Rimonabant precipitates anxiety in rats withdrawn from palatable food: role of the central amygdala

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Supporting Materials and Methods

Drug

Rimonabant (SR141716A, 5-(4-Chlorophenyl)-1-(2,4-dichloro-phenyl)-4-methyl-N-(piperidin-1-yl)-1*H*-pyrazole-3-carboxamide) HCl was synthesized as reported previously (Kumar *et al*, 2008). The base was converted to rimonabant HCl that was crystallized from acetone and a slight excess of 37% HCl and showed 234-242 °C melting point (Fang *et al*, 2012). It was chromatographically homogenous and gave satisfactory (± 0.3%) combustion analysis for carbon, hydrogen and nitrogen and satisfactory high resolution mass spectral analysis: calculated 463.0807, found 463.0859.

Intracranial surgeries and microinfusion procedure

Intracranial surgeries. Briefly, anaesthetized (isoflurane, 2–3% in oxygen) subjects were secured in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) and stainless steel, guide cannulas (Plastics One, Inc., Roanoke, VA, USA) were lowered bilaterally above the CeA. Four stainless steel jeweler's screws were fastened to the rat's skull around the cannula. Dental restorative filled resin (Henry Schein Inc., Melville, NY, USA) and acrylic cement were applied, forming a pedestal firmly anchoring the cannula. The cannula coordinates used were A/P -0.2 mm, M/L ±4.1 mm, D/V -6.5 mm. The interaural bar was set at +5; coordinates were based on the atlas of Pellegrino (Pellegrino et al, 1979). A stainless steel dummy stylet (Plastics One, Inc., Roanoke, VA, USA) maintained patency. After surgery, rats were allowed to recover from surgery for 1 week before the experimental procedure began.

Microinfusion procedure. For intracerebral microinfusion, the dummy stylet was removed from the guide cannula and replaced with a stainless steel injector projecting 2 mm beyond the tip of the guide cannula; the injector was connected via a PE 20 tubing to a Hamilton microsyringe (Hamilton Company, Reno, NV) driven by a microinfusion pump (Kd Scientifics/Biological Instruments, Holliston, MA, USA). Microinfusions were performed in 0.5 µl volume delivered over 2 min; injectors were left in place for an additional minute to minimize backflow. Cannula placement was verified at the conclusion of all testing. Briefly, anaesthetized (isoflurane, 2–3% in oxygen) subjects were and transcardially perfused with ice-cold 4% paraformaldehyde (PFA) in water (pH 7.4) and microinfused with Cresyl violet (0.5 µl over 2 minutes bilaterally). Brains were then fixed overnight in 4% PFA and equilibrated in 30% sucrose in water. Coronal sections of 40 µm were collected using a cryostat and placements were verified under a microscope. 24 subjects were excluded from analysis because they were 'misses'. Data from incorrect placements were analyzed to help interpret the site-specificity of effects.

Behavioral tests

Food intake and body weight measurements. Preweighed food was provided in the rats' home cage at the dark cycle onset. Food intake was measured 2h and 24h later. Rats' body weights were recorded immediately before and 24h following drug treatment. Rimonabant was administered intraperitoneally (0, 0.3, 1, 3 mg/kg) or was microinfused within the CeA (0, 0.5 and 1.5 µg/side. Treatments were given during the first switch to palatable diet (1st week, P Phase, 6th day of the cycle), after 7 weeks of cycling on the 4th day of withdrawal from palatable diet (C phase, 4th day of the cycle), or after renewing access to palatable diet (P Phase, 6th day of the cycle).

Defensive withdrawal test. The defensive withdrawal test (Cottone *et al*, 2009b; Zorrilla *et al*, 2002) apparatus was a walled, black polyvinylchloride open field (106 cm × 92 cm × 77 cm) containing a cylindrical "withdrawal" chamber (2-L Pyrex beaker wrapped in brown tape). The chamber was located 15 cm from a corner facing the open arena. *Chow/Palatable* rats were diet-cycled for at least 7 weeks and tested during the 4th day of withdrawal from palatable diet (C Phase). Rats were pretreated with either vehicle or rimonabant (i.p., 0.3, 1 and 3 mg/kg. *Chow/Chow* control rats were tested concurrently in a between-subjects design. Chow diet was available *ad libitum* until the time of testing. Rats were kept in the dark anteroom for ≥2 h before testing. For the 10-min test, rats were placed into the withdrawal chamber facing the rear, and behavior was video recorded. Blind raters scored the total time spent in the withdrawal chamber. Testing occurred under room light (~300 lux).

Elevated Plus Maze test. The elevated plus-maze (Cottone et al, 2009a; Cottone *et al*, 2009b; Cottone *et al*, 2007; Cottone *et al*, 2008b) apparatus was made of black Plexiglas and consisted of four arms (50 cm long 10 cm wide). Two arms had 40-cm-high dark walls (enclosed arms), and two arms had 0.5-cm-high ledges (open arms). The maze was elevated to a height of 50 cm. Open arms received 1.5–2.0 lux of illumination. Animals were habituated to the anteroom the day before testing. On the day of testing, rats were kept in the quiet, dark anteroom for at least 2 h before testing. White noise (70 dB) was present throughout habituation and testing. Previous experience in our laboratory indicates that control subjects spend similar time in the open and closed arms using the above procedures, allowing the sensitive detection of anxiogenic-like stimuli (Zorrilla *et al*, 2002). For testing, rats were placed individually onto the center of the maze facing a closed arm and removed after a 5-min period. The primary measures were the percentage of total arm time directed toward the open arms [i.e., 100*open arm/(open arm+closed arm)], a validated index of anxiety-related behavior (Fernandes *et al*, 1996) and the number of closed arm entries, a specific index of locomotor activity (Cruz *et al*, 1994) *Chow/Palatable* rats were diet-cycled for at least 7 weeks and tested on the 4th day of withdrawal from

palatable diet for spontaneous anxiety-like behavior or following microinfusion of rimonabant into the CeA (0.5 µg/side). *Chow/Chow* control rats were tested concurrently in a between-subjects design. Chow diet was available *ad libitum* until the time of testing.

Tissue endocannabinoid measurement

On the 4th day (C Phase) or on the 7th day (P Phase) rats were sacrificed right after the dark cycle onset and CeA, NAc, and LH punches were collected on an ice-cold stage and stored at -80°C. Tissues were then dounce homogenized and extracted with CHCl3:MeOH:Tris-HCl 50 mM (pH 7.4) (1:1:1 by volume) containing 20 pmol of d8-AEA and d5-2-AG as internal standards. Lipid-containing organic phase was dried down, weighed and purified by open-bed chromatography on silica gel, and analyzed by isotope dilution liquid chromatography (LC)-atmospheric pressure chemical ionization (APCI)-MS (LC-APCI-MS) using a Shimadzu HPLC apparatus (LC-10ADVP) coupled to a Shimadzu (LCMS-2010) quadrupole MS via a Shimadzu APCI interface. MS analyses were carried out in the selected ion-monitoring mode. AEA and 2-AG levels were quantified on the basis of their area ratio with the signal area of their respective deuterated internal standards.

Quantitative real-time PCR

Tissue samples collected in RNA later (Invitrogen) were homogenized by a rotor-stator homogenizer in 1.5 mL of Trizol (Invitrogen). Total RNA was extracted according to manufacturer recommendations, dissolved in RNAase-free water, and further purified by spin cartridge using the Micro-to-Midi total RNA purification system (Invitrogen). Total RNA was dissolved in RNA storage solution (Ambion, Austin, TX, USA), UVquantified by a Bio-Photometer (Eppendorf, Hamburg, Germany) and stored at -80°C until use. RNA

aliquots (6 μg) were digested by RNAse-free DNAse I (Ambion DNA-free™ kit) in a 20 μL final volume reaction mixture to remove residual contaminating genomic DNA. After DNAse digestion, the concentration and purity of RNA samples were evaluated by the RNA-6000 Nano microchip assay using a 2100 Bioanalyzer equipped with a 2100 Expert Software (Agilent, Santa Clara, CA, USA) following the manufacturer's instructions. For all samples tested, the RNA integrity number was >8 on a 0–10 scale. One microgram of total RNA, as evaluated by the 2100 Bioanalyzer, was reverse-transcribed in cDNA and analyzed as previously described (Grimaldi *et al*, 2009). This cDNA was used as the template in PCR reactions set up with specific primers and probes for CB_1 (TaqMan gene expression assay, Applied Biosystems, UK, ref. #Rn01637601_m1). The primers were designed so that the PCR product spans an intron–exon boundary in order to avoid amplification of any contaminating genomic DNA and the melting curves were analyzed to ensure the specificity of the PCR reactions. PCR was performed on 1 μl of cDNA (corresponding to 50 ng of input RNA) using Universal TaqMan Mastermix or SYBR Green Mastermix. The PCR reaction conditions were: 50 °C for 2 minutes, 95 °C for 10 minutes, followed by 40 cycles of amplification (95 °C for 15 seconds, 60 °C for 1 minute). Samples were assayed on the Applied Biosystems PRISM 7700 Sequence detection system, each sample being assayed in duplicate and a 6-point standard curve run in parallel. To confirm the absence of genomic DNA contamination, a control sample of non-reverse-transcribed RNA was also run for each set of RNA extractions. Relative quantification was obtained by calculating the ratio between the values obtained for each gene of interest and the house-keeping gene GAPDH.

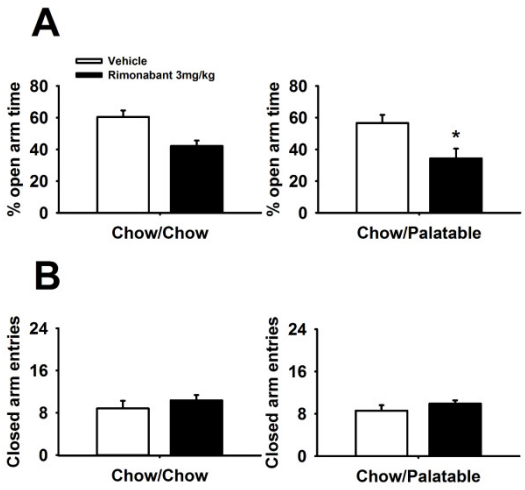
Plasma corticosterone RIA

Samples were collected into polypropylene tubes (1.6 ml, Eppendorf, USA Scientific) containing 5 μL of 0.5 M EDTA and centrifuged at 4 °C for 20 min at 3000 g. The plasma fraction was stored at −80 °C until

analysis. Plasma levels of corticosterone-like immunoreactivity levels were quantified by radioimmunoassay with a commercially available kit according to the manufacturer's instructions (MP Biomedicals, Inc). The area under the curve (AUC) was calculated using the trapezoidal rule.

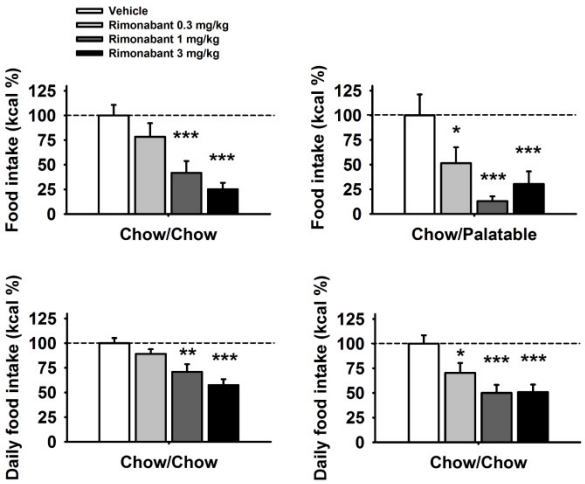
Supporting Figures and Figure legends

Supporting Figure 1



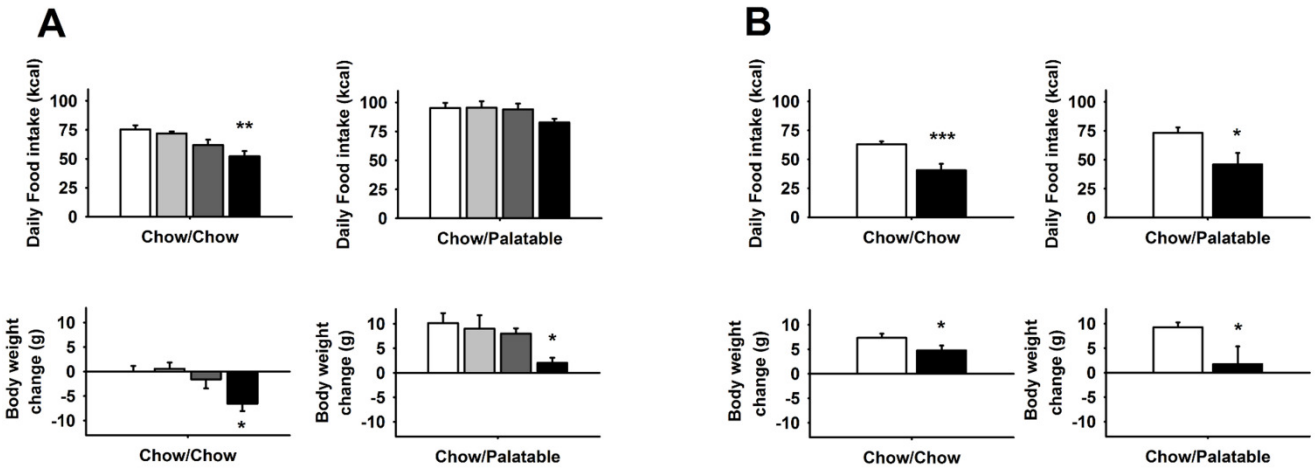
Effects of rimonabant (30 minutes pretreatment, 0, 3 mg/kg, *i.p.*) on anxiety-like behavior in female Wistar rats (n=41) during day 4th (C phase). **(A)** Rimonabant selectively precipitated anxiety-like behavior in rats withdrawn from palatable food, (*Chow/Palatable* group) but not in the *Chow/Chow* control group. **(B)** No significant effect, among groups, was detected in the closed arms entries analysis. Panels represent M±SEM. Symbols denote: * significant difference from vehicle- treated group p<0.05.

Supporting Figure 2



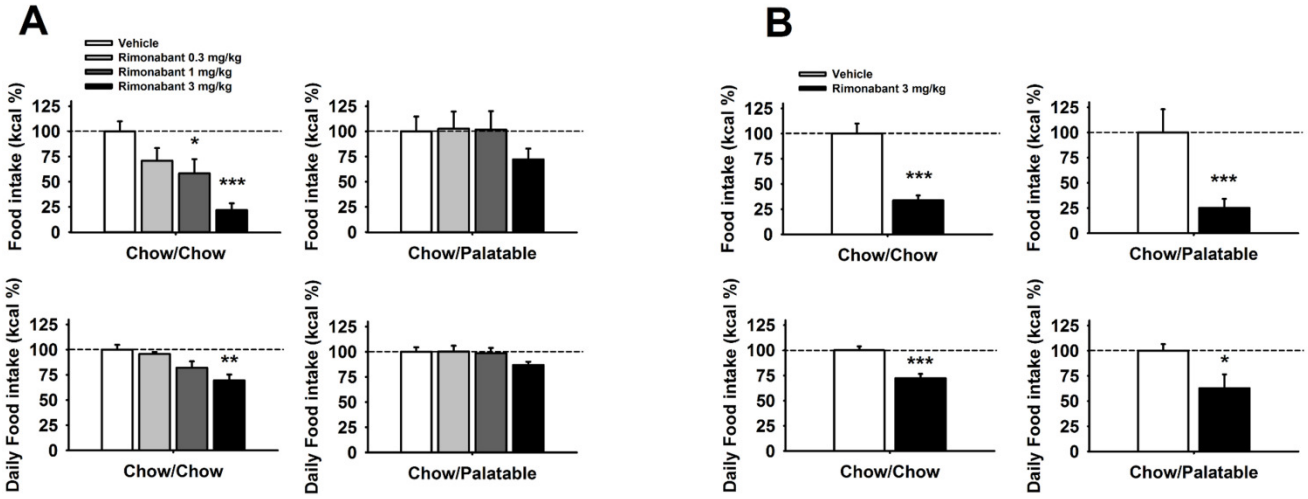
Effects of rimonabant (30 minutes pretreatment, 0, 0.3, 1, 3 mg/kg, *i.p.*) on food intake (expressed as % of vehicle-treated condition) in female Wistar rats (n=70) withdrawn for 4 days from chronic, intermittent access to a highly palatable diet. Rimonabant differentially precipitates chow anorexia and body weight loss in *Chow/Palatable* rats. (Top panels) 2h food intake, and (bottom panels) 24h food intake. Panels represent M±SEM. Symbols denote: * significant difference from vehicle- treated group $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Supporting Figure 3



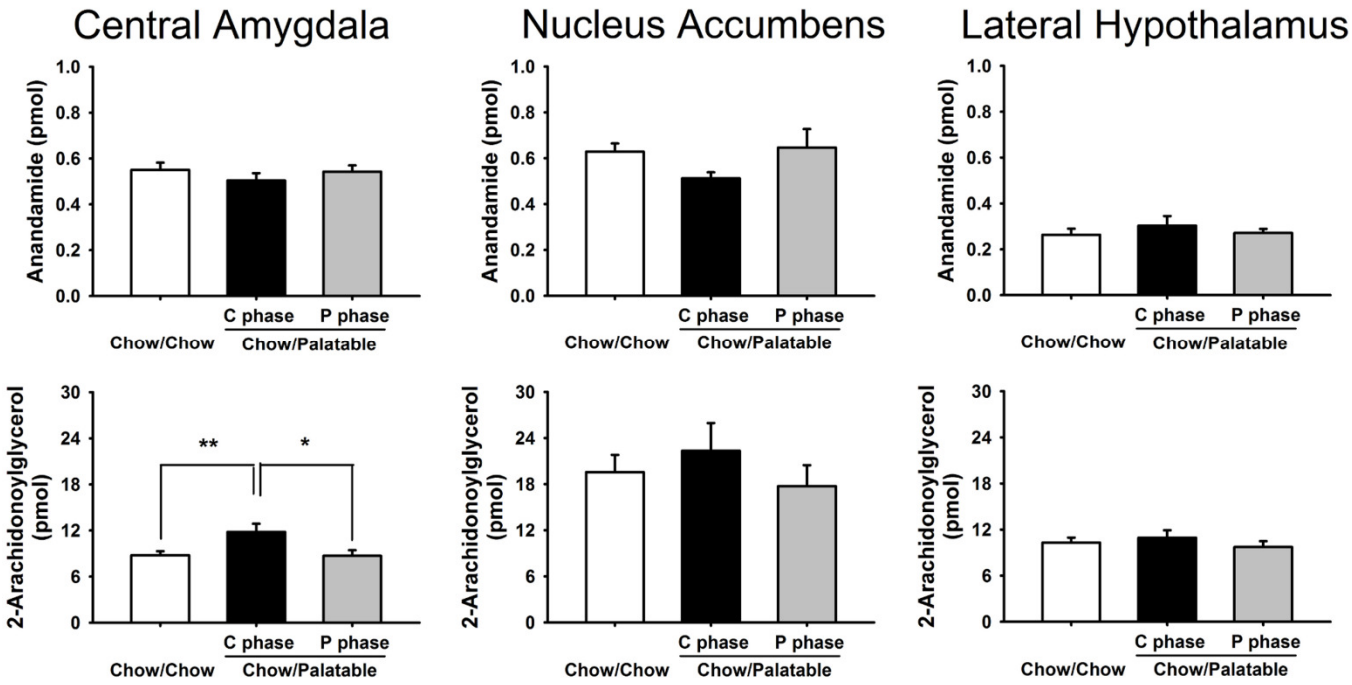
Effects of rimonabant (30 minutes pretreatment, 0, 0.3, 1, 3 mg/kg, i.p.) on food intake in female Wistar rats (n=48) during day 6th (P phase, when *Chow/Chow* rats are fed chow diet and *Chow/Palatable* rats are fed the highly palatable diet). **(A)** Rimonabant fails to reduce 2h intake of the highly palatable diet in *Chow/Palatable* rats which underwent chronic diet alternation (at least 7 weeks). (**B**) Rimonabant reduces 2h food intake during the first access to the palatable diet in *Chow/Palatable* rats. Panels represent M±SEM. Symbols denote: * significant difference from vehicle condition p<0.05, ** p<0.01, *** p<0.001.

Supporting Figure 4



Effects of rimonabant (30 minutes pretreatment, 0, 0.3, 1, 3 mg/kg, i.p.) on food intake (expressed as % of vehicle-treated condition) in female Wistar rats (n=48) during day 6th (P phase, when *Chow/Chow* rats are fed chow diet and *Chow/Palatable* rats are fed the highly palatable diet). (**A**) Rimonabant fails to reduce intake of the highly palatable diet in *Chow/Palatable* but reduces body weight gain in *Chow/Palatable* rats which underwent chronic diet alternation (at least 7 weeks). (Top panels) 24h food intake, and (bottom panels) body weight change. (**B**) Rimonabant reduces 24h food intake and body weight change during the first access to the palatable diet in *Chow/Palatable* rats. (Top panels) 24h food intake, and (bottom panels) body weight change. Panels represent M±SEM. Symbols denote: * significant difference from vehicle condition p<0.05, ** p<0.01, *** p<0.001.

Supporting Figure 5



Effects of palatable diet alternation on AEA and 2-AG levels in the CeA, NAc, and LH (n=23). (Top panels) AEA, and (bottom panels) 2-AG. Values are expressed in pmol. Panels represent M±SEM. Symbols denote: * significant difference from *Chow/Chow* group $p<0.05$, ** $p<0.01$.

References

1. Cota D, Marsicano G, Lutz B, Vicennati V, Stalla GK, Pasquali R *et al.* (2003) Endogenous cannabinoid system as a modulator of food intake. *Int J Obes Relat Metab Disord* 27(3):289-301.

2. Di Marzo V & Matias I (2005) Endocannabinoid control of food intake and energy balance. *Nat Neurosci* 8(5):585-589.

3. Akbas F, Gasteyger C, Sjodin A, Astrup A, & Larsen TM (2009) A critical review of the cannabinoid receptor as a drug target for obesity management. *Obes Rev* 10(1):58-67.

4. Pi-Sunyer FX, Aronne LJ, Heshmati HM, Devin J, & Rosenstock J (2006) Effect of rimonabant, a cannabinoid-1 receptor blocker, on weight and cardiometabolic risk factors in overweight or obese patients: RIO-North America: a randomized controlled trial. *JAMA* 295(7):761-775.

5. Van Gaal LF, Rissanen AM, Scheen AJ, Ziegler O, & Rossner S (2005) Effects of the cannabinoid-1 receptor blocker rimonabant on weight reduction and cardiovascular risk factors in overweight patients: 1-year experience from the RIO-Europe study. *Lancet* 365(9468):1389-1397.

6. Van Gaal LF, A J Scheen, H.P.F. Koppeschaar, Study Group RIO-Europe, C Hanotin, O Ziegler (2008) Long-term effect of CB1 blockade with rimonabant on cardiometabolic risk factors: two year results from the RIO-Europe Study. *Eur Heart J* 29(14):1761-1771.

7. Biala G, Budzynska B, & Kruk M (2005) Naloxone precipitates nicotine abstinence syndrome and attenuates nicotine-induced antinociception in mice. *Pharmacol Rep* 57(6):755-760.

8. Weaver MT, Sweitzer M, Coddington S, Sheppard J, Verdecchia N, Caggiula AR, *et al.* (2012) Precipitated withdrawal from nicotine reduces reinforcing effects of a visual stimulus for rats. *Nicotine Tob Res* 14(7):824-832.

9. Culpepper-Morgan JA & Kreek MJ (1997) Hypothalamic-pituitary-adrenal axis hypersensitivity to naloxone in opioid dependence: a case of naloxone-induced withdrawal. *Metabolism* 46(2):130-134.

10. Shoblock JR & Maidment NT (2006) Constitutively active micro opioid receptors mediate the enhanced conditioned aversive effect of naloxone in morphine-dependent mice. *Neuropsychopharmacology* 31(1):171-177.

11. Williams DA & Thorn BE (1984) Opiate withdrawal behavior after focal brain stimulation. *Pharmacol Biochem Behav* 21(5):699-703.

12. Sadee W, Wang D, & Bilsky EJ (2005) Basal opioid receptor activity, neutral antagonists, and therapeutic opportunities. *Life Sci* 76(13):1427-1437.

13. Eisenberg RM (1982) Further studies on the acute dependence produced by morphine in opiate naive rats. *Life Sci* 31(15):1531-1540

14. Martin WR & Jasinski DR (1969) Physiological parameters of morphine dependence in man--tolerance, early abstinence, protracted abstinence. *J Psychiatr Res* 7(1):9-17.

15. Nutt JG & Jasinski DR (1974) Methadone-naloxone mixtures for use in methadone maintenance programs. I. An evaluation in man of their pharmacological feasibility. II. Demonstration of acute physical dependence. *Clin Pharmacol Ther* 15(2):156-166.

16. Jin C, Araki H, Nagata M, Suemaru K, Shibata K, Kawasaki H, Hamamura T *et al.* (2004) Withdrawal-induced c-Fos expression in the rat centromedial amygdala 24 h following a single morphine exposure. *Psychopharmacology (Berl)* 175(4):428-435.

17. Celerier E, Laulin JP, Corcuff JB, Le Moal M, & Simonnet G (2001) Progressive enhancement of delayed hyperalgesia induced by repeated heroin administration: a sensitization process. *J Neurosci* 21(11):4074-4080.

18. Nylander I, Vlaskovska M, & Terenius L (1995) The effects of morphine treatment and morphine withdrawal on the dynorphin and enkephalin systems in Sprague-Dawley rats. *Psychopharmacology (Berl)* 118(4):391-400.

19. Olive MF, Bertolucci M, Evans CJ, & Maidment NT (1995) Microdialysis reveals a morphine-induced increase in pallidal opioid peptide release. *Neuroreport* 6(8):1093-1096.

20. Shoblock JR & Maidment NT (2006) Constitutively active micro opioid receptors mediate the enhanced conditioned aversive effect of naloxone in morphine-dependent mice. *Neuropsychopharmacology* 31(1):171-177.

21. Farrell M & Strang J (1995) Compressed opiate withdrawal syndrome and naltrexone. *J Psychopharmacol* 9(4):383-385.

22. Weerts EM, Ator NA, Kaminski BJ, & Griffiths RR (2005) Comparison of the behavioral effects of bretazenil and flumazenil in triazolam-dependent and non-dependent baboons. *European journal of pharmacology* 519(1-2):103-113.

23. Ronnback L, Eriksson PS, Zeuchner J, Rosengren L, & Wronski A (1987) Aspects of abstinence after morphine ingestion. *Pharmacol Biochem Behav* 28(1):87-93.

24. Chew M, White JM, Somogyi AA, Bochner F, & Irvine RJ (2001) Precipitated withdrawal following codeine administration is dependent on CYP genotype. *Eur J Pharmacol* 425(3):159-164.

25. Li T, Hou Y, Cao W, Yan CX, Chen T, Li SB. (2010) Naloxone-precipitated withdrawal enhances ERK phosphorylation in prefrontal association cortex and accumbens nucleus of morphine-dependent mice. *Neurosci Lett* 468(3):348-352.

26. Becker GL, Gerak LR, Li JX, Koek W, & France CP (2010) Precipitated and conditioned withdrawal in morphine-treated rats. *Psychopharmacology (Berl)* 209(1):85-94.

27. Anggadiredja K, Yamaguchi T, Tanaka H, Shoyama Y, Watanabe S, Yamamoto T. (2003) Prostaglandin E2 attenuates SR141716A-precipitated withdrawal in tetrahydrocannabinol-dependent mice. *Brain Res* 966(1):47-53.

28. Thornhill JA, Hirst M, & Gowdey CW (1978) Tolerance and evidence of physical dependence to daily codeine injections in the rat. *Pharmacol Biochem Behav* 9(4):433-438.

29. Kumar AA, Singh SG, Venkate SC & Lalit W. An Improved process for the preparation of rimonabant. Patent publication WO200862480 A2 May, 29, 2008.

30. Fang, Z, Xu, J-F., Guo, K., Wei, P & Yang, Z. (2012) A novel and practical synthesis of rimonabant. *Organic Preparations and Procedures International*, 44(2):164-168.

31. Cottone P, Sabino V, Roberto M, Bajo M, Pockros L, Frihauf JB *et al.* (2009a) CRF system recruitment mediates dark side of compulsive eating. *Proc Natl Acad Sci U S A* 106(47):20016-20020.

32. Cottone P, Sabino V, Steardo L, & Zorrilla EP (2009b) Consummatory, anxiety-related and metabolic adaptations in female rats with alternating access to preferred food. *Psychoneuroendocrinology* 34(1):38-49.

33. Iemolo A, Valenza M, Tozier L, Knapp CM, Kornetsky C, Steardo L, *et al.* (2012) Withdrawal from chronic, intermittent access to a highly palatable food induces depressive-like behavior in compulsive eating rats. *Behav Pharmacol* 23(5-6):593-602.

34. Cottone P, Sabino V, Steardo L, & Zorrilla EP (2008a) Intermittent access to preferred food reduces the reinforcing efficacy of chow in rats. *Am J Physiol Regul Integr Comp Physiol* 295(4):R1066-1076.

35. Hagan MM & Moss DE (1997) Persistence of binge-eating patterns after a history of restriction with intermittent bouts of refeeding on palatable food in rats: implications for bulimia nervosa. *Int J Eat Disord* 22(4):411-420.

36. Sabino V, Cottone P, Steardo L, Schmidhammer H, & Zorrilla EP (2007) 14-Methoxymetopon, a highly potent mu opioid agonist, biphasically affects ethanol intake in Sardinian alcohol-preferring rats. *Psychopharmacology (Berl)* 192(4):537-546.

37. Pellegrino LJ, Pellegrino AS, & Cushman AJ (1979) in *A stereotaxic atlas of the rat brain* 2d Ed pp 35 p., 122 (Plenum Press, New York) leaves of plates.

38. Zorrilla EP, Valdez GR, Nozulak J, Koob GF, & Markou A (2002) Effects of antalarmin, a CRF type 1 receptor antagonist, on anxiety-like behavior and motor activation in the rat. *Brain Res* 952(2):188-199.

39. Cottone P, Sabino V, Steardo L, & Zorrilla EP (2007) FG 7142 specifically reduces meal size and the rate and regularity of sustained feeding in female rats: evidence that benzodiazepine inverse agonists reduce food palatability. *Neuropsychopharmacology* 32(5):1069-1081.

40. Cottone P, Sabino V, Steardo L, & Zorrilla EP (2008b) Opioid-dependent anticipatory negative contrast and binge-like eating in rats with limited access to highly preferred food. *Neuropsychopharmacology* 33(3):524-535.

41. Fernandes C & File SE (1996) The influence of open arm ledges and maze experience in the elevated plus-maze. *Pharmacol Biochem Behav* 54(1):31-40.

42. Cruz AP, Frei F, & Graeff FG (1994) Ethopharmacological analysis of rat behavior on the elevated plus-maze. *Pharmacol Biochem Behav* 49(1):171-176.

43. Grimaldi P, Orlando P, Di Siena S, Lolicato F, Petrosino S, Bisogno T *et al*. (2009) The endocannabinoid system and pivotal role of the CB2 receptor in mouse spermatogenesis. *Proc Natl Acad Sci U S A* 106(27):11131-11136.