Bimodal control of stimulated food intake by the endocannabinoid system

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Supplementary Figures (pages 2-16)

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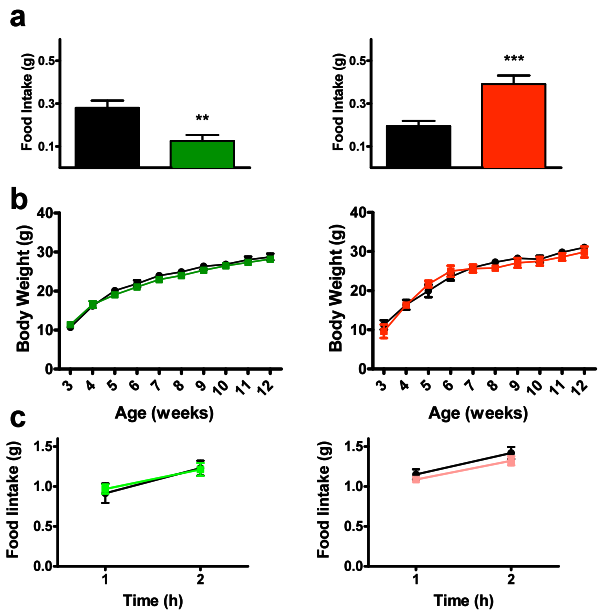

Supplementary Figure 1. (**a**) Deletion of *CB1* in cortical glutamatergic neurons (Glu-*CB1*-KO mice, green bar, n=13) or in GABAergic neurons (GABA-*CB1*-KO, red bar, n=18) results in opposite intakes of a novel palatable food. Black bars, respective wild-type littermates (Glu-*CB1*-WT, n=12; GABA-*CB1*-KO, n=18). **, $p<0.01$, ***, $p<0.001$ as compared to wild-type. (**b**) Deletion of *CB1* in cortical glutamatergic neurons (Glu-*CB1*-KO mice, green symbols) or in GABAergic neurons (GABA-*CB1*-KO, red symbols) does not alter body weight of mice under normal chow diet. N=8-13 per group. (**c**) Expression of the Cre recombinase in the specific neuronal populations is not involved in the phenotype of conditional *CB1* mutants. NEX-Cre and Dlx5/6-Cre mice, expressing only the Cre recombinase necessary for the deletion of *CB1* from specific neuronal populations, were tested in the fasting-refeeding protocol (see **Supplementary Methods**). Light green symbols, NEX-Cre mice (controls for Glu-*CB1*-KO mice); Light red symbols, Dlx5/6-Cre mice (controls for GABA-*CB1*-KO mice). No significant difference was observed as compared to the respective wild-type littermates (black symbols). N=5-7 per group.

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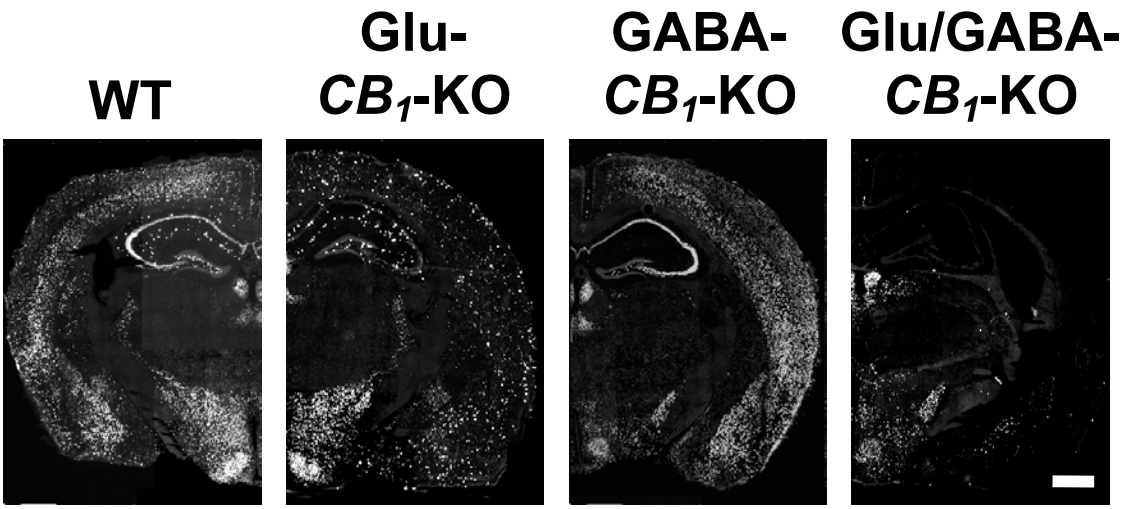

Supplementary Figure 2. Combined micrographs of fluorescent *in situ* hybridization (FISH) to detect CB_1 mRNA in wild-type, Glu-*CB1*-KO, GABA-*CB1*-KO and double Glu/GABA-*CB1*-KO mice. To detect the fluorescent signal, several adjacent pictures were taken with a Leica epifluorescent microscope at 20x magnification, using Cy3 filters. Single photos were combined using the programs Metamorph and Photoshop 10.0.1. Red images were changed to black and white for better visualization. As previously described[1], Glu-*CB1*-KO lack CB_1 expression mainly from cortical glutamatergic neurons, whereas GABA-*CB1*-KO lack the receptor mainly from GABAergic neurons. The double mutant Glu/GABA-*CB1*-KO mice almost completely lack CB_1 mRNA expression in cortical regions. Bar, 1 mm.

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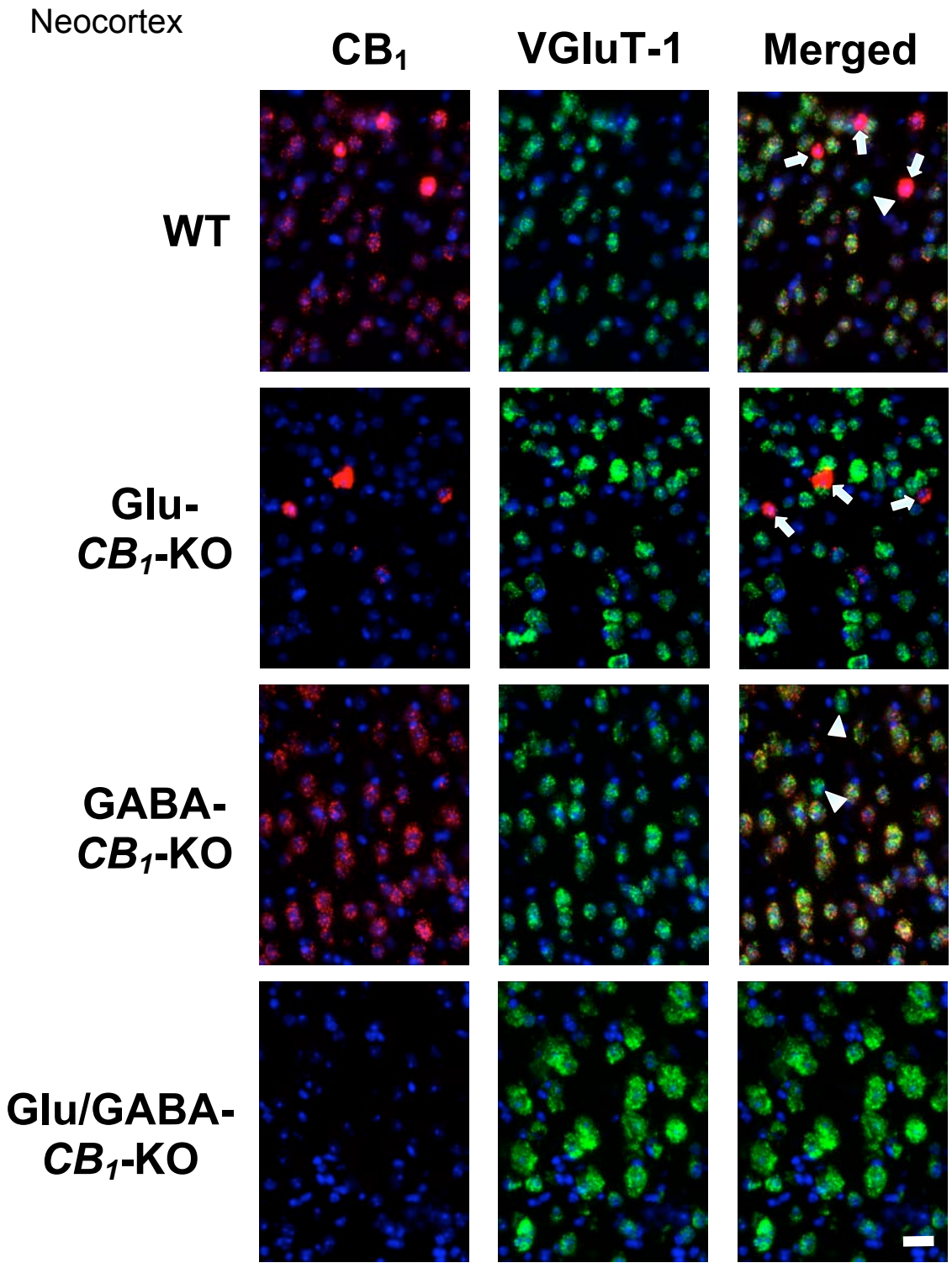

Supplementary Figure 3. Quantification of CB1 mRNA expression in glutamatergic neurons of the somatosensory area of the neocortex. See below for complete legend.

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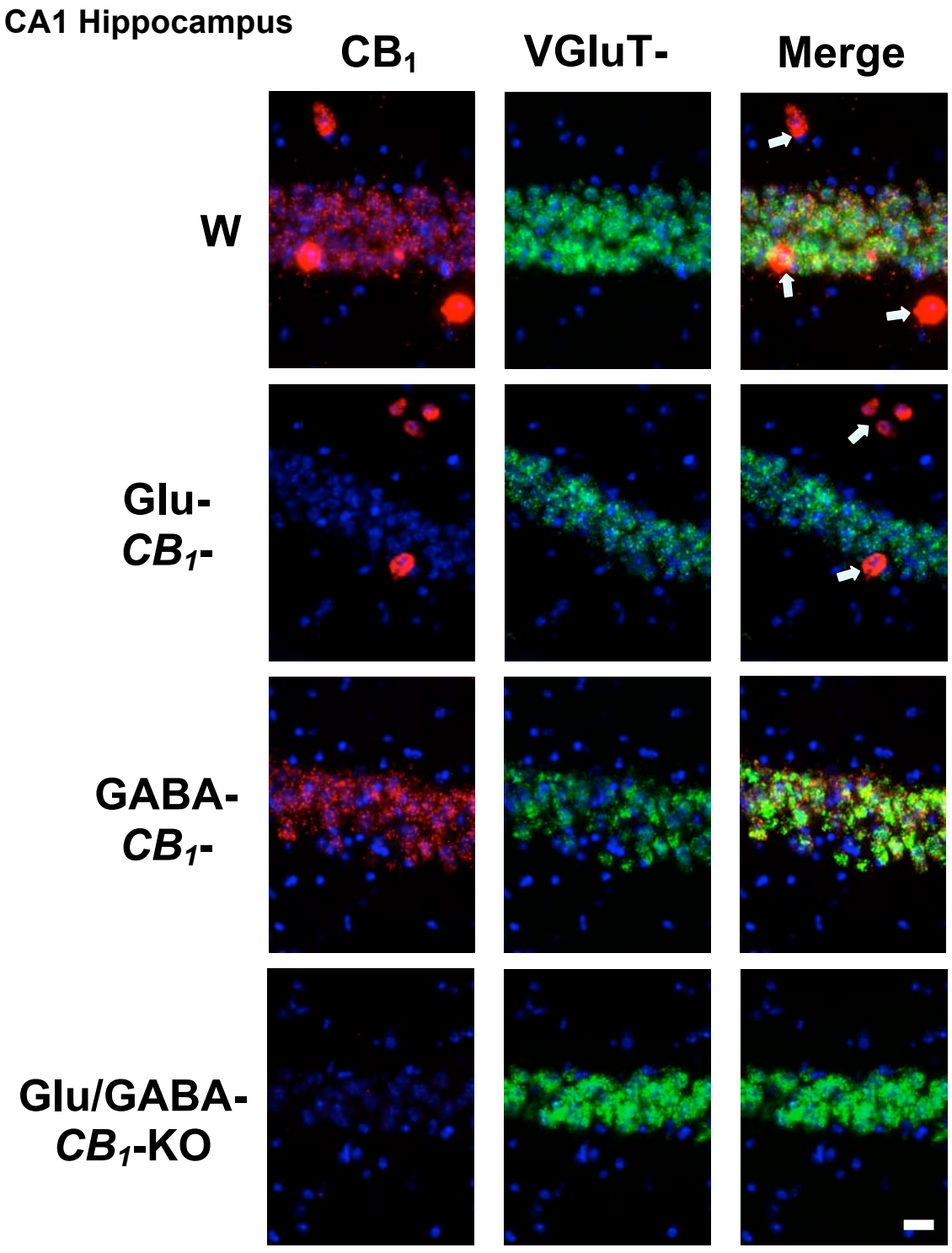

Supplementary Figure 4. Quantification of CB1 mRNA expression in glutamatergic neurons of the CA1 region of the hippocampus. See below for complete legend.

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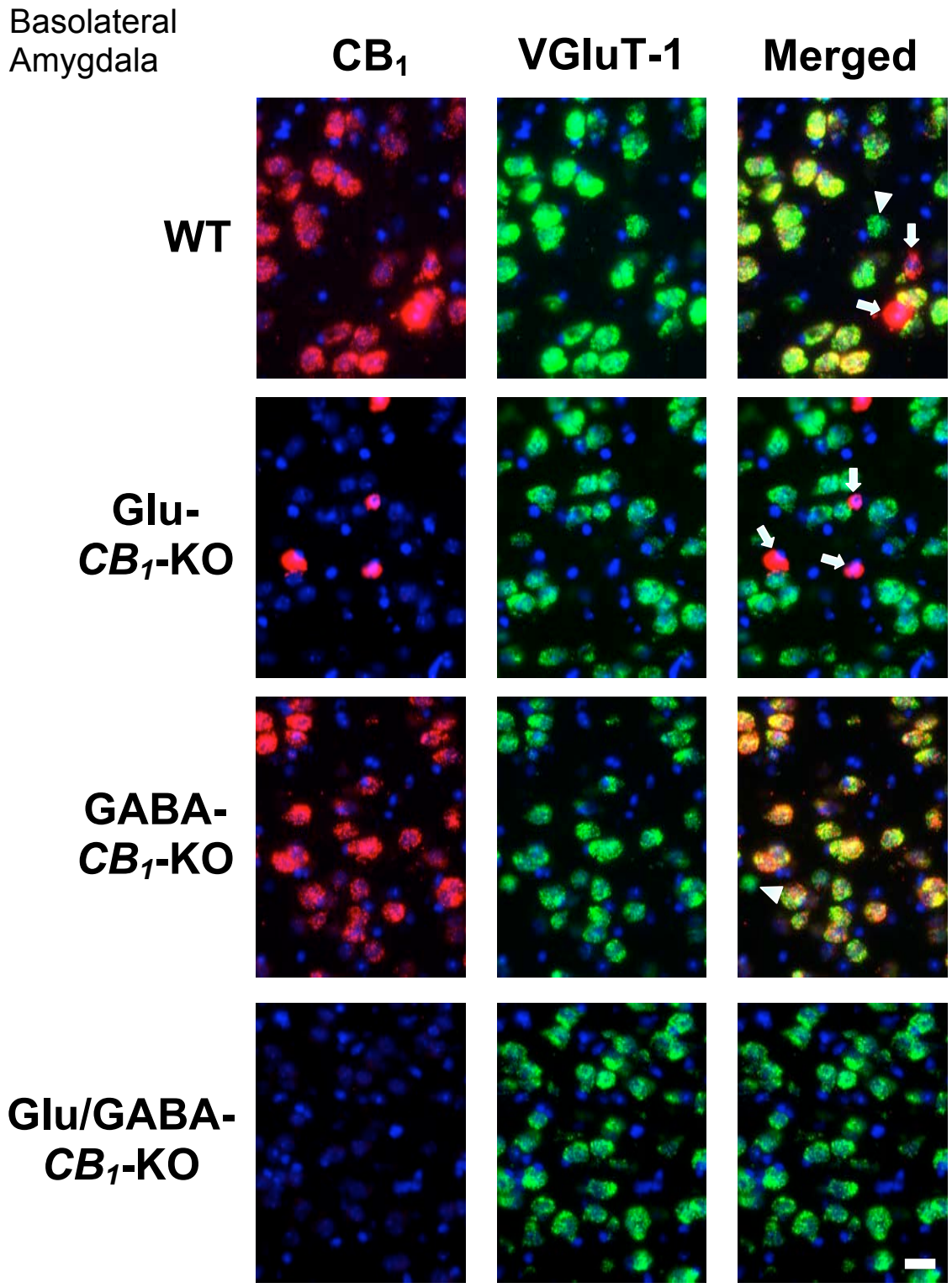

Supplementary Figure 5. Quantification of CB1 mRNA expression in glutamatergic neurons of the basolateral amygdala. See below for complete legend.

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Supplementary Figure 3-5. Representative micrographs of double fluorescent ISH (D-FISH) analysis, used to quantify the expression of CB_1 mRNA in glutamatergic neurons (identified by the expression of mRNA coding for the specific marker of glutamatergic cortical neurons, VGluT1)[2] in the different mutant mice. **Supplementary Figure 3**, Somatosensory area of neocortex. **Supplementary Figure 4**, CA1 region of the hippocampus. **Supplementary Figure 5**, basolateral nucleus of the amygdala. Red staining, CB_1 mRNA expression. Green staining, VGluT1 mRNA. Blue staining, nuclear counterstaining (DAPI). In the "merged" panels, co-expression is indicated by yellow colour and/or by the presence of intermingled red and green spots on the same cell. Arrows indicate neurons expressing only CB_1 mRNA. These generally contain high levels of the receptor (as evidenced in the micrographs representing single CB_1 mRNA stainings) and are known to be GABAergic interneurons (see Ref 3). Arrowheads represent neurons containing only VGluT1 mRNA. Arrowheads are omitted in images from the CA1, because of the impossibility to identify single pyramidal neurons clearly isolated from neighbouring ones. Arrowheads are also omitted from merged images from Glu-*CB_1*-KO and Glu/GABA-*CB_1*-KO mice, because in these mutants virtually all VGluT1-positive neurons do not contain CB_1 mRNA. Bars, 20 μm.

Quantification of CB_1 mRNA expression in cortical glutamatergic neurons

Semi-quantitative cell counting was performed in these cortical areas. In the neocortex (**Supplementary Figure 3**), cell counting in wild-type mice (WT) confirmed that 70-80% (78.0±3.9%) of VGluT1-positive neurons contain also CB_1 mRNA[4]. In Glu-*CB_1*-KO mice, this percentage dropped to 1.1±0.2%, whereas in GABA-*CB_1*-KO mice the amount of glutamatergic neurons expressing the receptor was similar as in WT animals (76.1±1.7%). In double mutants Glu/GABA-CB_1-KO mice CB_1 mRNA is almost undetectable. Due to their high density, it is not possible to count individual glutamatergic neurons in the pyramidal layers of the hippocampus (**Supplementary Figure 4**). However, as shown in the micrograph, low levels of CB_1 mRNA are present in almost the totality of hippocampal pyramidal neurons (see also Ref. 3). This expression is virtually lost in Glu-*CB_1*-KO and Glu/GABA-*CB_1*-KO and is fully preserved in GABA-CB_1-KO mice. In the basolateral amygdala (**Supplementary Figure 5**), 82.6±1.0% of glutamatergic neurons express CB_1 mRNA in WT animals. This percentage dropped to 3.3±0.2% in Glu-*CB_1*-KO. Again, no variation in the amount of glutamatergic neurons containing CB_1 mRNA was observed in GABA-*CB_1*-KO mice as compared to WT (83.1±3.4%).

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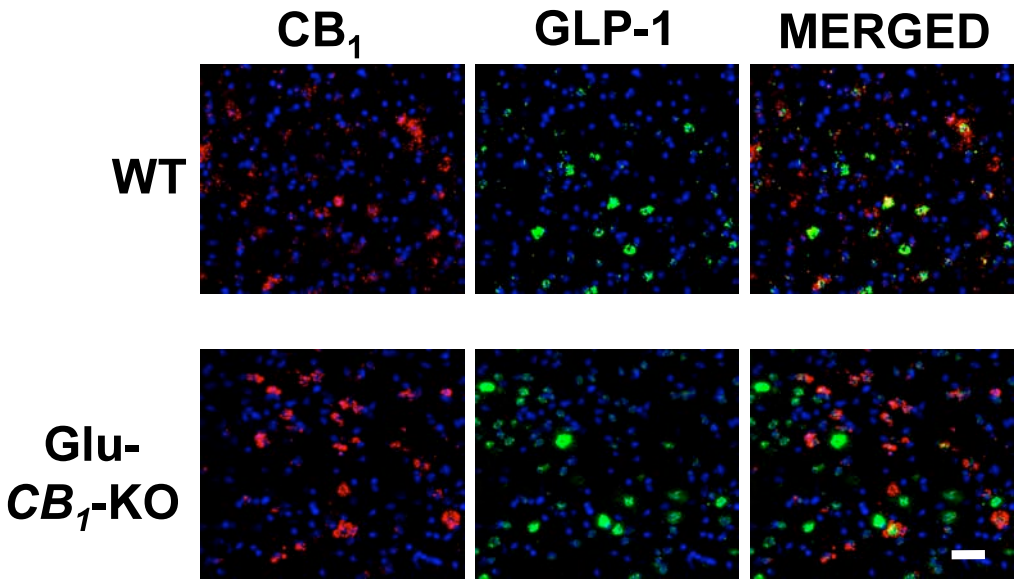

Supplementary Figure 6. The nucleus of solitary tract (NTS) contains similar levels of CB1 mRNA in Glu-*CB1*-KO mice as compared to wild-type littermates. NEX-Cre mice have been reported to drive Cre-mediated recombination in scattered cells and brain areas not belonging to the population of cortical glutamatergic neurons[5]. Among these "ectopic" recombination sites, the nucleus of the solitary tract (NTS) is involved in food intake and contains low levels of CB_1 receptors. Nevertheless, CB_1 deletion in this brain region might represent a confounding factor for the interpretation of the phenotype of Glu-*CB1*-KO mice. The NTS was identified by topo-anatomical localization and by the presence of clustered neurons expressing the mRNA coding for glucagone-like peptide-1 (GLP-1, green staining; Ref. 6) and neurons expressing CB_1 mRNA were identified in this brain area (red staining) by D-FISH. Due to the low levels of CB_1, quantitative evaluations of expression are extremely difficult in this brain area. However, qualitative evaluation did not reveal major alterations in CB_1 mRNA expression in the NTS of Glu-*CB1*-KO as compared to wild-type littermates. Bar, 40 μm.

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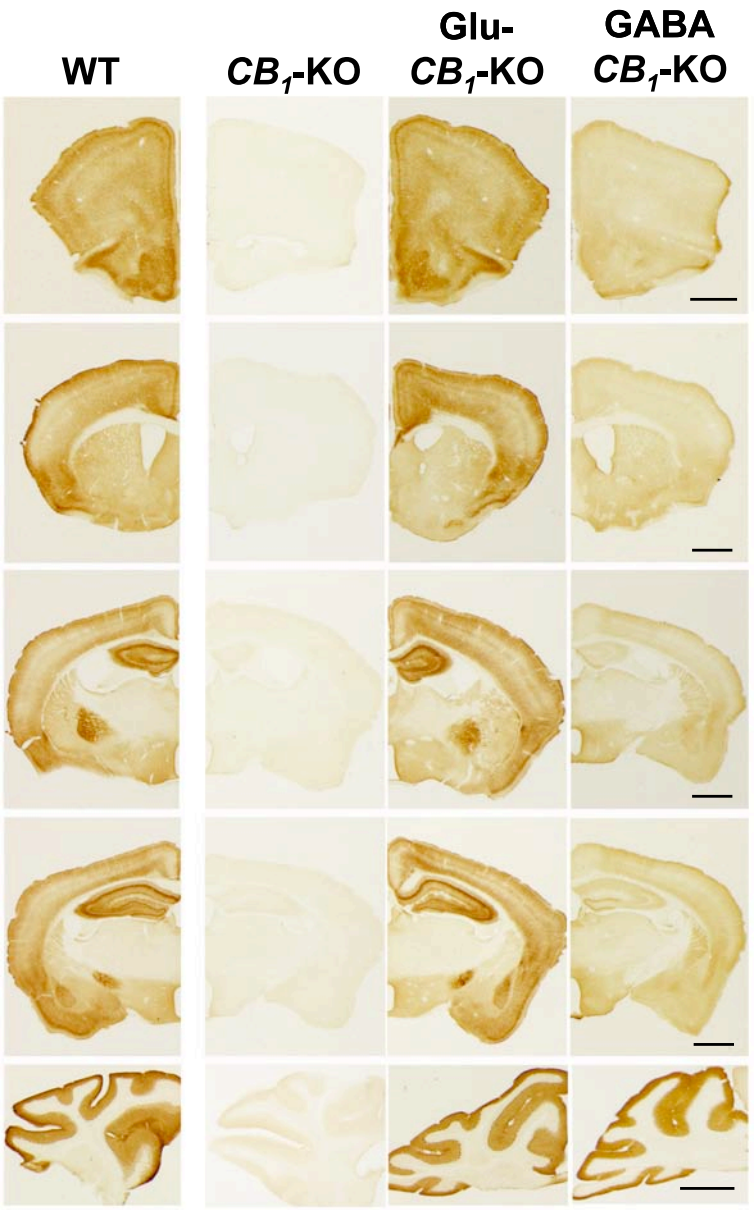

Supplementary Figure 7. Immunohistochemical analysis of CB_1 protein expression throughout the brain of wild-type (WT), *CB1*-KO, Glu-*CB1*-KO and GABA-*CB1*-KO mice, respectively. Note the almost undetectable decrease of the protein in Glu-*CB1*-KO, and the abundant reduction in GABA-*CB1*-KO mice. These observations suggest that the approximately 80% of cortical glutamatergic neurons expressing CB_1 mRNA (See **Supplementary Figure 2-5**) contain a very limited amount of the total CB_1 protein present in the brain. Conversely, GABAergic neurons contain a large majority of brain CB_1 protein. No signal is detected by the CB_1 antibody in *CB1*-KO tissue processed in parallel with wild-type and conditional mutant's brain tissue sections. Bars, 1 mm.

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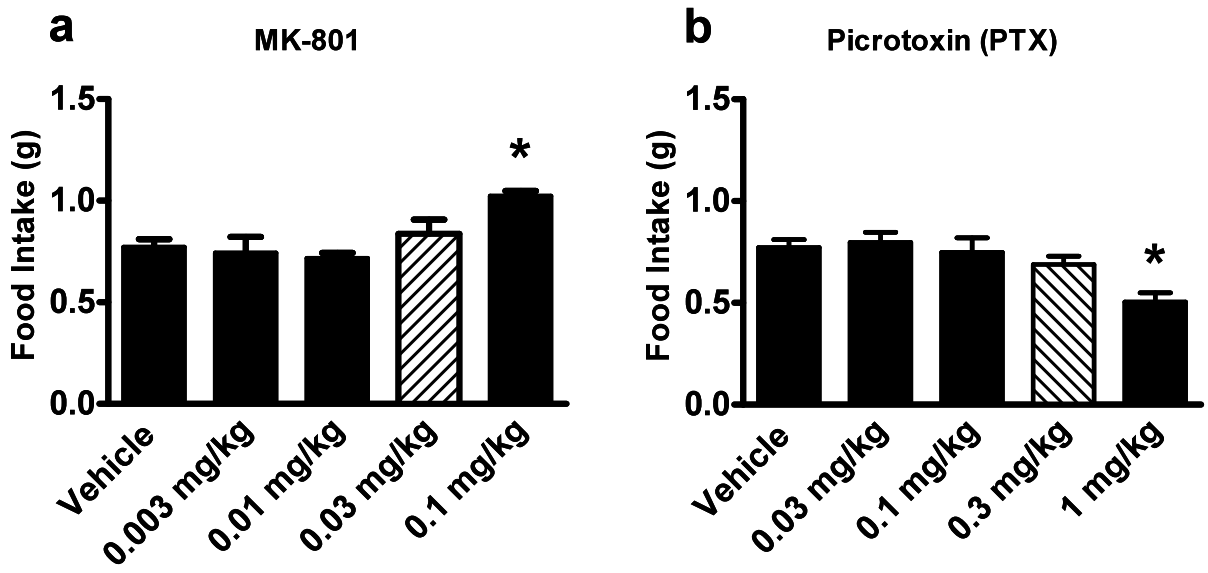

Supplementary Figure 8. Dose response studies of the effects of the NMDA receptor antagonist MK-801 (**a**) and of the $GABA_A$ receptor antagonist picrotoxin (PTX, **b**) on fasting-induced food intake in wild-type C57BL/6NCrl mice. Blockade of NMDA receptors bears a hyperphagic effect, whereas blockade of $GABA_A$ receptors decreases food intake. *, $p<0.05$ as compared to respective vehicle controls. Dashed bars, doses chosen for experiments in conditional mice as described in the main text. N=4-8 per group.

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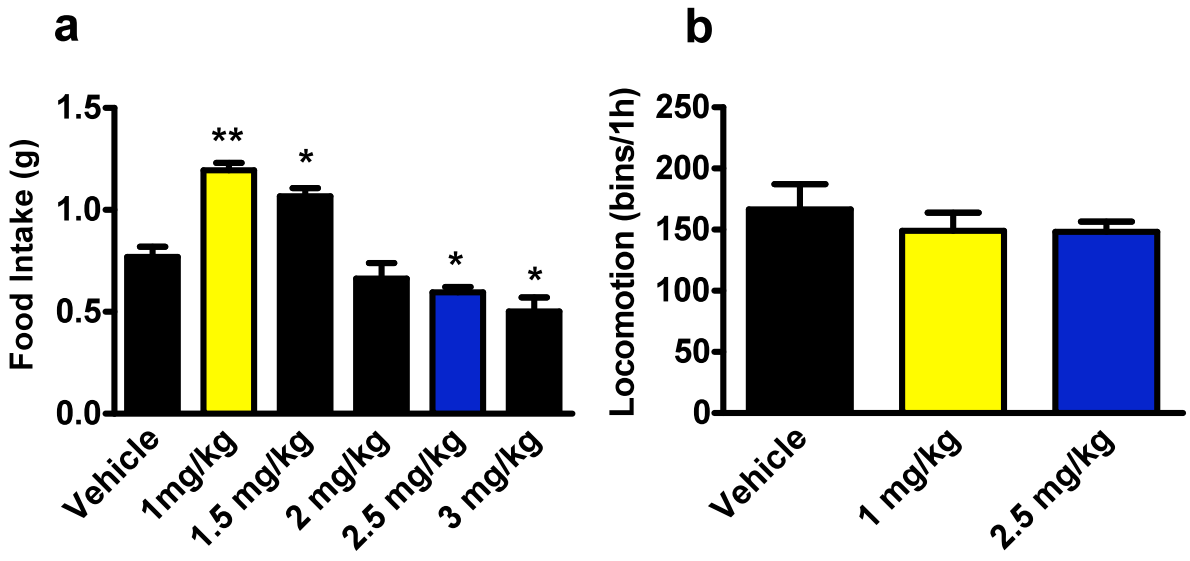

Supplementary Figure 9. The doses of 1 mg/kg and 2.5 mg/kg THC induce a hyperphagic and a hypophagic effect in wild-type C57BL/6NCrl mice, respectively, which do not depend on altered locomotion. (**a**) Dose response of THC effects in fasting-refeeding experiments in wild-type mice. Note the clear biphasic effect of the drug. N=4-6 per group. (**b**) The hyperphagic (yellow bar) and the hypophagic (blue bar) effects of THC are not accompanied by alterations in locomotor activity. N=8-9 per group. *, $p<0.05$, **, $p<0.01$ as compared to vehicle.

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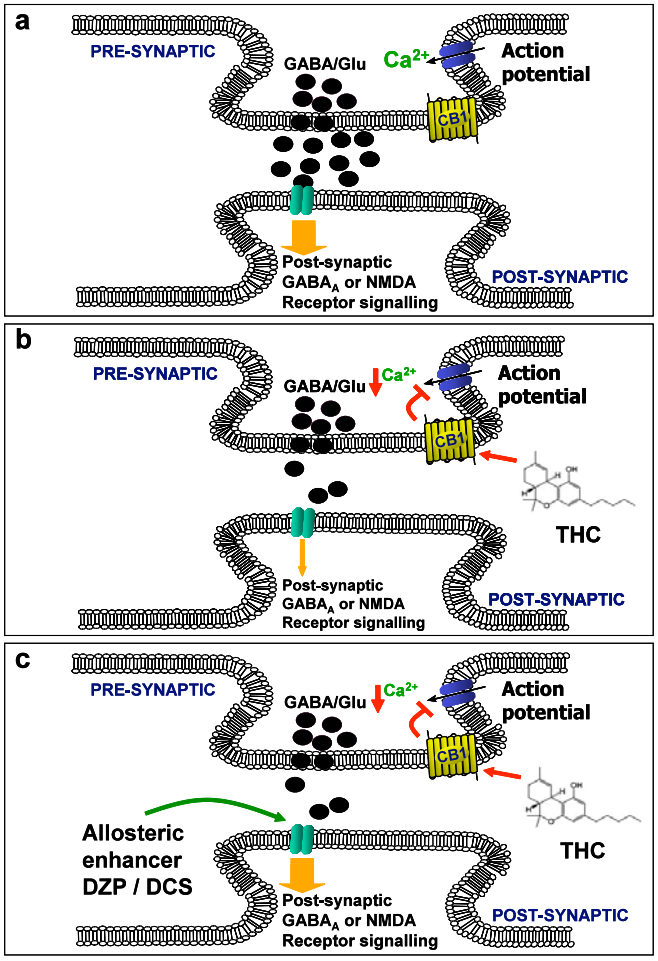

Supplementary Figure 10. Schematic representation of the rationale for pharmacological experiments using THC in combination with allosteric modulators of NMDA or $GABA_A$ receptors. (**a**) Under normal conditions, presynaptic action potentials and [Ca^{2+}] levels (black arrow) regulate the synaptic release of GABA or glutamate. The presynaptic release of the neurotransmitter (black circles) activates postsynaptic receptors (green symbol) and induces signalling in the postsynaptic neurons (large orange arrow). The intensity of this signalling depends on the amount of neurotransmitter released presynaptically. (**b**) The administration of THC activates presynaptic CB_1 receptors (red arrow), decreases presynaptic [Ca^{2+}] levels (red inhibitory arrow), and, thus, reduces the release of the neurotransmitter (rare black circles) and postsynaptic signalling (small orange arrow). (**c**) The co-administration of allosteric enhancers of NMDA and $GABA_A$ receptors (D-cyclo-serine, DCS, and diazepam, DZP, respectively, green arrow) would "restore" the postsynaptic levels of activity of these receptors (large orange arrow), thereby compensating the effects of the low and high doses of THC, respectively.

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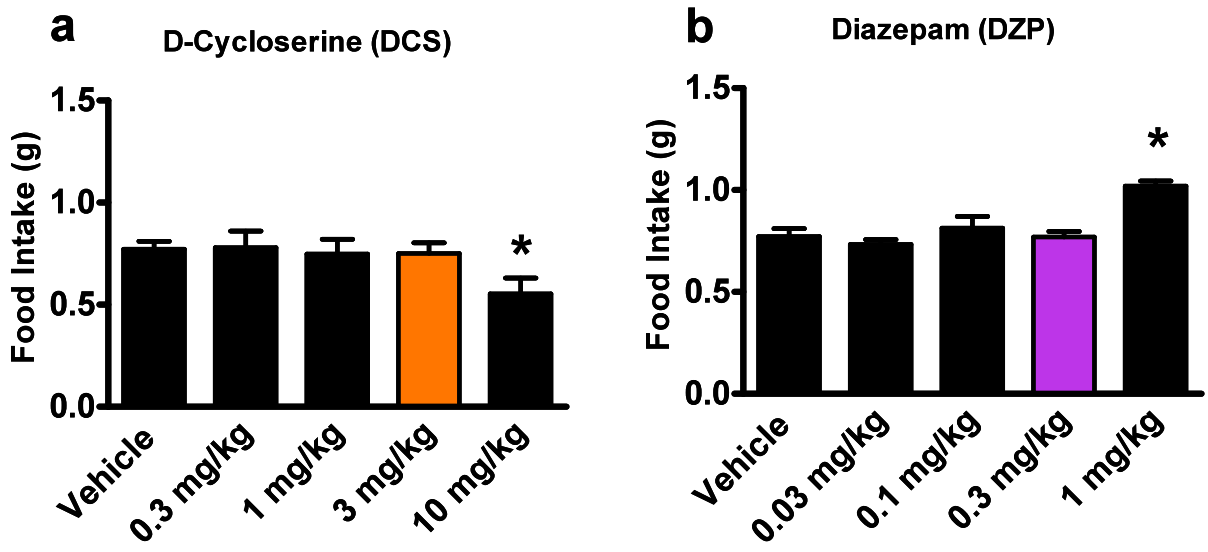

Supplementary Figure 11. Dose response studies of the effects of the NMDA receptor allosteric enhancer D-cyclo-serine (DCS, **a**), and of the $GABA_A$ receptor allosteric enhancer diazepam (DZP, **b**) on fasting-induced food intake in wild-type C57BL/6CtrlN mice. *, $p<0.05$ as compared to respective vehicle controls. Orange and violet bars, doses chosen for experiments in combination with THC, as described in the main text. N=4-8 per group.

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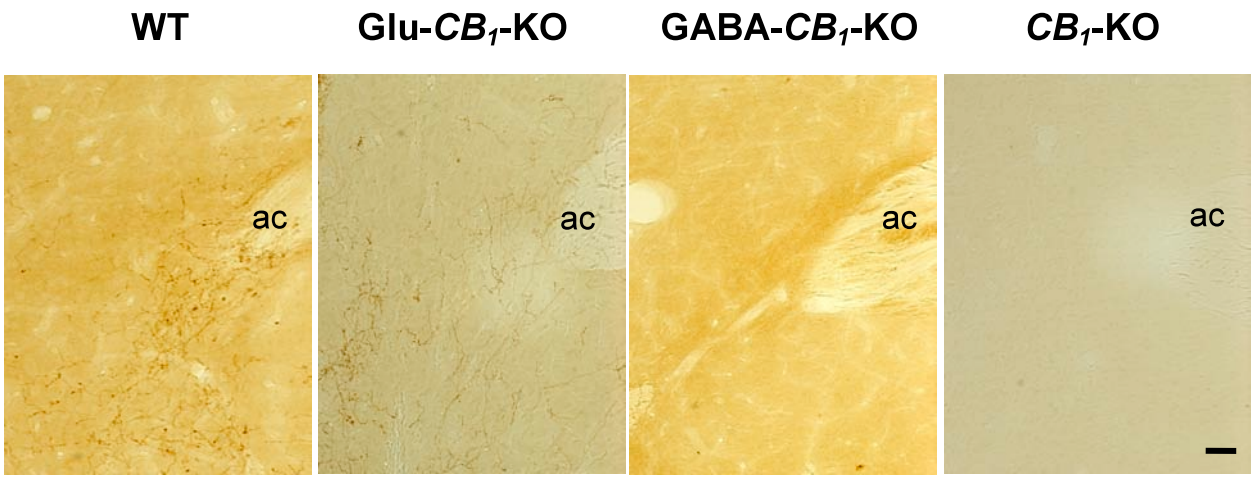

Supplementary Figure 12. Detailed analysis of CB_1 protein expression in the ventral striatum (medial nucleus accumbens) of wild-type (WT), *CB1*-KO, Glu-*CB1*-KO and GABA-*CB1*-KO mice, respectively. Also in this brain region, Glu-*CB1*-KO expression is very similar to the WT one, whereas GABA-*CB1*-KO mice display a strong reduction in the protein content. In particular, high magnification imaging reveals the presence of many CB_1 fibres (presumably axons) in WT sections. These fibres are conserved in Glu-*CB1*-KO, but are absent in GABA-*CB1*-KO mice. Interestingly, in addition to these fibres, detailed observation of WT sections reveals also the presence of a faint diffuse staining. This diffuse staining is above background levels, as it is not present in sections from global *CB1*-KO mice, and it is reduced in Glu-*CB1*-KO, but still present in GABA-*CB1*-KO sections. This suggests that this diffuse and low staining pattern reflects CB_1 expression in glutamatergic terminals in this brain region. Ac, anterior part of the anterior commissure. Bar, 50 μm.

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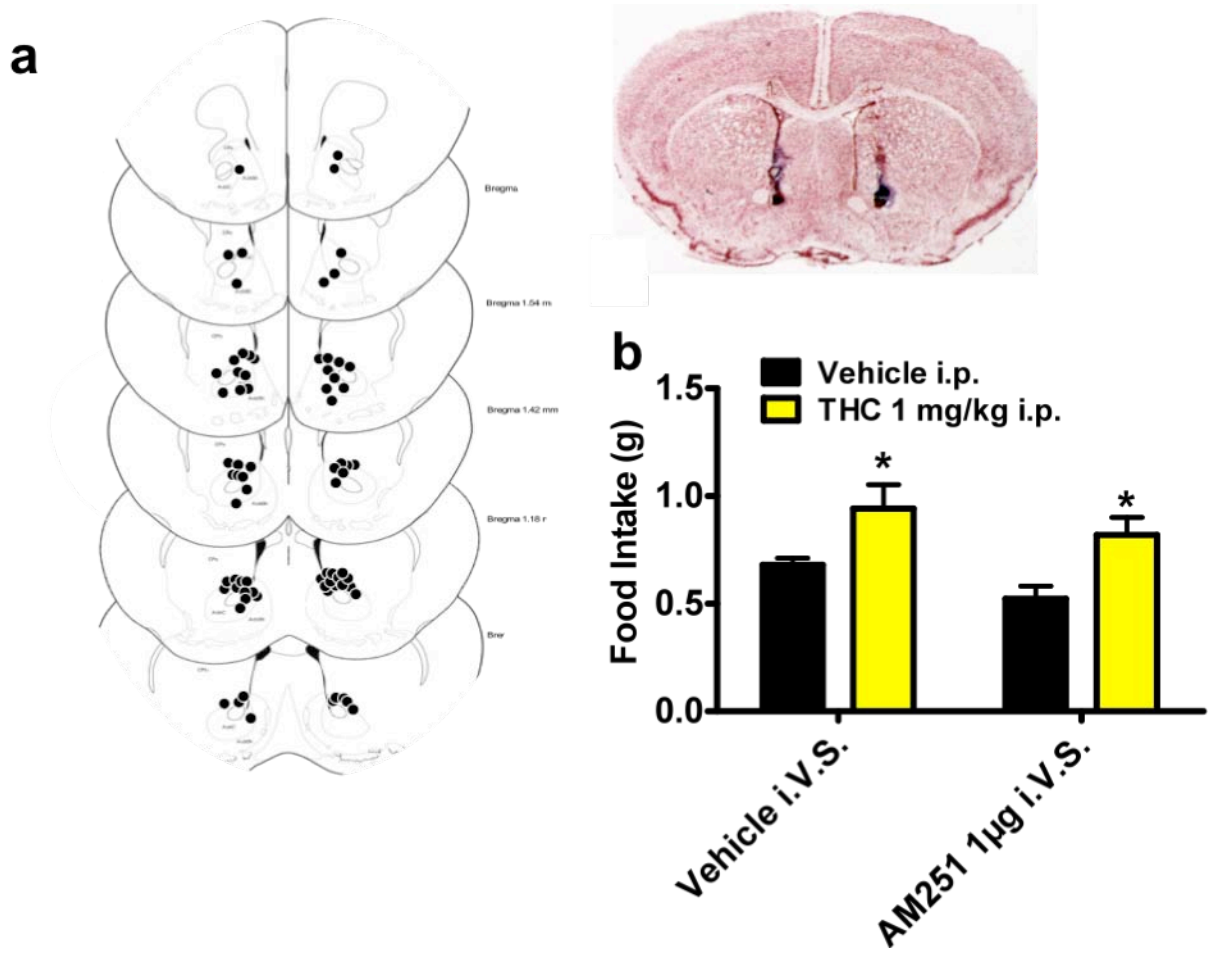

Supplementary Figure 13. Local intracerebral drug injections into the ventral striatum. (**a**) Schematic representations (left panel, black circles) and representative histological analysis (right panel) of the injection sites in the ventral striatum of wild-type animals. (**b**) Local injection of AM251 into the ventral striatum (i.V.S.) does not alter the hyperphagic effect of the systemic administration (i.p.) of THC 1 mg/kg. N=4-5 per group. *, $p<0.05$ as compared to systemic vehicle treatment.

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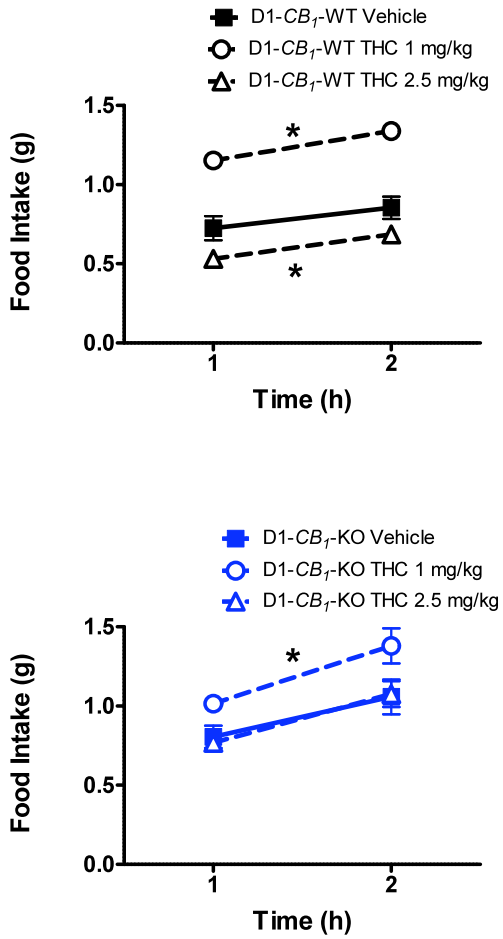

Supplementary Figure 14. The hypophagic effect of THC 2.5 mg/kg is abolished in D1-*CB1*-KO mice, lacking CB_1 expression from the large majority of striatal medium spiny neurons[7]. THC 1 mg/kg exerts a hyperphagic effect in D1-*CB1*-WT (black open circles, n=6), and in D1-*CB1*-KO littermates (blue open circles, n=5) as compared to vehicle-treated animals (solid squares; D1-*CB1*-WT, n=5; D1-*CB1*-KO, n=5). Conversely, the hypophagic effect of the highest dose of THC is present in D1-*CB1*-WT (black open triangles, n=5), but it is absent in D1-*CB1*-KO littermates (blue open triangles, n=6). *, $p<0.05$ as compared to vehicle group.

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Supplementary Methods

Animals

All experimental procedures were approved by the Committee on Animal Health and Care of INSERM and French Ministry of Agriculture and Forestry (authorization number, 3306369). Maximal efforts were made to reduce the suffering and the number of animals used. Male mice, aged 2–5 months, were maintained under standard conditions with food and water *ad libitum*. In the experiments with double Glu/GABA-CB_1 mutants, male and female mice were used, due to low number availability. No significant difference was observed between genders in this mouse line ($p>0.2$ for gender comparisons within each genotype). CB_1 mutant mice (CB_1-KO, Glu-CB_1-KO, GABA-CB_1-KO and D1-CB_1-KO) were obtained, maintained and genotyped as described[7-10]. All lines were in a mixed genetic background, with a predominant C57BL/6NCrl contribution. All animals used in experiments involving mutant mice were littermates. For total CB_1-KO mice, the parents of experimental animals were always heterozygous for the mutation. For conditional mutants, obtained using the Cre/loxP system, Cre-positive/$CB_1^{flox/flox}$ males were bred with Cre-negative/$CB_1^{flox/flox}$ females (phenotypically wild-type), in order to avoid potential influence of the mother's genotype on the adult phenotype of the experimental animals. To control for the potential influence of Cre expression on the observed phenotypes of conditional CB_1 mutant mice, Cre-positive and Cre-negative littermate mice were derived from Cre-positive/CB_1-WT males crossed with wild-type C57BL/6NCrl female mice. Dlx 5/6-Cre mice and NEX-Cre were the controls for the Cre effect of GABA-*CB1*-KO and Glu-*CB1*-KO, respectively[7-10]. Wild-type C57BL/6NCrl were purchased from JANVIER (France). Experimenters were always blind to genotypes and/or treatments.

Generation of double mutants Glu-GABA-*CB1*-KO

Glu-CB_1-KO mice were crossed with GABA-CB_1-KO mice in order to obtain a first generation with male mice bearing deletion in both glutamatergic and GABAergic neurons. These males were bred with $CB_1^{flox/flox}$ females. The resulting litters contained either wild-type, Glu-CB_1-KO, GABA-CB_1-KO, or Glu/GABA-CB_1-KO littermates, which were used for experiments.

The mice were genotyped by PCR, using the following primers: NEX-CRE forward primer for Glu-*CB1*-KO: `TCTTTTTCATGTGCTCTTGG`, Dlx5/6-CRE forward primer for GABA-*CB1*-KO:

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AGCAATCGCACTCACAACAGA, CRE reverse for both lines: CGCGCCTGAAGATATAGAAGA. *CB1*$^{f/f}$ alleles were detected as described[9].

Drugs

All drugs were purchased from SIGMA Aldrich (France), except AM251, which was purchased from Tocris (U.K.). Stocks of all drugs were prepared in ethanol for i.p. injections and in DMSO for intra-accumbens administration. For i.p. treatments, the injectable solutions were prepared just before the experiments, by diluting the stock solutions into sterile distilled H_2O (final ethanol concentration, 2%). The control vehicle solution was distilled H_2O with 2% ethanol. Drugs or vehicle were injected 30 minutes before refeeding (see below). For intra-accumbens injections, drugs were dissolved in a mixture of saline (0.9% NaCl) with 10% DMSO and 10% Chremophor EL.

Behavioural tests

Fasting-induced food intake. Animals were housed under a 12h/12h dark/light cycle (light on 7 A.M., light off 7 P.M.). Before the experiments, mice were singly housed for at least 7 days. 2 hours after the light onset, animals were food deprived for 24 hours and then given free access to a pre-weighed amount of standard chow (Standard Rodent Diet A03, SAFE, France). Food intake was recorded 1 and 2 hours after refeeding[11]. In these conditions, spillage of food was minimal. It was however controlled by inspection of litter and calculated as not eaten food.

THC effect on locomotor activity. Locomotor activity in THC dose-response experiments was recorded during the first hour of refeeding by an automated system (Mice Actimetry System, IMETRONIC, France) and expressed as number of bins/h.

Palatable food intake. 2 hours after the light onset, *ad libitum* fed animals were presented with a pre-weighed pellet of palatable food (Happycookies for rodents, Vitakraft, Germany), together with a pre-weighed pellet of normal chow. Both pellets were placed onto the floor of the mouse home cage. After 30 minutes, both pellets were removed and weighed, after controlling for spillage (minimal in all experiments). All animals showed an approximate 100% preference for the palatable food (data not shown). Therefore, only data related to the consumption of palatable food are presented.

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Local intra-ventrostriatal drug administration

Surgery. Mice were anesthetized by intraperitoneal injection of a mixture of ketamine/xylazine and placed into a stereotaxic apparatus (David Kopf Instruments) with mouse adapter and lateral ear bars. Mice were bilaterally implanted with 8-mm stainless steel guide cannulae targeting the ventral striatum with the following coordinates: AP +1.8, L ±1.0, DV –3.6, according to the atlas of Franklin and Paxinos (2001)[12]. Guide cannulae were secured in place with dental cement. Mice were allowed to recover for 7-10 days in individual cages before the beginning of the experiment. Mice were weighed daily and individuals that failed to regain the pre-surgery body weight were excluded from following experiments.

Local drug injections. Twenty-three hours after food deprivation, cannulae-implanted mice were randomly divided into three groups: one group received the ventrostriatal injection of vehicle (10% DMSO and 10% Chremophor EL in saline), one group received 1 μg/side of the CB_1 antagonist AM251, and one group received the injection of 5 μg/side of the $GABA_A$ allosteric enhancer diazepam. A 9-mm-long injector connected by polyethylene tubing to a 10-μL Hamilton syringe was lowered into the guide cannula and a volume of 0.5 μl of either vehicle, AM251 or Diazepam was then infused into each side with a micropump (Harvard Apparatus, France) at the rate of 0.2 μl/min. Consequently, each infusion lasted 2.5 min and was followed by 1 min of diffusion time with the injector left in place. After the injections, mice were returned to their home cage. Ten to fifteen minutes later, they were systemically injected (i.p.) with either vehicle or THC. Thirty minutes after the systemic treatment, animals were re-fed and the experiment was conducted as described above.

Analysis of cannula placement. At the completion of the behavioural testing, all mice were killed with an overdose of pentobarbital. In order to verify the sites of injection, mice were bilaterally injected with 0.5 μL of Trypan Blue 0.4%. Brains were then quickly removed and frozen on dry ice before being stored at –80°C until analysis. They were then cut into 30-μm sections in a cryostat (Microm HM 500M, Microm Microtech, France) and injection placements were determined by examination of coronal sections stained with Cresyl Violet or Neutral Red. The slides were analyzed with an Olympus SZX10 stereomicroscope (Olympus, France).

Single and double fluorescent *in situ* hybridization

Wild-type, GABA-CB_1-KO, Glu-CB_1-KO and double Glu/GABA-CB_1-KO mice (3 per genotype) were sacrificed by cervical dislocation. Their brains were isolated, quickly frozen on dry ice and

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stored at -80º C until sectioning in a cryostat (14 μm, Microm HM 500M, Microm Microtech, France). DIG-labeled riboprobes against mouse CB_1 receptor and FITC-labeled riboprobes against mouse VGluT1 were prepared as decribed[3,9,10]. For the generation of FITC-labelled riboprobes, GLP-1 cDNA (imaGenes, access number 1164409, Germany) was sub-cloned into pBluescript vector using the restriction enzymes EcoRI and NotI (New England Biolabs, Ipswich, MA, USA). After linearization with NotI, the antisense riboprobe was synthesized with T3 RNA polymerase (Roche, Basel, Switzerland). For the generation of the sense riboprobe, EcoRI was used for the linearization and T7 RNA polymerase (Roche) for the synthesis. For signal amplification we used the TSA™ Plus System Cyanine 3/Fluorescein (Perkin Elmer). Blocking buffer TNB and wash buffer TNT were prepared according to the manufacturer's protocol. Slides were analyzed by epifluorescence microscopy at 40X (Leica). Quantitative co-expression data were obtained using the program ImageJ with the "CellCounter plug-in" (http://rsbweb.nih.gov/ij/), by separately counting VGluT1 (green), CB1 (red) and co-expressing neurons in the somatosensory neocortex and basolateral amygdala (BLA) of WT, Glu-*CB_1*-KO and GABA-*CB_1*-KO mice (see **Supplementary Figures 3-5**). A total of 9579 neurons (4998 in the neocortex and 4581 in the BLA) were counted in 28 images acquired at a 20X magnification from the somatosensory cortex and BLA of two mice for each genotype (4-5 images/region/genotype).

Immunohistochemical detection of CB_1 receptors in the brain

Wild-type, Glu-*CB_1*-KO, GABA-*CB_1*-KO and *CB_1*-KO mice (3 of each condition) were deeply anesthetized with chloral hydrate (400 mg/kg body weight), transcardially perfused with phosphate-buffered solution (PB 0.1M, pH 7.4) and then fixed by 500 ml of 0.1% glutaraldehyde, 4% formaldehyde (freshly depolymerized from paraformaldehyde) and 0.2% picric acid in 0.1M PB (pH 7.4) prepared at 4°C. Tissue blocks were extensively rinsed in 0.1M PB (pH 7.4). Serial coronal vibrosections of all brain were cut at 50 μm and collected in 0.1M PB (pH 7.4) at room temperature (RT). Sections were pre-incubated in a blocking solution of 10% bovine serum albumin (BSA), 0.1% sodium azide and 0.5% Triton X-100 prepared in Tris-HCl buffered saline (TBS, pH 7.4) for 30 minutes at RT.

A pre-embedding immunocytochemistry for light microscopy was used to localize *CB_1* receptors in the brain of wild-type, Glu-*CB_1*-KO, GABA-*CB_1*-KO and *CB_1*-KO mice. Sections were incubated with goat *CB_1* polyclonal antibodies raised against a 31 aminoacid C-terminal sequence

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(NM007726) of the mouse CB_1 receptor (CB1-Go-Af450-1; 2µg/ml; Frontier Science Co. Ltd, 1-777-12, Shinko-nishi, Ishikari, Hokkaido, Japan) overnight at RT. The antibody was prepared in 10% BSA/TBS containing 0.1% sodium azide and 0.5% Triton X-100. After several washes, the tissue was incubated with biotinylated donkey anti-goat (1:200, Vector Laboratories) for 1 hour and then processed by a conventional avidin-biotin peroxidase complex method (ABC, Elite, Vector Laboratories). To visualize the immuno-reaction in the light microscope, sections were pre-incubated with 0.05% 3,3'-diaminobenzidine-tetrahydrochloride (DAB, Sigma Chemical Co., St. Louis, MO, USA) in 0.1 M PB for 5 min and subsequently incubated by adding 0.01% hydrogen peroxide to the same solution for 5 min. After several washes in 0.1 M PB at RT, stained sections were mounted, dried, dehydrated and coverslipped with DPX (Fluka Chemie AG, Buchs, Switzerland). The slides were analyzed with an Olympus SZX10 stereomicroscope (Olympus, France) and a Zeiss Axiophot 1 microscope.

Statistical analysis

Data were analysed using one- or two-way ANOVA (using genotypes and treatments as variables), followed by Newman-Keuls' and Bonferroni's *post-hoc* tests. Data were also analyzed by Student's *t*-test when appropriate. Graphs and statistics were generated by GraphPad Prism 4.03 (U.S.A.) and GBstat v10 (U.S.A.).

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Supplementary References

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