Supporting Information

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SI Methods

Materials

2-AG, d_5-2-AG, AEA, d_4-AEA, d_4-PEA, and pentadecanoic acid (PDA) were purchased from Cayman Chemicals. Monopentadecanoin and monoheptadecanoin were purachased from Nu-Chek-Prep, Inc. FP-rhodamine and FP-biotin were synthesized as described previously (1, 2). Rimbonabant was obtained from National Institute on Drug Abuse (Rockville, MD) and dissolved in a vehicle of 18:1:1 vol/vol/v saline, ethanol, and alkamuls-620 (Rhone-Poulenc, Princeton, NJ).

General Synthetic Methods. All reagents were purchased from Sigma-Aldrich, Acros, Fisher, Fluka, or Maybridge and used without further purification, except where noted. Dry solvents were obtained by passing commercially available predried, oxygen-free formulations through activated alumina columns. All reactions were carried out under a nitrogen atmosphere using oven-dried glassware unless otherwise noted. Flash chromatography was performed using 230–400 mesh silica gel. NMR spectra were recorded in $CDCl_3$ on a Varian Inova-400 or a Bruker DMX-600 spectrometer and were referenced to trimethylsilane (TMS) or the residual solvent peak. Chemical shifts are reported in ppm relative to TMS and J values are reported in Hz. High-resolution mass spectrometry (HRMS) experiments were performed at the Scripps Research Institute Mass Spectrometry Core on an Agilent mass spectrometer using electrospray ionization–time of flight (ESI-TOF). The synthesis, characterization, and inhibitory properties of other dual FAAH/MAGL inhibitors will be reported elsewhere.

Chemical Synthesis of JZL195. To a stirring solution of *N*-Boc piperazine (2.81 g, 15.1 mmol) in CH_2Cl_2 (30 ml) was sequentially added K_2CO_3 (3.7 g, 28 mmol) and 1-(bromomethyl)-3-phenoxybenzene (2.0 g, 7.6 mmol). After the reagent addition was complete, the reaction was heated to reflux overnight. After 24 h, the reaction was quenched with water and diluted with EtOAc. The layers were separated and the organic layer was washed once with water, once with brine, dried over Na_2SO_4, and concentrated in vacuo. Purification of the crude oil via flash chromatography (Hex:EtOAc = 9:1) gave the Boc-protected intermediate (1.31 g, 47% yield): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.36–7.26 (m, 3H), 7.10–6.99 (m, 5H), 6.89 (ddd, J_1 = 0.9 Hz, J_2 = 2.5 Hz, J_3 = 8.1 Hz, 1H), 3.47 (s, 2H), 3.42 (t, J = 5 Hz, 4H), 2.38 (t, J = 5 Hz, 4H), 1.62 (bs, 1H), 1.46 (s, 9H). To the Boc-protected intermediate (1.3 g, 3.5 mmol) was added 90% vol/vol TFA/CH_2Cl_2 (10 ml) at room temperature. After 12 h, the purple solution was concentrated in vacuo and used directly in the next step. To a stirring solution of the crude amine in CH_2Cl_2 (10 ml) was added DIPEA (6.5 ml, 37 mmol) and the color of the reaction changed from purple to pale yellow. 4-Nitrophenyl chloroformate (818 mg, 4.07 mmol) was subsequently added at room temperature. After stirring overnight, the reaction was quenched with 2 N aq. NaOH and diluted with EtOAc. The layers were separated and the organic layer was washed thrice with 2 N aq. NaOH, twice with brine, dried over Na_2SO_4, and concentrated in vacuo. Purification of the crude oil via flash chromatography (Hex:EtOAc = 7:1 then 3:1) gave JZL195 as a pale yellow solid (600 mg, 40% yield over two steps): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.24–8.22 (m, 2H), 7.36–7.27 (m, 5H), 7.13–7.01 (m, 5H), 6.93–6.91 (dd, J = 2 Hz, 8 Hz, 1H), 3.68 (bs, 2H), 3.59 (bs, 2H), 3.55 (s, 2H), 2.51 (t, J = 5 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 157.4, 157.2, 156.3, 152.1, 144.8, 139.8, 129.8, 129.7, 125.1, 123.9, 123.3, 122.3, 119.4, 118.9, 117.7, 62.5, 52.7, 52.5, 44.8, 44.2; HRMS calculated for $C_{24}H_{24}N_3O_5$ $[M+H]^+$ 434.1711, found 434.1710.

Chemical Synthesis of JZL184. JZL184 was synthesized using an alternative procedure, described below, that was found to be higher yielding than the original route (3).

To a stirring solution of ethyl isonipecotate (6.79 g, 43.2 mmol) in dry CH_2Cl_2 (100 ml) was added 1-[2-(trimethylsilyl)ethoxycarbonyloxy]pyrrolidin-2,5-dione (10.0 g, 38.9 mmol) and a catalytic amount of 4-dimethylaminopyridine. After stirring for 2 h at room temperature, TLC indicated complete consumption of the starting material. The reaction was poured onto water and the layers were separated. The organic layer was washed once with water, once with brine, dried over Na_2SO_4, and concentrated in vacuo. The crude oil was flushed through a pad of silica to afford the Teoc-protected intermediate as a yellow oil (11.2 g, 89% yield): ^{1}H NMR ($CDCl_3$, 400 MHz) δ4.14 (m, 4H), 4.05 (bs, 2H), 2.88 (t, J = 12 Hz, 2H), 2.44 (m, 1H), 1.88 (d, J = 13 Hz, 2H), 1.64 (m, 2H), 1.25 (t, J = 7 Hz, 3H), 0.99 (t, J = 9 Hz, 2H), 0.03 (s, 9H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ174.7, 155.8, 63.7, 60.7, 43.3, 41.3, 28.1, 18.0, 14.4, −1.2. To a stirring solution of 4-bromo-1,2-methylenedioxybenzene (12.0 g, 55.3 mmol) in dry THF (90 ml) was added *n*-BuLi (22 ml, 2.5M in hexanes, 55 mmol) at −78 °C. After 30 min, the reaction turned into a white slurry, and a solution of Teoc-protected intermediate (8.66 g, 26.8 mmol) in dry THF (20 ml) was added dropwise at −78 °C. An additional portion of dry THF (10 ml) was used to quantitate the transfer. After stirring for 1 h at −78 °C, TLC indicated complete consumption of starting material, and the reaction was poured into a separatory funnel containing EtOAc and water. The layers were separated and the organic layer was washed twice with water, once with brine, dried over Na_2SO_4, and concentrated in vacuo. Purification of the crude oil via flash chromatography (Hex:EtOAc = 5:1 then 3:1) gave the product as a flocculent white solid (11.3 g, 85% yield): ^{1}H NMR ($CDCl_3$, 400 MHz) δ6.93 (s, 2H), 6.91 (d, J = 8 Hz, 2H), 6.73 (d, J = 8 Hz, 2H), 5.30 (s, 4H), 4.20 (bs, 2H), 4.14 (t, J = 8 Hz, 2H), 2.74 (t, J = 12 Hz, 2H), 2.40 (t, J = 12 Hz, 1H), 1.54 (d, J = 9 Hz, 2H), 1.28 (m, 2H), 0.97 (t, J = 9 Hz, 2H), 0.02 (s, 9H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 155.7, 147.8, 146.3, 140.0, 119.0, 109.0, 106.9, 101.2, 79.5, 63.6, 44.7, 44.2, 26.6, 17.9, −1.2. To a stirring solution of JZL184-Teoc (8.47 g, 16.9 mmol) in dry DMF (35 ml) was added CsF (11.3 g, 74 mmol) and the reaction was heated to 80 °C. After stirring for 2 h, TLC indicated complete consumption of the starting material. The DMF was evaporated under reduced pressure, and the crude was passed through a plug of silica (DCM:MeOH = 9:1) to afford the intermediate, which was used without further characterization. To a stirring solution of the intermediate in dry CH_2Cl_2 (30 ml) was sequentially added TEA (5 ml, 36 mmol) and 4-nitrophenyl chloroformate (3.8 g, 18.9 mmol) portion-wise. After stirring overnight, TLC indicated complete consumption of the starting material, and the reaction was quenched by addition of water and EtOAc. The layers were separated and the organic layer was washed twice with water, twice with brine, dried over Na_2SO_4, and concentrated in vacuo. Purification of the crude oil via flash chromatography (Hex:EtOAc = 4:1) gave JZL184 as a slightly yellow solid (6.9 g, 79% yield over two steps): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.23 (d, J = 9 Hz, 2H), 7.26 (d, J = 9 Hz, 2H), 6.91–6.93 (m, 4H), 6.75 (d, J = 9 Hz, 2H), 5.93 (s, 4H), 4.30 (t, J = 10 Hz, 2H), 3.1 (t, J = 13 Hz,

1H), 2.88 (t, J = 13 Hz, 1H), 2.48 (t, J = 12 Hz, 1H), 2.16 (s, 1H), 1.62–1.69 (m, 2H), 1.40–1.48 (m, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 156.5, 152.3, 148.0, 146.5, 145.0, 139.7, 125.2, 122.5, 119.0, 108.1, 106.9, 101.3, 79.6, 45.2, 44.8, 44.5, 26.9, 26.5; HRMS calculated for $C_{27}H_{24}N_2NaO_9$ $[M+Na]^+$ 543.1374, found 543.1368.

Preparations of Mouse and Rat Tissue Proteomes. Tissues were Dounce-homogenized in PBS, pH 7.5, followed by a low-speed spin (1,400 *g*, 5 min) to remove debris. The supernatant was then subjected to centrifugation (64,000 *g*, 45 min) to provide the cytosolic fraction in the supernatant and the membrane fraction as a pellet. The pellet was washed and resuspended in PBS buffer by sonication. Total protein concentration in each fraction was determined using a protein assay kit (Bio-Rad). Samples were stored at −80 °C until use.

Recombinant Expression in COS7 or HEK293T Cells. Full-length cDNAs encoding mouse serine hydrolases were purchased from OpenBioSystems (Huntsville, AL). cDNAs were either transfected directly (if available in a eukaryotic expression vector) or subcloned into pcDNA3 (Invitrogen). COS7 or HEK293T cells were grown to ≈70% confluence in 10-cm dishes in complete medium (DMEM with L-glutamine, nonessential amino acids, sodium pyruvate, and FCS) at 37 °C and 5% CO_2. The cells were transiently transfected by using the appropriate cDNA or empty vector control ("mock") and the FUGENE 6 (Roche Applied Science) transfection reagents following the manufacturers' protocols. After 48 h, the cells were washed twice with phosphate-buffered saline (PBS), collected by scraping, resuspended in 0.25 ml PBS, and lysed by sonication. The lysates were used in assays as whole-cell homogenates.

Competitive ABPP Experiments. Tissue proteomes were diluted to 1 mg·ml^{-1} in PBS and FP-rhodamine was added at a final concentration of 1 μM in a 50 μl total reaction volume. After 30 min at 25 °C, the reactions were quenched with 4× sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) loading buffer, boiled for 5 min at 90 °C, subjected to SDS/PAGE and visualized in-gel using a flatbed fluorescence scanner (Hitachi). For experiments involving a preincubation with inhibitor, the reactions were prepared without FP-rhodamine. JZL195 was added at the indicated concentration and incubated for 30 min at 37 °C. FP-rhodamine was then added and the reaction was carried out exactly as described above. For ABPP-MudPIT studies, a portion of the brain membrane proteome (1 ml, 1 mg·ml^{-1} in PBS) from the mice treated with JZL195 or vehicle as described below was labeled with 5 μM FP-biotin for 2 h at room temperature and prepared for ABPP-MudPIT analysis as described previously (4), except that the Lys-C digestion step was omitted. MudPIT analysis of eluted peptides was carried out as previously described on a coupled Agilent 1100 LC-ThermoFinnigan LTQ-MS instrument. All data sets were searched against the mouse IPI database using the SEQUEST search algorithm and the results were filtered and grouped with DTASELECT. Peptides with cross-correlation scores greater than 1.8 (+1), 2.5 (+2), 3.5 (+3), and delta CN scores greater than 0.08 were included in the spectral counting analysis. Spectral counts are reported as the average of three samples with the standard error of the mean (SEM).

Enzyme Activity Assays. 2-AG hydrolysis activities were measured as follows: 2-AG (100 μM) was incubated with tissue membrane (25 μg) or recombinant MAGL in COS7 cells (1 μg) in PBS (100 μl) at room temperature for 5 min. The reactions were quenched by the addition of 300 μl 2:1 vol/vol $CHCl_3$:MeOH, doped with 5 nmol PDA, vortexed, then centrifuged (1,400 *g*, 3 min) to separate the phases. A 30-μl quantity of the resultant organic phase was injected onto an Agilent 1100 series LC-MSD SL instrument. LC separation was achieved with a Gemini reverse-phase C18 column (5 μm, 4.6 mm × 50 mm, Phenomonex) together with a precolumn (C18, 3.5 μm, 2 mm × 20 mm). Mobile phase A was composed of a 95:5 vol/vol H_2O:MeOH, and mobile phase B was composed of a 65:35:5 vol/vol/v *i*-PrOH:MeOH:H_2O. 0.1% ammonium hydroxide was included to assist in ion formation in negative ionization mode. The flow rate was 0.5 ml·min^{-1} and the gradient consisted of 1.5 min 0% B, a linear increase to 100% B over 5 min, followed by an isocratic gradient of 100% B for 3.5 min before equilibrating for 2 min at 0% B (12 min total per sample). MS analysis was performed with an electrospray ionization (ESI) source. The capillary voltage was set to 3.0 kV and the fragmentor voltage was set to 70 V. The drying gas temperature was 350 °C, the drying gas flow rate was 10 l min^{-1}, and the nebulizer pressure was 35 psi. Hydrolysis products were quantified by measuring the area under the peak in comparison to the PDA standard. For assays using recombinant MAGL, concentration-dependence inhibition curves were obtained and were fit using Prism software (GraphPad) to obtain effector concentration for half-maximum response values with 95% confidence intervals. AEA hydrolysis activities were measured as follows: AEA (100 μM) was incubated with tissue membrane (50 μg) or recombinant FAAH in COS-7 cells (10 μg) in PBS (100 μl) at 37 °C for 30 min. The reactions were quenched and analyzed in exactly the same way as described above. For assays involving a preincubation with inhibitor, the reactions were prepared without substrate. JZL195 was added at the indicated concentration and incubated for 30 min at 37 °C. The appropriate substrate was then added and the assay was carried out exactly as described above.

Acetyl Cholinesterase Activity Assays. Acetyl cholinesterase (AChE) activity was measured using a method similar to that described previously (5). Briefly, 50 μl of 10 mM acetyl thiocholine was added to 200 μl of PBS containing 2 mM DTNB and 20 μg of mouse brain membrane proteome. Absorbance was measured at 412 nm over 5 min, and the rate of product accumulation was calculated from the slope of the absorbance over time. For assays involving preincubation with JZL195, the reactions were prepared without acetylthiocholine and JZL195 was incubated at the indicated concentration for 30 min at 37 °C. Acetylthiocholine was then added and the assay was carried out exactly as described above.

Determination of k_{obs} [I]$^{-1}$ Values. Brain membrane proteomes (1 mg·ml^{-1}, 300 μl total) were incubated with JZL195 or PF-3845 (10, 25, or 50 nM, 10–40 min, 37 °C). Every 10 min, 50 μl of the reaction was removed and treated with FP-rhodamine (1 μM) for 30 min, quenched with 4× SDS/PAGE loading buffer, and boiled for 5 min at 90 °C. The combined reactions were subjected to SDS/PAGE and visualized in-gel using a flatbed fluorescence scanner. The percentage activity remaining was determined by measuring the integrated optical density (ImageJ) corresponding to the MAGL, FAAH, or ABHD6 bands, and the results were fit to an exponential curve to determine k_{obs}.

CB1 and CB2 Receptor Binding Assays. Chinese Hamster Ovary (CHO) cells stably expressing the human CB1 or CB2 receptor were cultured in a 50:50 mixture of DMEM and Ham F-12 supplemented with 100 U·ml^{-1} penicillin, 100 μg·ml^{-1} streptomycin, 0.25 mg·ml^{-1} G418, and 5% FCS. Cells were harvested by replacement of the media with cold PBS containing 0.4% EDTA followed by agitation. Membranes were prepared by homogenization of cells in 50 mM Tris-HCl, 3 mM $MgCl_2$, 1 mM EGTA, pH 7.4, centrifugation at 50,000 *g* for 10 min at 4 °C, and resuspension in the same buffer at 1.5 mg·ml^{-1}. Membranes were stored at −80 °C until use. For CB1 receptor binding, compe-

tition curves were generated by incubating 8 mg of membrane protein with 1 nM [^{3}H]rimonabant in the presence or absence of JZL195 (0.2 nM-20 μM), or WIN 55,212-2 (10 μM), which served as a positive control, for 90 min at 30 °C. Nonspecific binding was determined in the presence of 5 μM nonradiolabeled rimonabant. The reaction was terminated by vacuum filtration although Whatman GF/B glass fiber filter that was presoaked in Tris buffer containing 5 g·l^{-1} BSA (Tris-BSA), followed by three washes with 4 °C Tris-BSA. Bound radioactivity was determined by liquid scintillation spectrophotometry at 45% efficiency after extraction in ScinitSafe Econo 1 scintillation fluid. For CB2 receptor binding, CB2 receptor CHO cell membranes (8 mg protein), were incubated with 1.5 nM [^{3}H] CP-55940 in the presence or absence of JZL195 (0.5 nM–5 μM), or CP 55,940 (3 μM) positive control for 90 min at 30 °C. Nonspecific binding was determined in the presence of 5 mM SR144528. The reaction was terminated by vacuum filtration although Whatman GF/B glass fiber filter that was presoaked in Tris buffer containing 5 g·l^{-1} BSA (Tris-BSA), followed by three washes with 4 °C Tris-BSA. Bound radioactivity was determined by liquid scintillation spectrophotometry at 45% efficiency after extraction in ScinitSafe Econo 1 scintillation fluid. For the CB1 and CB2 receptor binding assays, data are expressed as mean ± standard error for the percentage radiolabeled ligand displaced for each concentration of JZL195, which was calculated as follows: (specific radiolabeled ligand in the absence of JZL 195-specific radiolabeled ligand in the presence of JZL 195)/ specific radiolabeled ligand in the absence of JZL 195) × 100.

Agonist-Stimulated [^{35}S]GTPgS Binding. CB1 receptor or CB2 receptor transfected CHO cell membranes were thawed on ice, centrifuged at 50,000 *g* for 10 min at 4 °C, and resuspended in Assay Buffer A (50 mM Tris-HCl (pH 7.4), 3 mM $MgCl_2$, 0.2 mM EGTA, and 100 mM NaCl). Reactions containing 10 μg of membrane protein were incubated for 90 min at 30 °C in Assay Buffer A containing 10 μM GDP, 0.1 nM [^{35}S]GTPγS, 0.1% BSA, and various concentrations of agonist or JZL 195. Nonspecific binding was determined in the presence of 20 μM unlabeled GTPγS. Reactions were terminated by rapid vacuum filtration through GF/B glass fiber filters, and radioactivity is measured by liquid scintillation spectrophotometry at 95% efficiency for ^{35}S. For [^{35}S]GTPγS data, percent stimulation is defined as ([agonist-stimulated stimulated binding-basal binding]/basal binding) × 100%.

Guanine Triphosphate Stimulation Assays. CB1 receptor or CB2 receptor transfected CHO cells, or mouse cerebellar homogenates (CB1 receptor) were thawed on ice, centrifuged at 50,000 *g* for 10 min at 4 °C, and resuspended in Assay Buffer A (50 mM Tris-HCl (pH 7.4), 3 mM $MgCl_2$, 0.2 mM EGTA, and 100 mM NaCl). Reactions containing 10 μg of membrane protein were incubated for 90 min at 30 °C in Assay Buffer A containing 10 μM GDP, 0.1 nM [^{35}S]GTPγS, 0.1% BSA, and various concentrations of test compounds. Nonspecific binding was determined in the presence of 20 μM unlabeled GTPγS. Reactions were terminated by rapid vacuum filtration through GF/B glass fiber filters, and radioactivity is measured by liquid scintillation spectrophotometry at 95% efficiency for ^{35}S.

In Vivo Studies with Inhibitors. JZL184, JZL195, PF-3845, or rimonabant were prepared as saline-emulphor emulsions by vortexing, sonicating, and gentle heating neat compound directly into an 18:1:1 vol/vol/vol solution of saline:ethanol:emulphor. Both PF-3845 and rimonabant dissolved completely in the 18:1:1 vehicle, whereas JZL184 and JZL195 were administered as milky suspensions. Male C57BL/6J mice (< 6 months old, 20–28 g) were i.p. administered compound or an 18:1:1 vol/vol/vol saline:emulphor:ethanol vehicle at a volume of 10 μl·g^{-1} weight. Animal experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Scripps Research Institute.

Measurement of Brain Lipids. Mice were anesthetized with isofluorane and killed by decapitation. Brains were removed, hemisected along the midsaggital plane, and flash frozen in liquid nitrogen. One half brain was weighed and subsequently Dounce homogenized in 2:1:1 vol/vol/vol $CHCl_3$:MeOH:Tris pH 8.0 (8 ml) containing standards for AEA, 2-AG, and FFA (2 pmol d_4-AEA, 0.5 nmol d_5-2-AG, and 100 nmol PDA). The mixture was vortexed and then centrifuged (1,400 *g*, 10 min). The organic layer was removed, dried under a stream of N_2, resolubilized in 2:1 vol/vol $CHCl_3$:MeOH (120 μl), and 10 μl of this resolubilized lipid was injected onto an Agilent G6410B QQQ instrument. For AEA and 2-AG measurements, LC separation, mobile phase A, and mobile phase B were exactly the same as for the enzyme activity assays, except 0.1% formic acid was included to assist in ion formation in positive ionization mode. The flow rate for each run started at 0.1 ml·min^{-1} with 0% B. At 5 min, the solvent was immediately changed to 60% B with a flow rate of 0.4 ml·min^{-1} and increased linearly to 100% B over 10 min. This was followed by an isocratic gradient of 100% B for 5 min at 0.5 ml·min^{-1} before equilibrating for 3 min at 0% B at 0.5 ml·min^{-1} (23 min total per sample). The following MS parameters were used to measure the indicated metabolites (precursor ion, product ion, collision energy in V): AEA (348, 62, 11), OEA (326, 62, 11), PEA (300, 62, 11), d_4-AEA (352, 66, 11), 2-AG (379, 287, 8), d_5-2-AG (384, 287, 8). MS analysis was performed with an ESI source. The dwell time for each lipid was set to 60 ms. The capillary was set to 4 kV, the fragmentor was set to 100 V, and the delta EMV was set to + 300. The drying gas temperature was 350 °C, the drying gas flow rate was 11 l min^{-1}, and the nebulizer pressure was 35 psi. Lipids were quantified by measuring the area under the peak in comparison to the deuterated standards. For FFA measurements, the LC parameters were exactly the same as for AEA/2-AG measurements except 0.1% ammonium hydroxide was included instead of formic acid to assist in ion formation in negative ionization mode. The MS analysis was performed with an ESI source in MS2 scan mode from 100-1000 *m/z* in negative polarity. The capillary was set to 4 kV, the fragmentor was set to 100 V, and the delta EMV was set to 0. The drying gas temperature, pressure, flow rate, and nebulizer pressure was the same as described previously (3). Arachidonic acid was quantified by measuring the area under the peak in comparison to the PDA standard.

Mouse Tetrad Experiments. Mice were housed on a normal 6 AM/6 PM light/dark phase, had ad libitum access to water and food, and were tested during the day. Testing was performed blinded to genotype and drug treatment. The animals were housed individually overnight, and baseline tail immersion latencies and rectal temperatures were obtained the morning of the testing. The tetrad was assessed in the following order: catalepsy, locomotion, tail flick, and rectal temperature. Catalepsy was assessed on a bar 0.7 cm in diameter placed 4.5 cm off of the ground. The mouse was placed with its front paws on the bar and a timer (Timer #1) was started. A second timer (Timer #2) was turned on only when the mouse was immobile on the bar, with the exception of respiratory movements. If the mouse moved off the bar, it was placed back on in the original position. The assay was stopped when either Timer #1 reached 60 s, or after the fourth time the mouse moved off the bar, and the cataleptic time was scored as the amount of time on Timer #2. Locomotor activity was assessed in a Plexiglas cage (18 × 10 × 8.5 in) that was marked by 7 cm × 7 cm grids on the bottom of the cage. The number of grids traversed by the hind limbs was counted for 5 min. Nociception was then assessed in the tail immersion assay,

where each mouse was hand-held and 1 cm of the tail was submerged into a 56 °C water bath. The latency for the mouse to withdrawal its tail was scored. Rectal temperature was assessed by inserting a thermocouple probe 1.2 cm into the rectum and temperature was determined using a telethermometer.

Mouse Drug Discrimination Experiments. Male FAAH knockout and matched littermate wild type mice (20–25 g) were housed individually in clear plastic cages in a temperature-controlled (20–22 °C) vivarium. Water was available ad libitum except while the mice were in the operant chambers. Training and test sessions were conducted at similar times during the light phase of a 12 h light/dark cycle. Mice were maintained at 85–90% of free-feeding body weights by restricting daily ration of standard rodent chow. Eight standard mice operant conditioning chambers that were sound- and light-attenuated (MED Associates, St. Albans, VT) were used for behavioral training and testing. Each operant conditioning chamber (18 × 18 × 18 cm) was equipped with a house light, two nose poke apertures (left and right), and a recessed well centered between the two apertures. A sweetened pellet served as reinforcement and was delivered to the recessed well according to the reinforcement schedule. Fan motors provided ventilation and masking noise for each chamber. House lights were illuminated during all operant sessions. A computer with Logic "1" interface and MED-PC software (MED Associates) were used to control schedule contingencies and to record data. Mice were trained to respond in one aperture following administration of 5.6 mg·kg^{-1} THC and to respond in the opposite aperture following vehicle administration according to a FR10 schedule of reinforcement. Each incorrect response reset the response requirement. Daily injections were administered on a double alternation sequence of THC and vehicle (e.g., drug, drug, vehicle, vehicle). Daily 15-min training sessions were held Monday–Friday until the mice had met two criteria during eight of 10 consecutive sessions: (*i*) correct completion of the first FR10 (e.g., first 10 consecutive responses on condition-appropriate aperture) and (*ii*) ≥80% of the total condition-appropriate responding. When these two criteria were met, acquisition of the discrimination was established and substitution testing began. Stimulus substitution tests were conducted on Fridays during 15-min test sessions and training continued on all other days. During test sessions, responses in either aperture delivered reinforcement according to an FR10 schedule. To be tested, mice must have completed the first FR10 on the correct aperture and ≥80% of the total condition-appropriate responding on the preceding drug and vehicle sessions. Control tests were then conducted with the training dose (5.6 mg·kg^{-1} THC) and vehicle. For substitution tests, 40 mg·kg^{-1} JZL184 or 40 mg·kg^{-1} JZL195 were administered i.p. 2 h before the test session. For antagonism studies, mice were injected with rimonabant (3 mg·kg^{-1} i.p.) 10 min before administration of either 40 mg·kg^{-1} JZL184, 40 mg·kg^{-1} JZL195, or vehicle.

Mouse Acetic Acid Abdominal Stretching Experiments. Mice were given an i.p. injection of 0.6% acetic acid (in saline) in a total volume of 10 μl·g^{-1} body weight and were then placed in observation chambers for a 3-min acclimation period. The number of abdominal stretches that included extension of the hind limbs, belly pressing, and curling were tabulated during a 20-min observation period. Mice were given a s.c. injection of JZL184 (16 mg·kg^{-1}), JZL195 (20 mg·kg^{-1}), or vehicle 2 h before acetic acid. Rimonabant (3 mg·kg^{-1}) or vehicle was given s.c. 10 min before either drug or vehicle. Upon completion of the test, subjects were killed with CO_2.

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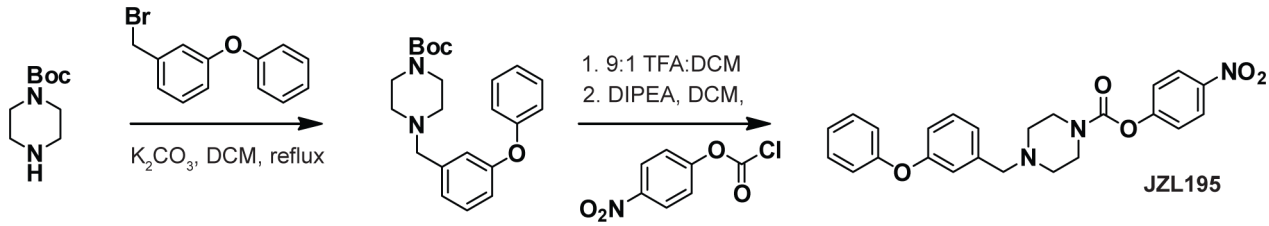


Fig. S1. Chemical synthesis of JZL195. Other dual FAAH/MAGL inhibitors were synthesized by a similar route.

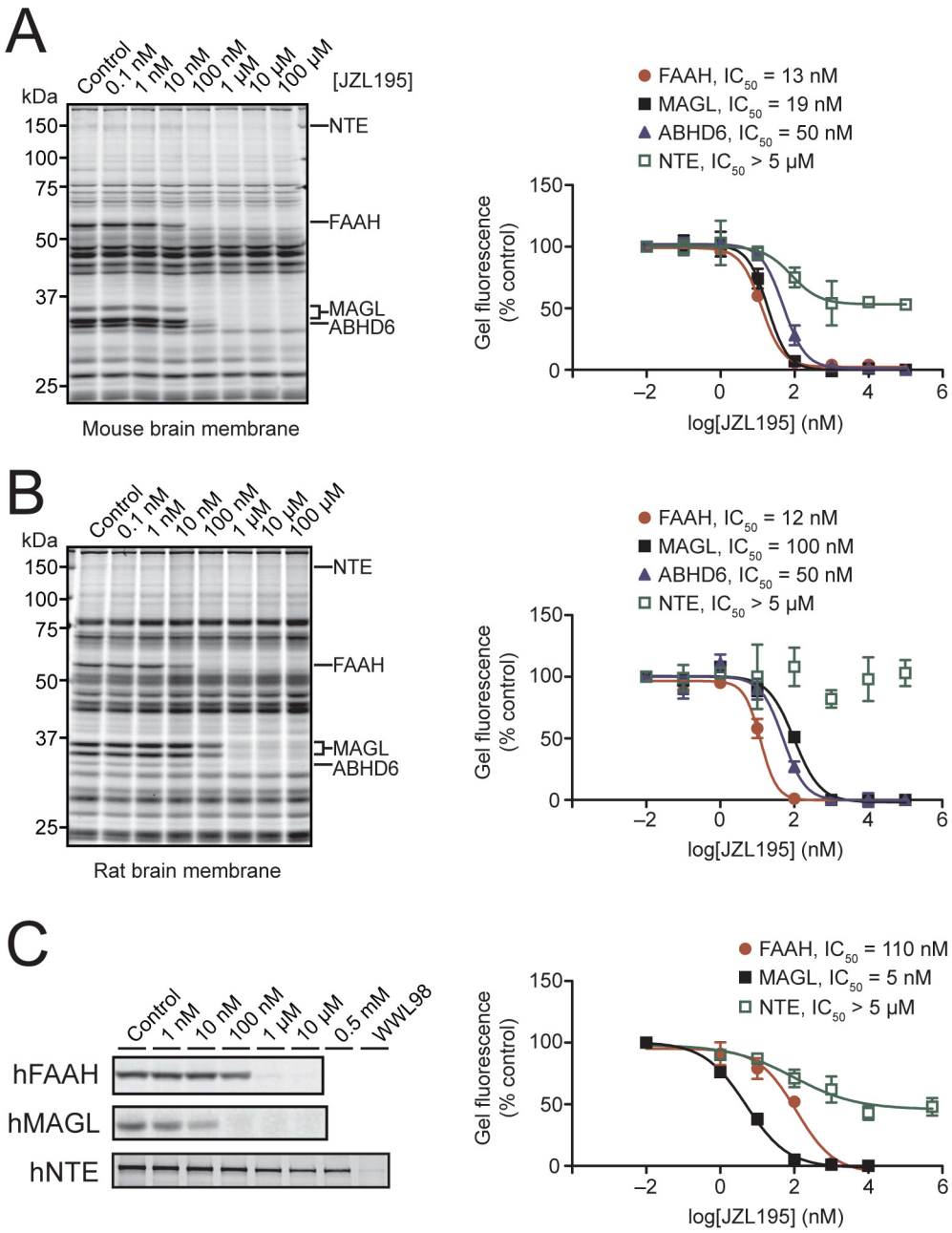


Fig. S2. Inhibitory activity of JZL195 against mouse, rat, and human endocannabinoid hydrolases. (*A* and *B*) Concentration-dependent effects of JZL195 on mouse (*A, left panel*) or rat (*B, left panel*) brain membrane serine hydrolases activities as judged by gel-based ABPP. (*C*) Concentration-dependent effects of JZL195 on recombinant human FAAH and MAGL overexpressed in HEK293T cells. Samples were treated with inhibitor for 30 min at 37 °C before addition of the serine hydrolase-directed ABPP probe FP-Rh (1 μM, 30 min, room temperature). For *C*, WWL98 (10 μM), a nonselective carbamate inhibitor (3) was used as a positive control for NTE inhibition. Gel band intensity quantifications and IC_{50} values for FAAH, MAGL, ABHD6, and NTE are shown in the *right panels.* Data are shown as means ± SEM of three independent experiments.

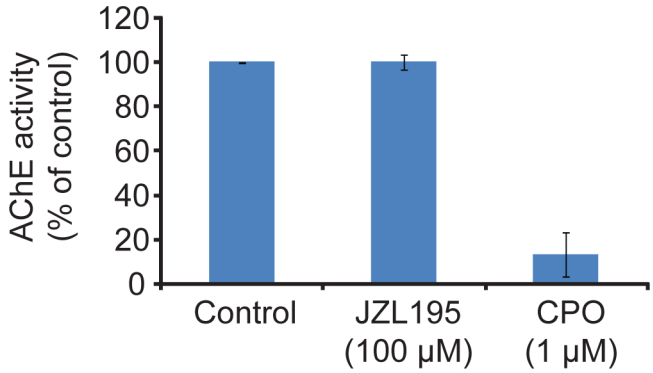


Fig. S3. JZL195 does not inhibit mouse brain acetylcholinesterase activity. Mouse brain membrane proteome was incubated with either DMSO or inhibitor for 30 min at 37 °C before the addition of acetylthiocholine substrate. Chlorpyrifos oxon (CPO), a known acetyl cholinesterase inhibitor, was used as a positive control. Data are shown as means ± SEM of three experiments.

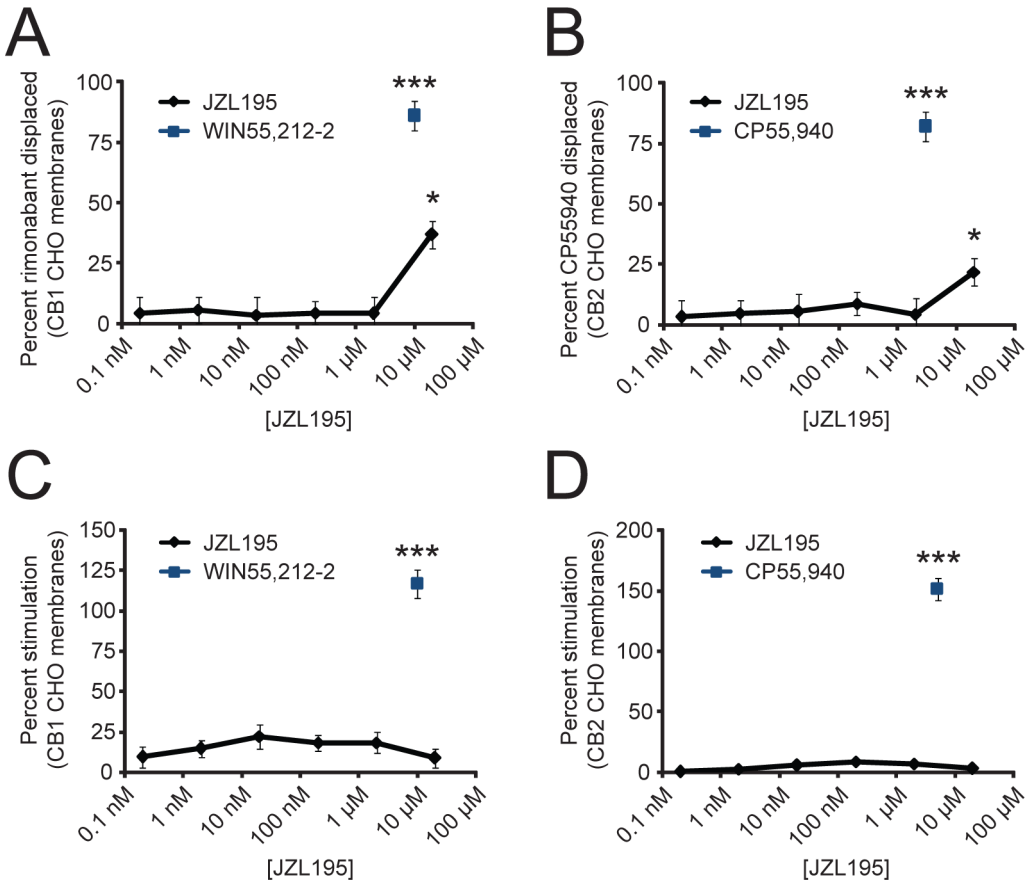


Fig. S4. JZL195 does not activate the cannabinoid receptors. (*A* and *B*) JZL195 displacement of ^{3}H-rimonabant binding to CB1 (*A*) or ^{3}H-CP-55,940 binding to CB2 (*B*) receptors. (*C* and *D*) JZL195 stimulation of GTPγS binding to CB1 (*C*) or CB2 (*D*). WIN55,212–2 (*A* and *C*) and CP55,940 (*B* and *D*) was used as positive controls. *, $P < 0.05$; ***, $P < 0.001$. Data are shown as means ± SEM of four independent experiments.

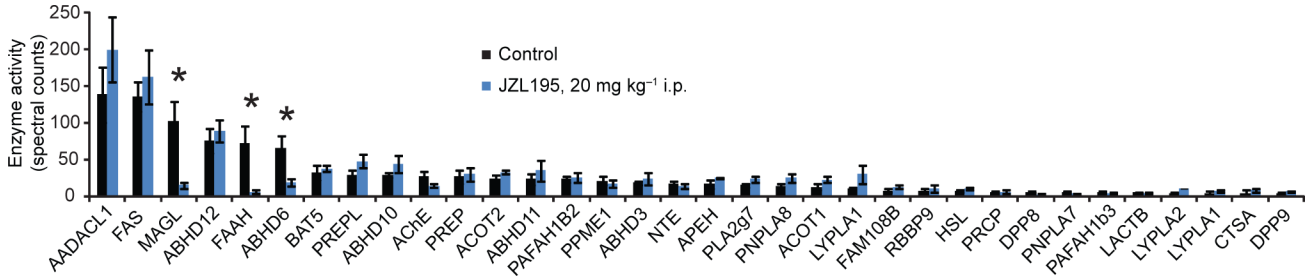


Fig. S5. ABPP-MudPIT analysis of serine hydrolase activities in brain membrane proteomes from mice treated with vehicle or JZL195 (20 mg·kg^{-1}, i.p.) for 2 h. *, $P < 0.05$, for inhibitor-treated versus vehicle-treated samples. Data are presented as means ± SEM. $n = 4$ mice per group.

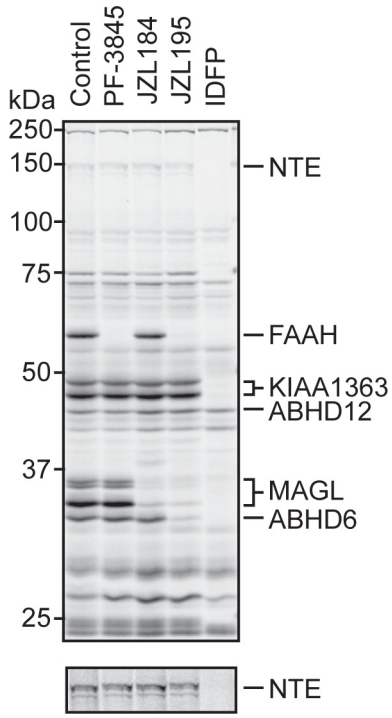


Fig. S6. Comparison of selectivity profiles of various endocannabinoid hydrolase inhibitors following in vivo administration. (*Top panel)* Gel-based ABPP analysis of serine hydrolase activities in mouse brain membrane proteomes from mice treated with vehicle, PF-3845 (10 mg·kg^{-1}, i.p.), JZL184 (20 mg·kg^{-1}, i.p.), JZL195 (20 mg·kg^{-1}, i.p.), or isopropyldodecylfluorophosphonate (IDFP, 10 mg·kg^{-1}, i.p.) for 3 h. (*Bottom panel*) Gel band corresponding to NTE, shown at a darker exposure, underscoring its complete blockade by IDFP, but not by any of the other endocannabinoid hydrolase inhibitors.

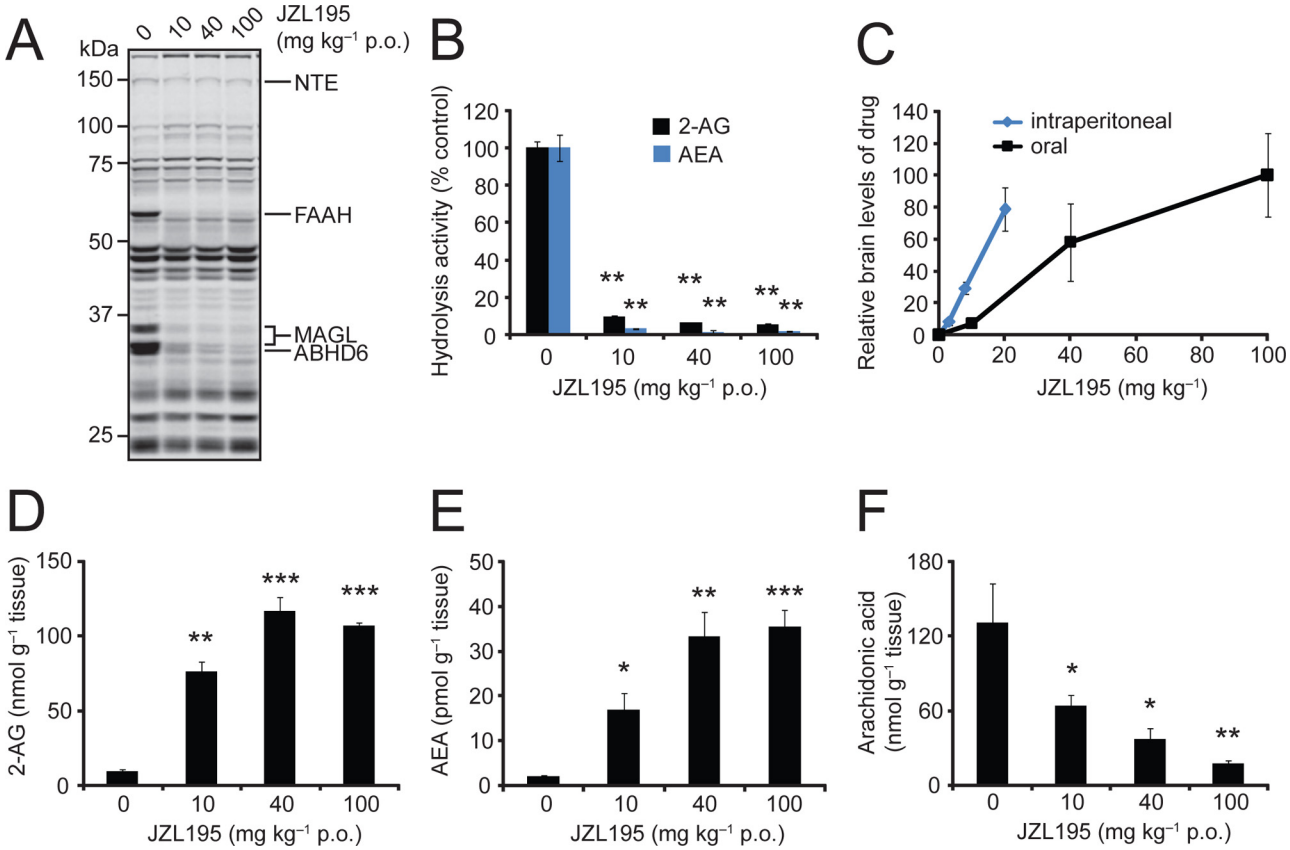


Fig. S7. JZL195 can be administered orally to inactivate FAAH and MAGL in vivo. (*A* and *B*) Serine hydrolase activity profiles (*A*) and 2-AG and AEA hydrolytic activities (*B*) of brain membranes prepared from mice treated with JZL195 at the indicated doses (3–20 mg·kg^{-1}, p.o.) for 4 h. (*C*) Comparison of JZL195 levels in the brain following i.p. versus oral administration. (*D–F*) Brain 2-AG (*D*), AEA (*E*), and arachidonic acid (*F*) levels from mice treated with JZL195 at the indicated doses (10–100 mg·kg^{-1}, p.o.) for 4 h. For *B–F*, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ for inhibitor-treated versus vehicle-treated animals. Data are presented as means ± SEM. $n = 3$–5 mice per group.

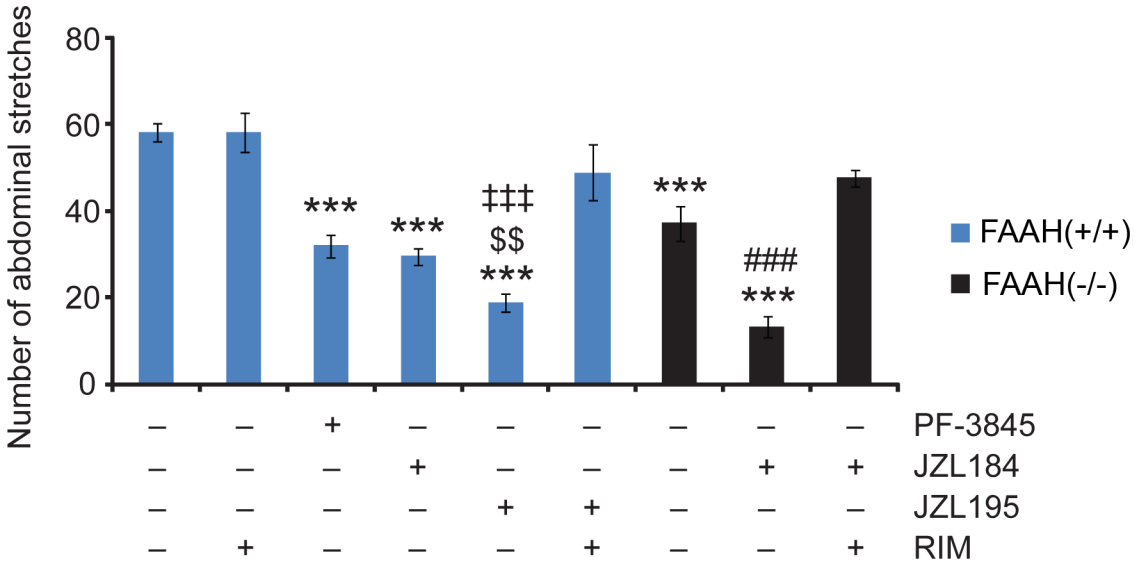


Fig. S8. Dual blockade of FAAH and MAGL produces enhanced antinociceptive effects in the acetic acid model of visceral pain sensation through a CB1 receptor mechanism of action. Subjects were treated with rimonabant (RIM, 3 mg·kg^{-1}), JZL195 (20 mg·kg^{-1}), JZL184 (16 mg·kg^{-1}), or vehicle. ***, $P < 0.001$ vs. FAAH(+/+) vehicle group (Scheffé test); ###, $P < 0.001$ vs. FAAH(−/−), JZL184, and FAAH(−/−) + JZL184 + RIM (Scheffé test); ‡‡‡, $P < 0.001$ vs. JZL195 + RIM (Scheffé test) or FAAH(−/−) mice (planned comparison); $$, $P < 0.01$ vs. JZL184 or PF-3845 (planned comparison). Data are presented as means ± SEM. Data in this figure were compiled from four different experiments (7–23 mice per group), with each experiment consisting of 7–8 mice per group.

A

Hyperreflexia

Genotype	Vehicle/Vehicle 2 h	Vehicle/Vehicle 4 h	Vehicle/JZL184 2 h	Vehicle/JZL184 4 h	RIM/JZL184 2 h	RIM/JZL184 4 h
FAAH(-/-)	0/11	0/11	4/12	1/12	0/12	0/12
FAAH(+/+)	0/10	0/10	5/11	4/11	0/10	0/10

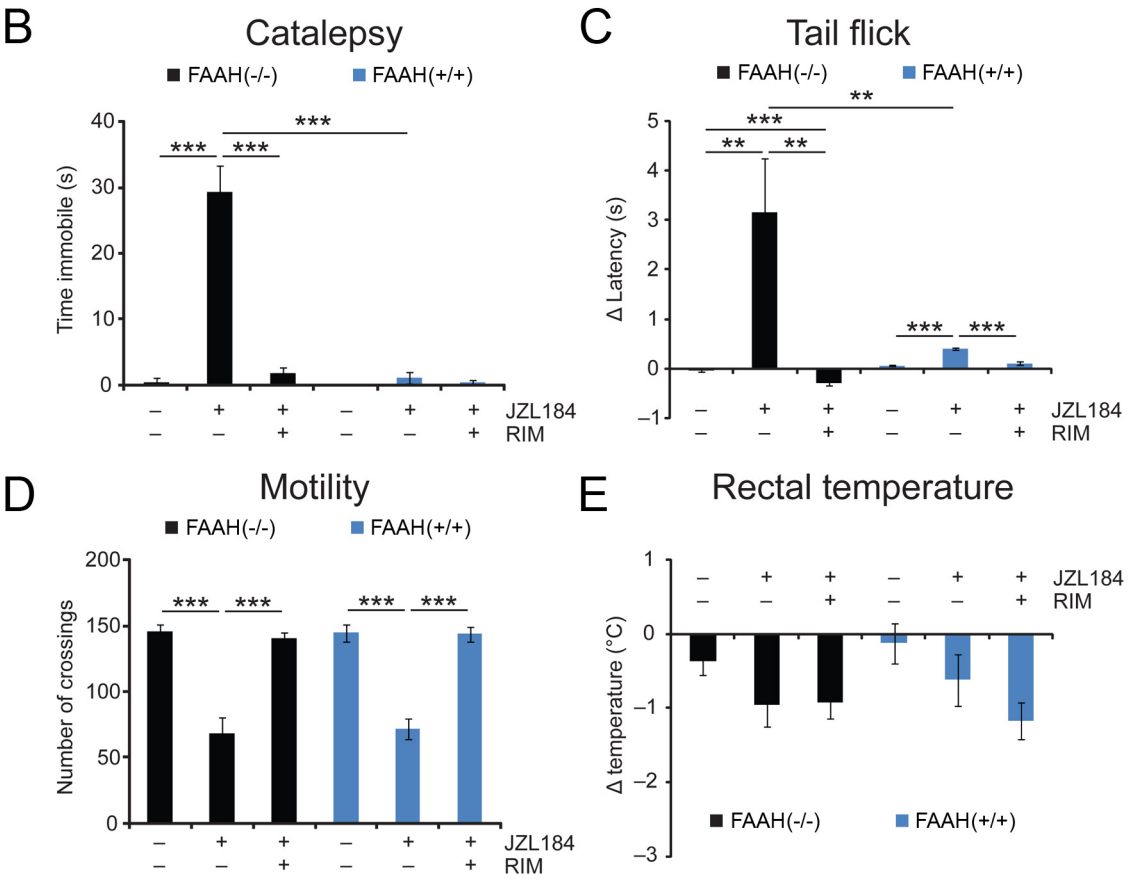


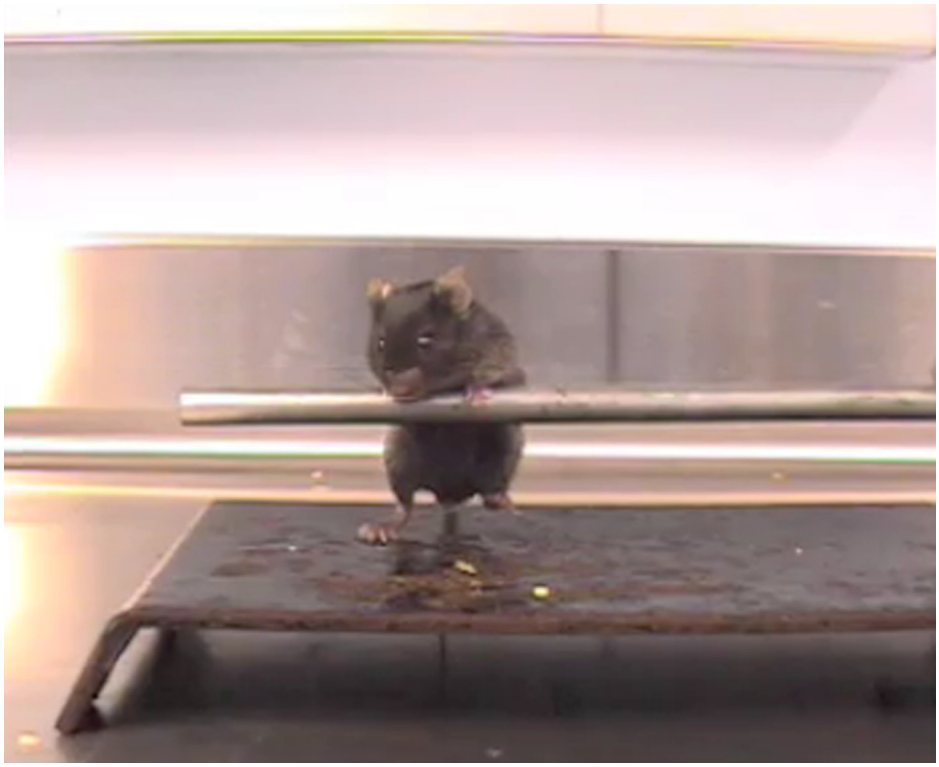
Fig. S9. Behavioral effects of JZL184 in FAAH(+/+) and FAAH(−/−) mice. (*A–D*) FAAH(−/−) mice treated with JZL184 (40 mg·kg^{-1}, i.p.) for 4 h showed hyperreflexia (*A*), catalepsy in the bar test (*B*), antinociception in the tail immersion assay (*C*), and hypomotility in an open field (*D*). FAAH(+/+) mice treated with JZL184 (40 mg·kg^{-1}, i.p.) also showed hyperreflexia (*A*) and hypomotility (*D*) but exhibited significantly less antinociception in the tail immersion assay than JZL184-treated FAAH KO animals (*C*) and were not cataleptic (*B*). The behaviors observed in both genotypes were significantly attenuated by pretreatment with rimonabant (RIM, 3 mg·kg^{-1}, i.p.). No hypothermia was observed in any treatment group. The baseline tail immersion and rectal temperature values for FAAH(+/+) mice were 0.50 ± 0.02 s and 37.6° ± 0.2 °C, respectively, and for FAAH(−/−) mice were 0.80 ± 0.02 s and 37.8° ± 0.1 °C, respectively. **, $P < 0.01$, ***, $P < 0.001$. For *B–E*, data are presented as means ± SEM; $n = 11–12$ mice per group.

Movie S1. Blockade of MAGL causes hyperreflexic behavior. A mouse displaying hyperreflexia on the bar test after administration of JZL184 (40 $mg^{-1}{\cdot}kg^{-1}$, i.p., 2 h). The number of mice exhibiting such behavior is quantified in Table S3 and Fig. S6.

Movie S1 (MOV)

Movie S2. A mouse treated with the CB1 agonist WIN55,212–2 (20 $mg^{-1}{\cdot}kg^{-1}$, i.p., 45 min) displays stereotypical cannabinoid behavioral phenotypes such as immobility in the home cage, flattened posture, unresponsiveness to handling, and catalepsy.

Movie S2 (MOV)



Movie S3. Effect of dual FAAH/MAGL blockade on behavior in the home cage and in the catalepsy bar test. A FAAH(−/−) mouse treated with JZL184 (40 $mg^{-1}{\cdot}kg^{-1}$, i.p., 4 h) has relatively normal home cage movement but exhibits catalepsy on the bar test.

Movie S3 (MOV)

TableS1. Gel-based IC_{50} values (nM) for dual FAAH/MAGL inhibitors

Inhibitor	FAAH	MAGL	ABHD6	NTE
JZL190	380	70	150	1100
JZL192	5	70	180	570
JZL193	4600	15	1650	4270
JZL194	3600	670	2900	330
JZL195	13	19	50	>5000

Brain membrane proteomes were treated with the indicated compounds at increasing concentrations (1 nM–100 μM) for 30 min at 37 °C and subsequently labeled with the serine hydrolase-directed probe FP-Rh (1 μM, 30 min, room temperature). Gel band intensities were quantified (ImageJ) and fit using Prism software (GraphPad) to obtain effector concentrations for half-maximum response values.

Table S2. Full names and Ensembl identifier codes for brain membrane serine hydrolases identified in ABPP-MudPIT experiments (shown in Fig. S5)

ENSEMBL Identifier	Common name	Abbreviation	Vehicle	JZL195	*P* value
ENSMUSG00000027698	Arylacetamide deacetylase-like 1	AADACL1	139 ± 38	200 ± 44	0.349
ENSMUSG00000025153	Fatty acid synthase	FAS	136 ± 20	163 ± 37	0.543
ENSMUSG00000033174	Monoacylglycerol lipase	MAGL	103 ± 26	15 ± 4	0.020
ENSMUSG00000032046	Abhydrolase domain-containing protein 12	ABHD12	76 ± 16	89 ± 15	0.588
ENSMUSG00000034171	Fatty acid amide hydrolase	FAAH	72 ± 24	6 ± 2	0.048
ENSMUSG00000025277	Abhydrolase domain-containing protein 6	ABHD6	65 ± 16	19 ± 5	0.042
ENSMUSG00000007036	HLA-B associated transcript 5	BAT5	32 ± 10	38 ± 4	0.630
ENSMUSG00000024127	Prolyl endopeptidase-like	PREPL	30 ± 5	48 ± 9	0.133
ENSMUSG00000033157	Abhydrolase domain-containing protein 10	ABHD10	29 ± 4	44 ± 11	0.248
ENSMUSG00000023328	Acetylcholinesterase	AChE	27 ± 7	14 ± 3	0.147
ENSMUSG00000019849	Prolyl endopeptidase	PREP	26 ± 9	30 ± 9	0.808
ENSMUSG00000021226	Acyl-CoA thioesterase 2	ACOT2	24 ± 5	33 ± 3	0.207
ENSMUSG00000040532	Abhydrolase domain-containing protein 11	ABHD11	23 ± 8	35 ± 14	0.489
ENSMUSG00000030718	Protein phosphatase methylesterase 1	PPME1	23 ± 4	26 ± 6	0.723
ENSMUSG00000003131	Platelet-activating factor acetylhydrolase IB beta	PAFAH-1b2	21 ± 6	17 ± 5	0.609
ENSMUSG00000002475	Abhydrolase domain-containing protein 3	ABHD3	18 ± 2	24 ± 8	0.526
ENSMUSG00000004565	Neuropathy target esterase	NTE	17 ± 4	14 ± 3	0.581
ENSMUSG00000032590	Acylamino-acid-releasing enzyme	AARE	17 ± 6	25 ± 1	0.300
ENSMUSG00000023913	Phospholipase A2, group VII	PLA2 g7	15 ± 3	23 ± 4	0.147
ENSMUSG00000036257	Calcium-independent phospholipase A2-gamma	iPLA2-gamma	13 ± 3	25 ± 6	0.126
ENSMUSG00000072949	Acyl-CoA thioesterase 1	ACOT1	12 ± 4	23 ± 4	0.133
ENSMUSG00000025903	Acyl-protein thioesterase 1	LYPLA1	10 ± 2	30 ± 13	0.173
ENSMUSG00000047368	Abhydrolase domain-containing protein FAM108B1	FAM108B1	8 ± 3	13 ± 2	0.176
ENSMUSG00000027428	Retinoblastoma-binding protein 9	RBBP9	8 ± 4	11 ± 5	0.636
ENSMUSG00000003123	Horomone sensitive lipase	HSL	7 ± 1	11 ± 2	0.120
ENSMUSG00000061119	Prolylcarboxypeptidase	PRCP	6 ± 2	6 ± 2	0.988
ENSMUSG00000032393	Dipeptidyl peptidase 8	DPP8	6 ± 2	2 ± 2	0.219
ENSMUSG00000036833	Patatin-like phospholipase domain-containing protein 7	PNPLA7	6 ± 2	3 ± 1	0.316
ENSMUSG00000005447	Platelet-activating factor acetylhydrolase IB subunit gamma	PAFAH-1b3	5 ± 2	4 ± 1	0.679
ENSMUSG00000032370	Serine beta-lactamase-like protein	LACTB	5 ± 1	4 ± 1	0.665
ENSMUSG00000028670	Acyl-protein thioesterase 2	LYPLA2	4 ± 1	10 ± 0	0.001
ENSMUSG00000039246	Lysophospholipase-like protein 1	LYPLAL1	4 ± 3	7 ± 2	0.436
ENSMUSG00000017760	Cathepsin A	CTSA	4 ± 4	8 ± 2	0.443
ENSMUSG00000001229	Dipeptidyl peptidase 9	DPP9	4 ± 1	7 ± 1	0.111

P values were calculated using Student's unpaired *t* test.

Table S3. k_{obs} $[I]^{-1}$ values ($M^{-1}{\cdot}s^{-1}$) for inhibition of FAAH, MAGL, and ABHD6 by JZL195, JZL184, and PF-3845

Compound	FAAH	MAGL	ABHD6
JZL195	8000 ± 500	5700 ± 600	3600 ± 500
JZL184	13 ± 3	4400 ± 300	26 ± 5
PF-3845	11800 ± 800	N.D.	N.D.

ND, no inhibition detected. k_{obs} $[I]^{-1}$ values for JZL184 against FAAH and MAGL are from Long *et al.* (23). Determination of k_{obs} $[I]^{-1}$ values is described in *Materials and Methods*.

Table S4. Hyperreflexic responses of mice treated with JZL195 (20 mg·kg^{-1}, i.p.), JZL184 (40 mg·kg^{-1}, i.p.), and PF-3845 (10 mg·kg^{-1}, i.p.) at 2 h and 4 h after administration of compounds

Treatment group	2 h	4 h
Vehicle/Vehicle	0/10	0/10
Vehicle/PF-3845	0/7	0/7
Vehicle/JZL184	5/11	4/11
JZL184/RIM	0/10	0/10
Vehicle/JZL195	6/7	3/7
JZL195/RIM	0/9	0/9
JZL184/PF-3845	3/7	4/10
JZL184/PF-3845/RIM	0/7	0/7

RIM, rimonabant. Rimonabant (3 mg·kg^{-1}, i.p.) or vehicle was administered 10 min before administration of other compounds. Data are shown as the number of mice exhibiting behavior/total number of mice tested.