SI Appendix

METHODS

Behavioral experiments. For behavioral experiments 2-AG was purchased from Tocris (TOC-1298-M010) dissolved in a cocktail of ethanol, Cremophor (Sigma) and saline (1:1:18), and administered to the animals intravenously (10 $mgKg^{-1}$). Noladin ether (NE) was purchased from Tocris (TOC-1411-M005) dissolved in a cocktail of ethanol, Cremophor (Sigma) and saline (1:1:18), and administered to the animals intravenously (10 mg/Kg and 5 mg/Kg). JZL 184 obtained from Cayman Chem USA was suspended in a cocktail of dimethylsulfoxid, Tween 20, and water (1:1:8), and administered to the animals intraperitoneally (16 $mgKg^{-1}$) 2 hours before performing the open field test. THDOC was purchased from Sigma Aldrich, dissolved in ethanol and water (1:19) and injected intravenously (2 $mgkg^{-1}$).

CB_1/CB_2 receptor deficient mice ($Cnr1^{-/-}/Cnr2^{-/-}$) (1), $GABA_A$ receptor β_2 subunit KO mice (2) and WT control C57BL6/J ($Cnr1^{+/+}/Cnr2^{+/+}$) and C57BL6/J/129SvEv animals were bred in our animal facility. During the experiments the animals had free access to food and water. Each animal was used only once and was naïve to the test. Experiments were carried out in the active (dark) phase between 10 and 17 hours. Animal procedures followed the guidelines of the German Animal Protection Law and approved by LANUW. Locomotor activity was assessed as previously described (3). Briefly, the animals were placed directly after 2-AG injection in the center of dimly illuminated (20 lux at the ground level of the arena) open-field arena (45 x 45 x 22 cm) in a sound-attenuated room. The loss of righting reflex after 2-AG and NE treatment was tested placing the animals on their back onto the arena. Exploratory locomotor activity was

continuously recorded and evaluated in 1-minute intervals for 10 minutes with an automatic system (TSE Systems GmbH, Germany).

Statistical analyses were performed using the STATISTICA software package. For animal experiments, statistical differences between treated and vehicle control groups were determined by repeated measurements (ANOVA) and post hoc least square difference tests. Differences between the analyzed samples were considered as significant at $P \leq 0.05$. Nonlinear regression analysis (curve fitting) was performed with Kaleidagraph software.

Quantification of 2-AG and arachidonic acid. The extraction and purification protocol was adapted from Muccioli and Stella (4). Shortly, immediately frozen brains were cut along their longitudinal axis and weighted. Chloroform was added (1ml/100mg) the half-brains were homogenized with a polytron (15,000 rmp for 1 min) and sonicated twice for 10 sec. The homogenate was added to 10 ml ice cold chloroform containing the internal standards (328 pmol AA-d8, 529 pmol 2-AG-d5). Folch extraction was performed by adding 5 ml methanol and 2.5 ml PBS. The mixture was vigorously vortexed and sonicated for 5 min at 4°C followed by a centrifugation for 5 min at 800 g. The organic phase was recovered into a glass vial and dried under N_2. The dried organic phase was reconstituted into 1 ml of EtOH, vortexed and 9 ml of water were added. Solid phase extraction was performed with Sep-Pak cartridges (Waters). Columns were conditioned with 3 ml methanol 3 ml of 10 % ethanol, then the samples were applied and washed with 3 ml of 10 % ethanol. The arachidonic acid and 2-AG were eluted with 3 ml of ethyl acetate/Acetonitrile 8:2. Eluates were dried under N_2 and reconstituted in 50 ul of ACN. 20 ul of N,N-diisopropylethylamine (Sigma) and

20 ul of PFB bromide ((Sigma) 1g in 3ml ACN were added and incubated for 25 min at 45°C to derivatize arachidonic acid. Excess of reagent was evaporated the sample reconstituted in 25 ul of DMIPSI (TCI Europe) and 2-AG was derivatized with it for 1 h at room temperature. Samples were stored at -20°C until GC/MS analysis.

GC/MS analysis. Samples were injected in splitless mode into an Agilent 6890N GC (HP-5MS column 30 m). The oven temperature program was as follows: Initial temp. 150°C for 1 min, followed by an 8°C/min increase up to 280°C which was hold for 10 min. Helium was used as a carrier gas at a flow rate of 1.5 ml/min. Coupled to the GC was an Agilent 5975C MSD. The following ions were used for selected ion monitoring (SIM) analysis: 2-AG/1-AG, m/z 535; 2-AG-d5/1-AG-d5, m/z 540; AA, m/z 386, 484; AA-d8, m/z 392. Because of a significant amount of spontaneous acyl group migration (isomerisation) of 2-AG during extraction the peak areas of 1(3)-AG and 2-AG were combined for quantitative analysis.

1. Karsak M, et al. (2007) Attenuation of allergic contact dermatitis through the endocannabinoid system. *Science* 316:1494-1497.
2. Sur C, et al. (2001) Loss of the major $GABA_A$ receptor subtype in the brain is not lethal in mice. *J Neurosci* 21:3409-3418.
3. Rácz I, et al. (2008) Anandamide effects on 5-HT(3) receptors in vivo. *Eur J Pharmacol* 596:98-101.
4. Muccioli GG, Stella N (2008) An optimized GC-MS method detects nanomolar amounts of anandamide in mouse brain. *Anal Biochem* 373:220-228.

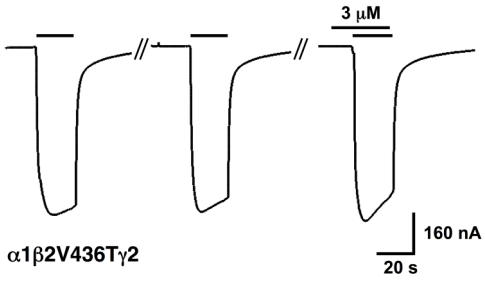

Figure S1 The point mutation $\alpha_1\beta_2$V436Tγ_2 abolishes potentiation by 2-AG. $\alpha_1\beta_2$V436Tγ_2 receptors were expressed in Xenopus oocytes. Electrophysiological traces show twice an application of 0.5 μM GABA followed by the same concentration GABA combined with 3 μM 1-AG.

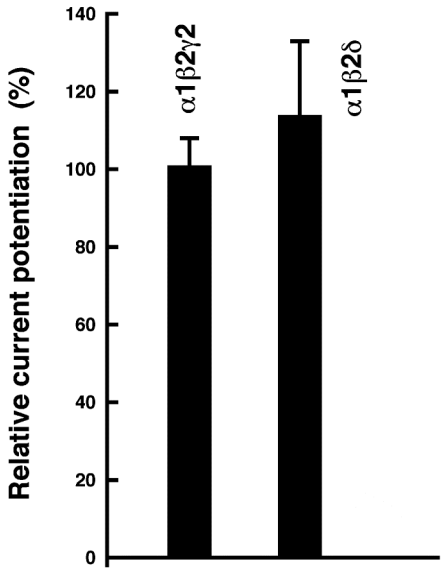

Figure S2 Current potentiation by 3 μM of 2-AG of recombinant $\alpha_1\beta_2\delta$ and $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors. Recombinant receptors were functionally expressed in Xenopus oocytes and investigated by electrophysiological techniques. Data are shown as mean values ± SD ($n \geq 4$). The GABA concentration was $EC_{1.0\text{-}3.3}$ for $\alpha_1\beta_2\gamma_2$ and $EC_{3\text{-}9}$ for $\alpha_1\beta_2\delta$.

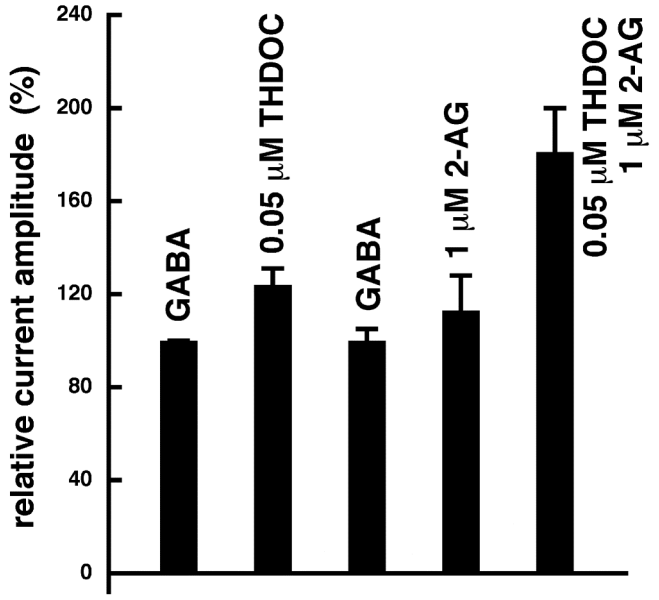

Figure S3 Super-additivity between 2-AG and the neurosteroid 0.05 μM THDOC. Recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors were functionally expressed in Xenopus oocytes. a) 0.5 μM GABA were applied twice alone followed by the same concentration of GABA in combination with 0.05 μM THDOC, GABA alone, GABA in combination with 1 μM 2-AG, and GABA in combination with 0.05 μM THDOC and 1 μM 2-AG. b) Three such experiments were averaged. Data are shown as mean values ± SD (n = 3).

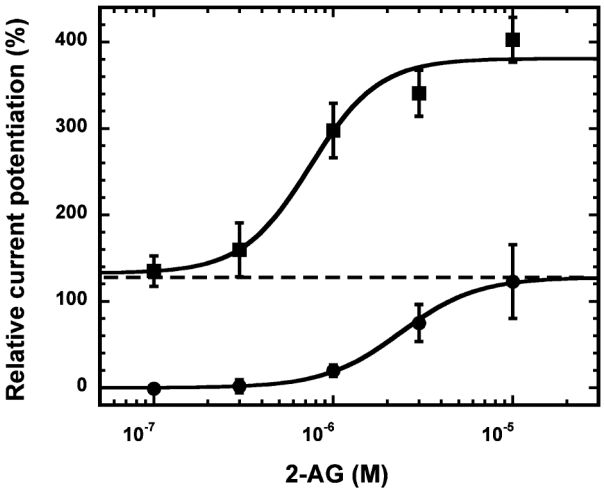

Figure S4 Super-additivity of the modulation by diazepam and 2-AG. Recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors were functionally expressed in Xenopus oocytes. Cumulative concentration response curve of 2-AG without potentiation by diazepam (lower curve) and after potentiation with 0.3 μM diazepam (upper curve).

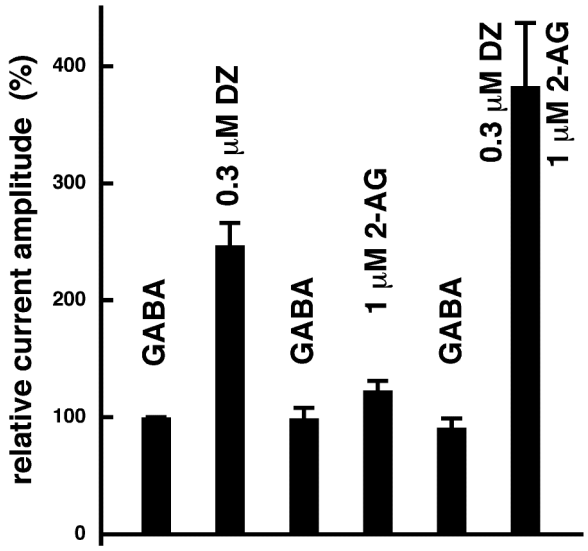

Figure S5 Super-additivity of the modulation by 2-AG and diazepam. Recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors were functionally expressed in Xenopus oocytes. A concentration of GABA eliciting $EC_{0.2\text{-}0.5}$ was applied twice alone followed by the same concentration of GABA in combination with 0.3 μM diazepam, GABA alone, GABA in combination with 1 μM 2-AG, GABA alone and GABA in combination with 0.3 μM diazepam and 1 μM 2-AG. Three such experiments were averaged. Data are shown as mean values ± SD (n = 3).

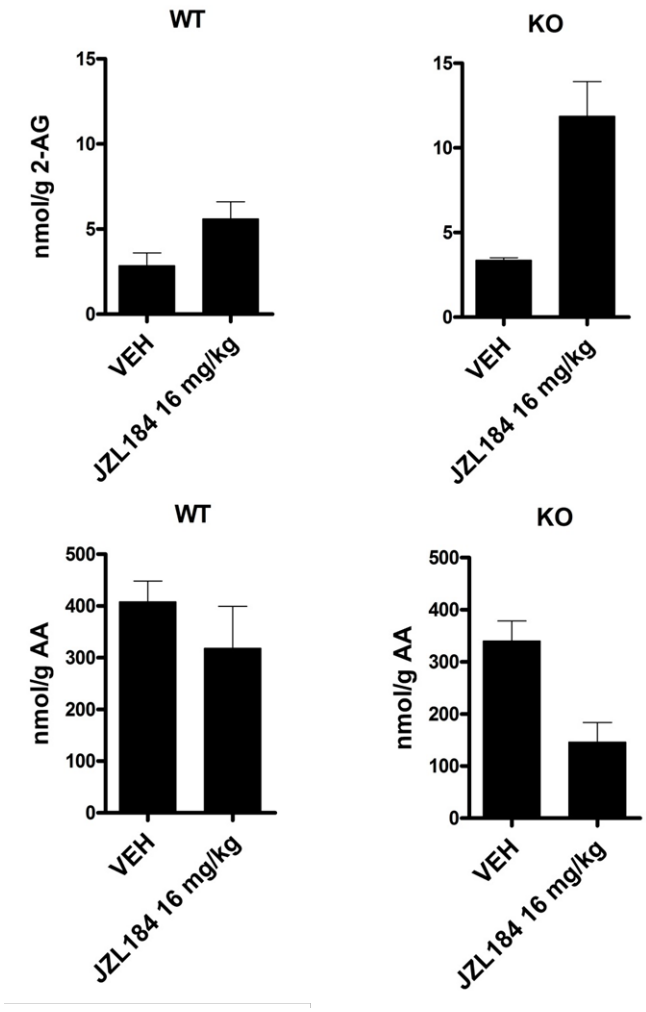

Figure S6 Relative levels of 2-AG and arachidonic acid (AA) in whole brain of wild type (WT) and cannabinoid receptor ($Cnr1^{-/-}/Cnr2^{-/-}$) double KO mice (KO) after treatment with JZL 184 (16 $mgKg^{-1}$ i.v.). Relative quantification was performed by GC/MS from brain tissue isolated after termination of locomotion experiments. Because of significant spontaneous acyl group migration (isomerisation) of 2-AG during extraction the peak areas of 1(3)-AG and 2-AG were combined for quantitative analysis. Data are shown as mean values ± SEM (n = 4).

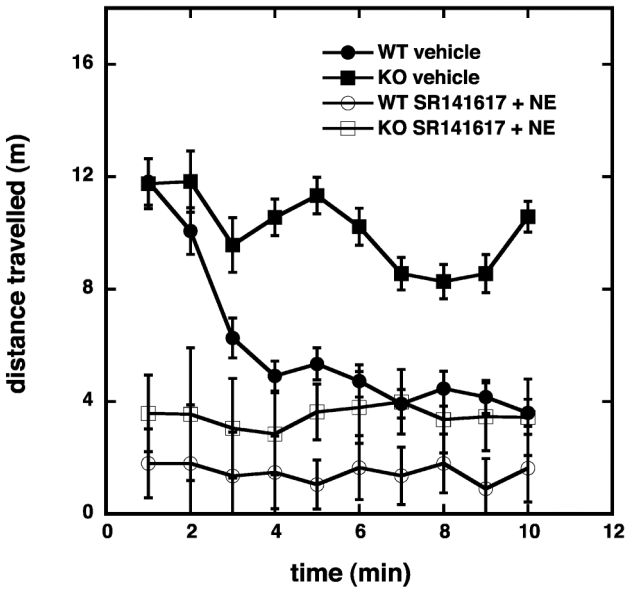

Figure 7S Combined effects of NE and SR141716 on motility. The locomotor activity was determined in WT and β_2 KO mice treated with vehicle or with NE (10 $mgKg^{-1}$ i.v.) + SR141716 (3 $mgKg^{-1}$ i.v.). Data are shown as the mean distance travelled in 1 min time bins ± SEM.