Anti-inflammatory lipoxin A_4 is an endogenous allosteric enhancer of CB_1 cannabinoid receptor

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Allosteric modulation of G-protein–coupled receptors represents a key goal of current pharmacology. In particular, endogenous allosteric modulators might represent important targets of interventions aimed at maximizing therapeutic efficacy and reducing side effects of drugs. Here we show that the anti-inflammatory lipid lipoxin A_4 is an endogenous allosteric enhancer of the CB_1 cannabinoid receptor. Lipoxin A_4 was detected in brain tissues, did not compete for the orthosteric binding site of the CB_1 receptor (vs. ^{3}H-SR141716A), and did not alter endocannabinoid metabolism (as opposed to URB597 and MAFP), but it enhanced affinity of anandamide at the CB1 receptor, thereby potentiating the effects of this endocannabinoid both in vitro and in vivo. In addition, lipoxin A_4 displayed a CB_1 receptor-dependent protective effect against β-amyloid (1–40)-induced spatial memory impairment in mice. The discovery of lipoxins as a class of endogenous allosteric modulators of CB_1 receptors may foster the therapeutic exploitation of the endocannabinoid system, in particular for the treatment of neurodegenerative disorders.

allosteric modulation | psychopharmacology | GPCR | inflammation | neuroprotection

The endocannabinoid system, comprising metabotropic cannabinoid receptors (CB_1 and CB_2), endogenous lipid ligands (endocannabinoids), and enzymes responsible for their synthesis and degradation, is a key regulator of neuronal function, being proposed as a therapeutic target for several diseases (1). Activation of CB_1 receptors reduces cAMP levels, inhibits voltage-dependent Ca^{2+} channels, and activates inward-rectifying K^+ channels, resulting in reduced neuronal excitability and presynaptic inhibition of neurotransmitter release (1, 2). The efficacy of endogenous CB_1 agonists varies according to the nature of the molecule (3). The endocannabinoid anandamide (AEA) is considered a partial agonist, whereas 2-arachidonoylglycerol (2-AG) is a full agonist inducing maximal responses (4, 5). At least three other endocannabinoids are known (noladin ether, virodhamine, and N-arachidonoyl dopamine) (1, 2). Each endocannabinoid has different affinities, efficacies, and sometimes distinct effects at the CB_1 receptor, which could also be cell type-dependent (1, 2). Cannabinoids have many effects on laboratory animals, but the prominent ones are known as the cannabinoid tetrad: analgesia, catalepsy, hypolocomotion, and hypothermia.

The selectivity of CB_1 agonists may be explained by multiple binding sites in the CB_1 receptor (6), in agreement with the current view of metabotropic receptors as dynamic macromolecules, rather than mere on/off switches of a transduction system (7). Allosteric modulators bind to additional site(s) on the receptor influencing the affinity and/or efficacy of endogenous molecules binding to the orthosteric or primary site (the orthosteric site is defined as the binding site for known endogenous ligand) (8).

Two synthetic compounds, Org27596 and Org29647, enhance the affinity and reduce the efficacy of CB_1 agonists, suggesting the existence of an allosteric binding site at CB_1 receptor (9). However, the existence of endogenous allosteric cannabinoid modulators has not yet been proved.

Synthetic and metabolic pathways of eicosanoids impact endocannabinoid levels, suggesting functional relationships among endocannabinoids, prostaglandins (10), and lipoxins (11). Lipoxin A_4 (LXA$_4$), the most studied endogenous lipoxin (12), is largely involved in immune system regulation and is linked to resolution of inflammation (13). The metabotropic ALX receptor (also called FPRL-1) is responsible for the immunological effects of LXA$_4$ and is expressed in peripheral organs, but has negligible occurrence in the central nervous system (CNS) (14). Nevertheless, LXA$_4$ is released in brain tissues during ischemia (15), suggesting the presence of non-ALX receptor targets in the brain.

Brain effects of LXA$_4$ include modulation of slow wave sleep (16), neuronal signaling (via PKCγ) (17, 18), and plasticity (19) through unknown mechanisms. Interestingly, these effects are similar to those of the endocannabinoid AEA (20, 21). We previously showed that intracerebroventricular (i.c.v.) injections of the aspirin-triggered LXA$_4$ (15-Epi-LXA$_4$) induce cannabinoid-like catalepsy in mice, which was prevented by the CB_1 antagonist SR141716A and not by an ALX antagonist. Altogether, these findings suggest that LXA$_4$ could have CB_1 receptor-dependent effects in the brain (22). Here we report that LXA$_4$ not only binds to CB_1 receptors to exert cannabimimetic effects in the brain, but does so by allosterically enhancing AEA signaling. This may have important implications for the therapeutic exploitation of the endocannabinoid system.

Results

LXA$_4$ Displays Cannabimimetic Effects in the Brain. The cannabinoid "tetrad" represents a prototypic signature of cannabinoid effects (23). Brain injection of LXA$_4$ (1 pmol/5 μL, i.c.v.) induced the

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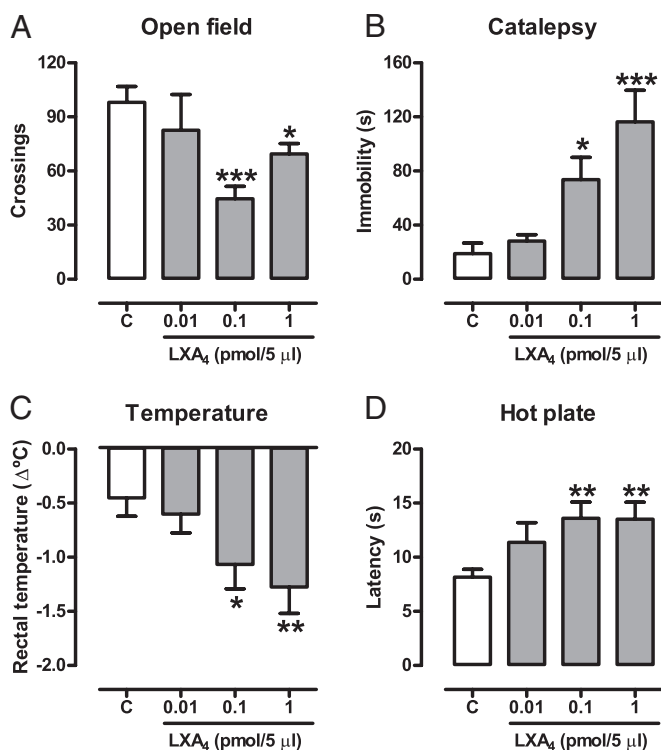**Fig. 1.** LXA$_4$ displays cannabimimetic effects in the brain. (*A–D*) Lipoxin A$_4$ (LXA$_4$ 0.01–1 pmol/5 μL, i.c.v.) or control (C) was injected in Swiss mice 5 min before the cannabinoid tetrad test (locomotion, catalepsy, body temperature, nociception). The treatment reduced the number of crossings in the open field [$F(3,43) = 4.56$, $P = 0.007$, $n = 9–14$/group], increased the immobility time in the bar catalepsy test [$F(3,24) = 9.07$, $P = 0.0003$, $n = 7$/group], reduced body temperature [$F(3,43) = 3.49$, $P = 0.02$, $n = 11–12$/group], and increased the nociceptive latency in the hot plate [$F(3,43) = 3.18$, $P < 0.03$, $n = 11–12$/group]. Data are mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. control (Duncan's post hoc).

full spectrum of tetrad cannabinoid effects in mice (Fig. 1). These effects were prevented by the CB$_1$ receptor antagonist SR141716A (1 mg/kg, i.p.), but not by the ALX receptors antagonist Boc-2 (10 μg/kg, i.p.) (Fig. 2*A*; Figs. S1 and S2) and were absent in CB$_1$-KO mice (Fig. 2*B*). Real-time PCR quantification revealed that the ALX receptor is present in negligible amounts in the mouse brain, compared with spleen and lung tissues (Fig. 2*C*). To determine whether LXA$_4$ binds directly to the CB$_1$ receptor, we measured the displacement of the CB$_1$-selective antagonist [^{3}H]SR141716A (0.5 nM) by LXA$_4$ using mouse brain membranes. LXA$_4$ partially displaced [^{3}H]SR141716A binding at concentrations up to 10 μM, reaching a maximum of 40% displacement (Fig. 2*D*). Also, LXA$_4$ did not change the amount of cAMP accumulated in CB$_1$ receptor-transfected HEK cells (Fig. 3*D*). These findings apparently conflicted with the in vivo results, in which i.c.v. administration of LXA$_4$ (2–200 nM) potently mimicked endocannabinoid actions. Therefore, a typical agonistic activation of the CB$_1$ orthosteric site by LXA$_4$ did not seem to be the likely mechanism supporting LXA$_4$'s cannabimimetic effects in vivo.

LXA$_4$ Potentiates AEA Effects. To understand the high potency of LXA$_4$ despite its relatively low affinity to CB$_1$ receptors, we investigated the interaction between LXA$_4$ and endocannabinoids in vivo. Subeffective doses of AEA and 2-AG (10 and 1 pmol, respectively; Fig. S2 *C and D*) were coinjected i.c.v. with a subeffective dose of LXA$_4$ in mice that were tested for catalepsy. Interestingly, LXA$_4$ potentiated the cataleptic effect of AEA

(Fig. 3*A*), but not the one of 2-AG (Fig. S3*A*) or significantly of CP55940 (Fig. S4). LXA$_4$ might potentiate the effect of AEA by inhibiting endocannabinoid degradation. However, LXA$_4$ did not alter the activity of the main AEA-degrading enzyme fatty acid amide hydrolase (FAAH; Fig. 3*B*), nor of the main 2-AG–degrading enzyme monoacylglycerol lipase (Fig. S3*B*). Consistently, i.c.v. administration of LXA$_4$ did not alter brain levels of AEA (Fig. 3*C*) or of 2-AG (Fig. S3*C*). The interaction between LXA$_4$ and AEA was further confirmed in HEK cells transfected with mouse CB$_1$ receptors. LXA$_4$ increased the potency of AEA in decreasing forskolin (FSK)-induced cAMP levels by ∼386 times (EC$_{50}$ AEA 1,547 nM × EC$_{50}$ AEA + LXA$_4$ 4 nM; Fig. 3*D*) at a subeffective concentration (100 nM; Fig. S3*D*). LXA$_4$ did not influence cAMP levels in concentrations ranging from 0.1 nM to 1 μM (Fig. S3*D*). LXA$_4$ slightly potentiated 2-AG–induced inhibition of FSK-induced cAMP accumulation (EC$_{50}$ 2-AG 147 nM; EC$_{50}$ 2-AG + LXA$_4$ 0.1 nM) in HEK-CB$_1$ cells, but apparently reduced 2-AG efficacy in half (Fig. S3*D*). Interestingly, the AEA–LXA$_4$ interaction was not observed in a GTPγS assay (Fig. S5).
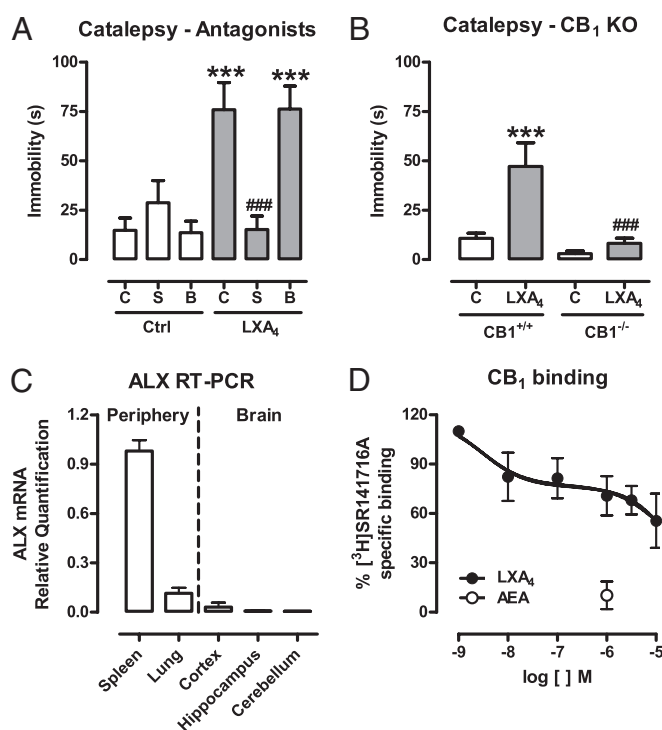

Fig. 2. Role of CB$_1$ cannabinoid receptor on LXA$_4$-induced catalepsy. (*A*) The CB$_1$ antagonist SR141716A (S; 1 mg/kg, i.p.), the ALX antagonist BOC-2 (B; 10 μg/kg, i.p.) or control (C) was injected 50 min before lipoxin A$_4$ (LXA$_4$, 1 pmol/5 μL, i.c.v.) or control (Ctrl) and tested in the catalepsy test 5 min later. LXA$_4$ induced catalepsy, which was prevented by the CB$_1$ antagonist (pretreatment vs. treatment) [$F(2,42) = 10.07$, $P = 0.0003$, $n = 8$/group]. (*B*) A selected dose of LXA$_4$ (1 pmol/5 μL, i.c.v.) or control (C) was injected in CB$_1$ knockout (CB$_1^{-/-}$) or wild-type mice (CB$_1^{+/+}$) 5 min before the bar catalepsy test. LXA$_4$ induced catalepsy in CB$_1^{+/+}$, but not in CB$_1^{-/-}$ mice (genotype vs. treatment) [$F(1,21) = 4.75$, $P = 0.04$, $n = 6–7$/group]. (*C*) Real-time PCR confirmed the negligible expression of ALX receptors in the brain. Spleen and lung tissues were used as positive controls for ALX mRNA ($n = 4$/group). (*D*) Competitive binding of LXA$_4$ (1 nM–10 μM) against the CB$_1$-selective radiolabeled antagonist [^{3}H]SR141716A (0.5 nM) in mouse brain membranes revealed very low affinity of LXA$_4$ for the CB$_1$ receptors ($K_i > 10$ μM, $n = 4$/group). AEA (1 μM) was used as a positive control and inhibited nearly 90% of [^{3}H]SR141716A binding. Binding curves were generated by nonlinear regression (curve fitting). Data are represented as mean ± SEM. ***$P < 0.001$ vs. Control; ###$P < 0.001$ vs. LXA$_4$ + C (*A*) or vs. LXA$_4$ in CB$_1^{+/+}$ mice (*B*) (Duncan's post hoc).
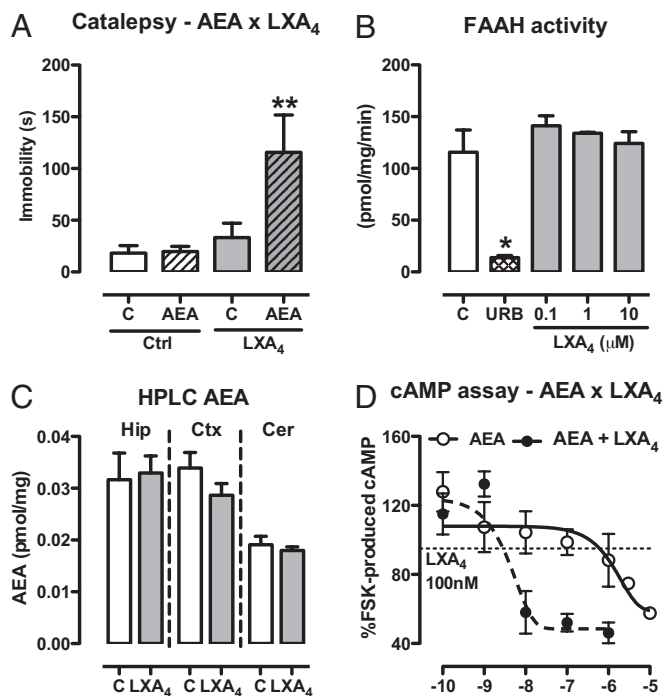
Fig. 3. LXA$_4$ interacts positively with the endocannabinoid AEA. (*A*) Selected pre-effective dose of AEA (10 pmol/2 µL, i.c.v.) was coinjected with LXA$_4$ (0.01 pmol/2 µL, i.c.v.) 5 min before the bar catalepsy test. LXA$_4$ interacted with AEA [$F(3,29) = 4.98$, $P = 0.007$, $n = 8$–9/group]. (*B*) Activity of the AEA-degrading enzyme FAAH was measured in the presence of LXA$_4$ (100 nM–10 µM) using [^{14}C]AEA (1.8 µM) as substrate. The FAAH inhibitor URB597 (URB, 50 nM) was used as positive control. LXA$_4$ did not interfere with FAAH activity [$F(3,7) = 0.73$, $P = 0.58$, $n = 3$/group], as opposed to the positive control URB597 ($t = 4.70$, $P < 0.05$). (*C*) AEA 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 signs 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 mouse CB$_1$ receptors. Cells were incubated with AEA (0.1 nM–10 µM) or AEA + LXA4 (100 nM), stimulated for 10 min with FSK for evaluation of the intracellular content of cAMP (~386 times potency increase in presence of LXA$_4$; EC$_{50}$: 1,547 × 4 nM). The results of the cAMP assay were normalized by the FSK group. 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).

LXA$_4$ Is a Positive Allosteric Modulator of CB$_1$ Receptors. LXA$_4$ might directly modulate AEA interaction with CB$_1$ receptors. Therefore, we investigated whether [^{3}H]SR141716A displacement by AEA would be affected by a subeffective concentration of LXA$_4$ (100 nM). The binding curve of AEA (1 nM–10 µM) was displaced to the left by LXA$_4$, suggesting enhancement of AEA-CB$_1$ affinity by LXA$_4$ (Fig. 4*A*). The best fitting of the AEA curve supported a two-site interaction, with a clearer LXA$_4$ effect at the high-affinity binding site (IC$_{50}$ 17 vs. 2 nM) compared with the low-affinity binding site (IC$_{50}$ 1,409 vs. 692 nM) (Fig. 4*A*). Using radiolabeled cannabinoid agonists (0.5 nM [^{3}H]CP55940 and 0.5 nM [^{3}H]WIN55212-2), and performing the binding assays with increasing concentrations of LXA$_4$, confirmed that LXA$_4$ enhances the affinity of these ligands to CB$_1$ receptors. LXA$_4$ enhanced 100% of [^{3}H]CP55940 binding (Fig. 4*B*) and nearly 30% of [^{3}H]WIN55212-2 binding (Fig. 4*B*), suggesting a functional selectivity in the LXA$_4$ effects (Fig. 4*B*). Kinetic dissociation-binding assays allow evaluating the influence of a given substance on the dissociation kinetics of a preformed orthosteric ligand–receptor complex. As the dissociation kinetic

is not altered if the interacting ligands recognize the same binding site, this assay is considered the method of choice for measuring allosteric modulation (9). The binding of [^{3}H]CP55940 was displaced by an excessive amount of WIN55,212 and followed over time. Addition of LXA$_4$ slowed down the agonist dissociation rate (k) (Control = 1.3 ± 0.48, $r^2 = 0.74$ vs. k LXA$_4$ 0.33 ± 0.13, $r^2 = 0.71$, $P < 0.05$; Fig. 4*C*), which is consistent with the notion that LXA$_4$ increases the affinity of the CB$_1$ receptor via an allosteric mechanism.

Allosteric modulation may be achieved by the binding of a compound to an allosteric site in a given receptor, but it can also indirectly result from protein–protein interactions, in either case changing the pharmacological properties of the main receptor (24). The fact that cells transfected with mouse CB$_1$ showed enhanced AEA-induced cAMP inhibition with coapplication of LXA$_4$, an effect not observed in nontransfected cells, suggests that the cannabinoid receptor is the site of the allosteric modulation of the CB$_1$ receptor. To investigate the dynamic action of LXA$_4$ at the CB$_1$ receptor, *Xenopus laevis* oocytes were injected with mouse CB$_1$ receptor and two GIRK subunit cDNAs to study the electrophysiological interactions between LXA$_4$ and AEA. AEA (100 nM) potently increased inward K$^+$ currents measured in the oocytes. LXA$_4$ (100 nM) alone had no effect on these
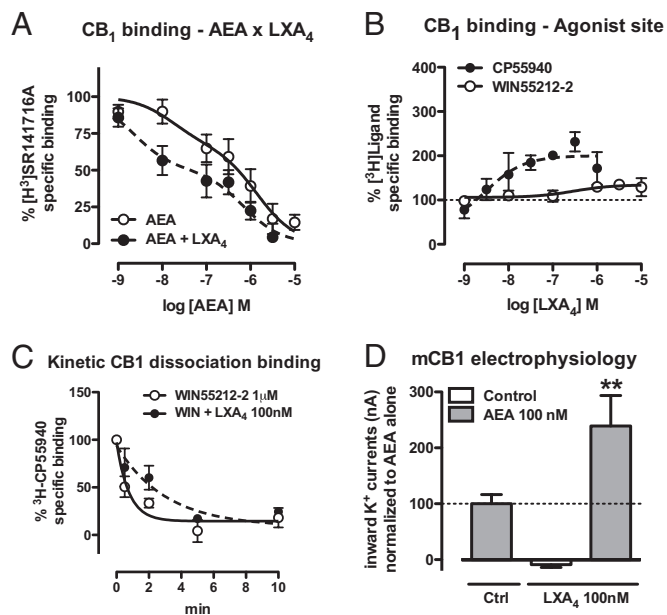

Fig. 4. Positive allosteric modulation of CB$_1$ receptors by LXA$_4$. (*A*) Competitive binding of AEA (1 nM–10 µM) against the CB$_1$-selective radiolabeled antagonist [^{3}H]SR141716A (0.5 nM) in mouse brain membranes performed in the presence or absence of LXA$_4$ (100 nM). LXA$_4$ increased the affinity of AEA for the CB$_1$ cannabinoid receptors ($n = 9$–10/group). (*B*) Competitive binding of LXA$_4$ (1 nM–10 µM) against the cannabinoid agonists [^{3}H]CP55914 and [^{3}H]WIN55212-2 in mouse brain membranes. LXA$_4$ increases twofold the affinity of [^{3}H]CP55914 to CB$_1$ receptors ($n = 4$–5/group). (*C*) Kinetic dissociation binding showing the displacement of the CB$_1$-ligand [^{3}H]CP55914 (0.5 nM) by an excess of the agonist WIN55212-2 (1 µM) over time. The decrease in dissociation rate (k Control = 1.3 ± 0.48, $r^2 = 0.74$ vs. k LXA$_4$ 0.33 ± 0.13, $r^2 = 0.71$, $P < 0.05$) confirms that LXA$_4$ increases the affinity to the CB$_1$ receptor via an allosteric mechanism. (*D*) Electrophysiological recording of *Xenopus* oocytes expressing mouse CB$_1$ receptors and K$^+$ channels was performed to confirm that the AEA–LXA$_4$ (100 nM) interaction occurs in fact at the CB$_1$ receptor protein ($n = 4$–9/group). LXA$_4$ increases twofold the potency of AEA to generate CB$_1$-dependent K$^+$ currents in the oocytes [$F(2,17) = 7.26$, $P = 0.04$]. Binding curves generated by nonlinear regression (curve fitting). Data are represented as mean ± SEM. **$P < 0.01$ vs. AEA 100 nM (Duncan's post hoc).

currents, but it strongly potentiated the effect of AEA (Fig. 4D). Thus, LXA$_4$ potentiates the effects of AEA at the level of the CB$_1$ receptor protein. Altogether, our results strongly suggest that LXA$_4$ is an endogenous allosteric modulator of the CB$_1$ receptor that specifically enhances AEA signaling.

LXA$_4$ Contributes to AEA in Vivo Effects. Exogenous LXA$_4$ causes in vivo and in vitro effects consistent with a positive allosteric modulation of the CB$_1$ receptor. As LXA$_4$ is an endogenous compound present at significant levels in the hippocampus, cortex, and cerebellum (Fig. 5A), we tested whether endogenous levels of LXA$_4$ in the brain would influence AEA effects in vivo. LXA$_4$ synthesis was reduced by the administration of the 5-lipoxygenase (LOX) inhibitor MK-886 (0.3–3 mg/kg, i.p.) before the i.c.v. injection of an effective dose of AEA (Fig. 5B). 5-LOX inhibition dose-dependently reduced the cataleptic effect of AEA up to roughly 50% (Fig. 5B). The MK-886 effect was reverted by exogenous i.c.v. LXA$_4$, suggesting that LXA$_4$ is the 5-LOX derivative that contributes to AEA effects in the brain (Fig. 5C). Very similar results were obtained in 5-LOX KO mice, which show decreased capacity to produce LXA$_4$ (25, 26). The effect of AEA is strongly reduced in the 5-LOX KO mice, and the coadministration of LXA$_4$ fully rescued the phenotype (Fig. 5D). Thus, endogenous LXA$_4$ in the brain is necessary for the full effect of AEA.
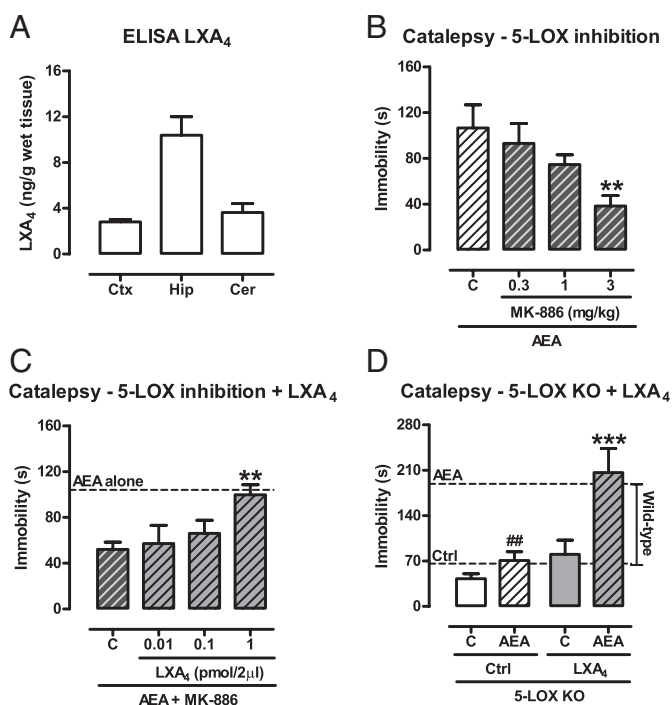

Fig. 5. Endogenous LXA$_4$ contributes to endocannabinoid signaling. (A) Presence of LXA$_4$ in the mouse brain assessed by ELISA in the cortex (Ctx), hippocampus (Hip), and cerebellum (Cer) (n = 5/group). (B) Inhibition of the LXA$_4$-synthesizing enzyme 5-LOX by MK-886 (0.3–3 mg/kg, i.p.) reduced the effects of AEA (200 pmol/2 µL, i.c.v.) in the bar catalepsy test [F(3,24) = 3.38, P = 0.04, n = 6–8/group]. (C and D) Supplementation of LXA$_4$ (0.01–1 pmol/2 µL, i.c.v.) restored the "normal" phenotype under 5-LOX inhibition (MK-886 3 mg/kg, i.p.) [F(3,19) = 3.27, P = 0.04, n = 5–6/group] or in the 5-LOX knockout mice (pretreatment vs. treatment) [F(1,17) = 4.96, P = 0.04, n = 5–6/group], indicating that the endogenous levels of LXA$_4$ contribute to endocannabinoid function. Dashed lines represent the mean of groups treated with AEA (200 pmol/2 µL, i.c.v.) or control, as indicated. Data are represented as mean ± SEM. **P < 0.01; ***P < 0.001 vs. Control; ##P < 0.01 vs. AEA wild-type mice (dashed lines) (Duncan's post hoc).

Neuroprotection Against β-Amyloid (1–40)-Induced Memory Impairments. To test for potential therapeutic application of LXA$_4$ as an allosteric enhancer of CB$_1$ receptors, we investigated the effects of LXA$_4$ in the β-amyloid (1–40)-induced spatial memory impairments in the water maze test, which is sensitive to endocannabinoid modulation (27, 28). It is already known that AEA is endogenously released in the first week after β-amyloid (1–40) i.c.v. injection (28). Therefore, LXA$_4$ (1 pmol/2 µL) was coinjected i.c.v. with β-amyloid (1–40) (400 pmol/2 µL, i.c.v.) 7 d before training of spatial memory retention in the water maze (Fig. 6 and Fig. S6). β-Amyloid (1–40) impaired spatial memory formation, which was prevented by coinjection of LXA$_4$ (Fig. 6). LXA$_4$ effects were only mildly inhibited by Boc-2 (BOC, 10 µg/kg, i.p.), but they were fully prevented by SR141716A (SR, 1 mg/kg, i.p.), showing that LXA$_4$-induced neuroprotection depends on CB$_1$ cannabinoid receptors.

Discussion

The present data show that the endogenous eicosanoid LXA$_4$ is an allosteric enhancer of CB$_1$ receptor signaling in the brain. The data do not preclude, nor reduce the importance of the extensive work done on LXA$_4$ effects in the periphery, showing that LXA$_4$ contributes to inflammation resolution (29). Rather, our data may impact those studies by suggesting a target for LXA$_4$ that may contribute to its therapeutic effects. For example, a convincing mechanism for LXA$_4$-induced analgesia was still an open question, and here we show that LXA$_4$ is a potent CB$_1$ receptor-dependent central analgesic in vivo. The interaction between cannabinoids and lipoxins was suggested earlier in a study showing that the nonpsychotropic cannabinoid ajulemic acid induces the release of LXA$_4$ (30). In addition to our previous study showing that aspirin-triggered LXA$_4$ enhances AEA effects (22), here we show that endogenous LXA$_4$ contributes to CB$_1$-mediated effects as a positive allosteric modulator, with physiological relevance for endocannabinoid-dependent regulation of brain functions and potential therapeutic utility.

Allosteric modulation of CB$_1$ receptor was originally described using synthetic compounds (9). The "Org" compounds (Org27596 and Org29647) and PSNCBAM-1 (31) enhance affinity and reduce efficacy of cannabinoid agonists acting at the orthosteric site of CB$_1$ receptors (9). These compounds have ligand-dependent effects, as they increase the affinity of [^{3}H]CP55940 but decrease the affinity of [^{3}H]SR141716A (9). Therefore, these compounds were considered as interesting negative regulators of (endo)cannabinoids, with potential therapeutically important regional and temporal selectivity (32). LXA$_4$ differs from the previously described compounds because (i) it promotes enhancement, rather than reduction of CB$_1$-mediated effects; (ii) it has apparent functional selectivity for AEA over 2-AG in vivo and in vitro; and (iii) it is physiologically present in the brain. The impact of LXA$_4$ on endocannabinoid affinity is more evident in the high-affinity binding site in a two-site interaction model, likely suggesting an increase of affinity toward the activated conformational state (R*) of CB$_1$ (9). The allosteric nature of the LXA$_4$–CB$_1$ interaction was confirmed with a dissociation-binding assay, where the dissociation kinetics of a preformed orthosteric ligand–receptor complex is evaluated. Therefore, our interpretation of the current results is that LXA$_4$ likely helps in stabilizing the pair formed by AEA and CB$_1$ receptors in a given conformation that favors AEA efficacy. For unknown reasons, the conformation stabilized by LXA$_4$ does not favor 2-AG as well. Considering the agonists tested in the present study, we may suggest that LXA$_4$ potentiates AEA = CP > WIN > 2-AG, although this phenomenon of LXA$_4$-induced functional selectivity certainly deserves further characterization.

Our findings may have an impact on the current interpretation of the role of AEA/2-AG as endocannabinoids. AEA has been described as an endocannabinoid (33), but lately it has been regarded as an endovanilloid, displaying higher affinity for TRPV$_1$
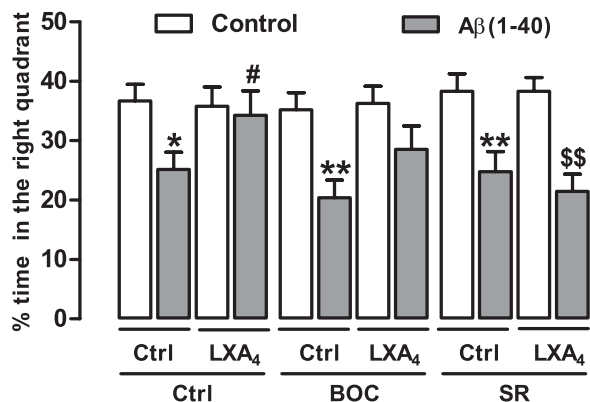
Water maze spatial memory test

Fig. 6. LXA$_4$ protects against β-amyloid (1–40)-induced memory impairment in a CB$_1$-dependent fashion. Coinjection with LXA$_4$ (1 pmol/5 μL, i.c.v.) prevents the β-amyloid (1–40) [Aβ (1–40)-induced; 400 pmol/2 μL, i.c.v.] spatial memory impairment in the water maze test observed 7 d later [F(2,88) = 4.82, P = 0.01, n = 7–10/group]. Neuroprotective effects of LXA$_4$ were prevented by the CB$_1$ cannabinoid receptor antagonist SR141716A (SR; 1 mg/kg, i.p.) and only partially by the ALX lipoxin receptor antagonist BOC-2 (BOC 10 μg/kg, i.p.) The figure indicates the percentage of time spent in the target quadrant, where the hidden platform was previously located (see Fig. S6 for water maze training and administration schedule). Data are represented as mean ± SEM. *P < 0.05, **P < 0.01 vs. Ctrl-Ctrl (white bars); #P < 0.05 vs. Aβ (1–40)-Ctrl; $$P < 0.01 vs. Aβ (1–40) + LXA$_4$ (Duncan's post hoc).

than for CB$_1$ receptors under certain conditions (34). Furthermore, AEA is only a partial agonist, whereas 2-AG is a full agonist of the CB$_1$ receptor (4, 35), suggesting that 2-AG is the "true" endocannabinoid in the CNS to which the retrograde messenger-mediated neuroplasticity can be attributed (36). According to the current results, there is an enhancement of AEA affinity/potency in the presence of LXA$_4$, which could help bring AEA back to the status of a "true" endocannabinoid, potentially reducing the efficacy of 2-AG effects. Thus, our data strongly suggest the existence of a functional selectivity between AEA and 2-AG, which is in good agreement with recent data proposing that these two molecules may mediate substantially distinct physiological effects and regulate each other (37–39).

We found that FSK-stimulated cAMP production is more strongly and more efficiently suppressed by the costimulation with AEA and LXA$_4$ compared with AEA alone. On the other hand, the costimulation with AEA and LXA$_4$ did result, unexpectedly, in a decrease in G-protein binding in the GTPγS-binding assay. At the moment, the reasons for this apparent discrepancy are not known. One possible methodological explanation may reside in the fact that the GTPγS assay is known to be biased toward the measurement of G$_{i/o}$ activation and is less efficient in determining receptor coupling to G$_s$ or G$_q$ proteins (40). The CB$_1$ receptor is able to couple to all three types of G proteins and, although G$_{i/o}$ is the most prominent one (23), coupling to G$_s$ (41) and G$_q$ protein (42) has also been reported. Thus, the costimulation of AEA with LXA$_4$ might reduce the coupling of the CB$_1$ receptor to G$_s$, which is more difficult to detect using GTPγS assays (40). In addition, G$_i$ protein-independent effects of CB$_1$ were recently proposed (43), which could also explain the different results obtained with cAMP and GTPγS assays. Notably, our results imply that nearly half of what we understand to be "pure" AEA effects are LXA$_4$-dependent, which may lead to further investigation of physiological and therapeutic effects previously attributed exclusively to AEA (44). Another interesting link may be suggested by the report that an unknown LOX derivative would be partially responsible for TRPV$_1$-mediated

AEA effects in the isolated bronchus (45). If this holds true for the CNS, the corelease of AEA with that "unknown entity" or with LXA$_4$ could be a kind of molecular switch driving AEA affinity toward TRPV$_1$ or CB$_1$ receptors. Thus, it will be very interesting to test the role of the "affinity switch" of lipoxins in endovanilloid and cannabinoid signaling.

Moreover, knowing that ajulemic acid induces the release of LXA$_4$ acting as a proresolving mediator in inflammation (30); that LXA$_4$ increases the affinity of AEA for the CB$_1$ receptors (this study); and that certain metabolites of AEA degradation by lipoxygenases retain affinity for the CB$_1$ receptor (46) or reduce AEA metabolism by FAAH (47), we may hypothesize that LOX derivatives such as LXA$_4$ might participate in a positive feedback loop sustaining endocannabinoid tonus under certain conditions, for example, brain inflammation (48), epilepsy (49, 50), or aging (51). This may be an interesting explanation for the observed neuroprotection against β-amyloid (1–40)-induced memory impairment, which is regarded as an important component of Alzheimer's disease pathophysiology (52). A recent report showed that the degradation of the endocannabinoid 2-AG by monoacyl glycerol lipase (MAGL) generates COX-derived neuroinflammatory eicosanoids that negatively impact the development of symptoms in a Parkinson's disease mouse model (10). This may suggest two different pathways of responses after neuronal injury. On one hand, a LOX–AEA–FAAH pathway may contribute to inflammation resolution, whereas a COX-2–AG–MAGL pathway may worsen the neuroinflammation process.

Pharmacological blockade or genetic inactivation of the LXA$_4$-synthesizing enzyme 5-LOX decreases AEA effects in vivo, strongly suggesting that the lipoxin acts as an endogenous modulator of AEA signaling. Our data show that LXA$_4$ exerts an endogenous role in AEA-related signaling, as shown by pharmacological inhibition or genetic deletion of the synthesizing enzyme 5-LOX. An alternative interpretation is that a LOX-derived molecule resulting from AEA metabolism could underlie the observed effect (53). Interestingly, 5-LOX inhibition abolishes long-term potentiation induction in the hippocampus (19), and recent data suggest a role for the CB$_1$ receptor in this phenomenon (20). Thus, it is possible that endogenous LXA$_4$ enhancement of AEA signaling might participate in long-term synaptic plasticity. Notably, previous studies describing CNS-related LOX functions did not associate these effects with any known receptor (14, 15, 17). Therefore, by linking LXA$_4$ to enhancement of CB$_1$ receptor function, the present study might provide a potential mechanism to make those observations.

Although the LXA$_4$-driven functional selectivity for endocannabinoids needs further characterization, our data clearly show that LXA$_4$ is necessary for some cannabinoid effects of AEA through a likely allosteric modulation mechanism at the CB$_1$ receptor. These results add a player in brain endocannabinoid signaling, which might help in clarifying unsolved issues in the field, such as the differential mechanism of action of different endocannabinoids (54). Moreover, the endogenous coagonist nature of LXA$_4$ for AEA actions at CB$_1$ receptors might pave the way to developing therapeutic concepts to exploit the potentialities of the endocannabinoid system in brain diseases.

Materials and Methods

A complete description of materials and methods is provided in *SI Materials and Methods*.

Experiments were conducted in Swiss albino mice, inbred C57BL/6 mice, CB$_1$ knockouts (CB$_1$$^{-/-}$) and controls (CB$^{+/+}$), and 5-LOX knockouts. Behavioral tests included tetrad test screening for cannabinoid effects and water maze spatial memory task for long-term memory. Ligand affinity was studied by competitive and dissociation binding assays using cannabinoid ligands ([^{3}H]SR141716A, [^{3}H]CP55914 and [^{3}H]WIN55212-2) in mouse whole-brain membranes. Endocannabinoid metabolism was studied by enzymatic activity of FAAH and MAGL using [^{14}C]AEA and [^{3}H]2-AG in brain homogenates and quantification of AEA and 2-AG levels by HPLC. In vitro functional assay of FSK-induced cAMP accumulation in CB$_1$-transfected HEK293T cells and

investigation of G protein–CB_1 receptor interaction by agonist-stimulated [^{35}S]GTPγS binding were conducted. Immunodetection of LXA_4 levels in the brain using an ELISA kit and real-time PCR for ALX receptors. Electrophysiology (voltage-clamp) in *Xenopus* oocytes containing CB_1 receptor linked G-protein–gated K^+ channels (Kir 3.1 and Kir 3.4).

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