked and washed as described in ELISA 1. Indicated concentration of biotin-morphine or biotin-morphine and LPS mix was added and incubated at room temperature for 1 h. After 5 washings, streptavidin coupled HRP conjugate was diluted at the ratio of 1:2000 and added into the wells and incubated at room temperature for 1 h. After a further 7 washings, the color reaction was developed and measured as described in ELISA 1.

ELISA 3: 2 μg/mL of rabbit anti-MD-2 antibody was coated onto the 96-well ELISA microplate (BD Bioscience, San Jose, CA, USA) in 0.1 M carbonate-bicarbonate buffer (pH 9.6) at room temperature for 2 h as the capturing antibody. The wells were blocked and washed as described in ELISA 1. 5 μg/mL of MD-2 (protein A tag were removed) and different concentration of morphine or roxithromycin (served as the control) were added and incubated at room temperature for 1 h. After washing with PBST 5 times, 0.1 μg/mL of mouse anti-MD-2 antibody (9B4), which specifically recognizes free MD-2 (1), was added and incubated at room temperature for 1 h. After further 5 washings, goat anti-mouse IgG-HRP conjugate was diluted at the ratio of 1:2000 and added and incubated at room temperature for 1 h. After a further 7 washings, the color reaction was developed and measured as described in ELISA 1.It should be noted that the absorbance at 450 nm here represents the free MD-2, not morphine/roxithromycin bound MD-2.

ELISA 4: To investigate whether morphine can block LPS, a well known MD-2 ligand, binding to MD-2, ELISA 4 was developed. Biotin-LPS (0.1 μg/mL) was immobilized on streptavidin coated plates in PBS buffer at room temperature for 2 h as the capturing probe. The wells were blocked and washed as described in ELISA 1. 10 μg/mL of human MD-2-protein A and different concentration of morphine or roxithromycin were added and incubated at room temperature for 1 h. After washing with PBST 5 times, mouse IgG-HRP conjugate was diluted at the ratio of 1:4000 and added into the wells and incubated for 1 h at room temperature. After a further 7 washings, the color reaction was developed and measured as described in ELISA 1.

We have designed two TLR4 antagonists that disrupt the TLR4-MD-2 interaction (8, 9). Compound **1** was designed to target TLR4 and compound **2** was designed to target MD-2. In order to further biophysical characterization of the compound **1** and **2** and their targets interactions, ELISA 5 and ELISA 6 were developed.

ELISA 5: ELISA 5 was developed to detect compound **1** and **3** binding to TLR4. Our previous work identified a peptide MD-2–I, which showed strong affinity to TLR4 (10, 11). We employed this peptide as the probe for capturing TLR4. MD-2–I (CRGSDDDYSFCRALKGE) was synthesized according to standardized solid phase synthesis and purified by HPLC. MALDI-MS was used to confirm the purity and identify of the peptide. MD-2-I peptide (4 μM) was coated onto the 96-well ELISA microplate (BD Bioscience, San Jose, CA, USA) in 0.1 M carbonate-bicarbonate buffer (pH 9.6) at room temperature for 2 h. The wells were blocked and washed as described in ELISA 1. Human TLR4-Fc (1 μM) and different concentrations of compound **1** or **3** reaction mix were added and incubated at room temperature for 1 h. After washing with PBST 5 times, mouse IgG-HRP conjugate was diluted at the ratio of 1:4000 and added into the wells and incubated at room temperature for 1 h. After further 7 washings, the color reaction was developed and measured as described in ELISA 1.

ELISA 6: ELISA 6 was developed to detect compound **2** binding to MD-2. We demonstrated here that morphine binds to MD-2. Thus morphine was used as the probe for capturing MD-2. Morphine (5 μM) was coated onto the 96-well ELISA microplate (BD Bioscience, San Jose, CA, USA) in 0.1 M carbonate-bicarbonate buffer (pH 9.6) at room temperature for 2 h. The wells were blocked and washed as described in ELISA 1. Human MD-2-protein A (1 μM) and different concentrations of compound **2** reaction mix were added and incubated at room temperature for 1 h. After washing with PBST 5 times, mouse IgG-HRP conjugate was diluted at the ratio of 1:4000 and added into the wells and incubated at room temperature for 1 h. After further 7 washings, the color reaction was developed and measured as described in ELISA 1.

Fluorescence titration

Fluorescence measurements were performed on a Fluorolog-3 spectrofluorimeter (Horiba Jobin Yvon, Edison, NJ, USA). All measurements were carried out under room temperature in a 2×10 mm quartz cell (Starna Cells, Atascadero, CA, USA). 430 nm was chosen as the excitation wavelength of extrinsic fluorescence probe curcumin and emission at 450-600 nm was recorded. Appropriate controls were **s**ubtracted from spectra obtained on the samples. Fluorescence was also corrected by the relation, $F_{corr} = F_{obs}$ anti-log $(OD_{ex} + OD_{em}/2)$ for the inner filter effect when necessary, where OD_{ex} and OD_{em} are the optical densities at excitation and emission wavelengths, respectively.

For displacement assay, different concentrations of morphine were titrated into MD-2 (0.5 μM) and incubated at room temperature for 1 h. Curcumin (0.5 μM, final concentration) was subsequently added. After overnight incubation at room temperature, the curcumin fluorescence intensity was measured. K_i was determined using the equation: $K_i = IC_{50}/ (1 + [curcumin]/K_d(curcumin\text{-}MD\text{-}2))$.

Morphine/MD-2 molecular dynamics simulation

The Langevin dynamics simulation of the MD-2-morphine system was performed using the Chemistry at Harvard Molecular Mechanics (CHARMM) software package (12). The initial position of morphine was chosen based on the position of paclitaxel, which is also known to bind to MD-2 (13). Morphine was placed near residues R90, E92, S127, K122 and F126 that are found near the opening of the MD-2 cavity. The fully atomistic models of morphine-MD-2 were simulated in a generalized Born implicit solvent (12). The PARAM27 force field (14) was utilized

with CMAP dihedral corrections (15) together with the CHARMM General Force Field for small molecules (16)

qRT-PCR and western blot

Total RNA was extracted by RNeasy Mini Kit (SABioscience, Frederick, MD, USA) according to manufacture's instruction. cDNA was synthesized by RT² Easy First Strand cDNA Synthesis Kit (SABioscience, Frederick, MD, USA) according to manufacture's instruction. The primers for MD-2, TLR4, IL-1β, iNOS and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from SABioscience (Frederick, MD, USA).

qPCR was performed on a CFX96™ Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA) using the SYBR Green method. The data were analysed by $\Delta\Delta C_t$ method (17).

Western blot was performed as following. The samples were first separated by 12% SDS-PAGE and then electroblotted to PVDF membrane. After blocking with 5% BSA, the membranes were incubated with 0.5 μg/mL of appropriate primary antibody at room temperature for 2 h. The membranes were washed 5 times in PBST for 5 min each and then incubated for 1 h at room temperature with secondary antibody-HRP conjugate (50 ng/mL). After extensive washing in PBST, the protein–antibody complexes were visualized by exposure to X-ray film after reacting with Super-Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA)

Co-immunoprecipitation (Co-IP) of MD-2 with morphine

HEK Blue hTLR4 cells, which over-express the human TLR4 and MD-2, were cultured in DMEM medium supplemented with 10% FBS, penicillin (50 unit/mL), streptomycin (50 μg/mL) and normocin (100 μg/mL). Cells were stimulated with morphine (300 μM) for 12 h. After 2 washings with cold PBS buffer, cells were lysed in mammalian protein extraction reagent (Pierce, Rockford, IL, USA) supplemented with 5 mM EDTA, 150 mM NaCl and protease inhibitor cocktails. After centrifugation, supernatant was kept and mixed with rabbit ant-morphine antibody at 4°C for 2 h. The lysates and anti-morphine antibody were then mixed with protein A/G sepharose beads at 4°C for 1 h. The beads were then washed with washing buffer (150 mM NaCl, 50 mM Tris–HCl pH 7.6, 0.1% Brij, 0.1% *n*-octyl-β-D-glucoside, 0.1% Triton X-100) three times and then boiled with 2×SDS-PAGE Laemmli buffer for 5 min. The samples were immuno-detected by anti-MD-2 antibody. Western blot with GAPDH was served as the lysates input control.

TLR4 receptor oligomerization assay

The TLR4 receptor oligomerization assay shown here was first developed by Miyake and colleagues (2). Ba/F3 cells, which over-express human TLR4-Flag, human TLR4-GFP, human CD14, and human MD-2, were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 μM 2-mercaptoethanol and recombinant murine IL-3 (~70 unit/mL). Cells were seeded at the concentration of 0.4×10^5 cells/mL and stimulated with morphine (300 μM) for 72 h. Cells were harvested and washed two times with cold PBS buffer and lysed in mammalian protein extraction reagent (Pierce, Rockford, IL, USA) supplemented with 5 mM EDTA, 150 mM NaCl and protease inhibitor cocktails. After centrifugation, supernatant was kept and mixed with rabbit anti-Flag antibody at 4°C for 2 h. The lysates and anti-Flag antibody were then mixed with protein A/G sepharose beads at 4°C for 1 h. The beads were then washed with washing buffer (150 mM NaCl, 50 mM Tris–HCl pH 7.6, 0.1% Brij, 0.1% *n*-octyl-β-D-glucoside, 0.1% Triton X-100) three times and boiled with 2×SDS-PAGE Laemmli buffer for 5 min. The samples were immuno-detected by anti-Flag and anti-GFP antibodies.

Dual luciferase reporter assay

NF-κB dual luciferase reporter glial BV-2 cell line was constructed by Cignal Lenti NF-κB Reporter kit (SABiosciences, MD, USA) according to manufacture's instructions. Firefly luciferase gene was placed under the control the NF-κB transcriptional response element and the constitutively expressing Renilla luciferase was placed under the control of CMV promoter. The internal control Renilla luciferase can overcome technical variability and obtain more reliable data.

NF-κB dual luciferase reporter BV-2 cells were cultured in DMEM medium supplemented with 10% FBS, penicillin (50 unit/mL), streptomycin (50 μg/mL) and puromycin (4 μg/mL). BV-2 reporter cells were seeded at a density of 1×10^4 cells/well in 96-well plates. After 24 h incubation, medium

was changed to Opti-MEM medium supplemented with 0.5% FBS, penicillin (50 unit/mL), streptomycin (50 μg/mL) and 1% of non-essential amino acid (NEAA) and indicated concentration of morphine or morphine and curcumin was added. After further 72 h treatment, the NF-κB activity was analysed by Dual-Glo Luciferase Assay System (Promega, Madison, MI, USA) according to manufacture's instructions. The ratio of Firefly luciferase activity to Renilla luciferase activity represents the NF-κB activity. It should be noted that NF-κB activity of the untreated control group was set as 1.

Secreted alkaline phosphatase (SEAP) Assay

HEK Blue hTLR4 cells, which were stably transfected with human TLR4, MD-2 and a secreted embryonic alkaline phosphatase (SEAP) reporter gene, were cultured in DMEM medium supplemented with 10% FBS, penicillin (50 unit/mL), streptomycin (50 μg/mL) and normocin (100 μg/mL). It should be noted that the SEAP reporter gene was placed under the control of NF-κB transcriptional response element. Cells were seeded at the concentration of 1×10^5 cells/mL. After 24 h incubation, medium was changed to Opti-MEM medium supplemented with 0.5% FBS, penicillin (50 unit/mL), streptomycin (50 μg/mL) and 1% of non-essential amino acid (NEAA) and 400 μM of morphine or morphine (400 μM) and LPS-RS were added. After further 72 h treatments, NF-κB activity was detected by Phospha-Light™ SEAP Reporter Gene Assay System (AppliedBiosystems, Foster, CA, USA.) according to manufacture's instruction.

RNA interference (RNAi)

Microglial BV-2 cells were grown in DMEM medium supplemented with 10% FBS. Cells were detached from flask by trypsin digestion when ~80% confluence was reached. Reverse RNAi was performed as following. 6 μL of SMARTpool siRNA (Dharmacon, Lafayette, CO, USA) stock solution (50 μM) was diluted with 14 μL D-PBS, and 8 μL of Lipofectamine LTX (Invitrogen, Grand Island, NY, USA) was diluted with 12 μL D-PBS. Subsequently TLR4 siRNA and Lipofectamine LTX solutions were gently mixed together in the multi-well plate and incubated at room temperature for 30 min. Then, cells were planted at a cell density of 4×10^4 cells per mL. After 72 h of RNAi, western blot was performed to confirm the knockdown efficiency (Fig. S15).

RNAi was performed as described above. After 48 h of RNAi, medium was changed to Opti-MEM+0.5% FBS and 400 μM of morphine was added. Plates were then incubated for 24 h. Following incubation 100 μl of media was removed and added to flat black 96-well microfluor plates (Thermo scientific, MA, USA). 10 μl of 2, 3-diaminonaphthalene (0.05 mg/ml in 0.62 M HCl) was added to each well and incubated for 15 min. The reaction was quenched by addition of 5 μl of 3 M NaOH and the plate was read on Beckman Coulter DTX880 reader (Beckman Coulter, CA, USA) with excitation at 365 nm and emission at 450 nm. Nitrite (a stable metabolite of nitric oxide) concentration was determined from a nitrite standard curve.

RNAi was performed as described above. After 48 h of RNAi, cells were stimulated with 200 μM of morphine and incubated for an additional 24 h. Cells were then collected and lysed by mammalian protein extraction reagent (Thermo Scientific, Rockford, IL, USA). IL-1 β and TNF-α levels were analyzed by ELISA (BD Bioscience, San Diego, CA, USA) according to manufacture's instructions. The IL-1 β and TNF-α result showed here have been normalized to total protein concentration.

Cell proliferation assay

Cells were seeded at 96-well plate with a density of 5×10^4 cells per mL. After 24 h incubation, different concentrations of morphine were added, After 72 h stimulation, cell proliferation was determined by Cell Proliferation Reagent WST-1 (Roche Diagnostics GmbH, Mannheim, Germany) according to manufacture's instruction. The absorbance at 450 nm was measured on a Beckman-coulter DTX 880 microplate reader and 620 nm was chosen as the reference wavelength. The $A_{450\ nm}$-$A_{620\ nm}$ for the control group was set as 100%.

Central nervous system endothelial cell isolation and culture

Primary cultures of central nervous system (CNS) endothelial cell (EC) were isolated from adult rat brain and spinal cord tissue, as described previously (18, 19). This method yields cultures that are > 98% pure, which we confirmed with positive immunostaining for von Willebrand factor, negative

immunostaining for markers of fibroblasts (prolyl 4-hydroxylase) and astrocytes (glial fibrillary acidic protein), and visual inspection of the cells, which had the typical spindle-shaped morphology of CNS EC and formed confluent monolayers that were longitudinally aligned and non-overlapping, as described previously (18). Rats were anesthetized with isoflurane then decapitated. The brain was dissected out of the skull and the spinal cord was removed by hydraulic extrusion with ice cold physiological saline. The tissue was then processed as follows (all procedures performed using sterile technique): the tissue was placed in an enzymatic digestion solution, containing collagenase type II (Invitrogen, Carlsbad, CA, USA) and DNase I (Sigma-Aldrich, St. Louis, MO, USA) in Hank's Balanced Salt Solution (HBSS) with calcium and magnesium, then chopped finely with a sterile scalpel. The tissue pieces were triturated in the collagenase II solution with a 25 mL pipette, then incubated at 37°C for 40 min. Following the incubation, the mixture was mixed thoroughly with a 10 mL pipette then a 5 mL pipette until there were no discernible pieces of tissue left. The mixture was centrifuged for removal of the supernatant, and the pellet was then centrifuged in a solution of 20% bovine serum albumin in Dulbecco's Modified Eagle Medium. This allowed removal of neurons and glia, leaving behind a pellet of microvessels. The microvessels were then placed in an enzymatic digestion solution, containing collagenase/dispase (Roche, Indianapolis, IN, USA) and DNase I in HBSS without calcium or magnesium, and incubated at 37°C for 30 min. The mixture was then centrifuged, and after removal of the supernatant, the pellet was centrifuged through a 33% Percoll gradient (Amersham Biosciences, Piscataway, NJ, USA) to purify the microvessel fragments. The microvessel fragments were washed once with DMEM then suspended in a basal medium (DMEM/F-12 containing 100 unit/mL penicillin-streptomycin, 50 μg/mL gentamicin, 2 mM GlutaMAX-I, 20% fetal bovine serum, and 1 ng/mL basic fibroblast growth factor (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 4 μg/mL puromycin (a translation inhibitor) which selectively kills microvessel cell types other than EC, due to high expression of the efflux pump P-glycoprotein selectively on EC (20, 21). This method does not alter EC viability (18). Microvessel fragments from the CNS tissues of 4 rats were pooled and seeded onto eight 10 cm petri dishes (coated with a mixture of extracellular matrix proteins including fibronectin, collagen type IV, and collagen type I, then maintained (37°C, 5% CO_2) for 48 hr in DMEM/F-12 with puromycin, followed by DMEM/F-12 without puromycin, until the EC reached confluence (1 week post-isolation), at which time there were approximately 2 million cells per dish. The cells were then passaged onto 96-well microplates (1 confluent dish of cells per microplate) for In-Cell Western blots or 24 well microplates (2 dishes of confluent cells per microplate) for qRT-PCR (microplates were again coated with the mixture of extracellular matrix proteins as above). The cells were used for experiments once they again reached confluence.

In-cell western blots

Cells were grown in 96-well microplates, and stimulated for 5 min or 30 min with LPS (10 ng/mL or 100 ng/mL) or (+) morphine (10 μM, 50 μM, or 100 μM). In-cell western blots were performed blind as to experimental condition. For each well, the medium was removed, and then 1 mL of fresh 4% paraformaldehyde was added and incubated for 20 min at room temperature. The cells were permeabilized by washing five times in PBS buffer + 0.1% Triton X-100 for 5 min per wash on a shaker at room temperature, then incubated in Odyssey blocking buffer (750 μl per well; LI-COR, Lincoln, NE) for 1 hr on a shaker at room temperature. The blocking buffer was removed and replaced with a solution containing primary antibody diluted in Odyssey blocking buffer. On each plate, 1 well was incubated with Odyssey blocking buffer that did not contain primary antibody, to serve as a negative control for the plate. Primary antibodies and dilutions used were: p-p38 MAPK (Thr180/Tyr182; Cell Signaling, Beverly, MA; 1:200); p-p44/42 MAPK (Erk1/2; Thr202/Tyr204; Cell Signaling; 1:400). The plates were incubated overnight at 4°C on a shaker, then washed the next morning 4 times with PBS + 0.1% Tween-20 for 5 min per wash at room temperature on a shaker. The cells were then incubated with a secondary antibody solution in Odyssey blocking buffer, containing secondary antibody, 0.2% Tween-20, and the cell stains DRAQ (Biostatus Limited, Leicestershire, UK), which emits fluorescence at 700 nm. The control well secondary antibody solution contained only 0.2% Tween-20 and secondary antibody in Odyssey blocking buffer. Secondary antibodies used were IRDye 800CW conjugates of goat-anti-rabbit-IgG and goat-anti-mouse-IgG (LI-COR Bioscience, Lincoln, NE, USA), which emit fluorescence at 800 nm. They were

diluted at 1:500 for p-p38 and p-ERK, The plates were incubated for 1 hr at room temperature on a shaker, protected from light, then washed 4 times with PBS + 0.1% Tween-20 for 5 min per wash at room temperature on a shaker while still protected from light. The wash buffer was then removed and the plate was tapped firmly against paper towels to remove as much buffer as possible. The plates were scanned and analyzed using an Odyssey IR scanner with Odyssey imaging software (LI-COR Bioscience, Lincoln, NE, USA). The antibody signals were analyzed for each well as the average 800 nm-channel integrated intensity normalized to the 700 nm-channel integrated intensity to correct for well-to-well variations in cell number. Results are expressed as percent relative responses compared to vehicle-treated control wells.

Behavioural assessment of responsivity radiant heat in rats

Pathogen-free adult male Sprague-Dawley rats (n=5-6 rats/group for each experiment; 300-375 g; Harlan Labs, Madison, WI, USA) were used for all experiments. Rats were housed in temperature (23 ± 3°C) and light (12h: 12h light: dark cycle; lights on at 07: 00) controlled rooms with standard rodent chow and water available *ad libitum*. All procedures were approved by the Institutional Animal Care and Use Committee of University of Colorado at Boulder. All testing was conducted blind with respect to group assignment. Rats received at least three 60 min habituations to the appropriate test environment prior to the behavioural testing. Thresholds for behavioural response to heat stimuli applied to the tail were assesses using modified Hargreaves test (22). Briefly, baseline withdrawal values were calculated from an average of two consecutive withdrawal latencies of the tail, measured at 15 min intervals. Latencies for the thermal stimulus at baseline ranged from 2 to 3 s and a cut-off time of 10 s was imposed to avoid tissue damage. Baseline withdrawal latency assessment were performed prior to, and again across a time course after drug administration. Vehicles were administrated equi-volume to the drugs under test. Data is shown as the percentage maximum effect which is (test - baseline/10s cut-off - baseline) ×100.

Behavioral assessment of responsivity radiant heat in TLR4-knockout and wild-type mice

Pathogen-free male Balb/c wild-type and TLR4 knockout mice, back crossed onto Balb/c 10 times were used for the TLR4 knockout studies (n=3-6 mice/group for each experiment; 24-32 g; kindly supplied by Dr. Simon Phipps and Prof Paul Foster and sourced from Prof. Akira). This TLR4 knockout strain has an established track record in the TLR4 literature (23). Mice were housed in temperature (23 ± 3 °C) and light (12 h: 12 h light: dark cycle; lights on at 07: 00) controlled rooms with standard rodent chow and water available *ad libitum*. All procedures were approved by the Animal Ethics Committee of the University of Adelaide. Mice received at least three 5 min habituations to the test environment prior to behavioural testing. Latencies for behavioural responses to the 50 ^{o}C hotplate were assessed. All testing was conducted blind to group assignment. A cut-off time of 60 s was imposed to avoid tissue damage. Latencies for the hotplate response ranged from 24 to 32 s. Baseline response latencies were recorded prior to drug administration. Examination of the effect of pharmacological TLR4 signalling blockade by Compound **1** and **2** in wild type and knockout mice was conducted by giving compound **1** (HCl salt, 82 mg/kg) or compound **2** (153 mg/kg) (intraperitoneal) 10 min prior to 2.5 mg/kg morphine (intraperitoneal) with hotplate latencies recorded prior to compound **1** and **2**, and morphine doses and 20 min after the morphine dose. Data are expressed as percent maximum potential effect (% MPE). For the construction of the dose response, mice (wild-type and TLR4-knockout) were injected twice daily (am & pm) with saline for 3 consecutive days. On day 4, hotplate latencies were assessed pre and 20 min post a challenge dose of morphine (0.1, 1, 2, 4, 10 and 50 mg/kg). Each opioid challenge dose consisted of 8 mice. Mice, which displayed freezing symptoms or urinated during hotplate latency testing, were excluded from data sets owing to errors in data collection. Mice behaviours that were two standard deviations from the mean were also excluded. Immediately following hotplate behavioural testing, mice were overdosed with sodium pentobarbital (400 mg/kg), perfused with 0.9% isotonic saline and then dissected to obtain spinal cord. The spinal cord tissue was homogenised in 1 mL of 1x denaturing buffer and then heated to 100°C in a heating block for 6 min to prevent protein aggregation and degradation. Spinal tissue were stored at -80 °C until use. Spinal lysates from wild-type and TLR4-knockout mice receiving 0.1, 2 and 50 mg/kg were tested for levels of p38, JNK and ERK phosphorylation. Total p38 was not measured owing to time limitations for transcription/translation.

Prior to analysis, spinal cord samples were diluted 1:4 using assay diluent and protein concentrations determined using BCA method. Cell lysates were prepared as per BD™ Cytometric Bead Array kit to label phosphorylated proteins. Following washing, the samples were analysed using a BD™ fluorescence-activated cell-sorting machine.

Statistical analysis

Analysis of variance was carried out using one-way ANOVA with Dunnett's *post hoc* test. Data are presented as mean ± standard deviation of the mean.

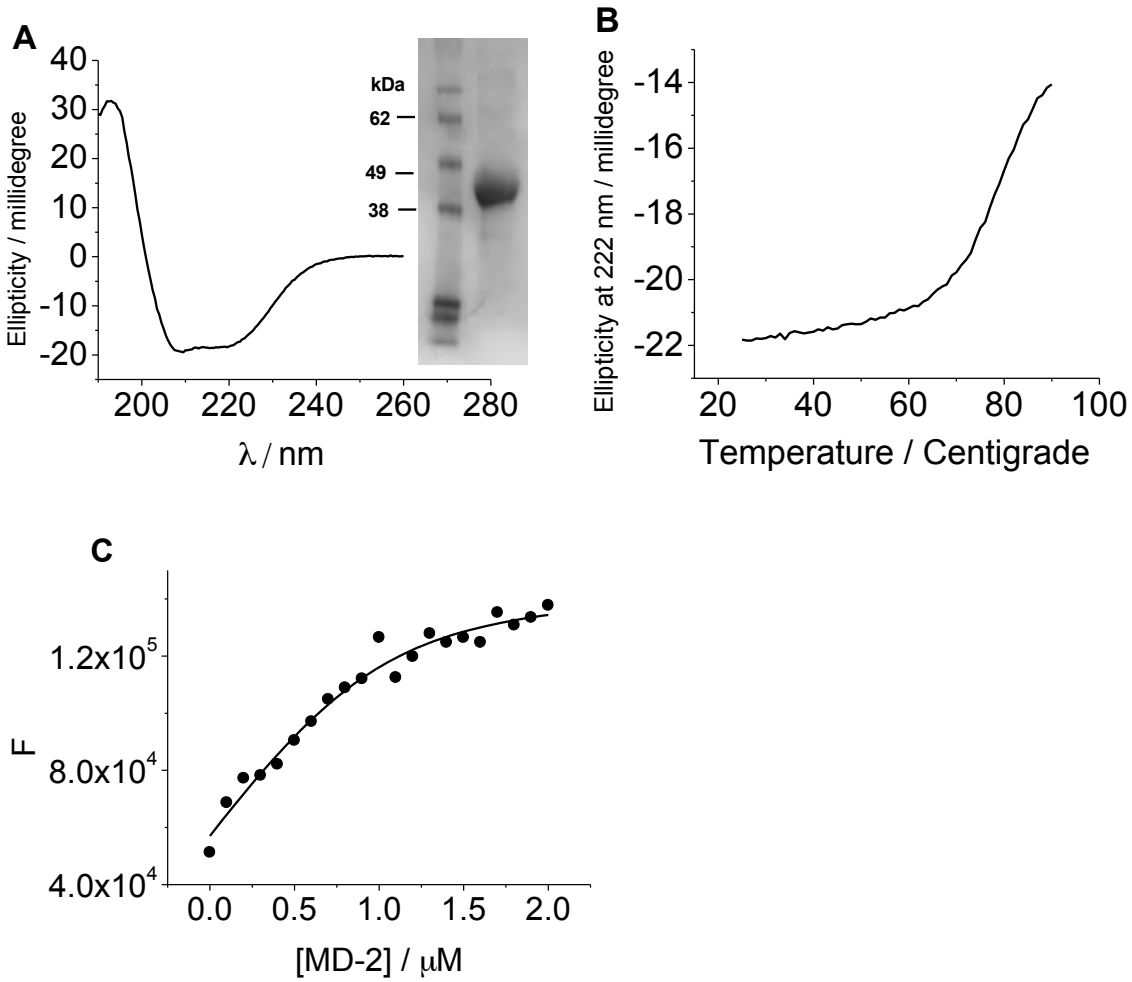

Fig. S1. Characterization of prepared human MD-2 (protein A tagged). (A), Far UV CD spectra of 5 μM MD-2. CD spectrum was measured on an Applied Photophysics Chirascan™-plus Circular Dichroism spectrometer. (B), Thermal denaturing curve of MD-2 (5 μM). CD signal at 222 nm was monitored as a function of temperature. (C), Binding curve of MD-2 with curcumin. Different concentrations of MD-2 were titrated into 1 μM curcumin (a known MD-2 ligand). 430 nm was chosen as the excitation wavelength and 494 nm was chosen as the emission wavelength. A K_d of 0.55 ± 0.36 μM was obtained by fitting the binding curve to one-site saturation model as previously reported (5).

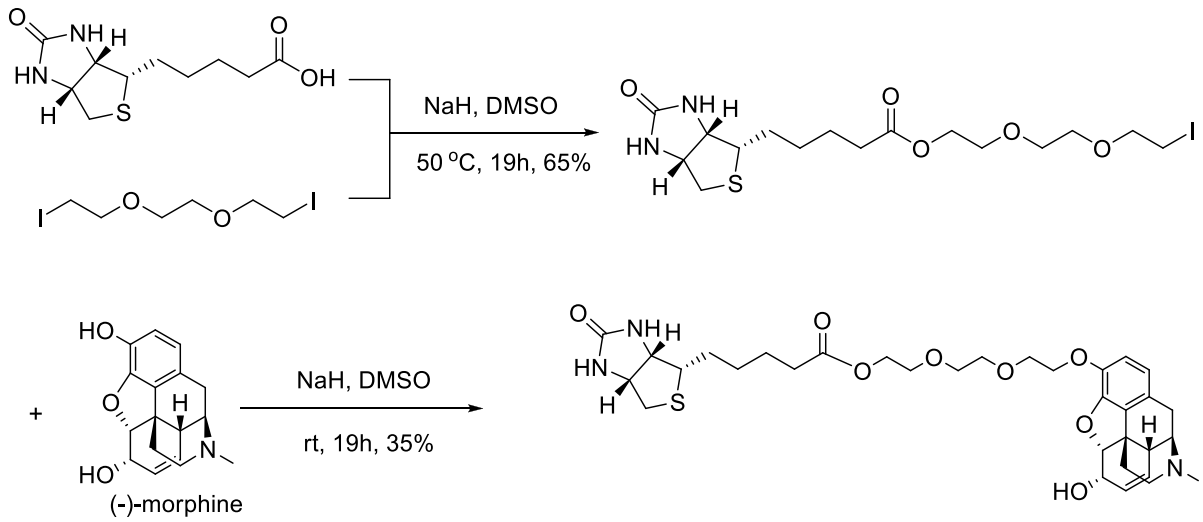

Fig. S2. Synthesis of biotin-morphine.

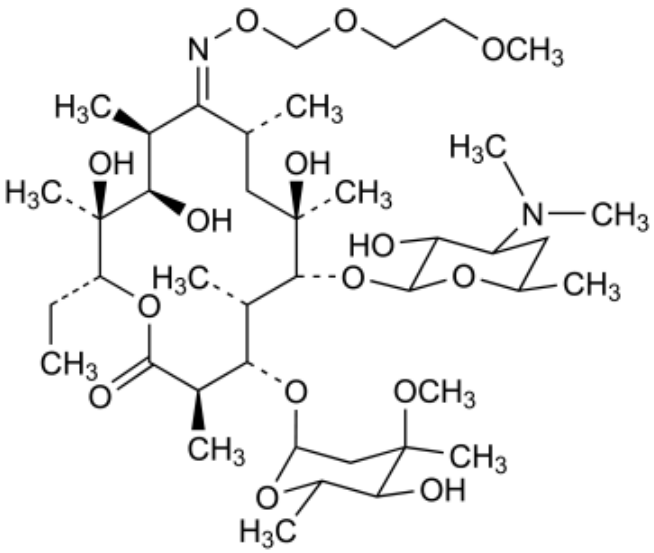

Fig. S3. Structure of roxithromycin.

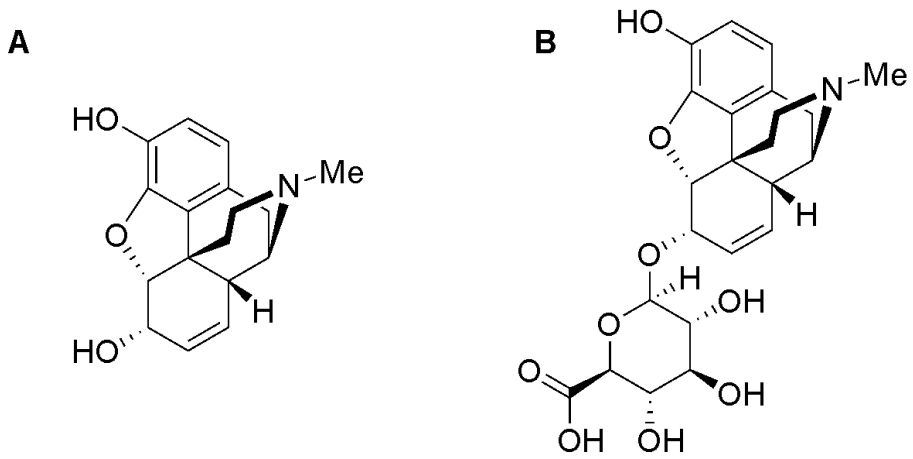

Fig. S4. Structure of morphine (A) and morphine-6-glucuronide (B, M6G).

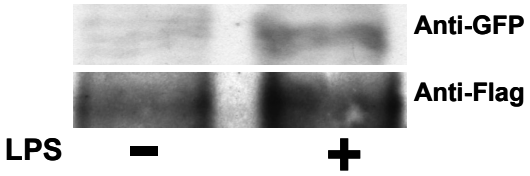

Fig. S5. LPS induces TLR4 receptor oligomerization. Ba/F3 cells simultaneously over-expressing human TLR4-Flag, human TLR4-GFP, human CD14, and human MD-2 were stimulated with LPS (1 μg/mL) for 30 min. Cells were then subjected to immunoprecipitation with anti-Flag antibody and immunoprobing with anti-GFP antibody (upper panel) and anti-Flag antibody (lower panel).

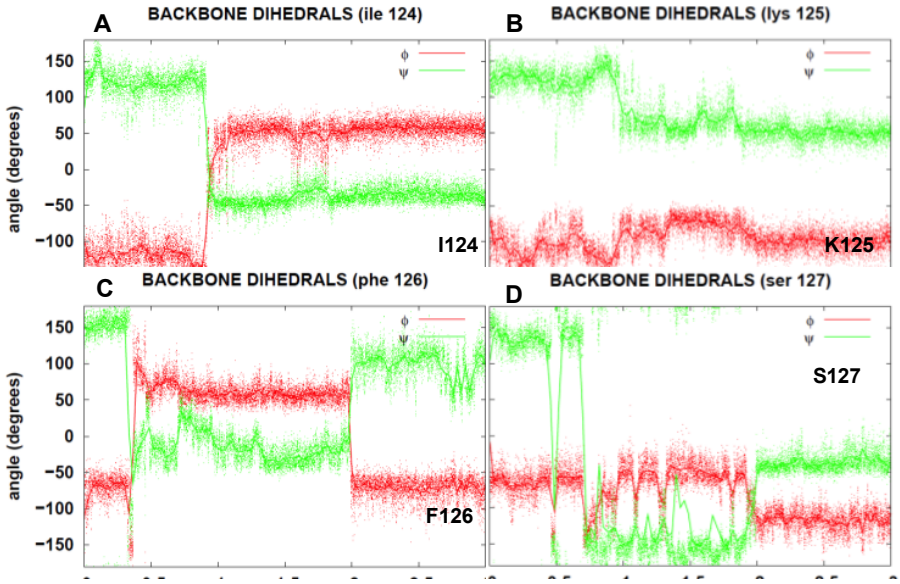

Fig. S6. The backbone dihedral angles during the course of simulation (3 ns) illustrating the transitions occurring in the backbone of the indicated residues in the F126 loop. ϕ is defined as the dihedral angle formed by the atoms C(O)-N-C(α)-C(O) while ψ is defined by the atoms N-C(α)-C(O)-N.

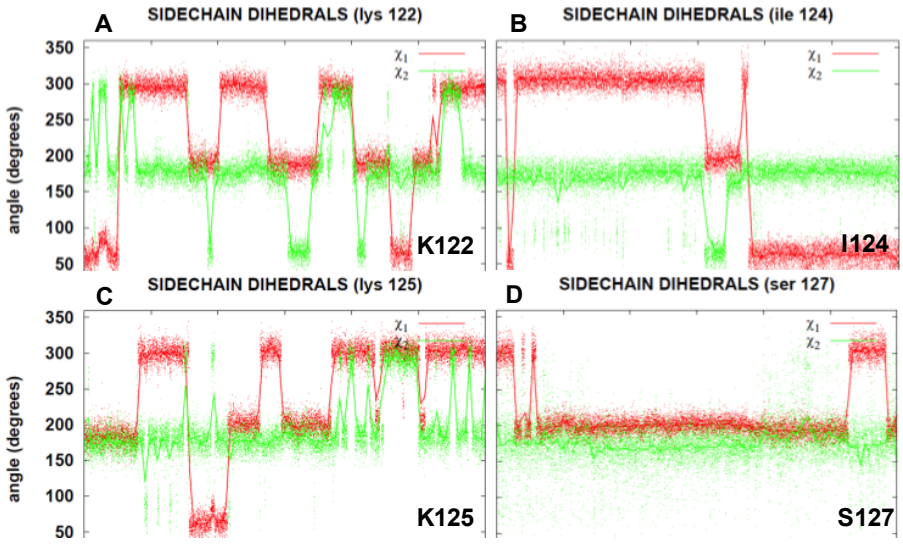

Fig. S7. The side chain dihedral angles χ_1 and χ_2 during the course of simulation illustrating the transitions occurring in the sidechains of the indicated residues in the F126 loop. χ_1 is defined as the dihedral angle formed by the atoms N-Cα –Cβ-Cγ while χ_2 is defined by the atoms Cα-Cβ-Cγ-Cδ.

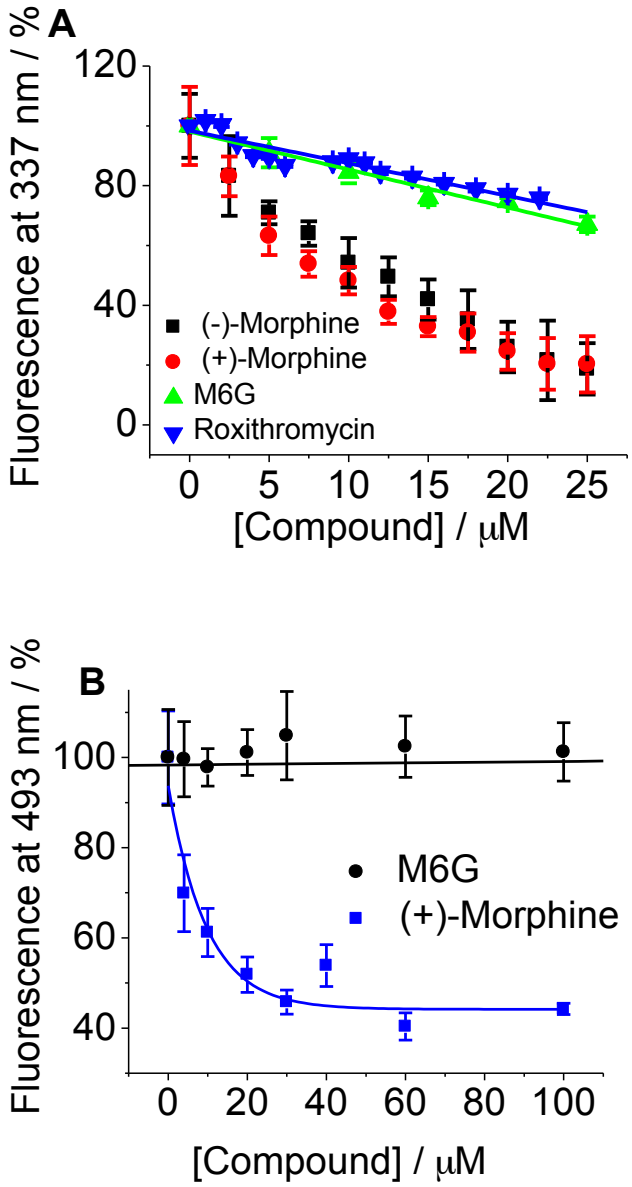

Fig. S8. (A), Titration of MD-2 with morphine and controls. MD-2 (1 μM) was titrated with different concentrations of (-)-morphine or (+)-morphine. Roxithromycin and morphine-6-glucuronide (M6G) were served as negative controls. The wavelength of 280 nm was chosen as the excitation wavelength of MD-2 intrinsic fluorescence. The fluorescence signal at 337 nm, after correction with the basal fluorescence subtracted, was plotted against titrated drug concentrations. Fluorescence intensity of MD-2 at the absence of ligand was normalized as 100%. (B), (+)-Morphine replaced curcumin binding to MD-2. Different concentrations of (+)-morphine or M6G were titrated into MD-2 (0.5 μM). Curcumin (0.5 μM) was added as the extrinsic fluorescence probe. To selectively excite curcumin, 430 nm was chosen as the excitation wavelength. Fluorescence signal at 493 nm was plotted against the titrated morphine concentration. Fluorescence intensity of curcumin-MD-2 complex at the absence of (+)-morphine was normalized as 100%. Data fitting to a one-site competitive model gives a K_i of 5.0 ± 3.3 μM.

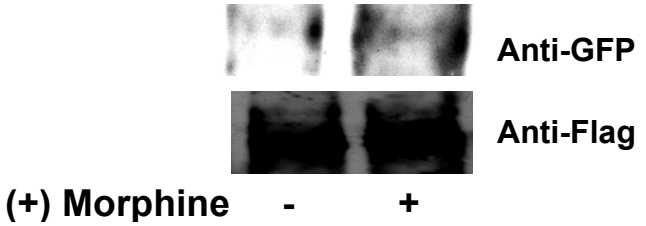

Fig. S9. (+)-Morphine induces TLR4 receptor oligomerization. Ba/F3 cells simultaneously over-expressing human TLR4-Flag, human TLR4-GFP, human CD14, and human MD-2 were stimulated with LPS (300 μM) for 72 h. Cells were then subjected to immunoprecipitation with anti-Flag antibody and immunoprobing with anti-GFP antibody (upper) and anti-Flag antibody (lower).

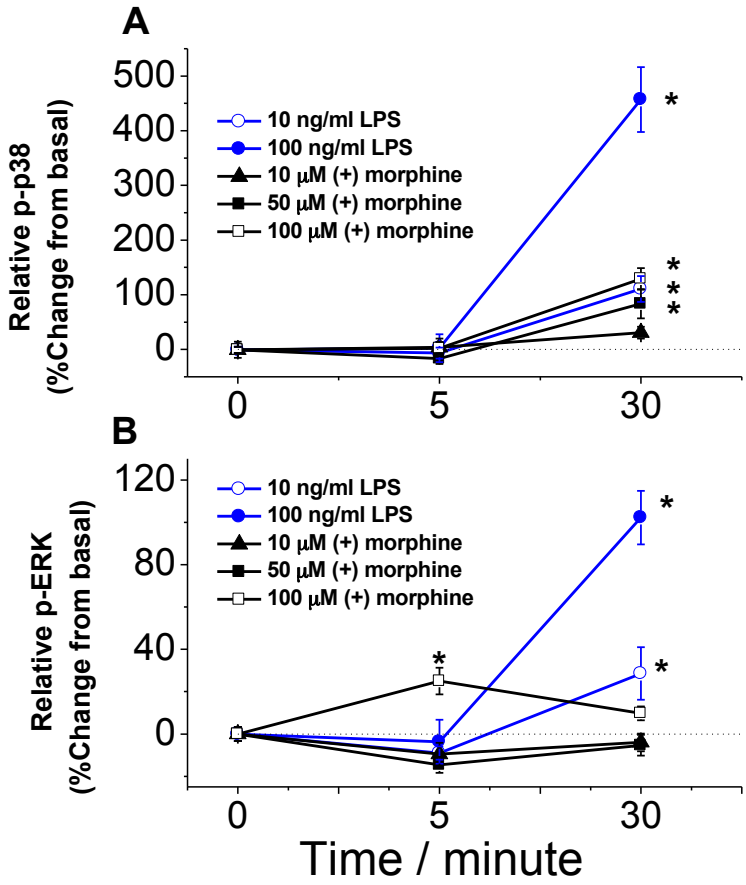

Fig. S10. Primary adult rat CNS endothelial cells exhibit rapid phosphorylation of the MAP kinases p38 and ERK in response to LPS or (+)-morphine. (A), LPS (100 ng/mL and 10 ng/mL) and (+)-morphine (100 μM and 50 μM) caused dose-dependent increases in p38 phosphorylation after 30 min but not 5 min. (B), LPS and (+)-morphine also caused dose-dependent increases in ERK phosphorylation. The effects of LPS (100 ng/mL and 10 ng/mL) were observed at 30 min but not 5 min, whereas the effect of (+)-morphine (100 μM) was observed at 5 min. 8 wells within a 96 well microplate were used for each dose/time point. * $P < 0.05$ versus 0 min.

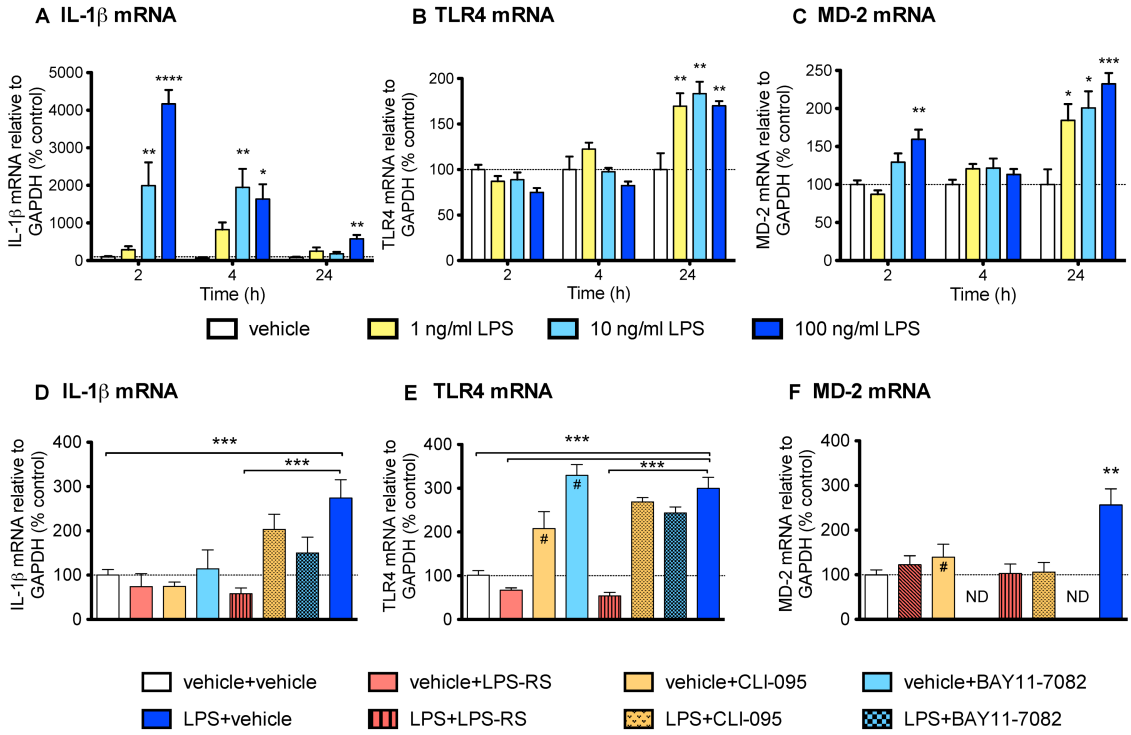

Fig. S11. Isolated primary CNS endothelial cells incubated with lipopolysaccharide (LPS, 0. 1 ng/mL, 10 ng/mL and 100 ng/mL) induce pro-inflammatory mediator (IL-1β) and TLR (TLR4 and the co-receptor MD-2) mRNA upregulation following 2 h, 4 h and 24 h incubations (n=6 per group per time interval). (A), 100 ng/mL LPS increases IL-1β mRNA at 2 h with a gradual decline in IL-1mRNA expression by 24 h. (B) and (C) show a gradual increase in TLR4 and MD-2 mRNA expression over time with the 100 ng/mL LPS compared to vehicle control. 100 ng/mL LPS was co-incubated for 24 h with 10 ng/mL LPS-RS, a competitive TLR-4 antagonist, 1 μM CLI-095, an intracellular TLR4 antagonist and 1 μM BAY11-7082, an IκB-α inhibitor. LPS-RS significantly attenuated LPS-induced IL-1β (panel D) and TLR4 (panel E) mRNA. CLI-095 significantly attenuated TLR4 (panel E) and MD-2 (panel F) mRNA. BAY11-7082 significantly attenuated IL-1β (panel D), TLR4 (panel E) and MD-2 (panel F) mRNA. Experimental procedures are described in the supplemental materials and methods sections. * $P< 0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$. 1-way ANOVA compared to vehicle control, with Bonferroni *post-hoc* comparisons.

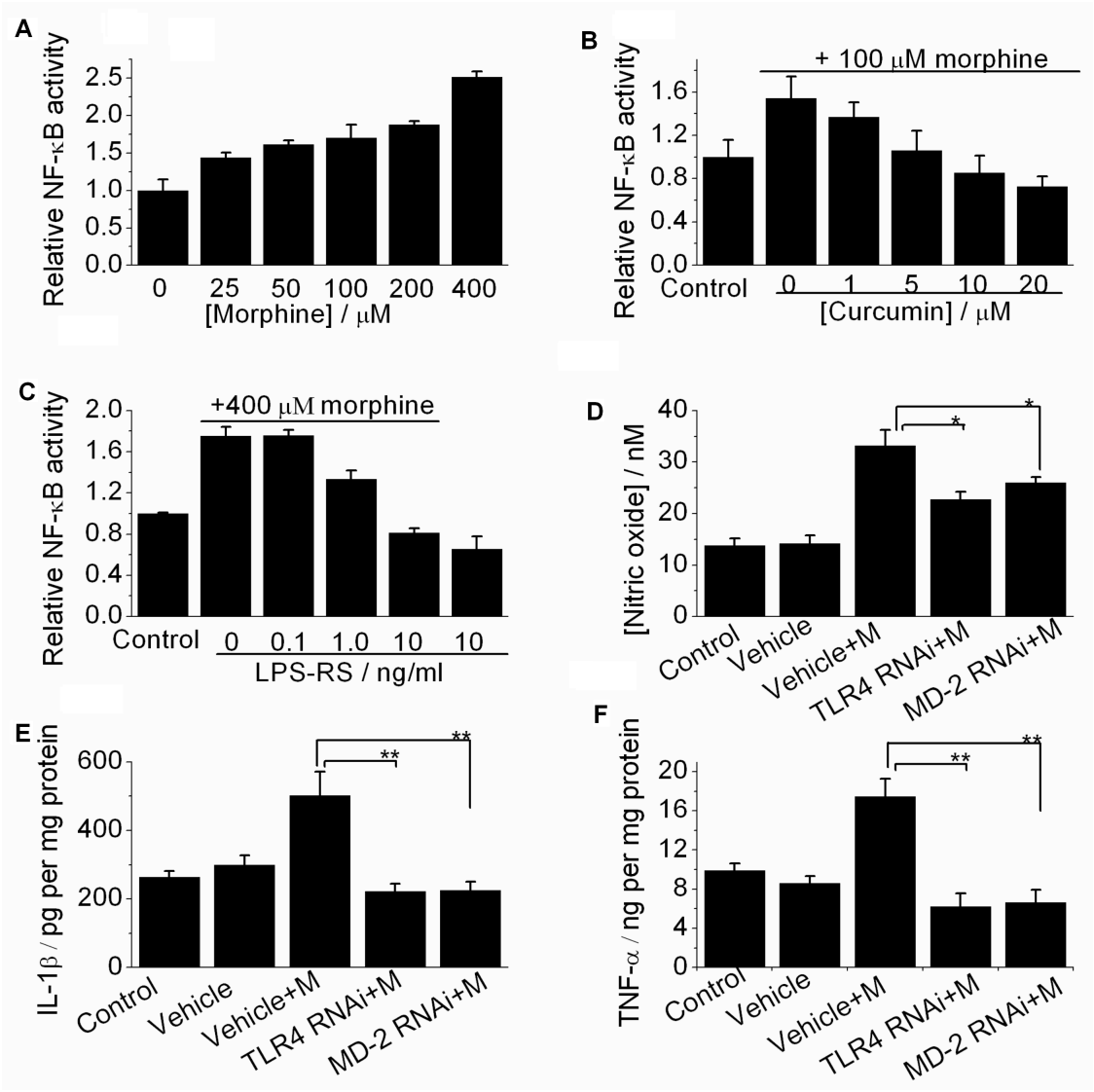

Fig. S12. Morphine induced proinflammatory activation is mediated by TLR4/MD-2. NF-κB activation and proinflammatory factors (including NO, IL-1β and TNF-α) over-production were monitored for proinflammatory microglial cell activation. (A), BV-2 dual luciferase NF-κB reporter cells were treated with different concentrations of morphine for 72 h. NF-κB activity was analyzed by Dual-Glo luciferase assay. (B), BV-2 dual luciferase NF-κB reporter cells were stimulated with morphine (100 μM) in the presence of different concentrations of curcumin for 72 h. NF-κB activity was determined as shown in (A). (C), NF-κB HEK 293 reporter cells, in which a secreted embryonic alkaline phosphatase (SEAP) reporter gene is placed under the control of NF-κB promoter, were stimulated with morphine (400 μM) or morphine (400 μM) and different concentrations of LPS-RS for 72 h. NF-κB activity was analyzed by SEAP assay and the NF-κB activity of the untreated control group was set as 1. (D), After 48 h RNAi, cells were stimulated by 400 μM of morphine for 24 h and NO produced in the media was measured by the well established 2,3-diaminonaphthalene assay. * $P<0.05$. (E, F), RNAi knockdown of TLR4/MD-2 inhibits morphine-induced IL-1β (E) and TNF-α (F) over-expression in microglial BV-2 cells. After 48 h RNAi, cells were stimulated by 200 μM of morphine for 24 h and IL-1β (E) and TNF-α (F) in the cell lysates were measured by ELISA. ** $P<0.01$.

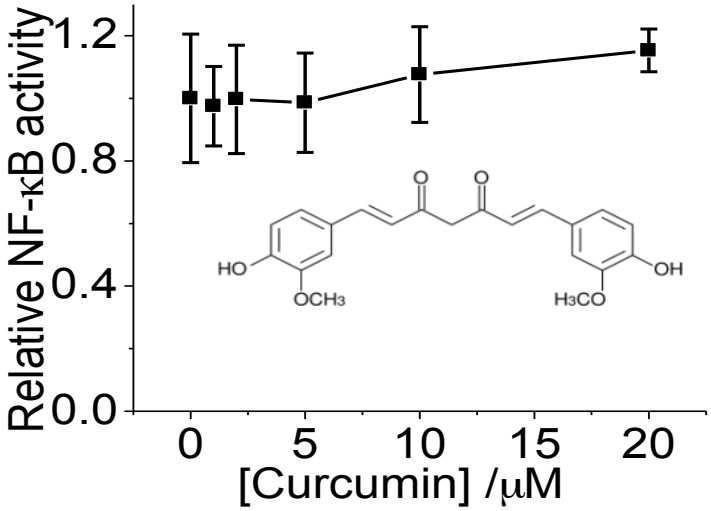

Fig. S13. Effect of curcumin on the NF-κB activity. NF-κB dual luciferase reporter BV-2 cells were cultured in DMEM medium supplemented with 10% FBS, penicillin (50 unit/mL), streptomycin (50 μg/mL) and puromycin (4 μg/mL). BV-2 reporter cells were seeded at a density of 1×10^4 cells/well in 96-well plates. After 24 h incubation, medium was changed to Opti-MEM medium supplemented with 0.5% FBS, penicillin (50 unit/mL), streptomycin (50 μg/mL) and 1% of non-essential amino acid (NEAA) and different concentrations of curcumin were added. After further 72 h treatment, the NF-κB activity was analysed by Dual-Glo Luciferase Assay System (Promega, Madison, MI, USA) according to manufacture's instruction. The ratio of Firefly luciferase activity to Renilla luciferase activity represents the NF-κB activity. It should be noted that NF-κB activity of the untreated control group was set as 1. Inset: the structure of curcumin.

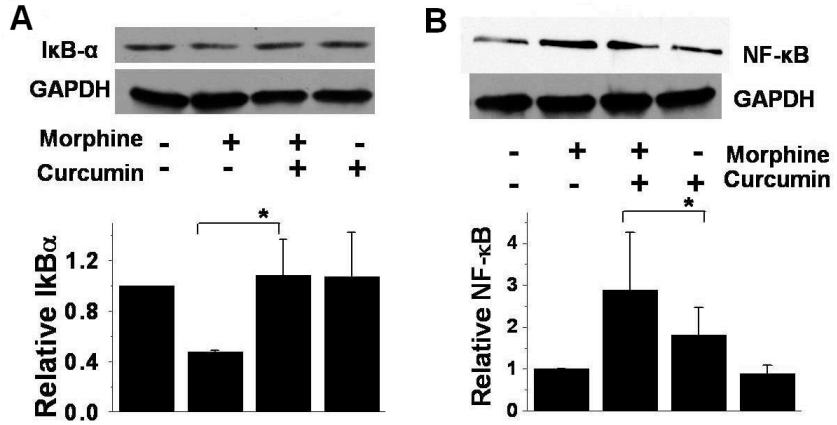

Fig. S14. Morphine induces TLR4 signaling in microglial BV-2 cells. (A), BV-2 cells were treated with morphine (200 μM), curcumin (20 μM) or morphine (200 μM) and curcumin (20 μM) for 72 h. Cell lysates were prepared and subjected to immunoprobing with anti-IκBα and anti-GAPDH antibodies. Upper panel: representative of three independent experiments. Lower panel: quantitative analysis of IκBα expression level relative to untreated control group. **P*<0.05 versus. morphine treated group by Student's t-test. (B), BV-2 cells were treated as described in (A) and cell lysates were subjected to immunoprobing with anti-NF-κB p65 and anti-GAPDH antibodies. Upper panel: representative of two independent experiments. Lower panel: quantitative analysis of NF-κB expression level relative to untreated control group. **P*<0.05 versus. morphine treated group. It should be note here that morphine used here is natural (-)-morphine.

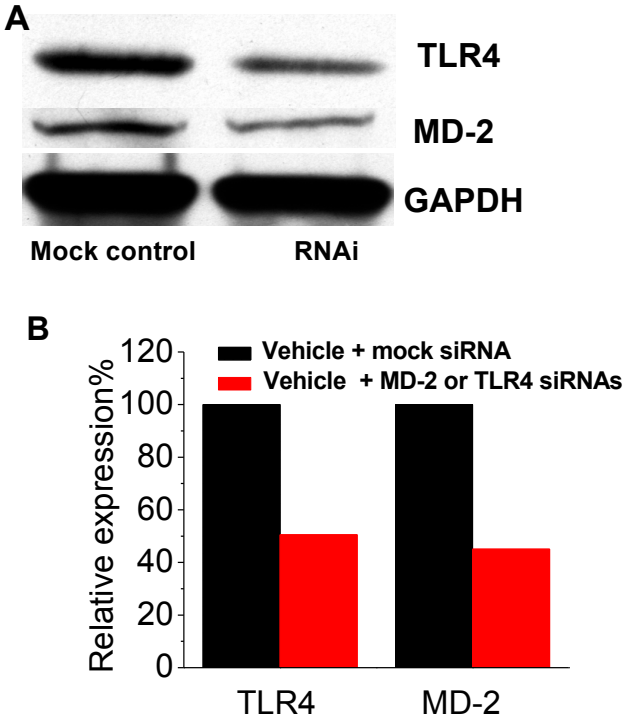

Fig. S15. Western blotting analysis of RNAi efficiency. RNAi was performed as described in the SI Materials and Methods section. After 72 h of RNAi, cells were harvested and lysed by mammalian protein extraction reagent (Thermo Scientific, Rockford, IL, USA). Western blot was performed as described in the SI Materials and Methods section. GAPDH was served as an internal control. (A), western blot image. (B), quantitative analysis.

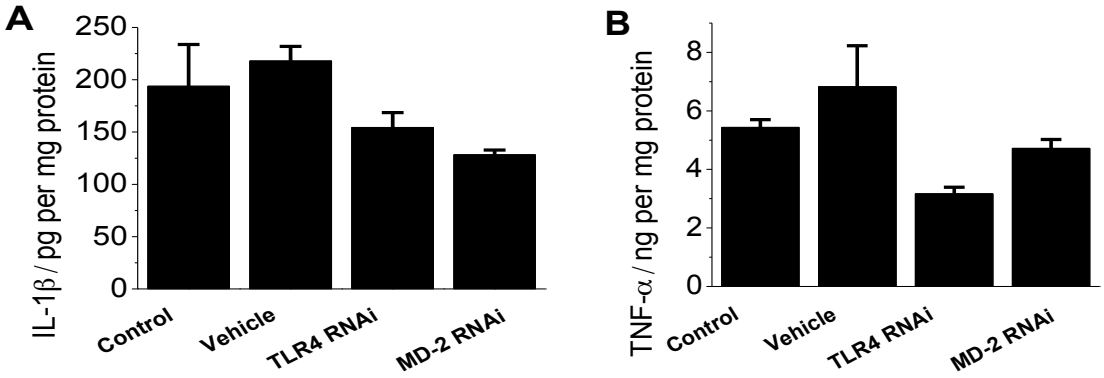

Fig. S16. Effects of TLR4 or MD-2 RNAi knockdown to the inflammatory factors IL-1β (A) and TNF-α (B). RNAi was performed as described in the SI Materials and Methods section. After 72 h of RNAi, cells were harvested and lysed by mammalian protein extraction reagent (Thermo Scientific, Rockford, IL, USA). IL-1 β and TNF-α levels were analyzed by ELISA (BD Bioscience, San Diego, CA, USA) according to manufacture's instructions. The IL-1 β and TNF-α result showed here have been normalized to total protein concentration.

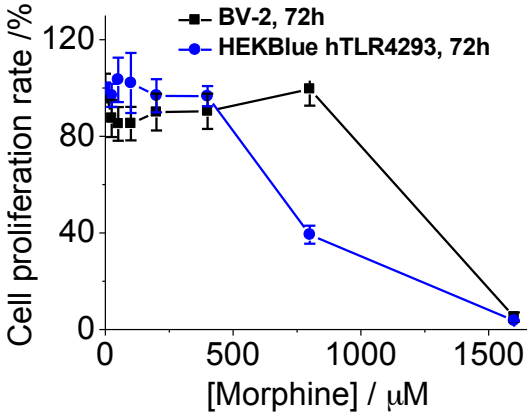

Fig. S17. WST-1 cell proliferation assay. Cells were seeded at a concentration of 4×10^4 cells/mL and grown for 24 h. After that, different concentrations of natural (-)-morphine were added and cultures were incubated for additional 72 h. 10 μL of WST-1 reagent (Roche, Mannheim, Germany) was then added into each well and incubated for additional 1 h. The absorbance at 450 nm was measured on a Beckman-coulter DTX 880 microplate reader and 620 nm was chosen as the reference wavelength. The $A_{450\ nm}$-$A_{620\ nm}$ for the control group was set as 100%.

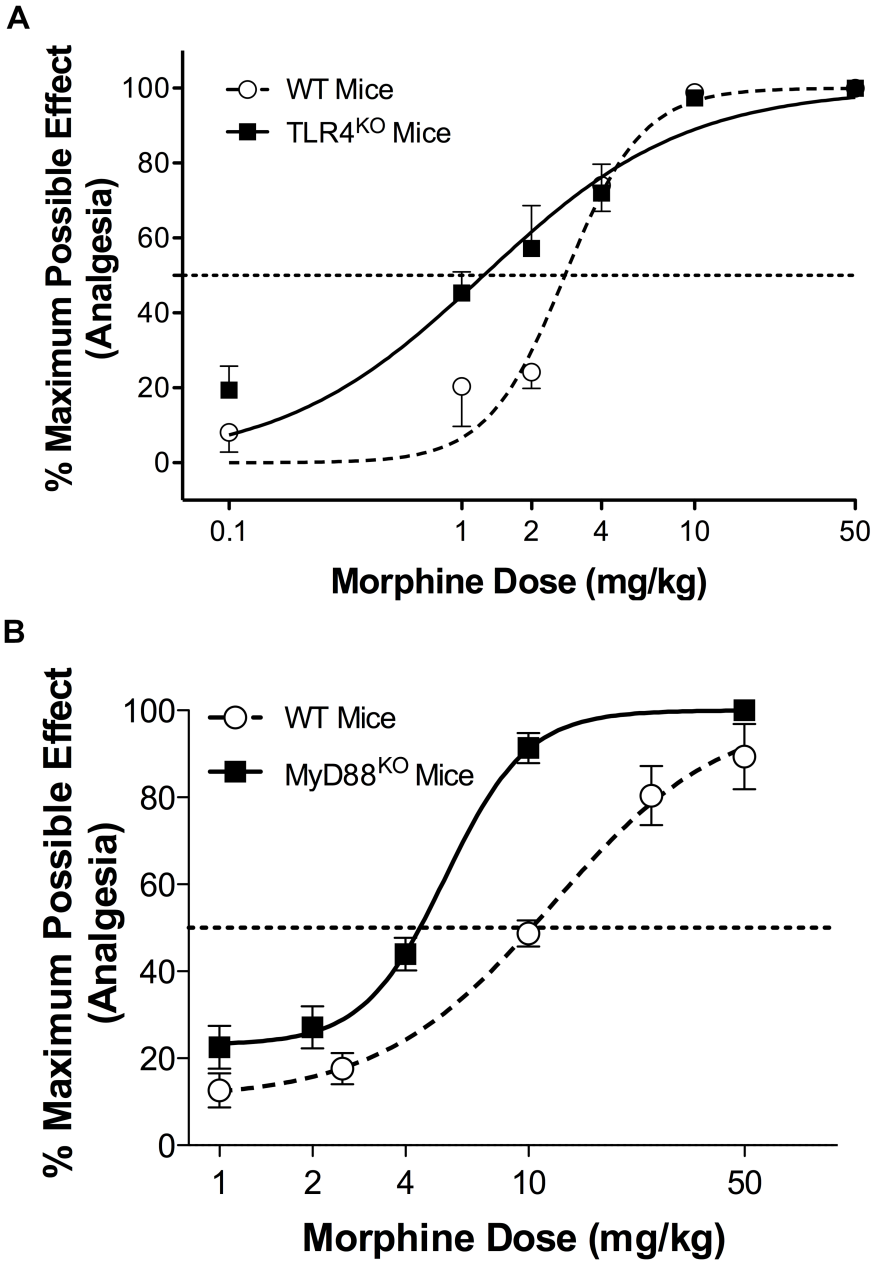

Fig. 18. TLR4^{KO} (A) and MyD88^{KO} (B) mice demonstrate significantly greater analgesia when administered with morphine compared to WT controls. Following pre-drug (baseline) assessment of responsivity to radiant heat (Hargreaves test) mice received 3 days of intraperitoneal saline injections. On day 4, mice received an acute single dose of morphine (0.1, 1, 2, 4, 10 or 50 mg/kg) and were tested for nociceptive response 20 min post dose. Data are expressed as mean %MPE taken from 8 animals.

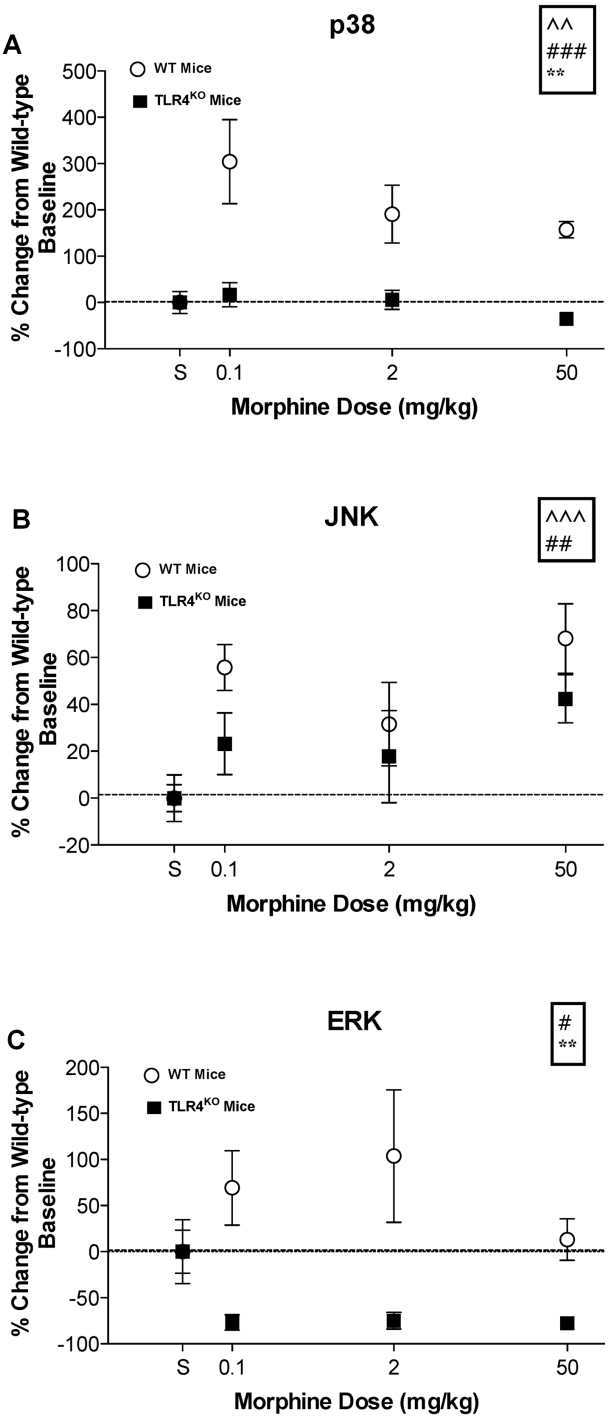

Fig. 19. TLR4-knockout significantly reduces morphine-induced MAP kinase signalling. MAP kinase signalling molecules (p38, JNK and ERK) were measured from wild-type and TLR4-knockout spinal cords for phosphorylation following acute morphine administration (0.1, 2 or 50 mg/kg). (A), Morphine had a main dose and strain effect and interaction on p38 phosphorylation. Post hoc analysis revealed at doses of 0.1 and 50 mg/kg of morphine, significant changes in phosphorylation occurred between wild-type and TLR4-knockout mice. (B), Morphine had a main dose and strain effect on JNK phosphorylation. (C), Morphine had a main strain effect and interaction on ERK phosphorylation. Main effect: Dose- ^ P<0.05, ^^ P <0.01, ^^^ P<0.001. Strain- # P<0.05, ## P<0.01, ### P<0.001. Interaction- + P<0.05, ++ P<0.01. Post hoc- * P<0.05, ** P<0.01, *** P<0.001. n=8/dose.

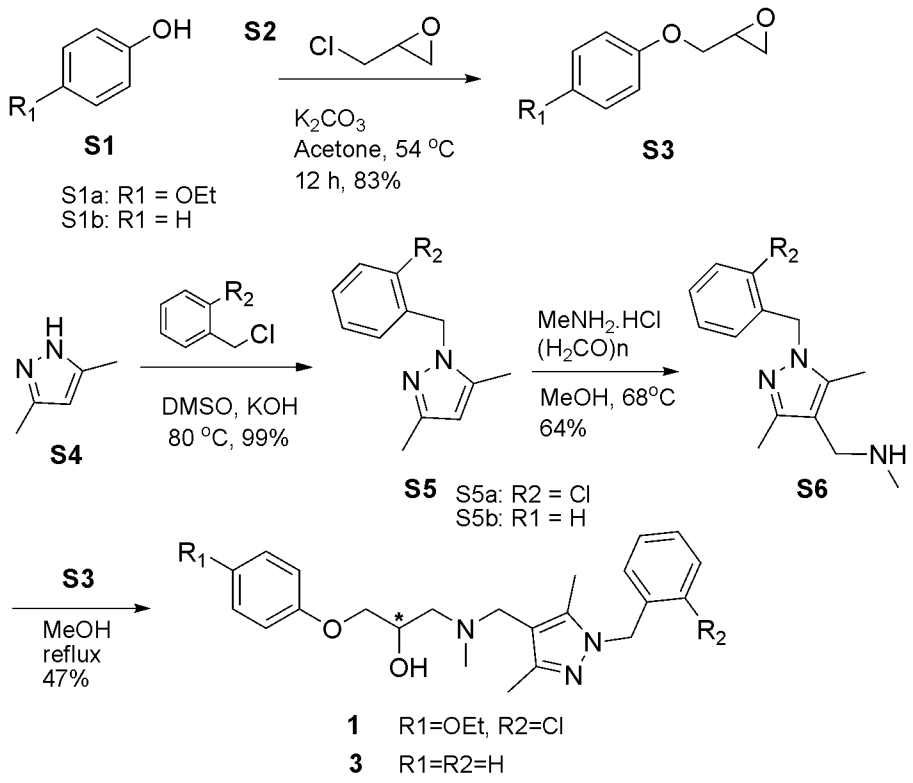

Fig. S20. Synthesis of racemic compounds **1** and **3**. Asterisk (*) represents the stereo-center.

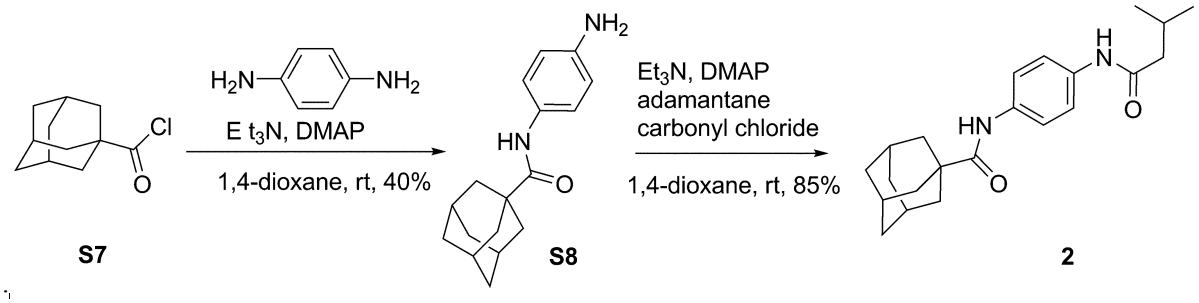

Fig. S21. Synthesis of compound **2**.

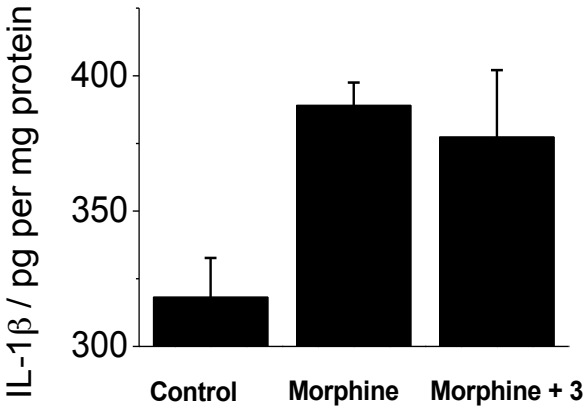

Fig. S22. Compound **3** does not inhibit morphine-induced microglial BV-2 cells inflammatory factor IL-1β over-production. BV-2 cells were incubated in the presence of 10 μM of compound **3**, morphine (200 μM), morphine (200 μM) and 10 μM of compound **3** for 24 h. Cell lysates from BV-2 cells were assayed for IL-1β protein using commercially available ELISA kit.

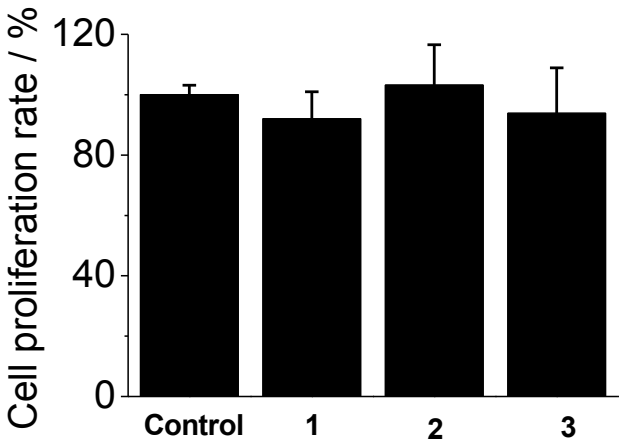

Fig. S23. Chemical probes demonstrating negligible cytotoxicity. WST-1 cell proliferation assay results that show 10 μM of compound **1,** compound **2** and compound **3** have no apparent growth inhibition in BV-2 cells. BV-2 cells were grown in DMEM medium supplemented with 10% FBS. Cells were detached from culture flask by trypsin digestion when 80% confluence. 100 μL of cell suspension was added to each well of 96-well plate (2500 cells per well) and grown for 24 h. After that, 10 μM compound **1,** compound **2** or compound **3** was added and culture was incubated for additional 24 h. 10 μL of WST-1 reagent (Roche, Mannheim, Germany) was then added into each well and incubated for additional 1 h. The absorbance at 450 nm was measured on a Beckman-coulter DTX 880 microplate reader and 620 nm was chosen as the reference wavelength. The $A_{450\ nm}$-$A_{620\ nm}$ for the control group was set as 100%.

SI References

1. Viriyakosol S*, et al.* (2006) Characterization of monoclonal antibodies to human soluble MD-2 protein. *Hybridoma (Larchmt)* 25:349-357.
2. Saitoh S*, et al.* (2004) Lipid A antagonist, lipid IVa, is distinct from lipid A in interaction with Toll-like receptor 4 (TLR4)-MD-2 and ligand-induced TLR4 oligomerization. *Int. Immunol.* 16:961-969.
3. Park BS*, et al.* (2009) The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. *Nature* 458:1191-1195.
4. Visintin A, Halmen KA, Latz E, Monks BG, & Golenbock DT (2005) Pharmacological inhibition of endotoxin responses is achieved by targeting the TLR4 coreceptor, MD-2. *J. Immunol.* 175:6465-6472.
5. Gradisar H, Keber MM, Pristovsek P, & Jerala R (2007) MD-2 as the target of curcumin in the inhibition of response to LPS. *J. Leukoc. Biol.* 82:968-974.
6. Youn HS, Saitoh SI, Miyake K, & Hwang DH (2006) Inhibition of homodimerization of Toll-like receptor 4 by curcumin. *Biochem. Pharmacol.* 72:62-69.
7. Li Y*, et al.* (2008) Design, Synthesis, and Evaluation of Biotinylated Opioid Derivatives as Novel Probes to Study Opioid Pharmacology. *Bioconjug. Chem.* 19:2585-2589.
8. Bevan DE*, et al.* (2010) Selection, preparation, and evaluation of small-molecule inhibitors of toll-like receptor 4. *ACS Med. Chem. Lett.* 1:194-198.
9. Joce CM*, et al.* (2010) Application of a novel in silico high throughput screen to identify selective inhibitors for protein-protein interactions. *Bioorg. Med. Chem. Lett.* 20:5411-5413.
10. Liu LP*, et al.* (2011) An MD2 hot-spot mimicking peptide that suppresses TLR4-mediated inflammatory response in vitro and in vivo. *ChemBioChem* 12:1827-1831.
11. Slivka PF*, et al.* (2009) A Peptide Antagonist of the TLR4–MD2 Interaction. *ChemBioChem* 10:645-649.
12. Im W, Lee MS, & Brooks CL, 3rd (2003) Generalized born model with a simple smoothing function. *J. Comput. Chem.* 24:1691-1702.

13. Zimmer SM, Liu J, Clayton JL, Stephens DS, & Snyder JP (2008) Paclitaxel binding to human and murine MD-2. *J. Biol. Chem.* 283:27916-27926.
14. MacKerell AD*, et al.* (1998) All-Atom Empirical Potential for Molecular Modeling and Dynamics Studies of Proteins†. *J. Phys. Chem. B* 102:3586-3616.
15. Mackerell AD, Jr., Feig M, & Brooks CL, 3rd (2004) Extending the treatment of backbone energetics in protein force fields: limitations of gas-phase quantum mechanics in reproducing protein conformational distributions in molecular dynamics simulations. *J. Comput. Chem.* 25:1400-1415.
16. Vanommeslaeghe K*, et al.* (2010) CHARMM general force field: A force field for drug-like molecules compatible with the CHARMM all-atom additive biological force fields. *J. Comput. Chem.* 31:671-690.
17. Nolan T, Hands RE, & Bustin SA (2006) Quantification of mRNA using real-time RT-PCR. *Nat. Protoc.* 1:1559-1582.
18. Perriere N*, et al.* (2005) Puromycin-based purification of rat brain capillary endothelial cell cultures. Effect on the expression of blood-brain barrier-specific properties. *J. Neurochem.* 93:279-289.
19. Verma S, Nakaoke R, Dohgu S, & Banks WA (2006) Release of cytokines by brain endothelial cells: A polarized response to lipopolysaccharide. *Brain Behav. Immun.* 20:449-455.
20. Beaulieu E, Demeule M, Ghitescu L, & Beliveau R (1997) P-glycoprotein is strongly expressed in the luminal membranes of the endothelium of blood vessels in the brain. *Biochem. J.* 326 (Pt 2):539-544.
21. Cordon-Cardo C*, et al.* (1989) Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood-brain barrier sites. *Proc. Natl. Acad. Sci. USA* 86:695-698.
22. Hargreaves K, Dubner R, Brown F, Flores C, & Joris J (1988) A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* 32:77-88.
23. Hoshino K*, et al.* (1999) Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J. Immunol.* 162:3749-3752.