A single *in-vivo* exposure to Δ9THC blocks endocannabinoid-mediated synaptic plasticity

Susana Mato[1,5], Vivien Chevaleyre[2,5], David Robbe[1,4], Angel Pazos[3], Pablo E Castillo[2] & Olivier J Manzoni[1]

Endogenous cannabinoids (eCB) mediate synaptic plasticity in brain regions involved in learning and reward. Here we show that in mice, a single *in-vivo* exposure to Δ9-tetrahydrocannabinol (THC) abolishes the retrograde signaling that underlies eCB-mediated synaptic plasticity in both nucleus accumbens (NAc) and hippocampus *in vitro*. This effect is reversible within 3 days and is associated with a transient modification in the functional properties of cannabinoid receptors.

There is strong evidence that a single exposure to addictive drugs can alter synaptic plasticity in the brain reward pathway[1–3]. Whether a single exposure to cannabis derivatives also modifies endocannabinoid-mediated synaptic plasticity has not been explored. Here we evaluated the consequences of a single *in-vivo* exposure to THC, the principal psychoactive ingredient of cannabis, in the NAc and the hippocampus, two brain areas where we have previously characterized eCB-mediated, long-lasting forms of synaptic plasticity[4–6].

Repetitive activation of prelimbic cortex afferents to the NAc induces an eCB-mediated long-term depression of excitatory transmission (eCB-LTD) that might be part of a negative feedback loop reducing the strength of glutamatergic synapses during sustained cortical activity[5]. In the hippocampus, eCBs mediate long-term depression of inhibitory synaptic transmission (I-LTD), a phenomenon that could underlie the effects of cannabinoids on learning and memory[4]. To test whether eCB-mediated retrograde signaling could be part of the early synaptic changes to THC exposure, we compared eCB-LTD and I-LTD in NAc and hippocampal slices prepared from vehicle- and THC-treated mice (**Fig. 1**). Tolerance to cannabinoids develops rapidly and behavioral tolerance is observed after one day of THC treatment[7,8]. Thus, mice were injected once with a non-aversive dose of THC (3 mg/kg)[7] or vehicle 15–20 h before the experiment. We found that eCB-LTD (**Fig. 1a**) and I-LTD (**Fig.1b**) were both abolished in THC-injected animals. This action could be due to a persistent change in eCB release or in the CB1 receptor (CB1R) itself. Because the eCB release that triggers eCB-LTD and I-LTD occurs as a result of the activation of postsynaptic metabotropic glutamate receptors (mGluR)[4,5], it is conceivable that THC-induced effects could be due to a modification of the mGluR-dependent release of eCBs. This possibility is unlikely because another CB1R-mediated phenomenon in which the release of eCBs does not require mGluR activation, the depolarization-induced suppression of inhibition (DSI)[9,10], was markedly reduced in the THC-injected animals (**Fig. 1c**). The THC-induced effects were reversible as eCB-LTD, I-LTD and DSI were entirely normal in slices prepared 3 d after injection (**Fig. 1d**). The effects of a single exposure to THC were completely prevented when the CB1 antagonist SR141716A (1 mg/kg) was injected 30 min before THC, demonstrating the role of CB1R in the THC-induced blockade of synaptic plasticity (**Fig. 1d**). THC injection did not cause a shift from an eCB-mediated to an eCB-independent form of synaptic plasticity, as 1 μM SR141716A bath application prevented eCB-LTD and I-LTD in slices prepared after 3 d or 1 week recovery (3 d after single THC, eCB-LTD was 77.1 ± 5.5% of baseline, $n = 3$; compared to 100.7 ± 3.0%, $n = 3$, in SR141716A, $P < 0.05$, and I-LTD was 76.7 ± 1.9%, $n = 4$, compared to 101.4 ± 1.4 %, $n = 5$ in SR141716A, $P < 0.05$; data not shown). Thus, a single *in-vivo* exposure to a low dose of THC transiently blocks eCB-mediated retrograde signaling in structures

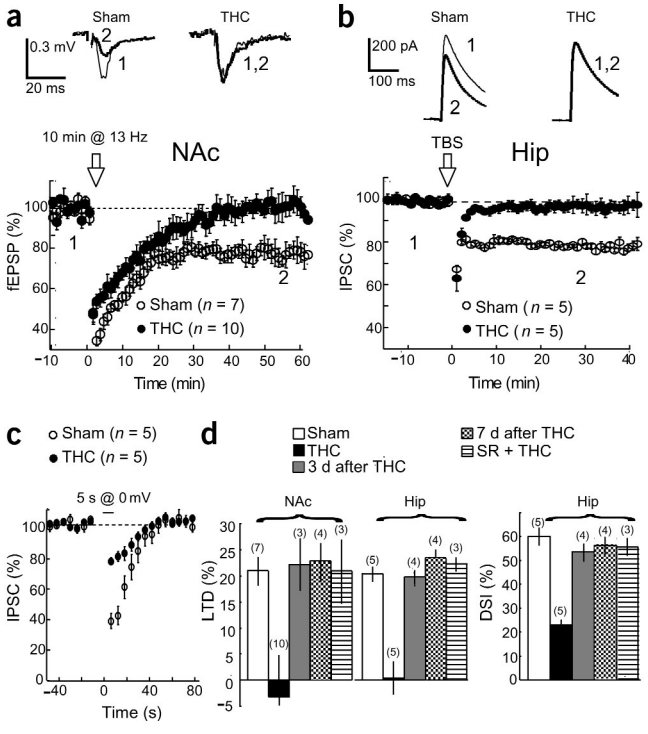

Figure 1 Single *in-vivo* administration of THC abolishes eCB-mediated synaptic plasticity in NAc and hippocampus. (**a**,**b**) Summary graphs (lower panels) of the time course of field excitatory postsynaptic potentials (fEPSPs) in the NAc (**a**) and IPSCs (inhibitory postsynaptic currents) from CA1 pyramidal cells (**b**), showing the effects of repetitive stimulation in THC-injected and sham animals. Upper panels show sample traces of representative experiments (numbers indicate the corresponding time point in the bottom graphs). (**c**) DSI was markedly reduced in THC-injected animals. (**d**) Bar histograms of the magnitude of eCB-LTD, I-LTD and DSI in sham and THC-treated animals, 1 d, 3 d and 1 week after injection. SR141716A (SR) injection (1 mg/kg) 30 min before THC abolished eCB-mediated plasticity in NAc and the hippocampus. Brain slices were prepared as previously described[4,5] (see **Supplementary Methods** online). All experimental procedures were in accordance with the Society for Neuroscience and European Union guidelines and were approved by the institutional animal care and use committees.

[1]Equipe INSERM Avenir, Plasticité synaptique: Maturation & Addiction, Institut Magendie des Neurosciences, Rue Camille Saint Saëns, 33077 Bordeaux, France. [2]Department of Neuroscience, Albert Einstein College of Medicine, Bronx, New York 10461, USA. [3]Department of Physiology and Pharmacology, University of Cantabria, Santander 39011, Cantabria, Spain. [4]Present address: Center for Molecular and Behavioral Neuroscience, Rutgers University, Newark, New Jersey 07102, USA. [5]These authors contributed equally to this work. Correspondence should be addressed to P.E.C. (pcastill@aecom.yu.edu) or O.J.M. (manzoni@bordeaux.inserm.fr).

Published online 16 May 2004; doi:10.1038/nn1251

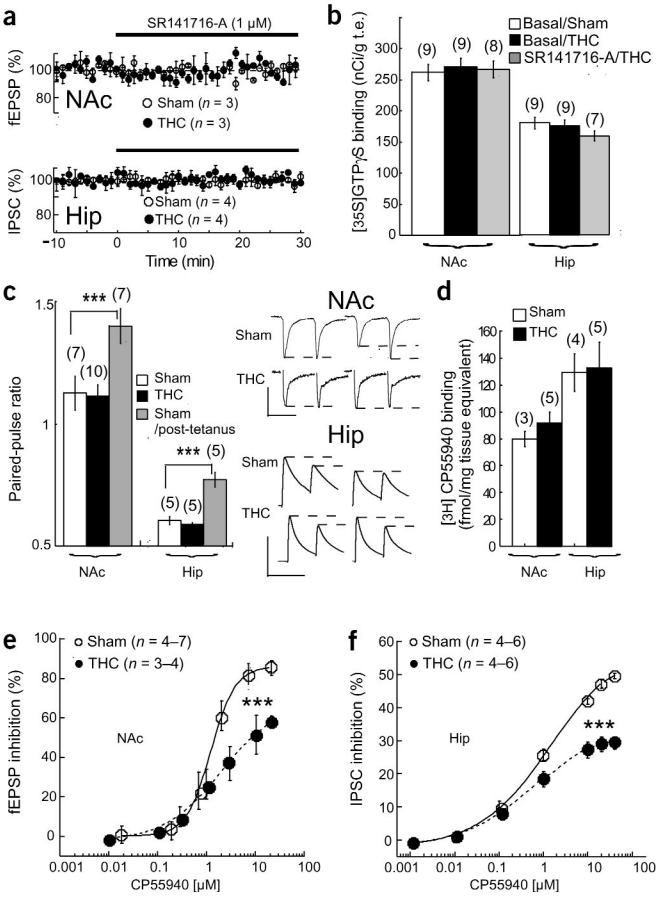

Figure 2 THC single injection causes functional tolerance. (**a**) CB1R blockade with SR141716-A did not affect basal synaptic transmission in NAc or hippocampus from vehicle- or THC-injected mice. (**b**) After THC single injection, basal [^{35}S]GTPγS binding was neither enhanced (vehicle-treated, white bar vs. THC-treated, black bar) nor reduced by SR141716-A (basal, black bar vs. SR141716-A, gray bar). (**c**) Paired-pulse ratio was identical in vehicle and THC-treated animals suggesting that basal probability of transmitter release was unchanged after THC injection. Right side: sample traces of representative experiments in NAc (scale bar: 0.2 mV, 40 ms) and hippocampus (scale bar: 1,000 pA, 200 ms), before and after LTD-induction from sham and THC-injected animals. (**d**) Specific binding of the cannabinoid agonist [^{3}H]CP55940 was similar in vehicle- and THC-treated animals. (**e**,**f**) Dose-response curves for CP55940 inhibition of fEPSP in the NAc (**e**) or IPSC in the hippocampus (**f**) from vehicle- or THC-treated mice. [^{35}S]GTPγS and [^{3}H]CP55940 autoradiography were as indicated elsewhere[11] (**Supplementary Methods**).

fundamental to reward-related behaviors (the NAc), and learning and memory (the hippocampus).

What cellular/molecular mechanism could account for the THC-induced effects on the eCB retrograde signaling? Persistent CB1R activation by residual THC could depress synaptic transmission, thereby occluding eCB-mediated changes in synaptic efficacy. This possibility was excluded based on electrophysiological and biochemical evidence. First, bath application of the selective CB1R antagonist SR141716-A (1 μM) did not increase baseline synaptic transmission in the NAc or the hippocampus of THC-injected mice (**Fig. 2a**). Second, [^{35}S]GTPγS autoradiography was performed in both structures to quantify the coupling efficiency between CB1R and Gi/o transduction proteins[11]. After THC single-injection, basal [^{35}S]GTPγS binding was unaltered and SR141716-A (1 μM) did not reduce basal [^{35}S]GTPγS binding (**Fig. 2b**).

Exogenous THC could trigger chemical LTD and occlude eCB-mediated synaptic plasticity. This possibility is also unlikely because paired-pulse ratio, a form of short-term plasticity that changes during eCB-LTD and I-LTD, was unaffected in THC-injected animals (**Fig. 2c**). It has been reported that multiple injections with high THC doses can trigger CB1R downregulation and uncoupling from Gi/o proteins[12,13]. However, we found no change in CB1 binding sites in the NAc and the hippocampus measured by *in-vitro* autoradiography[11], suggesting that a single THC treatment was not sufficient to cause significant internalization of CB1R (**Fig. 2d**). Moreover, CB1-agonist-stimulated [^{35}S]GTPγS autoradiography did not reveal significant uncoupling from Gi/o proteins after single THC treatment (**Supplementary Fig. 1** online)[14]. Finally, we explored whether a functional modification of the CB1R (tolerance) could explain the THC-mediated effects on synaptic plasticity[15]. We found that the depression induced by the CB1R-selective agonist CP55940 was clearly reduced in THC-treated mice, as compared to vehicle-treated mice, in both the NAc and the hippocampus (**Fig. 2e,f**). Taken together, these findings indicate that functional tolerance of the CB1R can account for the suppression of eCB-mediated synaptic plasticity after acute THC exposure.

In conclusion, our study shows that a single exposure to THC has profound repercussions—albeit transient—on synaptic plasticity in key brain areas that are involved in reward or learning. Thus, our findings reveal a mechanism by which cannabis derivatives may alter cognitive functions and motivational behaviors.

Note: Supplementary information is available on the Nature Neuroscience website.

ACKNOWLEDGMENTS

Work in O.J.M.'s laboratory is supported by grants from INSERM, Ministère de la Recherche, Fondation pour le Recherche Médicale and Région Aquitaine. P.E.C.'s laboratory is supported by grants from NIH (National Institute on Drug Abuse) and the Pew Charitable Trust.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 24 December 2003; accepted 22 March 2004
Published online at http://www.nature.com/natureneuroscience/

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Supplementary Methods

Animal treatment:

Mice (C57B1/6 strain) of 4 weeks old were housed, grouped and acclimatized to laboratory conditions (12 hr light/dark cycle) 1 week before the experiment and had *ad libitum* food and water access. Animals were injected once IP with Δ^9-THC (3 mg/kg) or vehicle the day before the experiment. Mice were sacrificed by decapitation 15-20 hours after the injection in order to avoid any interference due to the *in-vivo* prebound drug. The vehicle for Δ^9-THC was 5% cremophor-el/5% Ethanol in NaCl 0.9%.

Slice preparation and electrophysiology:

Nucleus accumbens

Extracellular field recordings were made from medium spiny neurons in parasagittal slices of mouse NAc. These methods have been described in details previously [5]. In brief, mouse (male C57BL6, 4-6 weeks) were anesthetized with isoflurane and decapitated according to institutional regulations. The brain was sliced (300µm) in the parasagittal plane using a vibratome (Integraslice, Campden Instruments, UK) and maintained in physiological saline at 4˚C. Slices containing the NAc were stored at least one hour at room temperature before being placed in the recording chamber and superfused (2 ml/min) with artificial cerebrospinal fluid (ACSF) that contained (in mM): 126 NaCl, 2.5 KCl, 1.2 MgCl2, 2.4 CaCl2, 18 NaHCO3, 1.2 NaH2PO4, and 11 Glucose, and was equilibrated with 95% O2/5% CO2. All experiments were done at room temperature. The superfusion medium contained picrotoxin (100 µM) to block GABA-A receptors. All drugs were added at the final concentration to the superfusion medium. The recording pipette was filled with ACSF. Both the field excitatory post-synaptic potential (fEPSP) slope (calculated with a least square method) and fEPSP amplitude were measured (graphs depict amplitudes). An Axopatch-1D (Axon Instruments) was used to record the data, which were filtered at 1-2 kHz, digitized at 5 kHz on a DigiData 1200 interface (Axon Instruments) and collected on a PC using ACQUIS-1 software (Bio-Logic, France). To evoke synaptic currents, stimuli (100-150µs duration) were delivered at 0.033 Hz through bipolar tungsten electrodes placed at the prefrontal cortex- NAc border. Recordings were made in the rostral-medial dorsal accumbens close to the anterior commissure.

Hippocampus

Transverse hippocampal slices were prepared from 3-5 weeks old C57BL6 mice. Animals were anesthetized with halothane and killed by decapitation in accordance with institutional regulations. Slices (400 µm thickness) were cut on a vibratome (Dosaka, Kyoto, Japan) in ice-cold extracellular solution containing (in mM): 238 Sucrose, 2.5 KCl, 10 glucose, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 $CaCl_2$ and 1.3 $MgCl_2$. The cutting medium was gradually switched to the recording solution (ACSF) that contained in mM: 124 NaCl, 2.5 KCl, 10 glucose, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 $CaCl_2$ and 1.3 $MgCl_2$. The slices were kept at room temperature for at least 1.5 hours before transfer to the recording chamber. Cutting and recording solutions were both saturated with 95% O_2 and 5% CO_2, pH 7.4. All experiments were performed at 25.0 ± 0.1 °C.

IPSCs were recorded in CA1 pyramidal neurons voltage clamped at –60mV for DSI or +10 mV for I-LTD and dose-response curve (Fig. 2f) with a pipette (3-5 MΩ) containing (in mM): 125 Cs gluconate, 1 $CaCl_2$, 10 EGTA, 10 Hepes, 10 glucose, 5 ATP, 0.4 GTP (pH 7.2; 280-290 mOsm). Series resistance (typically 8-15 MΩ) was monitored throughout each experiment with a -4 mV, 80 ms pulse and cells with more than 10% change in series resistance were excluded from analysis. IPSCs were evoked by

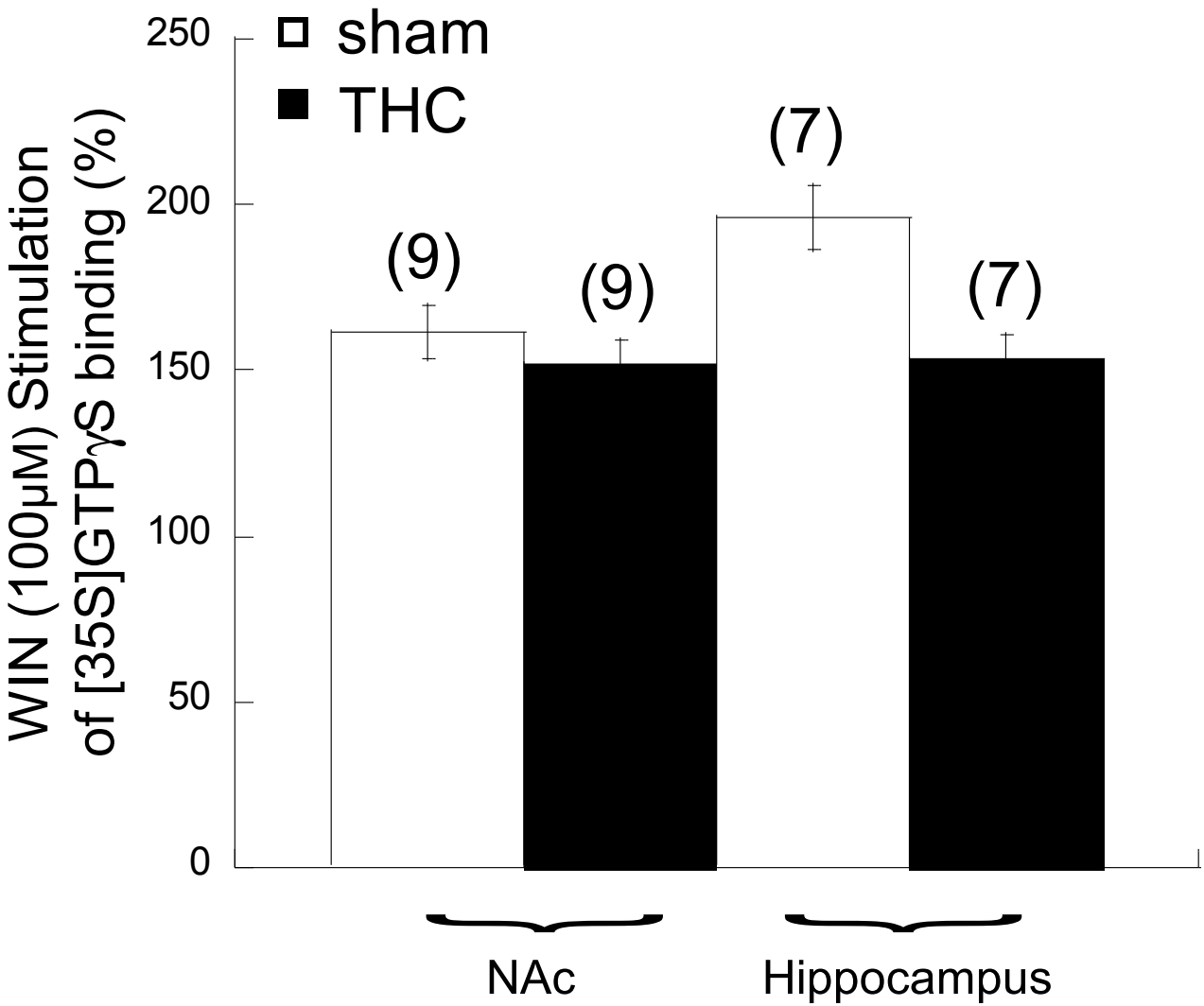
monopolar stimulation with a patch pipette filled with ACSF and placed in the middle third of *stratum radiatum.* Recordings were performed in the continuous presence of NMDA and AMPA/Kainate receptor antagonists (50 µM D-APV and 10 µM NBQX, respectively). I-LTD was induced after 20 minutes of stable baseline by theta burst stimulation (TBS) consisting of a series of 10 bursts of 5 stimuli (100 Hz within the burst, 200 ms inter-burst interval), which was repeated 4 times (5 s apart). Recordings were performed with a MultiClamp 700A (Axon Instruments Inc., Union City, CA) which output signals were filtered at 3 KHz. Data were digitized (5 kHz) and analyzed on-line using a macro written in IgorPro (Wavemetrics Inc., Lake Oswego, OR).
The magnitude of LTD was calculated by comparing averaged responses (50-60 min for eCB-LTD and 35-40 min for I-LTD) after induction to baseline-averaged responses before induction protocol.

Biochemical assays:
For autoradiographic studies the brains were rapidly removed and immersed in isopentane at -35°C. Twenty-micrometer coronal sections were obtained in a cryostat, mounted onto gelatin-coated slides, and stored at -20°C until use.
Agonist-stimulated [^{35}S]GTPγS autoradiography
Slides were incubated in assay buffer that contained (in mM) 50 Tris-HCl, 0.2 EGTA, 3 $MgCl_2$, 100 NaCl, 1 DTT, 2 GDP and 0.5% BSA (pH, 7.7) for 30 minutes at 25ºC. Basal [^{35}S]GTPγS binding was defined by subsequent incubation of the slides for 120 minutes at 25ºC in the same buffer containing 0.04 nM [^{35}S]GTPγS. CB_1 receptor-stimulated [^{35}S]GTPγS binding was evaluated by incubation of consecutive sections in the presence 100 µM WIN55212-2 and co-incubation with 10 µM SR141716A for the antagonism condition. Additional sections from treated mice were incubated with 1 µM SR141716A in order to assess the presence of residual Δ^9-THC. Non-specific binding was determined using 10 µM GTPγS. The slides were washed twice for 15 minutes each in 50 mM Tris-HCl buffer (pH, 7.4) at 4ºC, dried, and exposed for 48 hours at 4ºC to β radiation-sensitive films (Hyperfilm^{TM}-βmax) together with ^{14}C-polymer standards ([^{14}C] microscale standards). Autoradiograms were scanned and images analyzed as using the National Institute of Health IMAGE program (Bethesda, MA). Optical densities were corrected to nanocuries/g tissue equivalent (nCi/g te) by comparison with the microscale standards and WIN55,212-2 stimulation values expressed as percentage of over basal activity.
[^{3}H]CP55,940 receptor autoradiography
Slides were incubated for 2 hours at 37ºC in a buffer containing 50 mM Tris-HCl, 5% BSA (pH, 7.4) and 3 nM [^{3}H]CP55,940 (specific activity, 165 Ci/mmol). Non-specific binding was determined in adjacent sections by co-incubation with 10 µM WIN55,212-2. Unbound radioligand was removed by washing the slides twice for 2 hours each at 4ºC in a buffer containing 50 mM Tris-HCl and 1% BSA (pH, 7.4). After a drying on cold air-stream, autoradiograms were generated by apposing the tissues for 15 days at 4ºC to tritium-sensitive films (Hyperfilm^{TM}-^{3}H) together with ^{3}H-polymer standards. The films were scanned and images analyzed with NIH-IMAGE program. Optical densities were then evaluated by comparison with the microscale standards and expressed in femtomol/mg tissue equivalent (fmol/mg tissue equivalent).

Data analysis and materials:
All values are given as mean ± S.E.M. For all the experiments, the treatment or the absence of treatment was considered as the dependent variable. Thus, the data from all

the slices obtained from each animal tested were averaged and statistical significance tested with the Mann-Whitney U-test ($p<0.05$ was taken as indicating statistical significance) using Kyplot β13 (Koichi Yoshioka).

Drugs used: THC, picrotoxin, GDP, GTPγS and Cremophor-el from SIGMA; CP55,940 and WIN55,212-2 from Tocris. [^{3}H]CP55,940 and [^{35}S]GTPγS were obtained from New England Nuclear/Dupont. Radiation sensitive films and microscale standards were purchased from Amersham. SR141716A was a generous gift from Sanofi Recherche. All drugs were bath-applied following dilution into the external solution from concentrated stock solutions.



Supplementary Figure 1