Supporting Information

Pamplona et al. 10.1073/pnas.1202906109

SI Materials and Methods

Mouse Strains. Swiss albino mice were provided by the animal facility of Universidade Federal de Santa Catarina; inbred C57BL/6 mice were provided by Charles River; CB_1 knockout ($CB_1^{-/-}$) and control ($CB^{+/+}$) adult male mice were developed in the Max Planck Institute of Psychiatry and raised in-house (1); and 5-LOX knockouts were provided by Fundação Osvaldo Cruz and kept in the animal facilities of Universidade Federal do Rio de Janeiro. Adult male mice with ad libitum food and water were used throughout the study and tested during the light phase of the light cycle.

Behavioral Tests. The tetrad test has been classically used to evaluate cannabinoid effects (2). Locomotion in the open field was evaluated in a squared arena (30 × 30 × 15 cm) for 5 min; the bar catalepsy test consisted of evaluating the time spent in an upright position with the forepaws supported by a 5-cm high horizontal bar (maximum 300 s). Body temperature was assessed with a digital thermometer attached to a rectal probe; nociception was assessed by placing the mice on a heated surface (hot plate at 55 ± 0.5 °C) until forepaw licking occurred (nociceptive latency). Independent groups of animals were used for each test. Water maze training was carried out in 10 consecutive trials (maximum 60 s), followed by a 60-s probe trial of spatial memory performed 24 h after training. For a complete description of water maze procedure, see ref. 3.

CB_1 Receptor Binding. Competitive binding assays were performed using mouse whole-brain membranes (4). Lipoxin A_4 (LXA_4) (1 nM–10 μM) was incubated with membranes in assay buffer (50 mM Tris·HCl, 1 mM EDTA, 3 mM $MgCl_2$, 1 mg/mL BSA, 100 μM PMSF, pH 7.4) for 1 h at 37 °C with 0.5 nM of [^{3}H]SR141716A. To investigate the impact of LXA_4 on anandamide (AEA) binding, LXA_4 (100 nM) or control (ethanol 0.5%) were incubated together with increasing concentrations of AEA (1 nM–10 μM) strictly following the same conditions described. The results were confirmed by the investigation of the effects of LXA_4 (1 nM–1 μM) in the binding of the cannabinoid agonist [^{3}H]CP55914 and [^{3}H]WIN55212-2 in mouse whole-brain membranes. Dissociation binding was performed by the displacement of the CB_1 ligand [^{3}H]CP55914 (0.5 nM) by an excess of the agonist WIN55212-2 (1 μM) in the presence of LXA_4 (100 nM) or control (ethanol 0.5%). The kinetics of this reaction was followed by 120 min, during which aliquots were sampled over time. The reaction was terminated by filtration through GF/B filters, and the membrane-bound radioactivity was measured in a liquid scintillation counter. Nonspecific binding was calculated with 10 μM WIN55212-2.

Fatty Acid Amide Hydrolase and Monoacyl Glycerol Lipase Enzymatic Activity. The enzymatic assays were conducted in mouse whole-brain homogenates as previously described (5). LXA_4 (up to 10 μM) was incubated with the membrane fraction of the homogenate (100 μg of protein) with [^{14}C]AEA (1.8 μM; 5 mCi/mmol) (50 mM Tris·HCl, pH 9) for 30 min at 37 °C for fatty acid amide hydrolase and with the cytosolic fraction of the homogenate (100 μg of protein) with [^{3}H]2-arachidonoylglycerol (2-AG) (25 μM; 1 mCi/mmol) (50 mM Tris·HCl, pH 7) for 20 min at 37 °C for monoacyl glycerol lipase. The enzymatic activity was determined by the generation of the respective metabolite of AEA ([^{14}C]ethanolamine) or 2-AG ([^{3}H]glycerol) and normalized by the mass of fresh tissue.

Quantification of Endocannabinoid Levels. The levels of AEA and 2-AG in the brain (cortex, hippocampus, cerebellum) were quantified ex vivo after LXA_4 [1 pmol/2 μL, intracerebroventricularly (i.c.v.)] or control (ethanol 0.5% in PBS) injection, as previously described (6). The tissue was homogenized (50 mM Tris·HCl, pH 7.5) and extracted with 2 volumes of chloroform/methanol (2:1) containing 5 pmol of the internal standards d_4-AEA and d_5-2-AG and prepurified. The HPLC measurement was performed with a reversed-phase C18 column coupled to an MS. AEA and 2-AG were eluted with methanol/water/acetic acid (85:15:0.2) and a flow rate of 1 mL/min at 25 °C. Retention times were 14.5 min for AEA and 17 min for 2-AG (m/z tracking at 400 °C ionization temperature). Endocannabinoid levels were normalized by the mass of fresh tissue.

Forskolin-Induced cAMP Accumulation in CB_1-Transfected Cells. HEK293T cells plated on 35-mm culture dishes were transfected with 2 μg of murine CB_1 cDNA using Lipofectamine 2000 (Invitrogen). After 48 h in regular MEM cell culture media, they were incubated with Hepes 5 mM buffered MEM and 1 mg/mL BSA, with 200 μM 3-isobutyl-1-mehtylxanthine (IBMX). Drugs were preincubated with the HEK cells for 10 min, and 1 μM forskolin (FSK) was added to the media and incubated for 10 min. The reaction was stopped by aspiration of the cell culture media, followed by the addition 100 μL of 0.1 N HCl. The cAMP content in the supernatant was acetylated and measured by ELISA according to the kit instructions (Cayman).

Agonist-Stimulated [^{35}S]GTPγS Binding. G-protein activation was measured by agonist-stimulated [^{35}S]GTPγS-binding assay, as previously described (7). Membranes have been incubated with 0.05 nM [^{35}S]GTPγS (1,250 Ci/mmol) in assay buffer containing 30 μM GDP and 0.5% fatty acid-free BSA. HU-210, AEA, and/or LXA_4 (1 nM–100 μM) were added to the membranes and incubated for 50 min at 30 °C. Nonspecific binding was determined in the presence of 10 μM GTPγS. The reaction was terminated by filtration through GF/B filters, and the membrane-bound radioactivity was measured in a liquid scintillation counter.

Immunodetection of LXA_4 Levels in the Brain. Brains from Swiss mice were harvested and dissected (hippocampus, cortex, cerebellum), lipid was extracted with ethanol (5 μL/mg of wet tissue) and centrifuged for 5 min at 10,000 × g, and the supernatant was applied to preactivated Sep-Pak C18 columns. LXA_4 was eluted using the mobile phase of decreasing polarity (water:hexane: ethyl formiate) at 1 mL/min. The extracts were then applied into a double-sandwich ELISA kit, read at 650 nm and normalized by wet tissue weight (g).

Real-Time PCR for ALX Receptors. The presence of ALX receptors in the brain (cortex, hippocampus, cerebellum) was ruled out with real-time PCR (in comparison with RNA from spleen and lung). Total RNA (300 ng) was used for cDNA synthesis using the standard reverse transcriptase protocol, and the cDNA was amplified in duplicate using TaqMan Universal PCR Master Mix Kit with specific TaqMan Gene Expression target genes, the 3′ quencher Minor groove binder (MGB) and 6-carboxyfluorescein (FAM)-labeled probe for mouse FPR2 (Mm00484464_s1), and the 3′ quencher MGB and 2′-chloro-7′-phenyl-1,4-dichloro-6-carboxyfluorescein (VIC)-labeled probe for mouse GAPDH (NM_008084.2, internal control for normalization). Thermocycling parameters were 50 °C for 2 min, 95 °C for 10 min, 50 cycles of 95 °C for 15 s, and 60 °C for 1 min.

In Vitro Electrophysiology of CB_1 Receptors. The confirmation that LXA_4-induced potentiation of AEA effects occurred at the level of the CB_1 receptor protein was studied with in vitro electrophysiology using the heterologous expression of CB_1 receptors on *Xenopus* oocytes. The cRNA of CB_1 receptors in combination with the cRNA of G-protein–gated K^+ channels (Kir 3.1 and Kir 3.4), obtained by in vitro transcription (mMessage machine, Ambion), was injected into oocytes (total 1.5 ng of RNA in 50 nL of injection). The oocytes were surgically obtained from female *Xenopus*, defoliculated, and maintained in ND-96 (in mM: 96 NaCl; 2 KCl; 1.8 CaCl2; 1 MgCl2; 5 Hepes) supplemented with 40 mg/mL gentamicin, at 17 °C until use, as described before (8). Electrophysiological recording initiated 5–7 d after cRNA injection and was performed as described elsewhere (9). The oocytes were placed in a rectangular recording chamber (100 μL volume) and continuously perfused at a rate of 2 $mL \cdot min^{-1}$ with a solution containing (in mM) 70 KCl, 20 NaCl, 3 MgCl2, 5 Hepes, pH 7.4 (2 mL/min, room temperature) and impaled with two glass microelectrodes (0.5–2 MΩ) filled with 3 M KCl and voltage-clamped at 70 mV for current recording. Measurements were obtained before (basal) and after drug administration.

Statistical Analysis. Data are expressed as mean ± SEM, with the number of experimental subjects expressed between brackets (n). The results have been analyzed by one- or two-way analysis of variance (ANOVA), using treatment, pretreatment, strain, or time as independent variables, depending on the experimental design. Successful ANOVAs were followed by point-by-point analysis by Duncan's post hoc test. Water maze test data were analyzed by planned comparisons of least square means of meaningful groups. Two-tailed Student's t test was used for two-group comparisons. The competitive binding assays were analyzed by nonlinear curve fitting for one or two sites of interaction. The analyses are expressed in each figure legend. The minimum levels of significance accepted for all tests was $P < 0.05$. Statistical comparisons were done using the package Statistica 7 (StatSoft Inc.).

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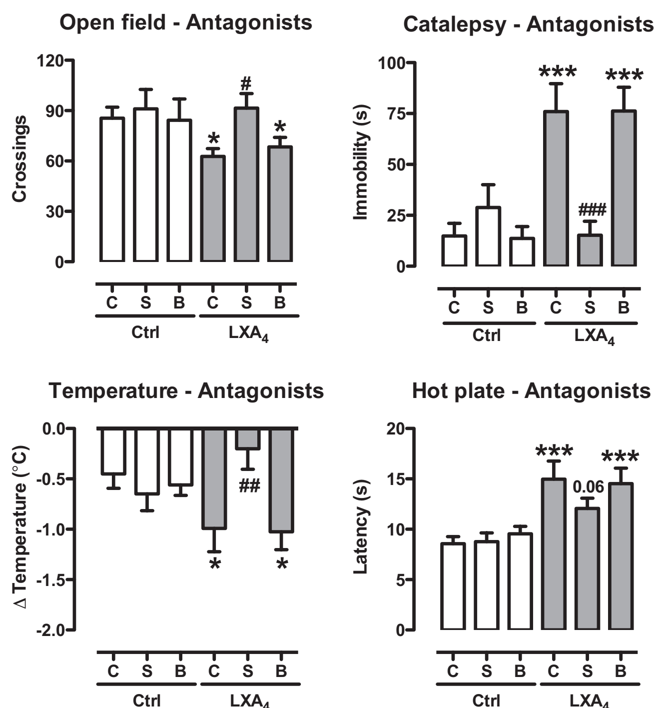

Fig. S1. LXA_4 displays CB1-dependent cannabimimetic effects in the brain. Lipoxin A_4 (LXA_4 0.01–1 pmol/5 μL, i.c.v.) or control (Ctrl) were injected in Swiss mice 5 min before the cannabinoid tetrad test (locomotion in the open field, catalepsy, body temperature, nociception in the hot plate). The CB_1 antagonist SR141716A (S; 1 mg/kg, i.p.), the ALX antagonist BOC-2 (B; 10μg/kg, i.p.), or control (C) were injected 50 min before LXA_4 (1 pmol/5 μL, i.c.v.) or control (Ctrl). Lipoxin induced the full pattern of cannabimimetic effects in the cannabinoid tetrad test, effects that were prevented by the CB_1 antagonist SR141716A. Data are represented as mean ± SEM. $^{*}P < 0.05$, $^{***}P < 0.001$ vs. Control $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. LXA_4 (Duncan's post hoc).

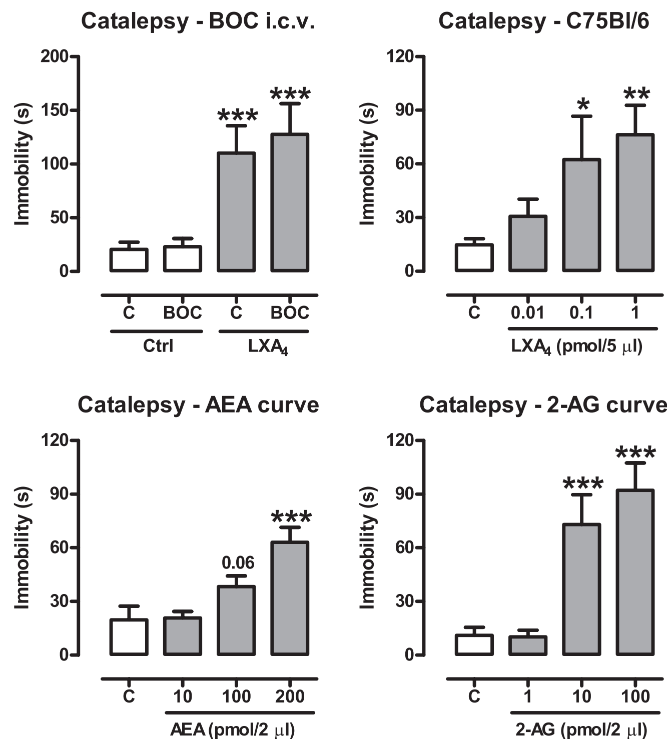

Fig. S2. Additional control experiments of catalepsy. (*Upper Left*) The ALX antagonist BOC-2 (B; 1,000 pmol/2 μL, i.c.v.) was coinjected with LXA_4 (1 pmol/2 μL, i.c.v.) or control (Ctrl) and tested in the catalepsy test 5 min later to confirm that LXA_4-induced catalepsy does not depend on ALX receptors, regardless of the BOC administration route. (*Upper Right*) LX_4 also induces catalepsy in C57BL/6 inbred mice. This dose–response curve was performed to confirm the dose for the experiment with CB1 knockout mice, which have been bred under the C5lBl/6 genetic background (Fig. 2). (*Lower* panels) Dose–response curve for the cataleptic effects of AEA and 2-AG via i.c.v route. This experiment defined the subdoses of AEA and 2-AG that were used to investigate whether LXA_4 would potentiate the effects of these molecules (Fig. 3). Data are represented as mean ± SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. Control (Duncan's post hoc).

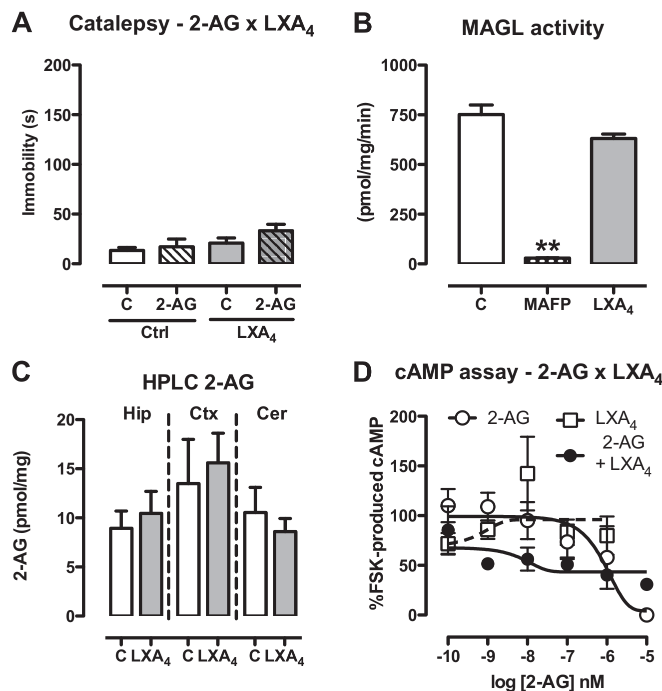

Fig. S3. LXA_4 do not potentiate 2-AG effects. (*A*) Selected pre-effective dose of 2-AG (1 pmol/2 μL, i.c.v.) was coinjected with LXA_4 (0.01 pmol/2 μL, i.c.v.) 5 min before the bar catalepsy test. LXA_4 did not interact with 2-AG [$F(3,27) = 2.37$, $P = 0.09$, $n = 7–9$/group]. (*B*) Activity of the 2-AG–degrading enzyme MGL was measured in the presence of LXA_4 (100 nM–10 μM) using [^{3}H]2-AG (25 μM) as substrate for monoacylglycerol lipase (MGL). Methyl arachidonyl fluorophosphonate (MAFP) (500 nM) was the enzymatic inhibitor used as positive control. LXA_4 did not interfere with MGL ($t = 2.26$, $P = 0.15$, $n = 3$/group) activity, as opposed to the positive control MAFP ($t = 14.96$, $P < 0.01$). (*C*) 2-AG levels in brain tissues were assessed by HPLC-MS 5 min after injection of LXA_4 (1 pmol/2 μL, i.c.v.) or control (C). There were no sign of treatment-related alterations of endocannabinoid content in the hippocampus (Hip), cortex (Ctx), or cerebellum (Cer) ($n = 6$/group). (*D*) cAMP production in response to FSK stimulation was investigated in HEK cells transfected with murine CB_1 receptors. Cells were incubated with 2-AG (0.1 nM–10 μM), LXA_4 (0.1 nM–1 μM), or 2-AG + LXA_4 (100 nM) and stimulated for 10 min with FSK for evaluation of the intracellular content of cAMP. LXA_4 did not influence cAMP levels by itself and apparently reduced 2-AG efficacy. Efficacy curves were generated by nonlinear regression (curve fitting). Data are represented as mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$ vs. Control (Duncan's post hoc or *t*-test for positive controls).

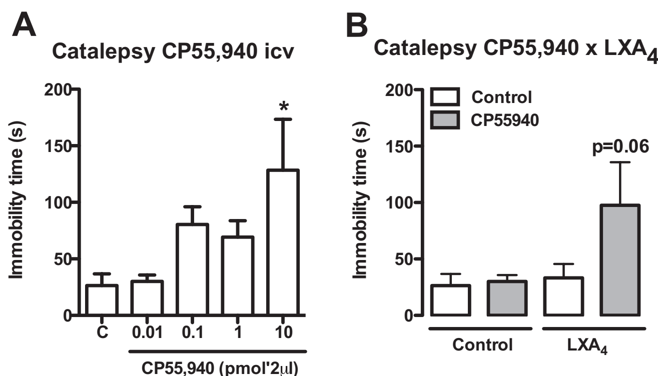

Fig. S4. LXA_4 also potentiates CP55,940-induced catalepsy. (*A*) Dose–response curve for the cataleptic effect of CP55,940 via i.c.v route. This experiment defined the subdose used to investigate whether LXA_4 would potentiate this cannabinoid agonist. (*B*) The cannabinoid agonist CP55,940 (0.01 pmol/2 μL, i.c.v.) was coinjected with LXA_4 (0.01 pmol/2 μL, i.c.v.) or control (Ctrl) and tested in the catalepsy test 5 min later to confirm that LXA_4 also potentiates CP55,940-induced catalepsy. This result is in line with the experiment depicted in Fig. 4*B* showing that LXA_4 increases the affinity of [^{3}H]CP55,940 for the CB_1 receptor in mouse brain membranes. Data are represented as mean ± SEM. $^*P < 0.05$ vs. Control (C); $P < 0.06$ vs. C/CP55,940 (Duncan's post hoc).

Fig. S5. Lack of potentiation on AEA-induced GTPγ[^{35}S] activation. (*A*) LXA_4 (0.01 nM–1 μM) or HU-210 (1 nM–1 μM) was incubated with mouse whole-brain membranes together with BSA, GDP, and a saturating amount of GTPγ[^{35}S] for 50 min. The amount of GTPγ[^{35}S] suggests the number of G proteins activated by the ligand. LXA_4 did not activate a relevant amount of GTPγ[^{35}S] to be considered a cannabinoid agonist (results of the synthetic cannabinoid agonist HU-210 are represented for comparison). (*B*) The same procedure was performed to investigate the interaction between LXA_4 (100 nM) and AEA (10 nM–100 μM). No relevant interaction was observed. Surprisingly, LXA_4 apparently reduces AEA-induced GTPγ[^{35}S] activation over time. (*C*) Same as *B* in cerebellar membranes. The reversal of AEA effect by the selective antagonist SR141716A (SR) suggests participation of CB_1 cannabinoid receptors. Binding curves were generated by nonlinear regression (curve fitting).

Experiment timeline:

Day 0 – IP treatment
(50 min before i.c.v.)
SR141716A 1 mg/kg
Boc-2 10 μg/kg)

Day 0 – ICV treatment
LXA_4 1 pmol
β-amyloid(1-40) 400 pmol

Day 7 – Water maze training
(10 consecutive trials, max 60 s)

Day 8 – Water maze spatial
memory test
(probe test, 60 s)

Fig. S6. Time line and training phase of the water maze test. The time line depicts the experimental protocol. The mice were coinjected with LXA_4 (1 pmol/5 μL, i.c.v.) and β-amyloid (1–40) peptide [Aβ(1–40); 400 pmol/2 μL, i.c.v.] and 7 d later trained in the spatial memory task of the water maze test. The CB_1 antagonist SR141716A (S; 1 mg/kg, i.p.), the ALX antagonist BOC-2 (10 μg/kg, i.p.), or control solution were injected 50 min before LXA_4 (1 pmol/5 μL, i.c.v.) or control solution (Ctrl). Water maze training consisted of 10 consecutive trials in which each mouse was placed in a pseudorandom location in the water maze and allowed to swim for 60 s to find a submerged transparent platform. The reduction in the escape latency is indicative of learning. According to this protocol, the performance improved from 60 s to nearly 30 s to reach the platform in the training phase. This improvement was clearly perceived in the memory test performed 24 h after training (Fig. 6). The figure represents the escape latency(ies), which is the time to reach the target platform. If an animal did not reach the platform in any given trial, it was gently guided to it. Data points represent mean ± SEM.