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Hippocampal CB_1 Receptors Control Incidental Associations

Highlights

- Hippocampal CB_1R is required for incidental associations leading to mediated learning
- Incidental associations enhance hippocampal CB_1R expression and inhibitory plasticity
- CB_1R-dependent control of hippocampal GABAergic cells controls mediated learning

Authors

Arnau Busquets-Garcia, José F. Oliveira da Cruz, Geoffrey Terral, ..., Pierre Trifilieff, Guillaume Ferreira, Giovanni Marsicano

Correspondence

guillaume.ferreira@inra.fr (G.F.), giovanni.marsicano@inserm.fr (G.M.)

In Brief

Busquets-Garcia et al. suggest that cannabinoid CB1 receptors signaling in the hippocampus, a brain structure involved in memory processes, underlines the mammalian ability to associate randomly encountered stimuli, allowing future inferred memories and possibly explaining seemingly ungrounded responses toward certain cues.

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Hippocampal CB_1 Receptors Control Incidental Associations

Arnau Busquets-Garcia,[1,2] José F. Oliveira da Cruz,[1,2,3,8] Geoffrey Terral,[1,2,8] Antonio C. Pagano Zottola,[1,2,8] Edgar Soria-Gómez,[1,2,4,5] Andrea Contini,[2,6] Hugo Martin,[2,6] Bastien Redon,[1,2] Marjorie Varilh,[1,2] Christina Ioannidou,[1,2] Filippo Drago,[3] Federico Massa,[1,2] Xavier Fioramonti,[2,6] Pierre Trifilieff,[2,6] Guillaume Ferreira,[2,6,7,*] and Giovanni Marsicano[1,2,7,9,*]

[1]INSERM, U1215 NeuroCentre Magendie, 33000 Bordeaux, France
[2]University of Bordeaux, 33000 Bordeaux, France
[3]Department of Biomedical and Biotechnological Sciences, Section of Pharmacology, University of Catania, I-95123 Catania, Italy
[4]Department of Neurosciences, University of the Basque Country UPV/EHU, Achucarro Basque Center for Neuroscience, 48940 Leioa, Spain
[5]IKERBASQUE, Basque Foundation for Science, 48013 Bilbao, Spain
[6]INRA, Nutrition and Integrative Neurobiology, UMR 1286, Bordeaux, France
[7]Senior author
[8]These authors contributed equally
[9]Lead Contact
*Correspondence: guillaume.ferreira@inra.fr (G.F.), giovanni.marsicano@inserm.fr (G.M.)
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SUMMARY

By priming brain circuits, associations between low-salience stimuli often guide future behavioral choices through a process known as mediated or inferred learning. However, the precise neurobiological mechanisms of these incidental associations are largely unknown. Using sensory preconditioning procedures, we show that type 1 cannabinoid receptors (CB_1R) in hippocampal GABAergic neurons are necessary and sufficient for mediated but not direct learning. Deletion and re-expression of CB_1R in hippocampal GABAergic neurons abolishes and rescues mediated learning, respectively. Interestingly, paired presentations of low-salience sensory cues induce a specific protein synthesis-dependent enhancement of hippocampal CB_1R expression and facilitate long-term synaptic plasticity at inhibitory synapses. CB_1R blockade or chemogenetic manipulations of hippocampal GABAergic neurons upon preconditioning affect incidental associations, as revealed by impaired mediated learning. Thus, CB_1R-dependent control of inhibitory hippocampal neurotransmission mediates incidental associations, allowing future associative inference, a fundamental process for everyday life, which is altered in major neuropsychiatric diseases.

INTRODUCTION

Direct associative memories, in which a sensory stimulus is explicitly paired with a negative or rewarding outcome, can determine daily behavioral choices. Very often, however, human behavior is governed by mediated learning, based on previous events implying incidental associations between low-salience sensory cues (Bornstein et al., 2017; Shohamy and Wagner, 2008; Wimmer and Shohamy, 2012). In other words, we are often repulsed or attracted by stimuli never explicitly paired with negative or positive outcomes but previously associated with other stimuli paired with a specific aversive or rewarding meaning (Bornstein et al., 2017; Shohamy and Wagner, 2008; Wimmer and Shohamy, 2012). These processes are well conserved in all mammals, including rodents (Gewirtz and Davis, 2000; Parkes and Westbrook, 2011). However, whereas the biological mechanisms underlying direct associative learning are under intense scrutiny (LeDoux, 2014), much less is known about the neural substrates mediating sensory stimulus-stimulus associations leading to higher-order mediated learning (Gewirtz and Davis, 2000; Parkes and Westbrook, 2011).

Sensory preconditioning is a typical behavioral procedure to study mediated learning (Gewirtz and Davis, 2000; Parkes and Westbrook, 2011). In this protocol, pairings of two low-salience stimuli (e.g., odors, tastes, lights, tones) are followed by classical conditioning of one of these stimuli with an aversive or appetitive unconditioned reinforcer (Parkes and Westbrook, 2011). As a result of these associations, subjects present aversion or preference to the stimulus never explicitly paired with the reinforcer, thereby allowing the evaluation of mediated learning (Gewirtz and Davis, 2000; Parkes and Westbrook, 2011; Wheeler et al., 2013). Thus, three distinct and temporally successive processes occur in sensory preconditioning. First, an incidental association is formed between low-salience stimuli during the preconditioning phase. Second, direct association with a reinforcer increases the salience of one of the stimuli during the conditioning phase. Finally, exposing the subjects to either of the original stimuli (the one directly associated with the reinforcer and the one never associated) reveals the retrieval of direct and mediated memories, respectively. The neurobiological mechanisms of these connected but distinct processes are poorly understood. The hippocampus has been suggested to play an important role in the conditioning and

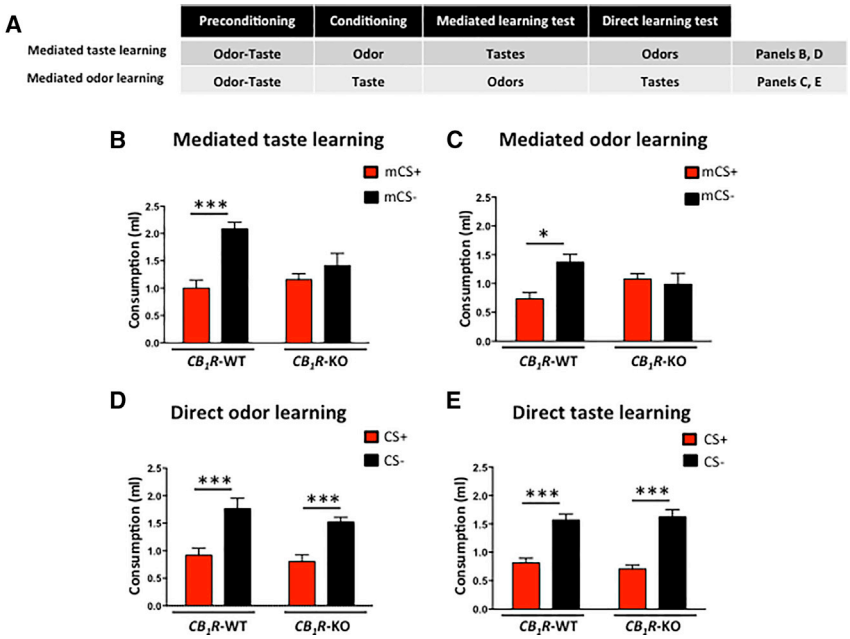

Figure 1. CB_1R Are Necessary for Odor-Taste-Mediated Learning

(A) Schematic table of the odor-taste sensory preconditioning protocol (see Star Methods and Figure S1 for more details).

(B and C) Liquid consumption under conditions of mediated taste (B) or odor (C) aversion in CB_1R-KO mice and wild-type littermates (CB_1R-WT).

(D and E) Liquid consumption under conditions of direct odor (D) or taste (E) aversion in CB_1R-KO mice and CB_1R-WT. *$p < 0.05$; ***$p < 0.001$ (mCS+ versus mCS− or CS+ versus CS−). For statistical details and *n*, see Tables S1 and S2.

retrieval phases of sensory preconditioning procedures (Iordanova et al., 2011; Talk et al., 2002; Wheeler et al., 2013; Wimmer and Shohamy, 2012). However, its involvement in the formation of initial incidental associations leading to mediated learning is unknown.

Type 1 cannabinoid receptors (CB_1R) are key neuromodulatory elements of synapses, and they are the main targets of endogenous signaling molecules, the endocannabinoids, forming the so-called endocannabinoid system (ECS) (Lu and Mackie, 2016; Piomelli, 2003). Through CB_1R, the ECS has been involved in direct conditioning such as fear conditioning (Marsicano et al., 2002; Metna-Laurent et al., 2012; Resstel et al., 2009) or conditioned taste aversion (Kobilo et al., 2007). Notably, the involvement of the ECS in direct conditioning appears to be more prominent in the modulation of behavioral expression of the acquired memory, rather than its formation (Kobilo et al., 2007; Marsicano et al., 2002; Metna-Laurent et al., 2012; Resstel et al., 2009). Regarding hippocampus, CB_1R are mainly expressed in GABAergic neurons (Marsicano and Kuner, 2008; Marsicano and Lutz, 1999), where they negatively control inhibitory neurotransmission (Katona et al., 1999), thereby modulating synaptic plasticity (Araque et al., 2017; Busquets-Garcia et al., 2018; Castillo et al., 2012; Kano et al., 2009) and cognitive processes (Busquets-Garcia et al., 2015). Although hippocampal CB_1R have been implicated in the cognitive impairment produced by exogenous cannabinoids (Busquets-Garcia et al., 2015; Puighermanal et al., 2009), no study has addressed the physiological role of the ECS in higher-order learning such as sensory preconditioning.

Thus, applying genetic, pharmacological, biochemical, imaging, electrophysiological, and chemogenetic approaches to sensory preconditioning procedures in mice, the present study shows how the physiological inhibition of specific hippocampal GABAergic neuronal populations by CB_1R is crucial for incidental associations between low-salience stimuli, eventually leading to mediated learning.

RESULTS

CB_1R Are Necessary for Mediated Learning

Mice were exposed to a preconditioning phase with three odor-taste pairings (sucrose and maltodextrin as tastes; banana and almond as odors), followed by a conditioning phase consisting in the devaluation of one of the two stimuli (Figures 1A, S1A, and S1B). First, we controlled that the potential salience of taste stimuli (Yiannakas and Rosenblum, 2017) was not sufficient to induce any type of observable direct conditioning in our experimental protocol (Figures S1C and S1D). Then we observed that wild-type mice consumed lower amounts of tastes or odors that were indirectly (preconditioned) or directly devaluated, indicating the formation of reliable mediated and direct aversion learning, respectively (Figures 1B–1E and S1E–S1H). Interestingly, global CB_1R knockout mice (CB_1R-KO) displayed impaired mediated aversion independently of the sensory modality (Figures 1B, 1C, S1E, and S1F), still maintaining normal expression of direct learning (Figures 1D, 1E, S1G, and S1H), thereby demonstrating that CB_1R are specifically required for mediated learning.

Activation of CB_1R during Preconditioning Is Necessary for Mediated Learning

Long-lasting absence of CB_1R in CB_1R-KO mice might induce chronic alterations, eventually causing the observed phenotype in mediated learning. To test whether CB_1R are acutely required during incidental associations, we administered the CB_1R antagonist Rimonabant (1 mg/kg, i.p.) before each session of preconditioning (Figures 2A, S1A, and S1B). This treatment impaired mediated learning, both in the taste and odor modalities, without affecting either direct aversions (Figures 2B–2E and S2A–S2D). Importantly, Rimonabant treatment did not alter the total liquid consumption during preconditioning (Figure S2E), nor did it affect the lack of taste-induced odor conditioning during odor-taste pairings (Figures S2F–S2H). Importantly, Rimonabant administration immediately before the test did not affect mediated odor aversion (Figures 2F, 2G, S2I, and S2J) or mediated

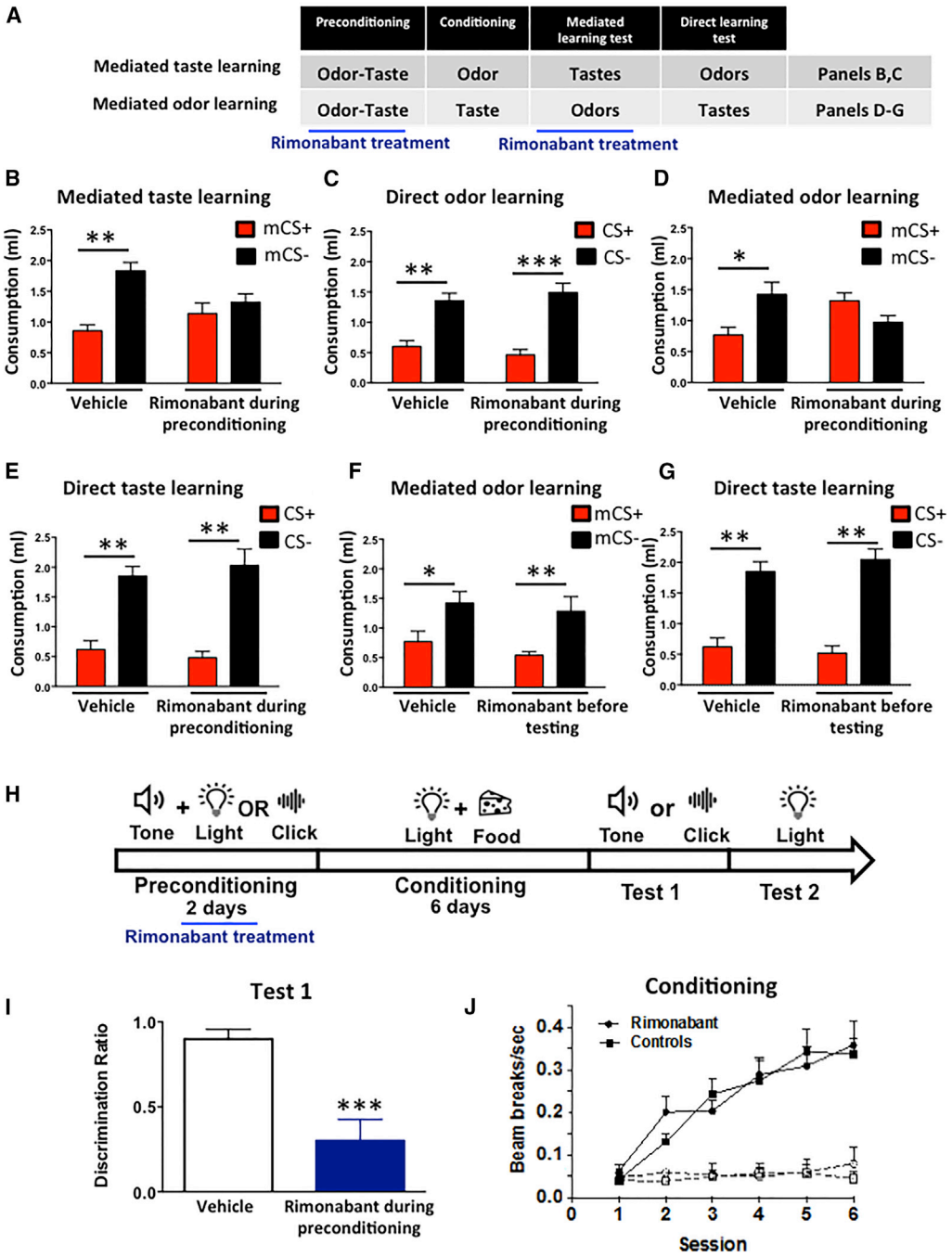

Figure 2. Activation of CB_1R during Preconditioning Is Necessary for Mediated Learning

(A) Schematic table of the odor-taste sensory preconditioning protocol (see Star Methods and Figure S1 for more details).

(B and C) Effect of daily preconditioning administrations of the CB_1R antagonist Rimonabant (1 mg/kg, i.p.) and its vehicle on mediated taste (B) and direct odor aversion (C) in C57BL/6-N mice.

(D and E) Effect of daily preconditioning administrations of Rimonabant (1 mg/kg, i.p.) and its vehicle on mediated odor (D) and direct taste aversion (E) in C57BL/6-N mice.

(F and G) Effect of acute administration of Rimonabant (1 mg/kg, i.p.) and its vehicle before testing on mediated odor (F) and direct taste aversion (G) in C57BL/6-N mice.

(H) Schematic representation of the light-tone sensory preconditioning protocol (see Star Methods for more details).

(I and J) (I) Effect of daily preconditioning administrations of Rimonabant (1 mg/kg, i.p.) and its vehicle on mediated tone learning, expressed as discrimination ratio, and (J) on the conditioning to light in C57BL/6-J mice (see Star Methods for further details). *$p < 0.05$; **$p < 0.01$; ***$p<0.001$ (mCS+ versus mCS- or CS+ versus CS-). ***$p < 0.001$ (vehicle- versus Rimonabant-treated mice). For statistical details and *n*, see Tables S1 and S2.

taste aversion (Busquets-Garcia et al., 2017b), suggesting that CB_1R activation is specifically required for the formation of incidental associations, but not for the expression of mediated aversion.

Next, we evaluated whether CB_1R activation was required also in another sensory preconditioning protocol using different sensory stimuli (visual and auditory cues) and unconditioned stimulus (food reward). To this aim, we adapted an existing paradigm originally developed in rats using operant chambers and visual/auditory cues (Robinson et al., 2014; Figure 2H). Mice treated with vehicle or Rimonabant (1 mg/kg, i.p.) were exposed to two preconditioning sessions consisting in pairings between a light and a tone (preconditioned cues), and to presentations of a click alone (unpaired cue). Next, the light was paired with food delivery and the visits of the food magazine in response to tone and click presentations were evaluated in the absence of the food reward (test session). Vehicle-treated mice displayed mediated conditioning as shown by a discrimination index indicating the increased visits of the food magazine during tone presentations as compared to the click (Figure 2I). Interestingly, mediated learning was specifically prevented by the Rimonabant treatment during the preconditioning phase (Figure 2I), whereas both vehicle- and Rimonabant-treated mice exhibited comparable levels of direct conditioning to the light (Figures 2J and S2K). Altogether, these results indicate that mediated learning relies on CB_1R activation specifically during the formation of incidental associations, regardless of the sensory modalities used and of the nature (aversive or appetitive) of the reinforcers.

Hippocampal CB_1R Are Required for Mediated Learning

Inactivation and functional imaging experiments have suggested the involvement of the hippocampus in mediated learning (Iordanova et al., 2011; Wheeler et al., 2013; Wimmer and Shohamy, 2012), but the role of hippocampal CB_1R in these processes have never been investigated. To address this issue, we injected adeno-associated viruses (AAVs) expressing Cre recombinase or control green fluorescent protein (GFP) into the hippocampus of CB_1R-flox mice (Marsicano et al., 2003) to generate mice lacking CB_1R in the hippocampus and control littermates with normal expression of the receptor (called HC-*CB_1R*-KO and HC-*CB_1R*-WT, respectively; Monory et al., 2006; Figures 3A and S3A). HC-*CB_1R*-KO mice displayed an impairment of mediated, but not direct, aversion in the odor-taste protocol, regardless of the sensory modality (Figures 3B,3C, and S3B–S3E). The lack of mediated learning after three odor-taste pairings was not due to slower incidental learning as mice undergoing extended preconditioning using six odor-taste pairings (Busquets-Garcia et al., 2017b) did not show mediated aversion (Figures S3F and S3G). We next investigated whether hippocampal CB_1R are sufficient to allow mediated learning. Notably, the viral re-expression of CB_1R in the hippocampus of *CB_1R*-KO mice (HC-*CB_1R*-RS mice; Figures 3D and S3A; Hebert-Chatelain et al., 2016) fully rescued mediated learning (Figures 3E, S3H, and S3I). Altogether, these data show that hippocampal CB_1R are necessary and sufficient for mediated learning.

GABAergic CB_1R Are Necessary and Sufficient for Mediated Learning

The large majority of hippocampal CB_1R are present in a specific subpopulation of GABAergic interneurons, namely the cholecystokinin (CCK)-expressing perisomatic basket cells (Kano et al., 2009; Marsicano and Kuner, 2008). To study the role of this specific population of CB_1R, we first used conditional mutant mice lacking the receptor from forebrain GABAergic neurons (Dlx5/6-*CB_1R*-KO mice, generally and hereafter called GABA-*CB_1R*-KO; Bellocchio et al., 2010; Monory et al., 2006). Notably, these animals displayed a specific impairment of mediated aversion independently of the sensory modality (Figures 4A, 4B, S4A, and S4B), accompanied by normal direct aversion (Figures 4C, 4D, S4C, and S4D). This impairment might be due to a delayed formation of mediated learning. However, when exposed to an extended preconditioning training (six odor-taste pairings; Busquets-Garcia et al., 2017b), GABA-*CB_1R*-KO mice were still unable to display mediated aversion (Figures S4E and S4F), strongly suggesting that "GABAergic" CB_1R are necessary for the formation of incidental learning independently of the training intensity. In addition, "rescue" mice carrying expression of the CB_1R protein exclusively in forebrain GABAergic neurons (GABA-*CB_1R*-RS) (Gutiérrez-Rodríguez et al., 2017; Remmers et al., 2017) displayed normal mediated aversion, both in the taste or odor modalities, in contrast to littermates globally lacking CB_1R expression (STOP-*CB_1R* mice; Figures 4E, 4F, S4G, and S4H; Ruehle et al., 2013), with no effect on direct aversion (Figures 4G, 4H, S4I, and S4J).

Altogether, these data indicate that, within distinct cell type-specific populations of CB_1R (Busquets-Garcia et al., 2015; Marsicano and Kuner, 2008), those present in GABAergic neurons are specifically involved in odor-taste mediated learning.

CB1R-Dependent Hippocampal Alterations Induced by Odor-Taste Pairings

The results showed above suggest a key role of the hippocampus and of GABAergic neurons in the modulation of odor-taste mediated learning. Thus, we next aimed at determining whether preconditioning recruits classical hippocampal mechanisms associated with memory formation, such as protein synthesis and synaptic plasticity, and the involvement of inhibitory transmission in these processes.

First, we investigated whether incidental learning specifically modulates CB_1R expression in the hippocampus. Immunoblotting experiments revealed that 3 odor-taste pairings, but not the same number of presentations of odor alone, taste alone, or unpaired odor-taste, specifically resulted in enhanced hippocampal expression of CB_1R protein (Figures 5A, S5A, and S5B). Extended preconditioning training is known to suppress the expression of mediated learning (Busquets-Garcia et al., 2017b), suggesting that increasing the number of stimuli associations could trigger cellular and molecular changes. Intriguingly, we observed that hippocampal CB_1R expression was normalized by extended preconditioning training (six odor-taste pairings) (Figures 5A and S5B), further suggesting that increased levels of hippocampal CB_1R protein are reliably associated to the expression of mediated learning. To investigate the

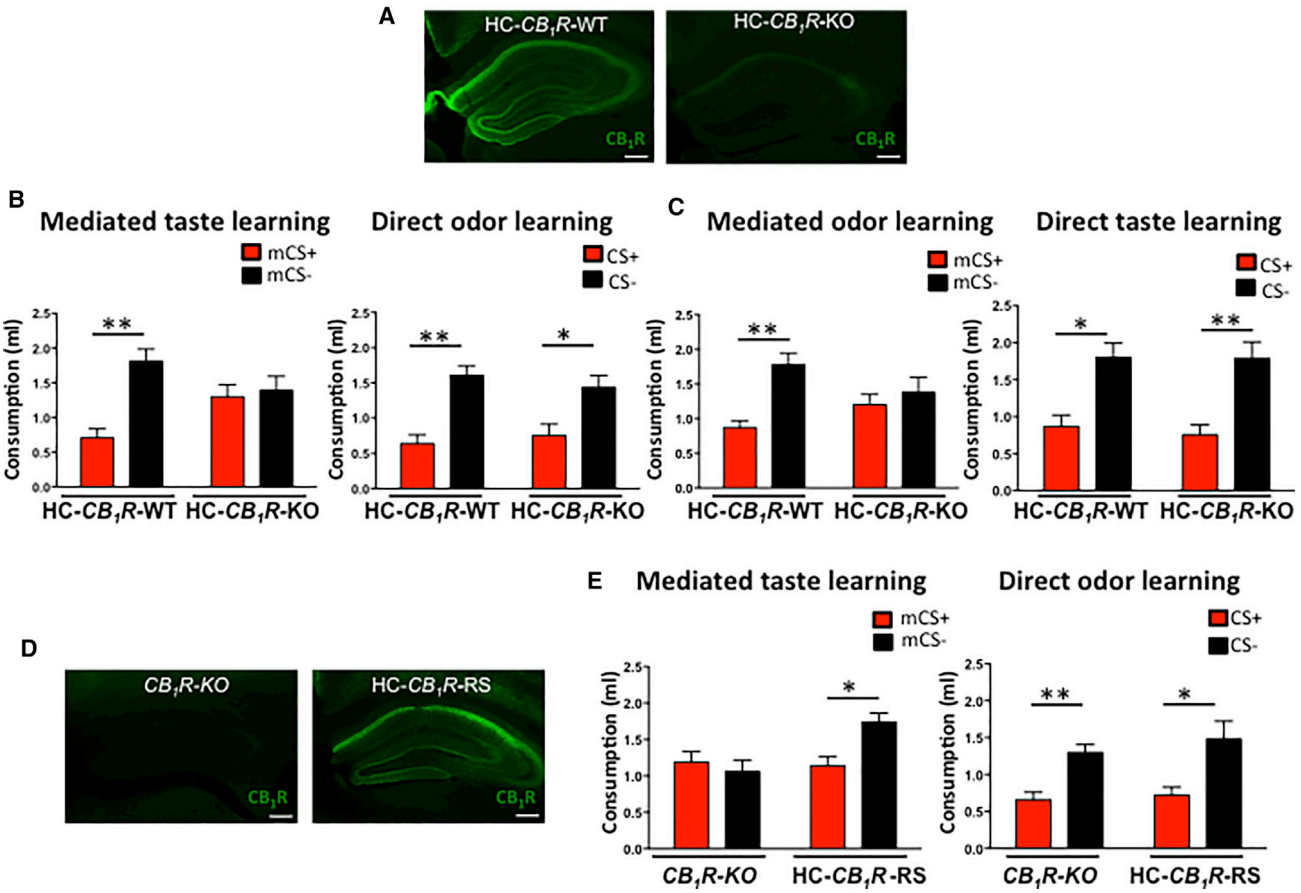

Figure 3. Hippocampal CB_1R Are Required for Mediated Learning

(A) Representative images showing hippocampal expression of CB_1R in control mice (HC-*CB_1R*-WT) and in mice carrying virally induced deletion of the receptor (HC-*CB_1R*-KO). Scale bar, 300 μm.

(B and C) (B) Effects of specific hippocampal deletion of CB_1R on mediated taste (left) and direct odor (right) learning and (C) on mediated odor (left) and direct taste (right) learning.

(D) Representative images showing hippocampal expression of CB_1R in *CB_1R*-KO mice injected with a control (Stop-*CB_1R*-KO) or with a CB_1R-expressing virus (HC-*CB_1R*-RS). Scale bar, 300 μm.

(E) Effects of exclusive hippocampal re-expression of CB_1R on mediated taste (left) and direct odor (right) learning. *$p < 0.05$; **$p < 0.01$ (mCS+ versus mCS− or CS+ versus CS−). For statistical details and *n*, see Tables S1 and S2.

molecular mechanisms of this effect, we asked whether the enhancement of CB_1R expression was due to new protein synthesis during the preconditioning phase. The administration of the protein synthesis inhibitor Anisomycin (18 mg/kg, i.p.; Puighermanal et al., 2009) before each odor-taste pairing blunted the increase of CB_1R expression (Figure S5C).

Intracellular CB_1R signaling involves many different pathways, including extracellular-regulated kinases (ERKs), mechanistic target of rapamycin (mTOR), and the cAMP response element-binding protein (CREB) (Piomelli, 2003; Puighermanal et al., 2009). Neither the phosphorylation of ERKs nor the activation of the mTOR pathway was affected by exposure to three odor-taste pairings (data not shown). Conversely, CREB phosphorylation was enhanced after three pairings, but not after extended training (Figure S5D). However, whereas exposure to taste alone did not affect CREB phosphorylation, odor alone promoted it (Figure S5D).

CB_1R activation participates in the regulation of synaptic transmission and plasticity (Busquets-Garcia et al., 2018; Castillo et al., 2012; Chevaleyre et al., 2006; Kano et al., 2009). Therefore, we asked whether odor-taste pairings impacted *in vivo* hippocampal long-term potentiation (LTP) induced by high-frequency stimulation (HFS) of the Schaffer collateral–CA1 pathway (Robin et al., 2018). Exposure to three odor-taste pairings enhanced *in vivo* hippocampal LTP in wild-type mice, but not in GABA-*CB_1R*-KO (Figures 5B, 5C, and S5E). However, control experiments indicated that exposure to the taste alone also increased *in vivo* hippocampal LTP in WT, but not in GABA-*CB_1R*-KO mice (Figures 5B and 5C).

Hippocampal CB_1R in GABAergic neurons control inhibitory transmission and plasticity, such as short-term depolarization-induced suppression (DSI) or long-term depression (I-LTD) of hippocampal evoked inhibitory post-synaptic currents (eIPSCs; Araque et al., 2017; Busquets-Garcia et al., 2018; Castillo et al., 2012;

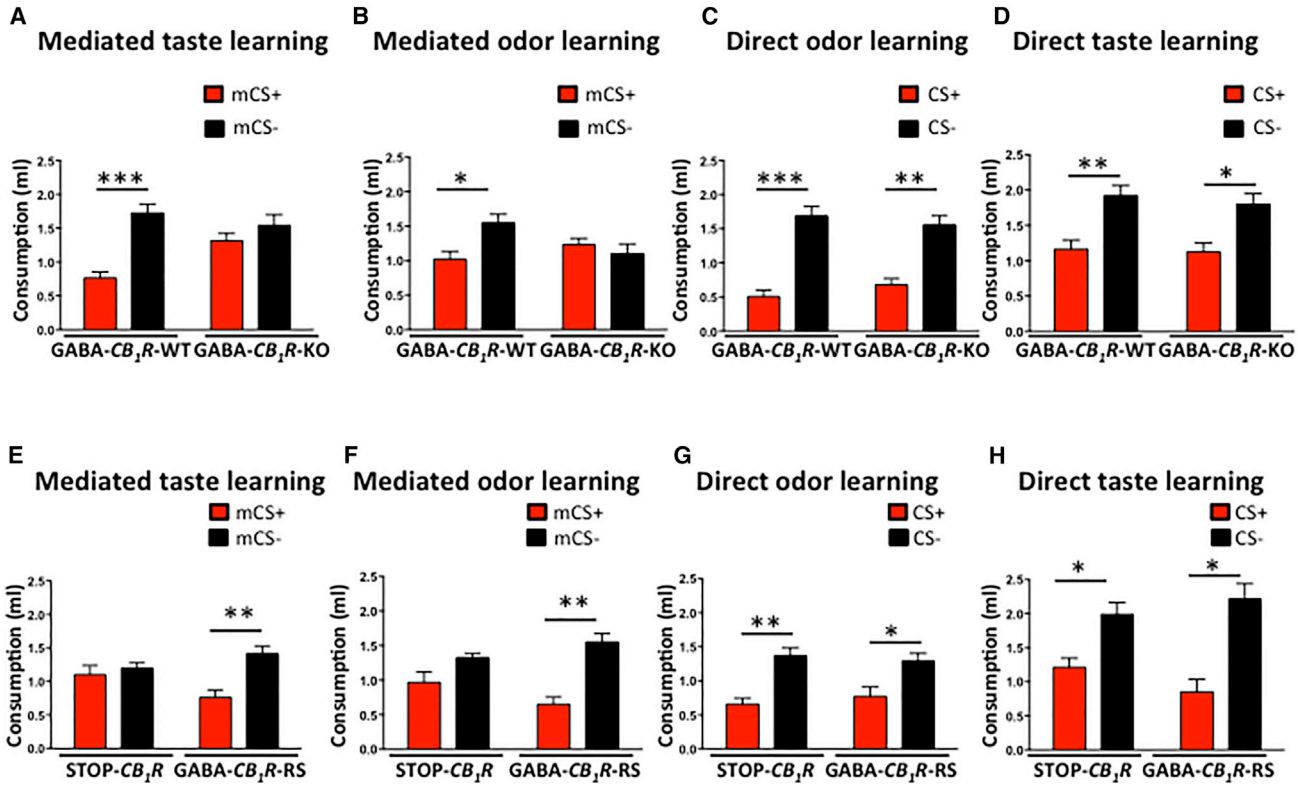

Figure 4. GABAergic CB_1R Are Necessary and Sufficient for Mediated Learning

(A–D) Liquid consumption under conditions of mediated taste (A) and odor (B) aversion and direct odor (C) and taste (D) aversion in mice lacking CB_1R in forebrain GABAergic neurons (GABA-*CB_1R*-KO) and wild-type littermates (GABA-*CB_1R*-WT).

(E–H) Liquid consumption under conditions of mediated taste (E) or odor (F) aversion and direct odor (G) or taste aversion (H) in mice carrying exclusive expression of CB_1R in forebrain GABAergic neurons (GABA-*CB_1R*-RS) and in littermates lacking CB_1R expression (STOP-*CB_1R*). *p < 0.05; **p < 0.01; ***p < 0.001 (mCS+ versus mCS− or CS+ versus CS). For statistical details, see Tables S1 and S2.

Chevaleyre et al., 2006; Kano et al., 2009). Hippocampal slices were prepared from mice exposed to three odor-taste pairings or control conditions, and inhibitory synaptic plasticity was analyzed. Preconditioning altered neither basal inhibitory neurotransmission as assessed by measuring miniature IPSCs (Figure S5F) nor short-term plasticity as determined by DSI measurements (Figure S5G). As previously described (Chevaleyre and Castillo, 2003), we observed that the application of two HFS trains induced a reliable I-LTD in 71% of pyramidal neurons from naive control mice (Figures 5D and 5E). Odor-taste preconditioning procedures involve limited access to water (1 hr per day; Busquets-Garcia et al., 2017a, 2017b; see Star Methods). Surprisingly, only 26% of pyramidal neurons in hippocampal slices from water-restricted control mice only exposed to plain water underwent I-LTD after HFS (Figure 5E), indicating an impact of limited access to water on this form of synaptic plasticity. However, paired odor-taste exposures in equally water-restricted mice rescued I-LTD expression to levels undistinguishable from naive animals in both amplitude (Figure 5D) and percentage of responsive cells (Figure 5E). Importantly, these effects were not present in mice exposed either to taste or odor alone (Figures 5D and 5E).

Altogether, these results show that exposure to three odor-taste pairings increases protein synthesis-dependent expression of CB_1R, CREB phosphorylation, *in vivo* LTP, and cellular sensitivity to endocannabinoid-dependent I-LTD in the hippocampus. However, whereas the effects on CREB phosphorylation and LTP are also triggered by the mere exposure to low-salience stimuli such as odors or tastes alone, the enhancements of CB_1R expression and I-LTD amplitude and sensitivity are specifically related to preconditioning odor-taste pairings, suggesting their involvement in the formation of incidental associations and the expression of mediated learning.

Specific Hippocampal GABAergic Interneurons Control the Formation of Incidental Associations

Considering the inhibitory role of CB_1R on hippocampal GABAergic neurotransmission (Chevaleyre et al., 2006; Katona et al., 1999; Ohno-Shosaku et al., 2012) and the enhancement of I-LTD sensitivity induced by preconditioning training, lack of CB_1R-dependent inhibition of hippocampal GABAergic transmission during incidental associations might be responsible for the impairment of mediated learning found in *CB_1R*-KO, HC-*CB_1R*-KO, and GABA-*CB_1R*-KO mice. In other words, the lack of CB_1R-dependent control might induce excessive inhibitory transmission, impairing incidental learning. To test this hypothesis, we aimed at reducing hippocampal GABAergic transmission

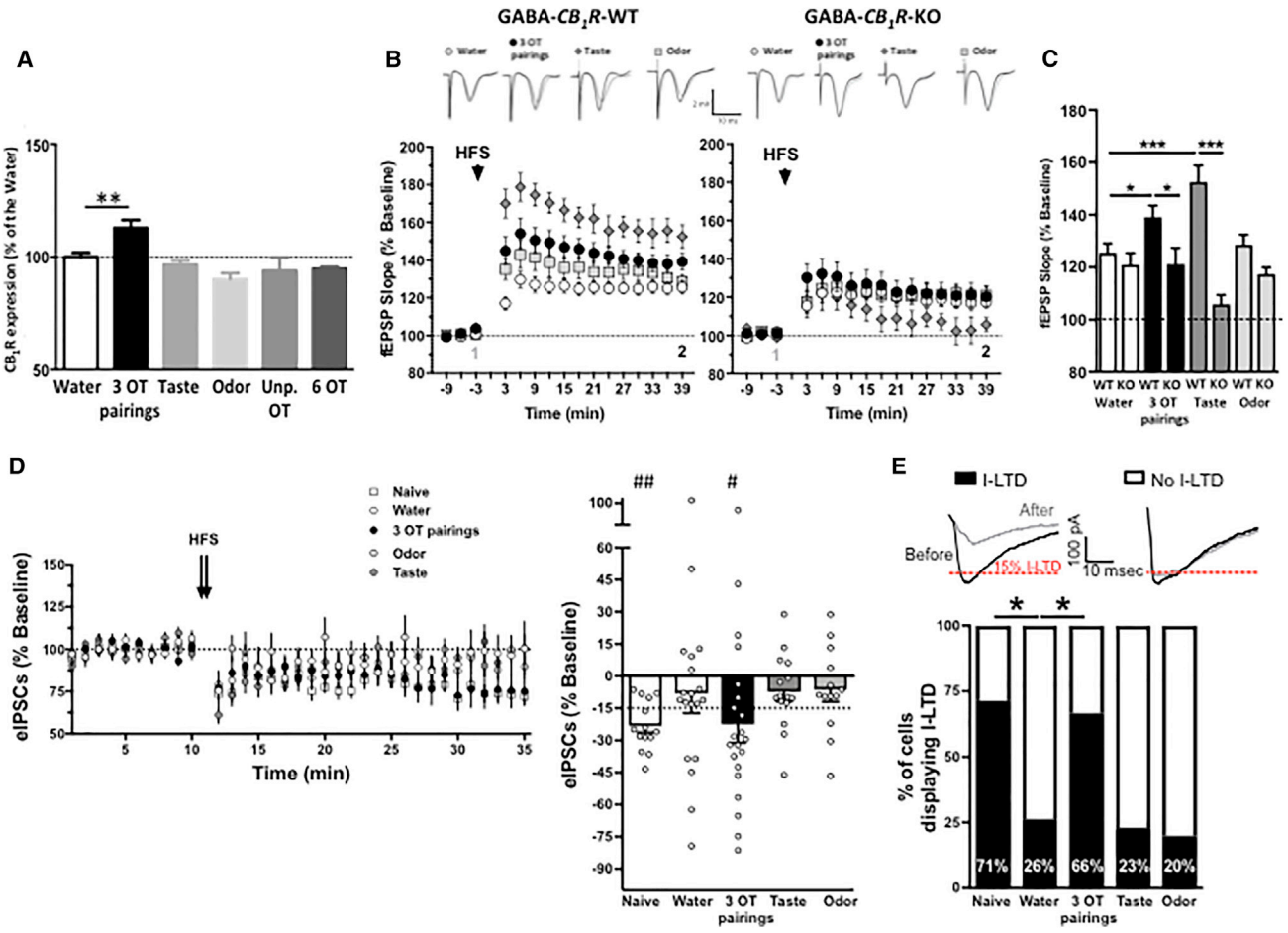

Figure 5. Hippocampal Alterations Induced by Odor-Taste Pairings

(A) Optical densitometric quantification of CB_1R levels in mice that received water, three odor-taste pairings (three OT pairings), odor alone (Odor), taste alone (Taste), unpaired odor-taste exposures (Unp. OT), or six odor-taste pairings (6 OT) (see Figure S5 for the representative gels and controls).

(B) Summary traces (top) and time course plots of normalized fEPSPs recorded *in vivo* before (1) and after (2) high-frequency stimulation (HFS) in the CA1 hippocampal region of GABA-*CB_1R*-WT mice (left) and GABA-*CB_1R*-KO littermates (right). Animals were anesthetized for recording immediately after the last exposure to water, odor-taste (3 OT pairings), taste alone, or odor alone, respectively.

(C) Bar histogram representing LTP amplitude during the last 3 min of recording in the same groups as described in (B).

(D) Left, time course plots showing eIPSCs amplitude before and after HFS in hippocampal slices obtained from non-water-restricted mice (Naive), or mice that received water, three odor-taste pairings (3 OT pairings), odor alone (Odor), or taste alone (Taste). Right, averaged eIPSCs recorded 15–20 min after HFS in the same groups.

(E) Top, traces of eIPSCs before and after HFS in representative recordings resulting in I-LTD (left) or no I-LTD (right). I-LTD was defined as a $\geq$15% reduction of eIPSCs as compared to baseline. Bottom, black bar histograms representing the proportion of cells displaying I-LTD in the same groups as described in (D). $^{*}p < 0.05$; $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared to Water or KO conditions. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ as compared to baseline. NS, not significant. For statistical details and *n*, see Tables S1 and S2.

in GABA-*CB_1R*-KO mutant mice during preconditioning, i.e., during each odor-taste pairing, using a chemogenetic approach (Figure 6A). An adeno-associated viral vector carrying Cre-dependent expression of an inhibitory DREADD (DIO-hM4DGi, hereafter called DREADD-Gi) (Robinson and Adelman, 2015) was infused into the hippocampi of GABA-*CB_1R*-KO mice, leading to specific expression of DREADD-Gi in hippocampal GABAergic neurons to generate GABA-*CB_1R*-KO (DREADD-Gi) (Figure 6B). To control for the potential effects of CNO in virally infected mice lacking DREADD receptors (Gomez et al., 2017), GABA-*CB_1R*-WT mice were infused with a control AAV expressing the mCherry protein (GABA-*CB_1R*-WT [mCherry]). The DREADD ligand clozapine-N-oxide (CNO) enhanced hippocampal *in vivo* LTP in GABA-*CB_1R*-KO (DREADD-Gi) mice as compared to saline-treated mice (Figure S6A), thereby indicating the effectiveness of the chemogenetic approach. Importantly, however, CNO injections into GABA-*CB_1R*-WT (mCherry) mice did not affect the consumption during preconditioning (Figure S6B) and had no effect on mediated (Figures 6B and S6C) or direct aversion (Figures 6B and S6D), showing that the potential unspecific effects of the drug or its metabolites (Gomez et al., 2017) were not present in our experimental conditions. Strikingly,

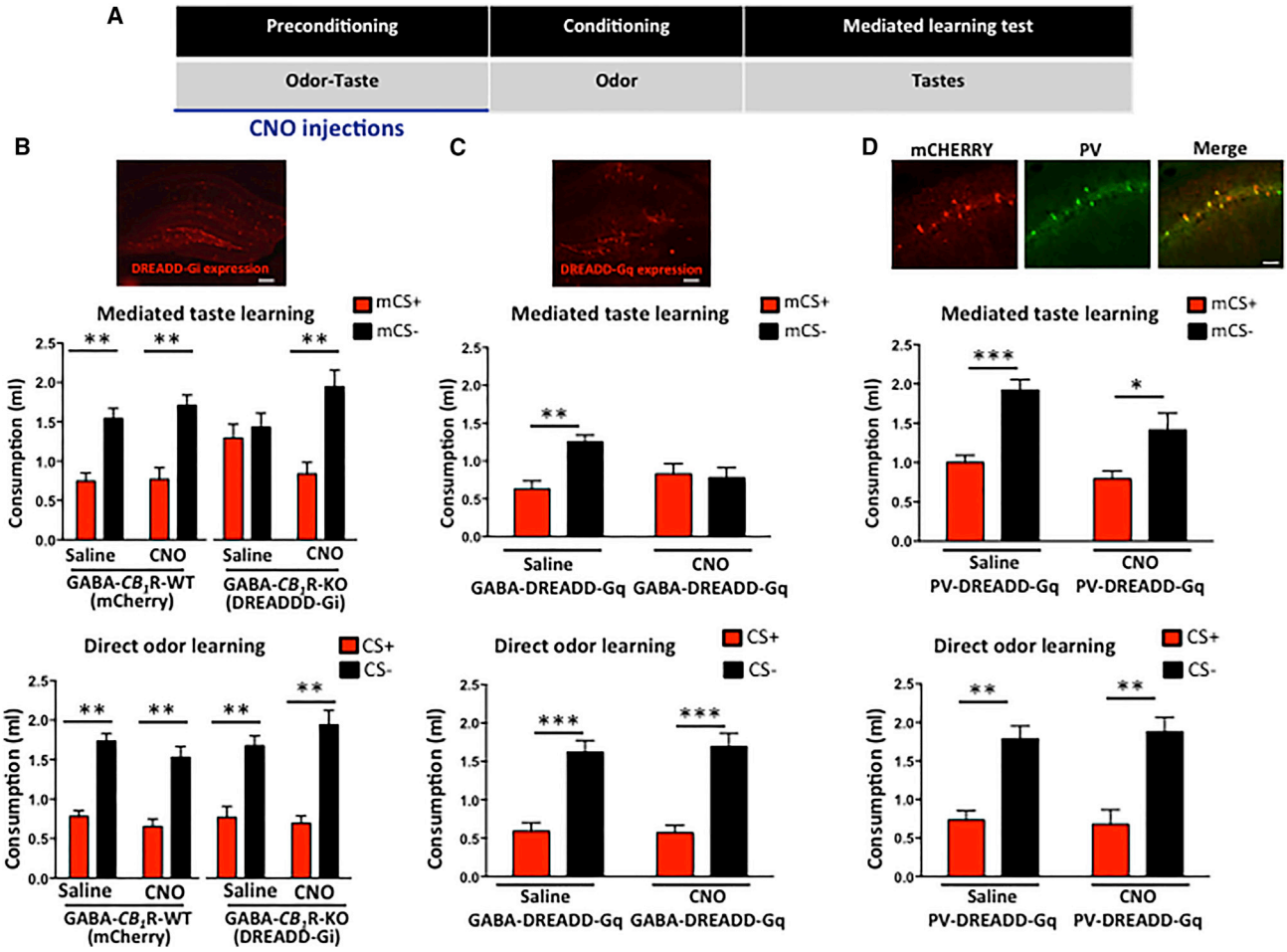

Figure 6. Specific Hippocampal GABAergic Neurons Control the Formation of Incidental Associations

(A) Scheme of CNO treatments in mice injected with different DREADD-expressing viruses.

(B) Top, representative micrograph image showing the viral expression of Cre-dependent DREADD-Gi in the hippocampus of GABA-CB_1R-KO mice. Middle bottom, effect of chemogenetic inhibition of hippocampal GABAergic neurons during preconditioning on mediated (middle) and direct (bottom) aversion in GABA-CB_1R-KO mice (see Figure S6 for further details; scale bar, 300 μm). Note the reversal of the mediated learning impairment in the mutants after CNO treatment.

(C) Top, representative micrograph image showing the viral expression of Cre-dependent DREADD-Gq in the hippocampus of GABA-CRE mice. Middle bottom, effect of chemogenetic activation of hippocampal GABAergic neurons during preconditioning on mediated (middle) and direct (bottom) aversion in GABA-DREADD-Gq mice (see Figure S6 for further details; scale bar, 300 μm). Note mediated learning impairment after CNO treatment.

(D) Top, representative micrograph image showing the mCHERRY viral expression, the PV interneurons and the merged image in the CA1 region of the hippocampus of PV-CRE mice (see Figure S6 for further details; scale bar, 100 μm). Middle bottom, effect of chemogenetic activation of hippocampal PV neurons during preconditioning on mediated (middle) and direct (bottom) aversion in PV-DREADD-Gq mice. Note the lack of impairment after CNO treatment. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (mCS+ versus mCS− or CS+ versus CS). For statistical details, see Tables S1 and S2.

however, the same treatment in GABA-CB_1R-KO (DREADD-Gi) mice fully rescued mediated learning (Figures 6B and S6C), without altering preconditioning consumption or direct aversion (Figures 6B, S6B, and S6D). These results suggest that an excess of inhibitory neurotransmission during the formation of incidental stimulus-stimulus associations might be the cause of the mediated learning impairment in GABA-CB_1R-KO mice.

The data presented so far imply a key role of the inhibition of hippocampal GABAergic transmission during the processing of incidental associations between low-salience stimuli and related synaptic plasticity. Therefore, stimulation of GABAergic transmission in the hippocampus of mice expressing normal levels of CB_1R should impair these processes and, thereby, block mediated learning. To test this idea, an adeno-associated viral vector carrying Cre-dependent expression of an excitatory DREADD (DIO-hM3DGq, hereafter called DREADD-Gq) (Robinson and Adelman, 2015) was infused into the hippocampi of mice expressing the Cre recombinase in Dlx5/6-positive cells (Figure 6C), encompassing all GABAergic interneurons (GABA-Cre mice; Monory et al., 2006) to obtain GABA-DREADD-Gq mice. We first verified that CNO administration was able to increase the number of cFos-positive neurons in the hippocampus of these mice (Figure S6E) and that CNO treatment did not alter mediated or direct aversion in GABA-Cre mice infused with a

control Cre-dependent virus (GABA-mCherry; Figure S6F). Notably, however, CNO administration during preconditioning fully blocked mediated, but not direct, aversion in GABA-DREADD-Gq mice (Figures 6C, S6G, and S6H).

These data are in agreement with the idea that hippocampal inhibitory neurotransmission plays a key role in the formation of incidental associations. However, generalized activation of GABAergic neurons by DREADD-Gq does not allow determination of whether there is a specific population of GABAergic neurons modulating mediated learning. In this context, it is important to note that CB_1R are expressed in specific interneuronal subpopulations in the hippocampus, CCK-positive but not in parvalbumin (PV)-positive basket cells (Katona et al., 1999; Marsicano and Kuner, 2008; Marsicano and Lutz, 1999). CCK- and PV-positive interneurons are two functionally and anatomically distinct cell populations, which together encompass the large majority of inhibitory basket cells innervating somas and proximal dendrites of hippocampal pyramidal neurons (Whissell et al., 2015). Due to the additional expression of CCK in pyramidal hippocampal neurons, the specific targeting of CCK- and CB_1R-positive interneurons is technically very challenging and laborious (Dimidschstein et al., 2016). Conversely, the use of PV-Cre mice (Courtin et al., 2014) allows a reliable targeting of PV-positive interneurons, which represent approximately half of the hippocampal basket cells (Whissell et al., 2015). Therefore, we injected adeno-associated viruses expressing Cre-dependent DREADD-Gq into the hippocampi of PV-Cre mice, obtaining the localization of the receptor in PV-positive neurons (>85% colocalization, Figure 6D), thereby generating PV-DREADD-Gq mice. First, we verified the functionality of the approach, by showing that CNO administration was able to increase the number of c-Fos-positive neurons in the hippocampus of these mice (Figure S6I). Strikingly, the specific activation of PV interneurons by CNO administration during preconditioning did not affect mediated learning (Figures 6D, S6J, and S6K). These data suggest that specific PV-negative subpopulations of GABAergic hippocampal interneurons are involved in the development of incidental associations between low-salience stimuli, eventually enabling the successive formation of mediated learning and memory.

DISCUSSION

Our daily behavioral choices are mainly based on past experiences. These behaviors can depend on direct associative memories, where sensory stimuli are directly associated with specific aversive or rewarding situations. However, they often originate from previous incidental stimulus-stimulus associations between low-salience sensory cues, which are able to assign new value to stimuli that were never directly reinforced (Bornstein et al., 2017; Shohamy and Wagner, 2008; Wimmer and Shohamy, 2012). Stimulus-stimulus associations can be evaluated through the sensory preconditioning paradigm in humans (Bornstein et al., 2017; Shohamy and Wagner, 2008; Wimmer and Shohamy, 2012) and animals (Gewirtz and Davis, 2000; Parkes and Westbrook, 2011). Using this task, our data strongly suggest that the CB_1R-dependent control of discrete subpopulations of hippocampal GABAergic interneurons is necessary and sufficient for the processing of incidental stimulus-stimulus associations. Accordingly, incidental associations induce a specific protein synthesis-dependent increase of hippocampal CB_1R expression, which is accompanied by an enhancement of hippocampal sensitivity to CB_1R-dependent synaptic plasticity. Thus, our data are compatible with a scenario in which incidental associations of different stimuli would trigger CB_1R activity and induce CB_1R-dependent synaptic plasticity, which would "prime" hippocampal circuits allowing mediated learning. In this context, our data reveal that the CB_1R-dependent control of hippocampal inhibitory synaptic neurotransmission is a key element of high-order cognitive processes, which allow the flexible use of the complex and changing patterns of sensory information that characterize daily individual experiences. Thus, the hippocampal ECS appears to enhance the repertoire of possible behavioral choices dictated by previous experiences, thereby increasing the survival potential of individuals.

Importantly, these mechanisms do not appear to be limited to specific sensory modalities of the stimuli. Despite the fact that tastes can also act as primary reinforcers (Yiannakas and Rosenblum, 2017), gustatory cues have been widely used in sensory preconditioning protocols as low-salience stimuli (Wheeler et al., 2013). Important control experiments showed that these cues are unable to elicit direct odor conditioning per se in our experimental conditions. Moreover, inhibition of CB_1R signaling impairs mediated learning when either tastes or odors are used as cues to elicit aversion. Thus, we can conclude that our odor-taste preconditioning paradigm is suitable to identify the formation of incidental associations between stimuli that are of low salience for the mice. In line with this idea, the general implication of the ECS in incidental learning extends also to other sensory preconditioning paradigms, in which low-salience auditory and visual stimuli are used and appetitive behavior is evaluated. Thus, the common involvement of the ECS in different experimental conditions suggests that similar mechanisms might underline higher-order cognitive processes independently of the sensory modalities used and of the nature (aversive or appetitive) of the reinforcer. Future studies will address this intriguing hypothesis that would lead to a unified vision of complex higher-order learning processes in the brain.

The hippocampus has been suggested to be necessary for associating and temporarily maintaining an internal record of different stimuli simultaneously presented (Voss et al., 2017), making it a key brain region for the integration of different sensory information. Accordingly, previous studies supported the importance of the hippocampus in sensory preconditioning both in humans and in animals (Iordanova et al., 2011; Talk et al., 2002; Wheeler et al., 2013; Wimmer and Shohamy, 2012). However, these studies focused on the role of the hippocampus during the second phase of the procedure (conditioning) or during the retrieval test, when incidental associations have already formed. Thus, these studies underlined the role of the hippocampus in enabling the value of the reinforcer to spread across the associated and not explicitly reactivated item, rather than in the processing and recording of incidental associations per se. Conversely, the present results reveal that the hippocampus is also involved in the preconditioning phase, when the coincidence of low-salience stimuli is recorded and stored for future

potential use. Specifically, our data provide an unforeseen physiological link between hippocampal GABAergic signaling and associative memory between low-salience events.

CB_1R blockade (or activation) before testing does not affect the expression of mediated aversion (Busquets-Garcia et al., 2017b), strongly arguing for a specific role of hippocampal endocannabinoid signaling in the processing of incidental stimulus-stimulus associations. Interestingly, recent work pointed to the importance of other brain regions, such as the orbitofrontal, retrosplenial, and perirhinal cortices for the processing of incidental associations between low-salience sensory cues (Holmes et al., 2013, 2018; Robinson et al., 2014; Sadacca et al., 2018). Interestingly, these regions are highly connected to the hippocampus (Agster and Burwell, 2013; Ritchey et al., 2015; Witter et al., 2017), suggesting that so-far-unexplored network activities between neocortical structures and CB_1R-controlled inhibitory circuits in the hippocampus allow the brain to record and store associations between different external stimuli that are constantly present in the environment.

The cellular and molecular mechanisms underlying ECS-dependent control of incidental learning in the hippocampus appear to be quite complex. Exposure to low-salience stimuli enhances *in vivo* hippocampal LTP and CREB phosphorylation. However, these effects are not specific to incidental associations, because they are also induced by the mere exposure to gustatory or olfactory stimuli alone, respectively. On the other hand, the simultaneous presentation of stimuli induces specific molecular and cellular effects. Incidental learning causes a protein synthesis-dependent increase of CB_1R expression and higher hippocampal sensitivity to ECS-dependent plasticity of inhibitory neurotransmission. The increased sensitivity of hippocampal pyramidal neurons to undergo I-LTD might be due to the increase of CB_1R expression. However, the fact that preconditioning does not alter other forms of ECS-dependent plasticity, such as DSI, suggests that the enhanced I-LTD sensitivity might result from other mechanisms, such as, for instance, increased endocannabinoid mobilization following HFS. Whereas enhancement of hippocampal CB_1R expression and I-LTD sensitivity are specific to odor-taste preconditioning (i.e., they do not occur under exposure to odors or tastes alone), the increased *in vivo* LTP induced by both odor-taste and taste alone is abolished in GABA-*CB_1R*-KO mice. Differently from I-LTD, this ECS-dependent phenomenon and the enhanced phosphorylation of CREB cannot be linked to increased CB_1R expression levels, because they occur in conditions where these levels are not changed (i.e., under exposure to single stimulus). Future experiments will address these complex molecular and cellular interactions. However, the present data allow speculating that incidental learning might occur via a "two-step" hippocampal CB_1R-dependent process: (1) First, animals might record the presence of stimuli independently of their possible association. CB_1R-dependent increase of LTP might underline this process, at least for taste-related information. (2) The simultaneous presence of odors and tastes then triggers additional processes, again involving the ECS (increased CB_1R expression and I-LTD sensitivity). In this frame, step (1) might represent a preliminary event (stimulus detection) that is necessary to further develop step (2), which would underline the "actual" incidental learning. Future studies will address these intriguing possibilities.

Our previous data revealed that extended preconditioning training (six odor-taste pairings instead of three) suppresses mediated learning, installing the so-called "reality testing," through which the animals are able to successfully discriminate between stimuli that they initially reacted to in the same way (Busquets-Garcia et al., 2017b; McLaren and Mackintosh, 2002). Notably, extended training also normalizes the hippocampal expression of CB_1R. Thus, it will be extremely interesting to investigate the causes and the consequences of these molecular adaptations in the training-dependent behavioral switch from mediated learning to "reality testing" responses (McDannald and Schoenbaum, 2009).

It has been suggested that the numerous and largely diverse types of hippocampal GABAergic interneurons are differentially required for specific functions and contribute to the computing of cognitive processes in different conditions (Caroni, 2015; Chevaleyre and Piskorowski, 2014; Klausberger and Somogyi, 2008). An important corollary of our data is that selective subpopulations of hippocampal interneurons are specifically involved in the processing of incidental stimulus-stimulus associations. Hippocampal CB_1R are abundantly expressed in a specific subset of basket cells, the inhibitory interneurons specialized in the innervation of somas and proximal dendrites of pyramidal neurons (Katona et al., 1999, 2000; Marsicano and Kuner, 2008; Marsicano and Lutz, 1999). The expression of PV or CCK characterizes two non-overlapping subpopulations of basket cells (Klausberger et al., 2005), with CB_1R almost exclusively expressed in CCK-positive cells (Katona et al., 1999, 2000; Marsicano and Kuner, 2008; Marsicano and Lutz, 1999). Notably, whereas global activation of GABAergic neurons in the hippocampus during preconditioning blocks mediated learning, selective activation of PV-positive hippocampal neurons does not alter sensory incidental associations nor mediated responses. Thus, our data suggest that the activity of CCK- and CB_1R-expressing cells plays a key role in the processing of incidental learning. According to the large inhibition exerted by both PV- and CCK-positive interneurons on the firing properties of pyramidal cells, this conclusion might appear surprising. However, the known wide functional differences between the microcircuits formed by these two interneuronal subpopulations (Armstrong and Soltesz, 2012; Bartos and Elgueta, 2012) might underline this intriguing dichotomy. Thus, our data suggest that activity- and endocannabinoid-dependent reduction of neurotransmission of a selected population of hippocampal interneurons is required during incidental associations of low-salience stimuli, to eventually enable mediated learning.

In conclusion, the tight regulation of hippocampal GABAergic interneurons by CB_1R can explain how people integrate and associate different low-salience stimuli randomly encountered to develop seemingly ungrounded attraction or aversion toward particular objects, places, or people. Importantly, alterations of mediated learning, also known as associative inference, can be found in several psychiatric diseases (Armstrong et al., 2012a, 2012b). Thus, these results suggest that modulating specific inhibitory hippocampal transmission via CB_1R might

represent a potential therapeutic target against cognitive dysfunctions in neuropsychiatric conditions.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- CONTACT FOR REAGENT AND RESOURCE SHARING
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Animals
- METHOD DETAILS
  - Drug preparation and administration
  - Chemical odors and tastes
  - Sensory preconditioning tasks
  - Western Blot analysis
  - Virus generation
  - Surgery and viral administration
  - Immunohistochemistry and fluorescence detection
  - *In vivo* electrophysiology in anesthetized mice
  - *Ex vivo* electrophysiology
- QUANTIFICATION AND STATISTICAL ANALYSIS
  - Data collection
  - Statistical analyses

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and two tables and can be found with this article at https://doi.org/10.1016/j.neuron.2018.08.014.

A video abstract is available at https://doi.org/10.1016/j.neuron.2018.08.014#mmc3.

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AUTHOR CONTRIBUTIONS

A.B.-G., G.F., and G.M. designed research; A.B.-G., J.F.O.C., E.S.-G., A.C.P.Z., G.T., A.C., B.R., M.V., and C.I. performed research; A.B.-G., G.F., and G.M. supervised research; A.B.-G., J.F.O.C., A.C.P.Z., G.T., P.T., G.F., and G.M. analyzed data; A.B.-G., G.F., and G.M. wrote the manuscript. All authors edited and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing financial interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-goat Alexa 488	Fisher Scientific	CAT #A21467; RRID: AB_10055703
Anti-Guinea pig Alexa 488	Fisher Scientific	CAT # A-11073; RRID: AB_2534117
Anti-rabbit Alexa 488	Fisher Scientific	CAT #PA5-23091; RRID: AB_2540618
Anti-rabbit Alexa 594	Fisher Scientific	CAT # A21211; RRID: AB_10375432
cAMP response element-binding protein (CREB)	Cell Signaling Technology	CAT #9104; RRID: AB_490881
CB1 receptor (Western Blot)	Abcam	CAT Ab23703, RRID:AB_447623
cFOS polyclonal antibody	Santa Cruz Biotechnology	CAT #Sc-52; RRID: AB_2106783
DAPI	Life Technologies	CAT #D3571; RRID: AB_2307445
DsRED	Clontech Laboratories	CAT #632496; RRID: AB_10013483
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Cell Signaling Technology Danvers	CAT #2118; RRID: AB_561053
Goat polyclonal CB1 receptor (Immunofluorescence)	Frontier Science Co	CB1-Go-Af450-1; RRID: AB_2571530
Guinea pig polyclonal parvalbumin	Synaptic Systems	CAT #1955004; RRID: AB_2156476
HRP-linked antibodies	Cell Signaling Technology	CAT #7074 (Anti Rabbit); RRID: AB_2099233; CAT #7076 (Anti Mouse); RRID: AB_330924
Phopho-cAMP response element-binding protein (pCREB)	Cell Signaling Technology Danvers	CAT #9198; RRID:AB_2561044
Bacterial and Virus Strains		
AAV-CAG-CRECre	Hebert-Chatelain et al., 2016	Virus n3 lab stock
AAV-CBA-GFP	Hebert-Chatelain et al., 2016	Virus n4 lab stock
AAV-CBA-mCB1	Hebert-Chatelain et al., 2016	Virus n6 lab stock
AAV-DIO-hM3Dq	UNC Vector Core	N/A
AAV-DIO-hM4Di	UNC Vector Core	N/A
AAV-DIO-mCherryCHERRY	UNC Vector Core	N/A
Chemicals, Peptides, and Recombinant Proteins		
2-Mercaptoethanol (as component of Laemmli Buffer 4X, 5%)	Sigma-Aldrich	CAT#3148
4-20% Mini-PROTEAN TGX Stain-Free Precast Gels	Bio-Rad	CAT#4568096
Anisomycin	Sigma-Aldrich	CAT#A9789
Anti-Fluorescein-POD	Sigma Aldrich	11426346910
Benzaldehyde	Sigma-Aldrich	CAT#418099
Bromophenol Blue (as component of Laemmli Buffer 4X, 0.02%)	Sigma-Aldrich	CAT#114391
CaCl2	Sigma-Aldrich	CAT#C3881
Clarity Western ECL Substrates	Bio-Rad	CAT#1705060
Clozapine-N-oxide (CNO)	Tocris Bioscience	CAT#4936
Complete Protease inhibitors cocktail	Roche	CAT#000000011697498001
Cremophor EL	Sigma-Aldrich	CAT#C5135
DAPI	Fisher Scientific	CAT #11530306
D-APV	Abcam	CAT# ab120003
DMSO	Sigma-Aldrich	CAT#D5879-M
Donkey serum	Sigma Aldrich	S30-100ML
EDTA	Sigma-Aldrich	CAT#EDS
EGTA	Sigma-Aldrich	CAT#E4378
Glucose	Sigma-Aldrich	CAT#G57-67

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Glycerol (as component of Laemmli Buffer 4X, 40%)	VWR	CAT#24386.298
HEPES	Sigma-Aldrich	CAT#H3375
Hydrochloric acid (HCl)	Sigma-Aldrich	CAT#
Isoamyl acetate	Sigma-Aldrich	CAT#W205508
Isoflurane (Vetflurane)	Virbac	N/A
KCL	Merck Millipore	CAT#104936
Ketamine (IMALGENE 1000)	Merial	N/A
Lidocaine (Lurocaine)	Vetoquinol	N/A
Lithium Chloride	Sigma-Aldrich	CAT#L4408
Maltodextrin	Sigma-Aldrich	CAT#419672
Membrane Immobilon-P, PVDF 0.45μm	Merck	CAT#IPVH00010
Mg-ATP	Sigma-Aldrich	CAT#A9187
MgCl2	VWR chemicals	CAT#36226
NaCl	Sigma-Aldrich	CAT#746398
NaCl	VWR	CAT#0241
Na-GTP	Sigma-Aldrich	CAT#G8877
NaH2PO4	Merck Millipore	CAT#106345
NaHCO3	Sigma-Aldrich	CAT#S6014
NBQX	Abcam	CAT#ab120046
Non-fat milk (skim milk powder)	Régilat	N/A
Paraformaldehyde	Sigma-Aldrich	CAT#HT501128
Phophatase inhibitors cocktail (PhosSTOP)	Roche	CAT#000000004906845001
Phosphocreatin	Sigma-Aldrich	CAT#P7936
Pontamine sky blue	Sigma-Aldrich	CAT#C8679
Roti-Nanoquant	Carl Roth	CAT#K880.2
SDS (as component of Laemmli Buffer 4X, 8%)	Carl Roth	CAT#2326.3
Sodium acetate	Sigma-Aldrich	CAT# S2889
Sodium chloride 0,9%	Cooper	N/A
SR101	Cayman Chemical	CAT#9000484
Sucrose	Sigma-Aldrich	CAT#S7903
Tris(hydroxymethyl)aminomethane (Tris)	Research Organics	CAT#30955T
Triton X-100	Sigma-Aldrich	CAT#X100
TSA Plus Fluorescein	Perkin Elmer	NEL741001KT
TTX	Tocris Bioscience	CAT#1069
Tween20	Sigma-Aldrich	CAT#P1379
Experimental Models: Organisms/Strains		
Mouse: C57BL/6	JANVIER Labs	C57BL/6
Mouse: *CB_1R*-flox	Marsicano et al., 2003	N/A
Mouse: *CB_1R*-KO	Marsicano et al., 2003	N/A
Mouse: DLX-*CB_1R*-KO	Monory et al., 2006, Bellocchio et al., 2010	N/A
Mouse: DLX-CRECre	Bellocchio et al., 2010	N/A
Mouse: GABA-*CB_1R*-Rescue	Ruehle et al., 2013	N/A
Mouse: PV-Cre	Courtin et al., 2014	N/A
Software and Algorithms		
Axograph	AxoGraph Software	N/A
Behavioral Scoring Panel	A. Dubreuq	N/A
Clampfit, pClamp10	Molecular devices	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Digidata 1440A	Molecular devices	N/A
GrabBee	N/A	N/A
GraphPad prism 6.0	GraphPad Software	N/A
ImageJ	NIH	N/A
Spike2	Cambridge Electronic Design	N/A
Other		
Amplifier	DAGAN Corporation	2400A
Concentric bipolar electrode model	FHC	CBARC50
Constant Current Stimulator	Digitimer	DS3
Data acquisition unit	Cambridge Electronic Design	CED 1401
Homeothermic system model	Harvard Apparatus	50-7087-F
Hydraulic micropositioner	Kopf instruments	Model 2650,
MultiClamp 700B amplifier	Molecular devices	N/A
Stereotaxic frame	Kopf instruments	Model 900
Vibratome VT1200S	Leica	N/A

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for sources should be directed to the Lead Contact, Giovanni Marsicano (giovanni.marsicano@inserm.fr).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals

All experimental procedures were approved by the Committee on Animal Health and Care of INSERM and the French Ministry of Agriculture and Forestry (authorization numbers, A501350, A3310035, C33063096). Maximal efforts were made to reduce the suffering and the number of mice used. All behavioral experiments were performed during the light phase (from 9am to 1pm) and animals were kept in individual cages.

Male wild-type C57BL/6 mice purchased from Janvier (France) were used for the pharmacological, biochemical and electrophysiological experiments. Male *CB_1R*-floxed mice (Marsicano et al., 2003), CB_1R full knockout (*CB_1R*-KO) mice (Marsicano et al., 2003), conditional knockout mice lacking CB_1R in forebrain GABAergic *Dlx*5/6 positive neurons (GABA-*CB_1R*-KO) (Bellocchio et al., 2010; Monory et al., 2006), mice expressing Cre recombinase under the regulatory elements of the *Dlx5/6* gene (GABA-Cre) or the *parvalbumin* (PV) gene (PV-Cre) and their wild-type littermates mice were obtained, maintained and genotyped as described before (Bellocchio et al., 2010; Courtin et al., 2014; Marsicano et al., 2003; Monory et al., 2006). The rescue line (GABA-*CB_1R*-KO) was generated as described before (Busquets-Garcia et al., 2016; Gutiérrez-Rodríguez et al., 2017; Ruehle et al., 2013; Soria-Gómez et al., 2014). All the mice used in this study were 9-10 weeks old at the beginning of the experiments.

METHOD DETAILS

Drug preparation and administration

Rimonabant (1 mg/kg) was purchased from Cayman Chemical (Michigan, USA) and was dissolved in a mixture of 4% ethanol, 4% Cremophor-EL and 92% of saline (NaCl 0.9%). Anisomycin (18 mg/kg) was purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France) and was dissolved in 2% DMSO 2% Tween and 96% saline (NaCl 0,9%). The exogenous DREADD ligand clozapine-N-oxide CNO (2 mg/kg) was purchased from Tocris Bioscience (Bristol, UK) and dissolved in saline after gently mixing with a vortex. All drugs were injected intraperitoneally (i.p.) in a volume of 10 ml/kg. Vehicle injection was composed of 4% ethanol, 4% Cremophor-EL and 92% saline for the experiment using Rimonabant, 2% DMSO, 2% Tween and 96% saline for the experiment using Anisomycin and saline injection for experiments with CNO.

Chemical odors and tastes

The solutions used in the sensory preconditioning task were presented in 50-mL drinking tubes in the home cage with either banana (0.05%, isoamyl acetate) or almond (0.01%, benzaldehyde) for odors, and sucrose (5%) or maltodextrin (5%) for tastes. All compounds were obtained from Sigma-Aldrich (St. Quentin Fallavier Cedex, France). The concentrations of odors and tastes were chosen to be equally preferred (Busquets-Garcia et al., 2017a, 2017b). In all experimental groups (see above) half of the animals

were conditioned with sucrose/banana and half of the mice with maltodextrin/almond. No differences were observed between both conditions in all the experiments performed.

Sensory preconditioning tasks

Odor-taste sensory precondition test. Mice were water deprived in the same room where the whole protocol occurred (Figure S1) as described before (Busquets-Garcia et al., 2017a, 2017b).

Habituation. All subjects received 1 hr access to water during three consecutive days.

Preconditioning phase. This consisted in 6 days of 3 odor-taste pairings (incidental associations). Each pairing consisted in two days: on the first day, the subjects had 1 hr access to a flavored solution containing a new taste (either 5% sucrose or 5% maltodextrin; Taste 1, T1) and a new odorant (0.05% Banana or 0.01% Almond; Odor 1, O1) mixed with water in order to pair T1 with O1. On day two, the animals received the taste and odor not given during the previous day, i.e., Taste 2 (T2) and Odor 2 (O2).

Conditioning or devaluation phase. This phase consisted in 6 days where O1 (or T1) was devaluated as to become the conditioned stimulus (CS+). On days 1, 3 and 5 of this phase, subjects received 1 hr access to O2 (or T2) followed by i.p. injection of saline (CS-). On days 2, 4 and 6, they received 1 hr access to O1 (or T1) immediately followed by an i.p. injection of lithium chloride (LiCl, 0.3 M, 1% b.w.) which induces gastric malaise to the mice (CS+). After conditioning, subjects were given a recovery day in which they could only drink water during 1 hr. In each experimental condition, half of the mice were conditioned with one stimulus (O1 or T1) or the other one (O2 or T2).

Test phase. On the next 2 days, mediated and direct aversions were assessed using a 1 hr two-choice test. Mediated aversion was always evaluated on the first test day with a choice between the stimulus T1 (or O1) previously associated with the CS+ but never directly paired with LiCl (thereby called mediated CS+, mCS+) and the stimulus T2 (or O2) previously associated with the CS- (mediated CS-, mCS-). On the second test day, the direct aversion was evaluated with a choice between the CS+ and CS-. Data are presented as absolute liquid intake.

The pharmacological treatments with Rimonabant (1 mg/kg, i.p.) and CNO (2 mg/kg, i.p.) and their respective vehicles were administered 1 hr before each session during the preconditioning phase. Moreover, Rimonabant (1 mg/kg, i.p.) and its vehicle were also administered 1 hr before the mediated odor learning test to check the effect of CB_1R blockade in this specific test phase. For the experiments using viral approaches, the behavioral protocol started five weeks after surgery.

Additional experiments

We evaluate whether sucrose and maltodextrin used during 3 odor-taste pairings (i.e., preconditioning sessions) induced appetitive conditioning to the odors and what is the effect of Rimonabant treatments during preconditioning on this response. Three different groups of C57BL6 mice underwent the 3-odor-taste pairings (one group injected with vehicle 1 hr before each session, one group receiving Rimonabant before all session and one group injected with Rimonabant before each O1/T1 session and vehicle before each O2/T2 session and). Then, different two-choice tests were performed in consecutive days: choice between odors (O1 versus O2) and between each odor and water (O1 versus water and O2 versus water).

In order to know whether impairment of mediated aversion could be due to delayed formation of mediated learning, mice with a specific deletion of CB_1R in the hippocampus and their controls (HC-CB_1R-KO and HC-CB_1R-WT, see below) and GABA-*CB_1R*-KO and their littermates underwent an extended preconditioning protocol receiving 6 odor-taste pairings instead of 3 (Busquets-Garcia et al., 2017a, 2017b).

Tone-light sensory preconditioning test

Twenty-two animals were used in this experiment. 10 animals were administered with Rimonabant (1 mg/kg, i.p.) and 12 with its vehicle. Mice were food-deprived to be maintained at 85%–90% of their *ad libitum* weight throughout the duration of the experiment.

Apparatus. Operant chambers (Imétronic, France) had internal dimensions 30x40x36 (cm) and located in a light- and sound-attenuated cabinet with a floor consisting of metal rods. Each operant chamber had two opaque panels at the right and left walls and two clear Plexiglas panels at the back and front walls. A feeder trough was centered on one wall of the chamber. Inside the trough, an infrared photocell detector was used to record head entries into the trough. Food pellets (dehydrated milk) were used as a reward.

Habituation. Animals were exposed to the operant chambers during 20 min for 2 consecutive days, 3 sessions a day, with free access to 4-5 pellets. Saline injection (i.p.) was performed 30 mi min nutes before the beginning of the first session.

Preconditioning phase. This consisted in two 30 min sessions per day during 2 consecutive days. The "preconditioning session" consisted in the simultaneous presentation of a 10 s tone (65 db, 3000 Hz) with a light (house light located at the top of the chamber), 6 times, with an average intertrial interval (ITI) of 5 min. This was followed 30 min later by a "control" session in which a 10 s click (65 db, 10 Hz) was presented 6 times (average ITI 5 min), with no light. Preconditioning and control sessions were counterbalanced between the 2 days of the preconditioning phase. Rimonabant (1 mg/kg, i.p.) or saline were injected 30 min before the preconditioning phase.

Conditioning phase. This consisted in 1 hr session per day for 6 consecutive days during which the light previously used during the preconditioning phase was presented 12 times (average ITI 5 min) and resulted in the delivery of a food pellet. Head entries in the food trough were recorded during the tone, the click and the ITI. The strength of sensory preconditioning was assessed using a discrimination ratio defined as the amount of responding observed during the first 4 presentations of the tone divided by the sum of responding during the first 4 presentations of the tone and the click.

Test phases. 1 hr session was performed during which the tone and the click were randomly presented 6 times each (average ITI 5 min) in the absence of any food reward. Head entries in the food trough were recorded.

The next day, response to the light cue was assessed through a 1 hr session during which the light was presented 12 times (average ITI 5 min) with no food delivery and head entries in the food trough were recorded.

Western Blot analysis

Just after the last preconditioning session (3 or 6 odor-taste pairings and control experimental groups), C57BL6 mice were sacrificed and used for the Immunoblot. For these experiments, different control groups were used: mice receiving water during all the "preconditioning" phase, mice receiving only taste presentations (sucrose on days 1, 3, 5 and maltodextrin on days 2, 4, 6 or vice versa), mice receiving only odor presentations (banana on days 1, 3, 5 and almond on days 2, 4, 6 or vice versa) and mice receiving unpaired associations of tastes and odors (tastes on days 1, 3, 5 and odors on days 2, 4, 6 or vice versa). Additional groups of animals were injected with vehicle or the protein synthesis inhibitor anisomycin (18 mg/kg, i.p.) 1 hr before each preconditioning session (3 odor-taste pairings) or water presentation. Immediately after the last 1 hr session of liquid presentation, mice were sacrificed and hippocampal tissues extracted, frozen in dry ice and kept in −80°C. Samples were homogenized in lysis buffer (0.05M Tris-HCl pH 7.4, 0.15M NaCl, 0.001M EDTA, 10% Glycerol, 1% Triton X-100) supplemented with protease and phosphatase inhibitors purchased from Roche (Basel, Switzerland), using the Tissue Lyser (Quiagen, Hilden, Germany). After 10 min incubation at 4°C, samples were centrifuged at 17 000 g for 20 min at 4°C to remove insoluble debris. The protein contents of the total solubilized fractions were determined with Roti-Nanoquant protein quantitation assay, following manufacturer's instruction (Carl Roth, Karlsruhe, Germany). Protein extracts were mixed with denaturing 4x Laemmli loading buffer and warmed for 30 min at 37°C. Samples (25μg per lane) were analyzed on 4%–20% precast polyacrylamide gels (Bio-Rad, Hercules, California) and transferred onto PVDF membranes 0.45μm (Merk Millipore, Billerica, MA). Membranes were blocked in a mixture of Tris-buffered saline and polysorbate 20 (20mM Tris-HCl pH 7.6, 150mM NaCl, 0.05% Tween 20) containing 5% of non-fat milk for 1 h at room temperature (RT). For immunoblotting we used antibodies against CB_1R, (ab23703; 1:200, 1h RT, Abcam, Cambridge, UK), phospho - cAMP response element-binding protein (pCREB, Ser133, #9198; 1:1000, overnight 4°C, Cell Signaling Technology Danvers, MA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (#2118; 1:1000, 30 min RT, Cell Signaling Technology Danvers, MA) or CREB (#9104; 1:1000 overnight 4°C, Cell Signaling Technology Danvers, MA). Bound primary antibodies were detected with HRP-linked antibodies (1:2000, Cell Signaling Technology, Danvers, MA) and visualized by enhanced chemiluminescence detection (Clarity Western ECL Substrate, Bio-Rad, Hercules, California). The Optical densities of immunoreactive bands were quantified by the Image Lab software (Bio-Rad, Hercules, California) after acquisition on ChemiDoc Touch (Bio-Rad, Hercules, California). The CB_1R expression levels normalized to the amount of GAPDH (loading control) in the same sample have been expressed as a percentage of control condition (water group). pCREB levels normalized to the amount of total CREB in the same sample have been expressed as a percentage of control condition (water group).

Virus generation

GFP (as control virus), CRE (to delete CB_1R) and CB_1R viral (to re-express CB_1R) constructs were of a mixed serotype AAV1/AAV2 and were generated by Calcium Phosphate transfection of HEK293T cells and purified as described (Bellocchio et al., 2016; Monory et al., 2006). Cre-dependent DIO-hM4Di (DREADD-Gi) and DIO-hM3Dq (DREADD-Gq) fused to mCherry (AAV8 serotype) was provided by UNC Gene Therapy Center (University of North Carolina). In the DREADD experiments, mCherry viral construct for GABA-CB_1R-WT and DIO-mCherry for GABA-Cre mice in order to check for the viral expression and to control the potential effects of the expression of a fluorescent protein in the hippocampus were used.

Surgery and viral administration

Mice were anesthetized by i.p. injection of a mixture of ketamine (100mg/kg, Imalgene 500®, Merial, France) and xylazine (10mg/kg, Rompun®, Bayer, France) and placed into a stereotaxic apparatus (Model 900, Kopf instruments, CA, USA) with mouse adaptor and lateral ear bars. For viral intra-hippocampus delivery, AAV vectors were injected with the help of a microsyringe attached to a pump (UMP3-1, World Precision Instruments, FL, USA). Mice were injected with AAV-GFP, AAV-Cre, AAV-CB_1R, AAV-DIO-hM4Di or AAV-DIO-hM3Dq and their control viral vectors (DIO-mCherry or AAV-mCherry) directly into the hippocampus (2 injections of 1 μl per side), with the following coordinates: AP −2, L ± 1, DV −2 (1st injection) and −1,5 (2nd injection), according to Paxinos and Franklin (Paxinos and Franklin, 2001). Animals were used for behavioral or electrophysiological experiments five weeks after injections in order to get an optimal expression of the viruses. CB_1R deletion or reexpression were verified by immunohistochemistry or fluorescent *in situ* hybridization (see below) and mCherry expression was checked by epifluorescence (see below). All mice used in the behavioral experiments were checked and representative images are shown in Figure 6.

Immunohistochemistry and fluorescence detection

After the behavioral experiment, mice were anesthetized with chloral hydrate (400 mg/kg body weight), transcardially perfused first with phosphate-buffered solution (PB 0.1M, pH 7.4) and then with 4% formaldehyde prepared at 4°C to fix tissues. After brain extraction, serial brain coronal sections were cut at 40 μm and collected in 0.1M PB (pH 7.4) at RT. Sections were permeabilized in a blocking solution of 10% donkey serum, 0.3% Triton X-100 and 0.02% sodium azide prepared in 0.1M PB for 1 hr at RT. Free-floating

sections were incubated with goat CB1R polyclonal primary antibodies raised against a 31 aminoacid C-terminal sequence (NM007726) of the mouse CB1R (CB1-Go-Af450-1; 1:2000, Frontier Science Co. Shinko-nishi, Ishikari, Hokkaido, Japan) for 48h at 4oC. The antibody was prepared in the blocking solution. After several washes, the tissue was incubated with a secondary antibody anti-goat Alexa Fluor 488 (10246392, 1:500, Fisher Scientific) for 2 hr and then washed in 0.1 M PB at RT. All sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI, 1:20000) to visualize cell nuclei, and then were mounted, dried and coverslipped. The sections were analyzed with an epifluorescence Leica DM6000 microscope (Leica, France). For c-FOS staining, GABA-Cre and PV-Cre mice infused with DREADD-Gq were sacrified 90 min after treatment with saline or CNO (2 mg/kg, i.p.) and the same protocol described above was used. After permeabilization, free-floating sections were incubated with rabbit c-FOS polyclonal primary antibody (sc-52; 1:500; Santa Cruz Biotechnology) and revealed with a secondary antibody anti-rabbit Alexa Fluor 488 (10424752; 1/500; Fisher Scientific). The quantitative analysis of c-FOS expression on sections was conducted on images using ImageJ software from black and white images captured by an epifluorescence Leica DM6000 microscope at 10X (Leica, France). Moreover, the merge between the expression of mCherry and the c-FOS staining was performed in some slices. Brains from animals expressing mCherry were processed in the same way, but omitting antibody incubations. For PV-Cre mice, the same protocol described above was used, with the following modifications. Briefly, after the perfusion, brains were cryoprotected in sucrose 30% PB 0.1M, pH 7.4, and were frozen and kept at −80°C. Free-floating frozen brain sections were collected with a cryostat (40 μm, Microm HM 500M, Microm Microtech). Slices were double stained with guinea pig parvalbumin (195004; 1:1000, Synaptic Systems) and rabbit DsRed (632496; 1:1000, Clontech Laboratories, Inc. Mountain View, CA, USA) polyclonal primary antibodies revealed respectively with secondary antibodies anti-guinea pig Alexa Fluor 488 (10193752, 1:500, Fisher Scientific) and anti-rabbit Alexa Fluor 594 (10266352, 1:500, Fisher Scientific).

Fluorescent *in situ* hybridization

The procedure was performed as described (Bellocchio et al., 2010; Marsicano and Lutz, 1999). After behavioral experiments, mice were killed by cervical dislocation. Their brains were isolated, quickly frozen on dry ice and stored at −80°C until sectioning in a cryostat (14 μm, Microm HM 500M, Microm Microtech). FITC-labeled riboprobes against mouse CB_1R were prepared as described (Kato et al., 2012; Kawamura et al., 2006). We used the tyramide signal amplification (TSA) method to improve the detection sensitivity of the *in situ* hybridization. Slides were incubated with FITC-conjugated tyramide (NEL741001KT, 1:80, PerkinElmer). Blocking buffer and wash buffer TNT were prepared according to the manufacturer's protocol. Slides were incubated in DAPI solution (1:20000, Fisher Scientific, NH, USA), washed, mounted, coverslipped and analyzed by an epifluorescence Leica DM6000 microscope (Leica, Germany).

In vivo electrophysiology in anesthetized mice

Experiments were performed as described in Robin et al. (2018). Immediately after the last session of preconditioning (3 odor-taste pairings), or control procedure (either water, odor alone or taste alone, see above Western Blot Analysis) mice were anesthetized in a box containing 5% Isoflurane (VIRBAC, France) before being placed in a stereotaxic frame (Model 900, Kopf instruments, CA, USA) in which 1.0% to 1.5% of Isoflurane was continuously supplied via an anesthetic mask during the whole duration of the experiment. The body temperature was maintained at ~36.5°C using a homeothermic system (model 50-7087-F, Harvard Apparatus, MA, USA) and the state of anesthesia was assessed by mild tail pinch. Before surgery, 100 μl of the local anesthetic lurocaine (vetoquinol, France) was injected in the scalp region. Surgical procedure started with a longitudinal incision of 1.5 cm in length aimed to expose Bregma and Lambda. After ensuring the correct alignment of the head, two holes were drilled in the skull for electrode placement. Electrodes were: a glass recording electrode, inserted in the CA1 stratum radiatum, and a concentric stimulating bipolar electrode (Model CBARC50, FHC, ME, USA) placed in the CA3 region. Coordinates were as follows: CA1 stratum radiatum: A/P −1.5, M/L −1.0, DV 1.20; CA3: A/P −2.2, M/L −2.8, D/V −1.3 (20° insertion angle). The recording electrode (tip diameter = 1–2 μm, 4–6 MΩ) was filled with a 2% pontamine sky blue solution in 0.5M sodium acetate. At first the recording electrode was placed by hand until it reached the surface of the brain and then to the final depth using a hydraulic micropositioner (Model 2650, KOPF instruments, CA, USA). The stimulation electrode was placed in the correct area using a standard manipulator. Both electrodes were adjusted to find the area with maximum response. *In vivo* recordings of evoked field excitatory postsynaptic potentials (fEPSPs) were amplified 1000 times and filtered (low-pass at 1Hz and high-pass 3000Hz) by a DAGAN 2400A amplifier (DAGAN Corporation, MN, USA). fEPSPs were digitized and collected on-line using a laboratory interface and software (CED 1401, SPIKE 2; Cambridge Electronic Design, Cambridge, UK). Test pulses were generated through an Isolated Constant Current Stimulator (DS3, Digitimer, Hertfordshire, UK) triggered by the SPIKE 2 output sequencer via CED 1401 and collected every 2 s at a 10 kHz sampling frequency and then averaged every 180 s. Test pulse intensities were typically between 40-250 μA with a duration of 50 μs. Basal stimulation intensity was adjusted to 30%–50% of the current intensity that evoked a maximum field response. All responses were expressed as percent from the average responses recorded during the 15 min before high frequency stimulation (HFS). HFS was induced by applying 3 trains of 100 Hz (1 s each), separated by 20 s interval. fEPSP were then recorded for a period of 30 or 39 min, depending on the experiment. C57BL/6 mice underwent this *in vivo* LTP induction immediately after the last exposition to odor-taste pairing, taste alone or odor alone of the behavioral protocol. Mice were exposed to control conditions (9 days of a daily presentation of water during 1 hr), ''odor-taste pairings'' (3 days of water habituation+6 days of odor-taste pairings as described above), ''taste alone'' (3 days of water habituation+6 days of taste presentations as described above) and ''taste alone'' (3 days of water habituation+6 days odor presentations as described above). On another group of GABA-*CB_1R*-KO mice, 5 weeks after the injection with the DIO-hM4Di virus into the

hippocampus, the following treatments were applied: CNO (2 mg/kg, i.p., 60-90 min before HFS) or vehicle (saline, i.p.). At the end of each experiment, the position of the electrodes was marked (recording area: iontophoretic infusion of the recording solution during 180 s at −20μA; stimulation area: continuous current discharge over 20 s at +20μA) and histological verification was performed *ex vivo* as shown in Supplementary information.

Ex vivo electrophysiology

Immediately after the last session of preconditioning (3 odor-taste pairings), or control procedure (either water, odor alone or taste alone, see above Western Blot Analysis) C57BL/6 mice were sacrificed by dislocation and the brain was immediately immerged in ice-cold oxygenated cutting solution containing in mM: 180 Sucrose, 26 $NaHCO_3$, 12 $MgSO_4$, 11 Glucose, 2.5 KCL, 1.25 NaH_2PO_4, 0.2 $CaCl_2$, oxygenated with 95% O_2-5%CO_2 ≈300mOsm. Parasagittal hippocampal slices (300μm thick) were obtained using a vibratome (VT1200S, Leica, Germany) and transferred for 30min into a 34°C bath of oxygenated ACSF containing in mM: 123 NaCl, 26 $NaHCO_3$, 11 Glucose, 2.5 KCL, 2.5 $CaCl_2$, 1.3 $MgCl_2$, 1.25 NaH_2PO_4 ≈305 mOsm. After a minimum of 1h recovery at room temperature (22-25°C), slices were transferred to a recording chamber in ACSF at 32°C.

Whole-cell recordings of IPSCs were made using a MultiClamp 700B amplifier (Molecular devices, UK) in CA1 pyramidal neurons voltage clamped at −70mV with a pipette (3-5 MΩ) containing in mM: 130 KCl, 10 HEPES, 1 EGTA, 2 MGCl2, 0.3 CaCl2, 7 Phosphocreatin, 3 Mg-ATP, 0.3 Na-GTP; pH = 7.2; 290mOsm. Evoked IPSCs were performed by a monopolar stimulating patch pipette filled with ACSF in *stratum radiatum* in presence of NMDA and AMPA/Kainate receptor antagonists (50μM D-APV and 10μM NBQX). Miniature IPSCs were isolated in presence of the voltage-gated sodium channels blocker, tetrodotoxin (1μM TTX) and collected for 5min.

Progressive DSIs were performed by depolarizing pyramidal neurons from −70mV to 0mV for 1 s, 3 s and 5 s. For every voltage step, DSIs' magnitude were measured as the average of 3 DSIs with 2min apart and represented the percentage of change between the mean of the 5 consecutive IPSCs preceding the depolarization and the first three IPSCs following the depolarization, with IPSCs evoked every 3 s.

iLTD were induced by High-Frequency-Stimulation (HFS) with 2 trains of 100 pulses at 100Hz with 20 s apart after a minimum of 10min of stable baseline, with IPSCs evoked every 20 s. The magnitude of iLTD was evaluated by the percentage of change between the mean of the 10min baseline with the percentage of responses averaged between 15 to 20min after HFS. Weak or no iLTD were assessed with a percentage of reduction less or equal to 15% whereas strong iLTD were estimated with a percentage of reduction higher than 15%.

Currents were filtered at 4kHz by a Digidata 1440A (Molecular devices, UK) and were analyzed using either Clampfit software (pClamp10) or Axograph for eIPSCs and mIPSCs, respectively.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data collection

No statistical methods were used to pre-determine sample sizes, but they are similar to those reported in previous publications. Data collection and analysis were performed blind to the conditions of the experiment. All mice were assigned randomly to the different experimental conditions.

Statistical analyses

For the behavioral experiments, we performed the D'Agostino&Pearson normality test using the Prism 6 Software. When all groups of an experiment passed the normality test, ANOVA (Two-way or Three-way, where appropriate) analysis was performed and when interaction was significant Bonferroniś post hoc analysis was used. When the normality was rejected, the non-parametric Wilcoxon test was used for within-group comparisons and Mann-Whitney test for inter-group comparisons. For the *in vivo* electrophysiological and western blot experiments, data were analyzed by One-way ANOVA followed, when it was significant, by Dunnet's post hoc test. For *ex vivo* electrophysiological experiments, data were analyzed by unpaired t test for DSI or by Chi-square test for I-LTD. For detailed statistical analysis, see statistical Tables S1 and S2.

Neuron, Volume *99*

Supplemental Information

Hippocampal CB_1 Receptors Control Incidental Associations

Arnau Busquets-Garcia, José F. Oliveira da Cruz, Geoffrey Terral, Antonio C. Pagano Zottola, Edgar Soria-Gómez, Andrea Contini, Hugo Martin, Bastien Redon, Marjorie Varilh, Christina Ioannidou, Filippo Drago, Federico Massa, Xavier Fioramonti, Pierre Trifilieff, Guillaume Ferreira, and Giovanni Marsicano

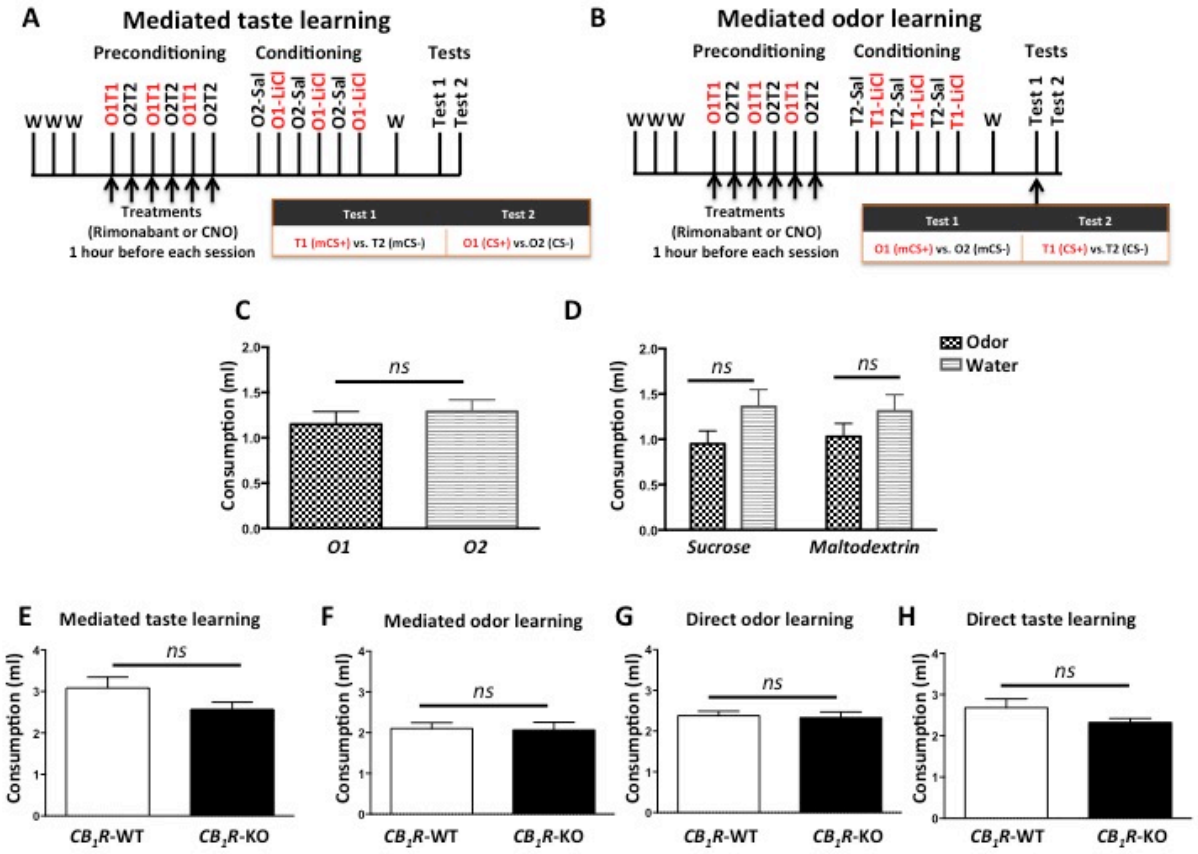

Figure S1 (Related to Main Figure 1). CB_1R are necessary for odor-taste mediated learning.
(A,B) Detailed representation of the paradigm to assess mediated taste aversion **(A)** and mediated odor aversion (**B**). All subjects received 1-hour access to water during three consecutive days as water habituation (W). During the following 6 days, the *preconditioning phase* consisted in 3 odor-taste pairings. Each pairing consists in two days: the first day the subjects received 1-hour access to a flavoured solution made of a new taste (T1) and a new odorant (O1). On day two, the animals received the taste and odor not given during the previous day, T2 and O2. Importantly, treatments such as Rimonabant or CNO (and their respective vehicle) were administered 1-hour before each preconditioning day. On the next 6 days, animals enter the *Conditioning or devaluation phase* where O1 **(A)** or T1 **(B)** was devaluated (CS+). On days 1, 3 and 5 of this phase, subjects received 1-hour access to O2 **(A)** or T2 **(B)** followed by an i.p. injection of saline (CS-) whereas on days 2, 4 and 6, they received 1-hour access to O1 **(A)** or T1 **(B)** immediately followed by an i.p. injection of the visceral malaise-inducing drug lithium chloride (CS+). After this conditioning, subjects were given a recovery day in which they received water (W). On the next 2 days, mediated and direct aversions were assessed using a 1-hour two-choice test. Mediated aversion was evaluated on Test 1 with a choice between the stimulus previously associated with the CS+ (mediated CS+: mCS+) and the stimulus previously associated with the CS- (mCS-). In the case of Rimonabant, it was administered 1-hour before the test of mediated learning. On the second day, the direct aversion was evaluated with a choice between the CS+ and the CS-. (**C**) Mean liquid consumption of O1 and O2 in a two choice test done after 3 odor-taste pairings exposition. (**D**) Mean liquid consumption of water and odorized water previously associated with either sucrose (left) or maltodextrin (right). Note that both tastes did not induce any appetitive conditioning after preconditioning. (**E,F,G,H**) Total liquid consumption during the mediated taste (**E**), mediated odor (**F**), direct odor (**G**) and direct taste (**H**) learning tests in full *CB_1R*-KO and their littermates. No differences were found in the total consumption between genotypes. *ns*, non significant.

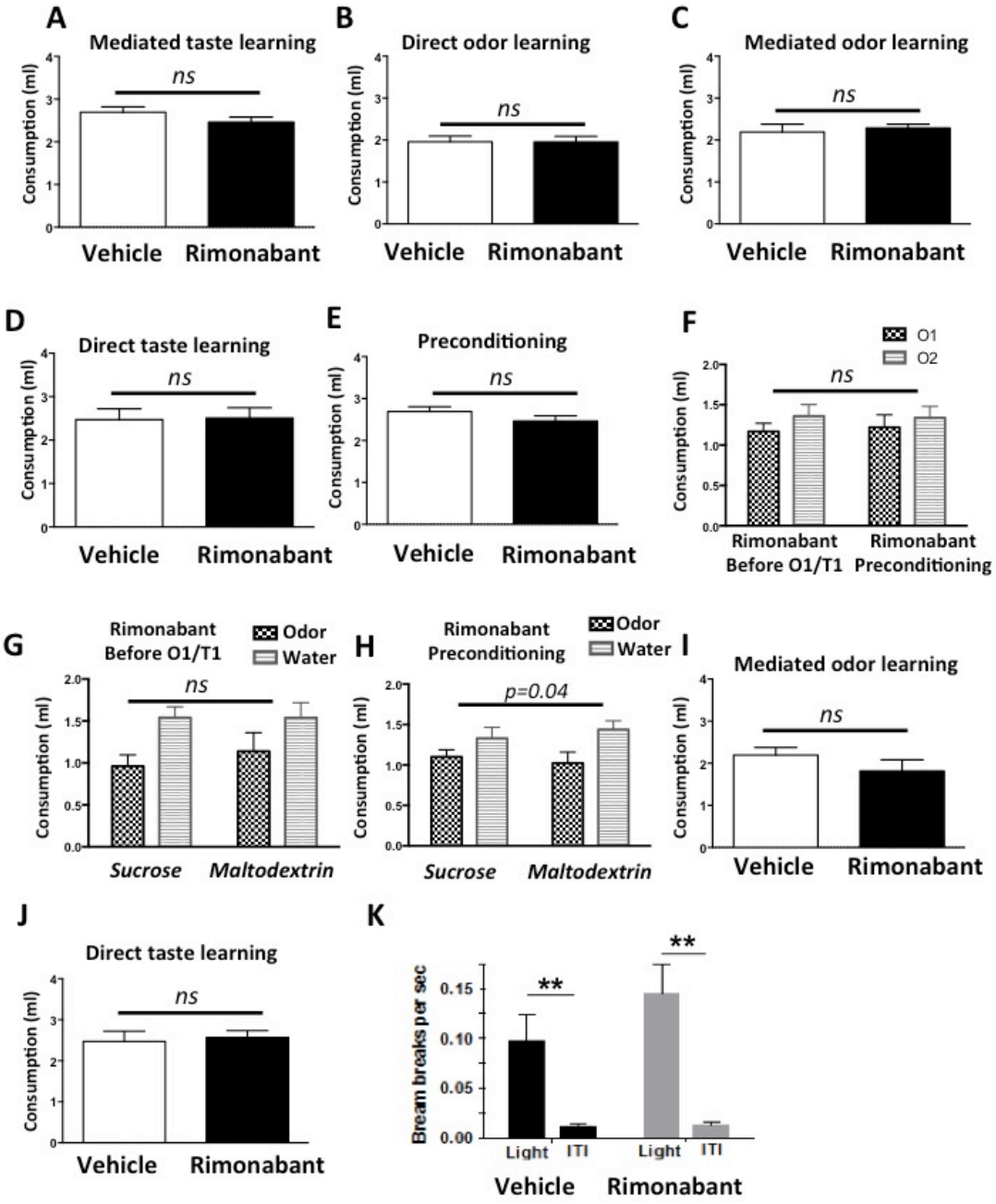

Figure S2 (Related to Main Figure 2). Activation of CB_1R during preconditioning is necessary for mediated learning.

(**A,B,C,D**) Total liquid consumption during mediated taste (**A**), direct odor (**B**), mediated odor (**C**) and direct taste (**D**) learning tests after Rimonabant treatment during preconditioning. (**E**). Mean liquid consumption during preconditioning in mice treated with vehicle or Rimonabant 1 h before each session. (**F**) Mean liquid consumption of O1 and O2 in a two choice test done right after 3 odor-taste pairings with Rimonabant injected 1 h before each O1/T1 pairing or before both O1/T1 and O2/T2 pairings. Note that Rimonabant injected before O1/T1 did not induce aversive or appetitive conditioning. (**G,H**) Mean liquid consumption of water and odorized water previously associated with either sucrose (left) or maltodextrin (right) for mice that receive Rimonabant before O1/T1 (**G**) or before both O1/T1 and O2/T2 pairings (**H**) Note that Rimonabant did not change the lack of taste-induced appetitive conditioning after preconditioning. (**I,J**) Total liquid consumption during mediated odor (**I**) and direct taste (**J**) learning tests when Rimonabant was injected 1-hour before the mediated aversion test. (**K**) Effects of Rimonabant (1 mg/kg) treatment during tone-light sensory preconditioning on the direct learning test assessed by beam breaks in response to the light cue. *p* refers to the general effect of water vs. odor in **H.** ***p*<0.01 (light vs. ITI)**.** For statistical details, see Supplementary Table 2. *ns*, non significant.

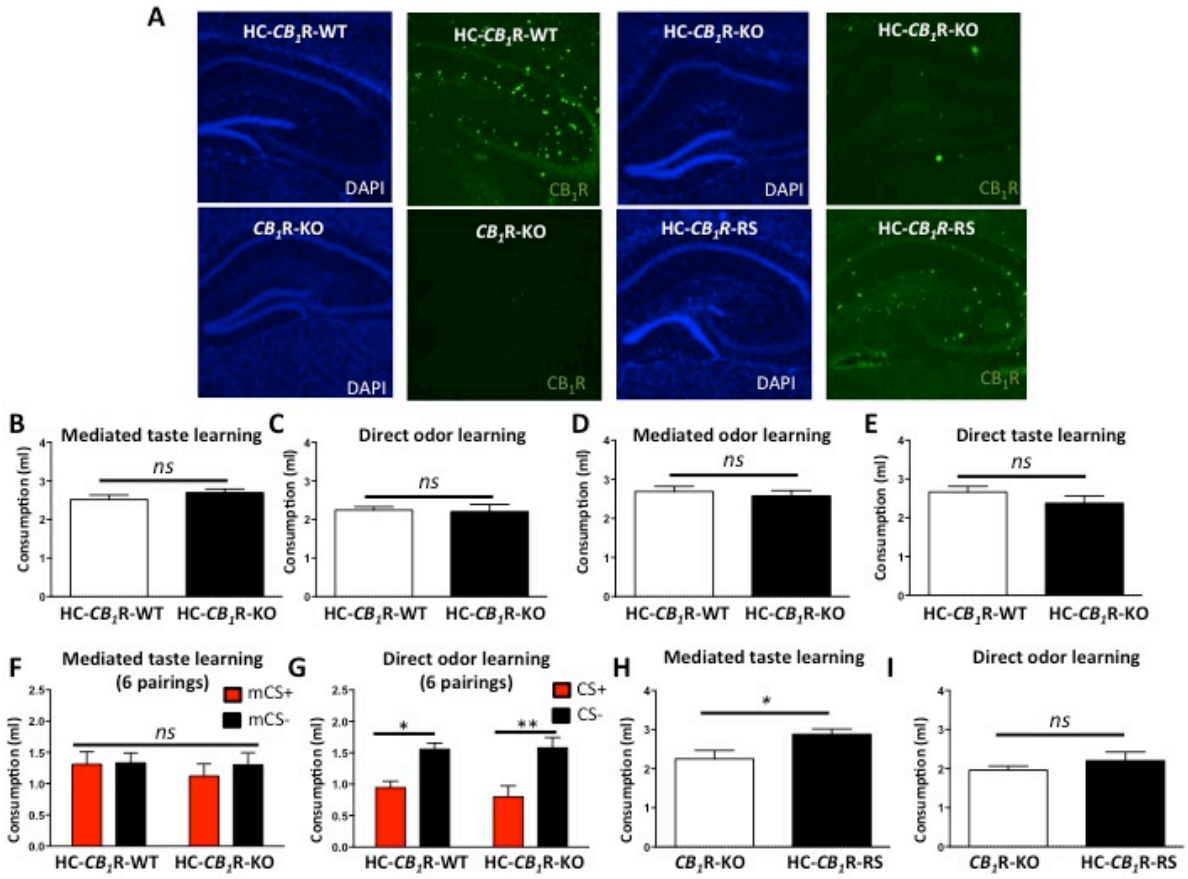

Figure S3 (Related to Main Figure 3). Hippocampal CB_1R are necessary and sufficient for mediated learning.

(**A**) Fluorescent *in situ* hybridization of CB_1R in *CB1*R-floxed mice injected in hippocampus with GFP (HC-*CB1*R-WT; up left) or with CRE virus (HC-*CB1*R-KO; up right) and in *CB1*R-KO mice injected in hippocampus with GFP (*CB1*R-KO; bottom left) or AAV-CB_1R virus (HC-*CB1R*-RS; bottom right). Blue images are DAPI staining. (**B,C,D,E**) Total liquid consumption during mediated taste (**B**), direct odor (**C**), mediated odor (**D**) and direct taste (**E**) learning tests in HC-*CB1*R-KO and HC-*CB1*R-WT mice. (**F,G)** Liquid consumption during mediated taste (**F**) and direct odor (**G**) aversion in HC-*CB1*R-KO and HC-*CB1*R-WT mice after the extended preconditioning procedure (6 pairings). (**H,I**) Total liquid consumption during mediated taste (**H**) and direct odor (**I**) learning tests in HC-*CB1*R-RS and *CB1*R-KO mice. *<0.05, **<0.01 (CS- vs. CS+ or *CB1*R-KO vs. HC-*CB1R*-RS). For statistical details, see Supplementary Table 2. *ns*, non significant.

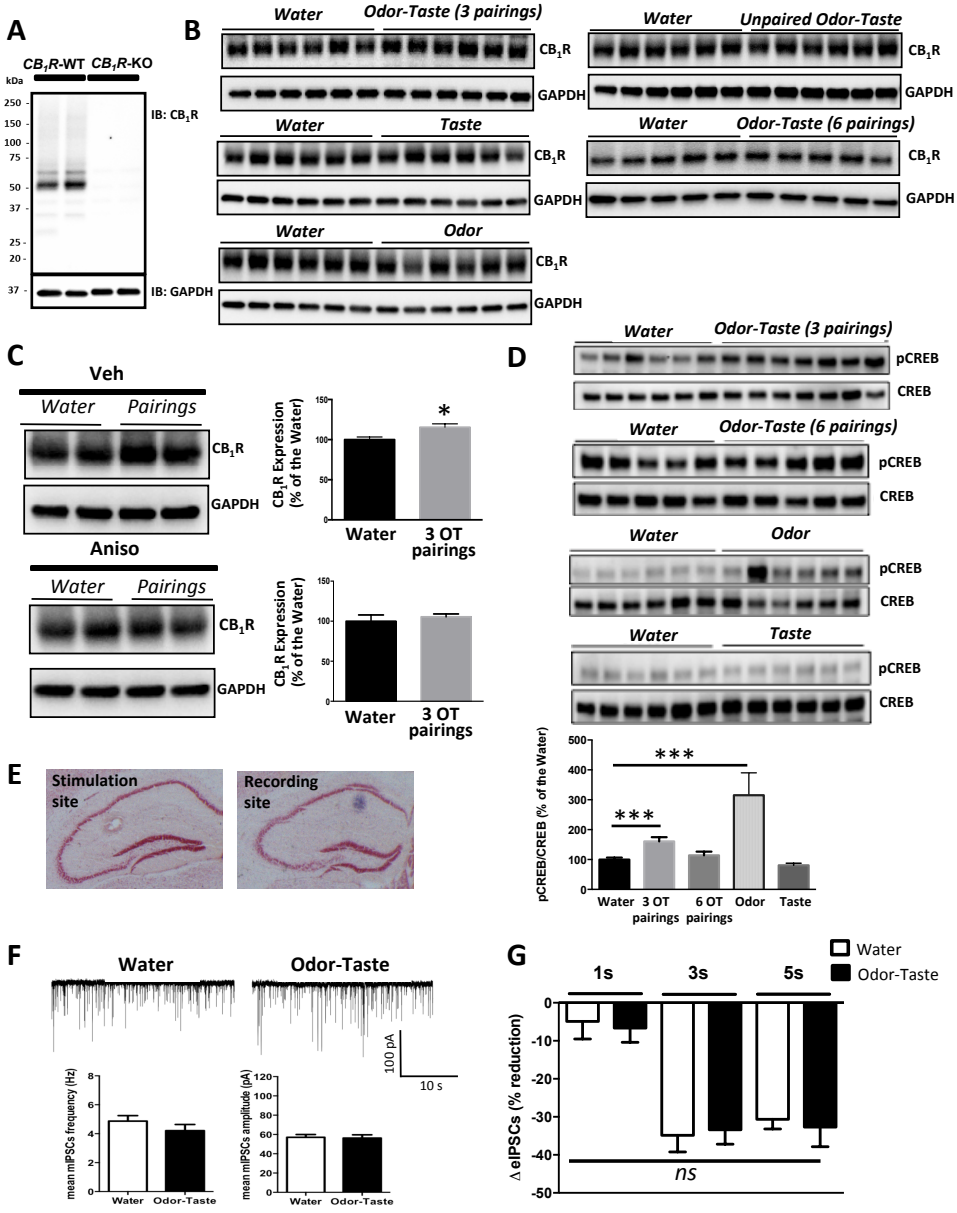

Figure S4 (Related to Main Figure 4). Hippocampal alterations induced by odor-taste pairings
(**A**) Representative immunoblot of a control experiment using hippocampal samples from *CB1*R-WT and *CB1*R-KO mice in order to check for specificity of the anti-CB_1R antibody. (**B**) Representative gels for the Western blot analysis of CB_1R expression levels in mice receiving 3 odor-taste pairings compared with mice receiving water (Water), taste alone (Taste), odor alone (Odor) or unpaired presentations of taste and odor (Unpaired odor-taste). An additional group received the extended preconditioning protocol with 6 odor-taste pairings. (**C**) Representative immunoblot (left) and optical densitometric quantification (right) of CB1R levels in mice receiving water or 3 odor-taste pairings (3 OT pairings) after a treatment with vehicle or the protein synthesis inhibitor anisomycin (18 mg/kg, i.p.) injected 1 h before each water or odor-taste presentations. (**D**) Representative gels for the Western blot analysis of CREB phosphorylation levels in mice receiving 3 or 6 odor-taste pairings, taste alone (Taste), or odor alone (Odor) compared with mice receiving water (Water). Bar graph at bottom represents optical densitometric quantification of phospho-CREB levels in these groups. (**E**) Histological controls illustrating the placement of the recording and stimulation electrodes for *in vivo* electrophysiological recordings. (**F**) Mean of mIPSCs frequency (left) and amplitude (right) in *ex vivo* hippocampal slices after water or 3 odor-taste pairings. On top, representative traces of mIPSCs. (**G**) DSI expression in *ex vivo* hippocampal slices from animals receiving water or odor-taste pairings using different timings of depolarization (1, 3 or 5 seconds). **<0.05, ***<0.001* (water vs. experimental condition). For statistical details, see Supplementary Table 2. *ns*, non significant.

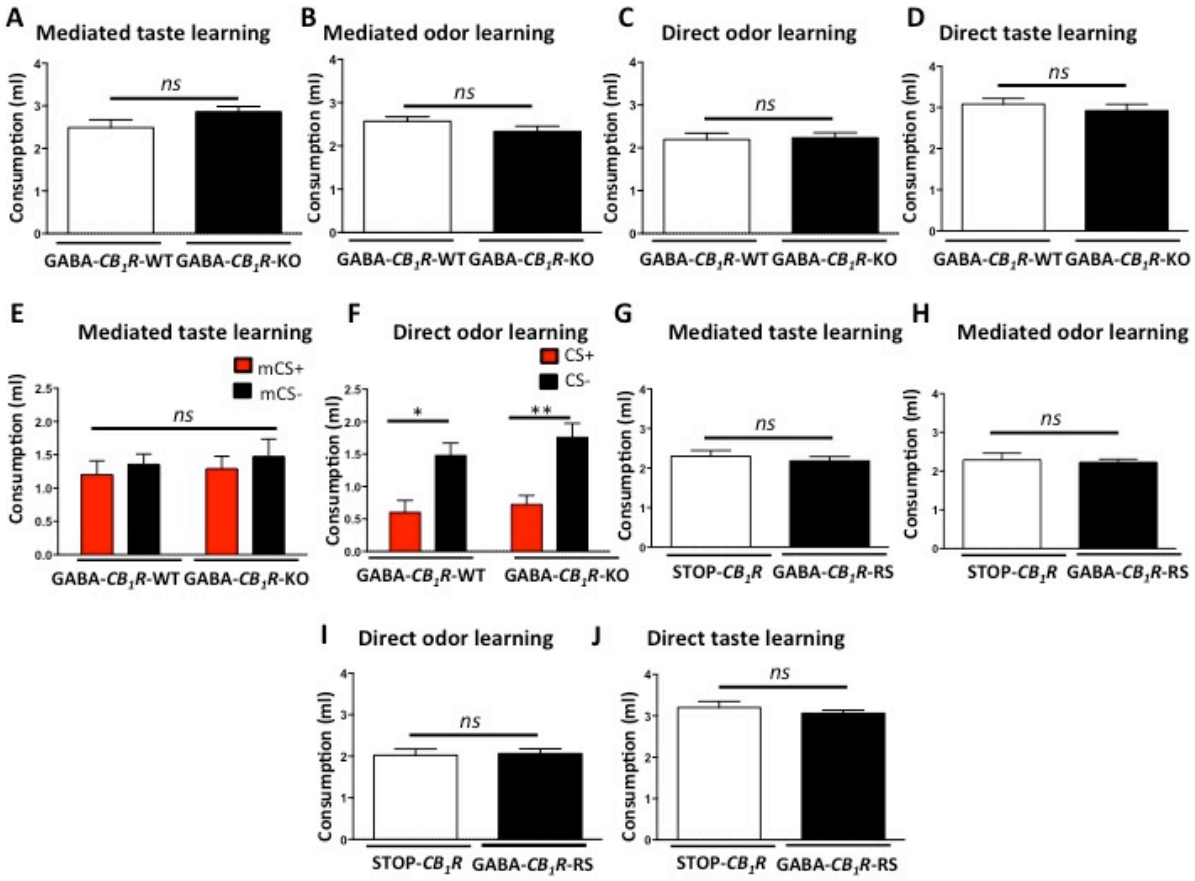

Figure S5 (Related to Main Figure 5). GABAergic CB_1R are necessary and sufficient for mediated learning formation

(**A,B,C,D**) Total liquid consumption during mediated taste (**A**), mediated odor (**B**), direct odor (**C**) and direct taste (**D**) learning tests in GABA-*CB1R*-KO mice and wild-type littermates. (**E,F**) Liquid consumption during mediated taste (**E**) and direct odor (**F**) aversion in GABA-*CB1*R-KO mice and GABA-*CB1*R-WT after the extended preconditioning procedure (6 odor-taste pairings). (**G,H,I,J**) Total liquid consumption during mediated taste (**G**), mediated odor (**H**), direct odor (**I**) and direct taste (**J**) learning tests in GABA-*CB1R*-RS mice and STOP-*CB1*R mice. *<0.05, **<0.01 (CS- vs. CS+). For statistical details, see Supplementary table 2. *ns*, non significant.

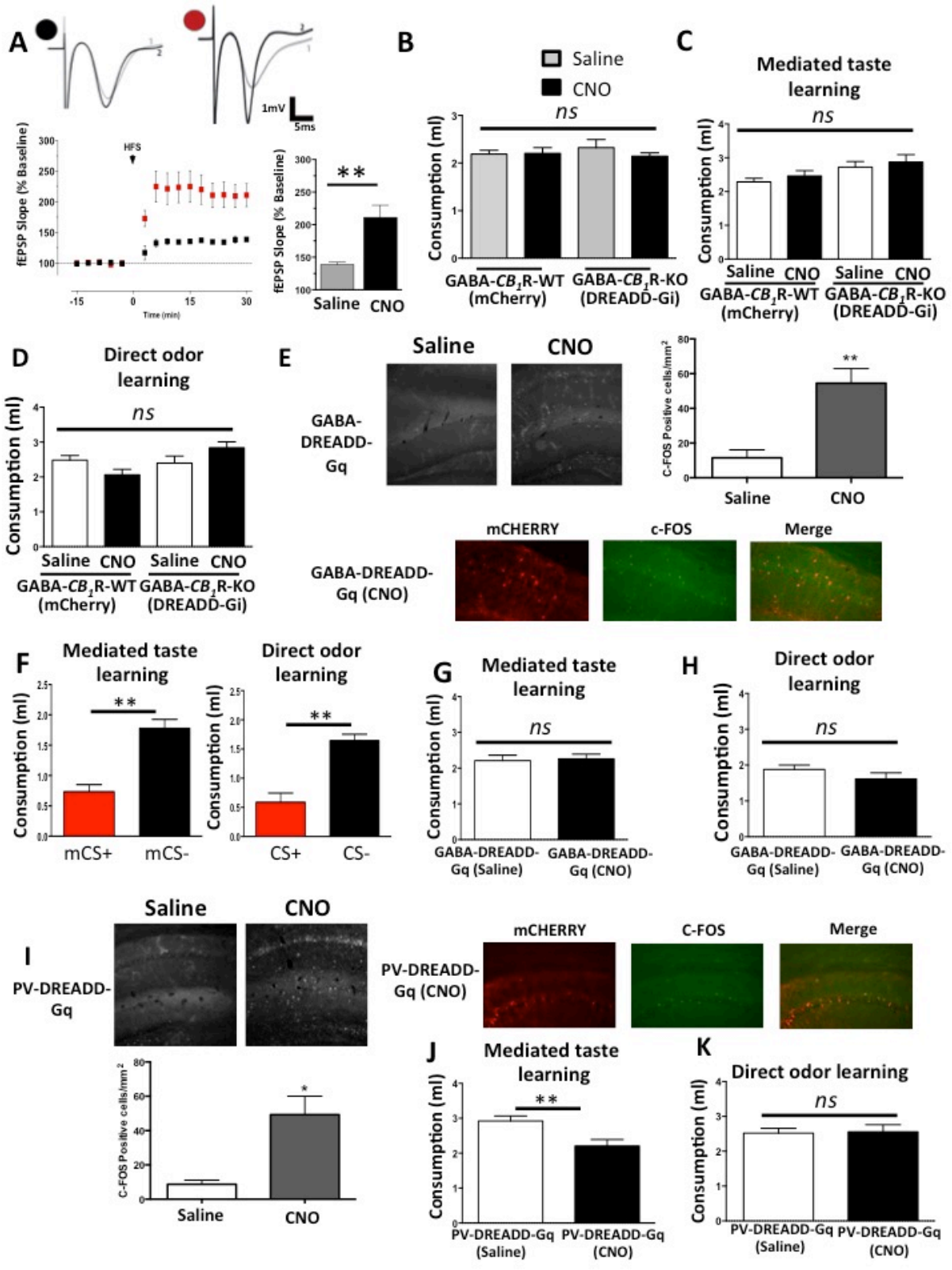

Figure S6 (Related to Main Figure 6). CB_1R in specific GABAergic neurons control the formation of incidental associations

(A) Summary time course plot, traces and bar histogram of normalized fEPSPs in CA1 of hippocampus recorded 30 minutes after high frequency stimulation (HFS) in anesthetized GABA-*CB*$_1$R-KO mice carrying expression of the inhibitory DREADD-Gi in hippocampal GABAergic interneurons under vehicle or CNO treatment. Note the stronger potentiation upon chemogenetic inhibition of hippocampal GABAergic neurons validating the DREADD approach using an inhibitory

DREADD. (**B**) Chemogenetic manipulation (with the Cre-dependent inhibitory DREADD-Gi) of hippocampal GABAergic transmission in GABA-CB_1R-KO mice during preconditioning did not affect the mean liquid consumption during the 3 odor-taste pairings. (**C,D)** Total liquid consumption during mediated taste (**C**) and direct odor (**D**) learning tests in GABA-CB_1R-KO and their littermates receiving CNO or saline during preconditioning. **(E)** Representative images (left) and quantification of c-FOS positive cells (right) in the hippocampus of GABA-CRE mice carrying the excitatory DREADD-Gq and treated with saline or CNO. Bottom, representative images of mCHERRY and c-FOS labelings (separated and merge) in GABA-DREADD-Gq mice injected with CNO. Note that around 85% of mCHERRY positive cells are c-FOS positive. **(F)** Liquid consumption during mediated taste (left) and direct odor (righ) aversion in GABA-Cre mice infused by a DIO-mCHERRY control virus (GABA-mCherry) and injected with CNO during the preconditioning phase. (**G,H**) Total liquid consumption during mediated taste (**G**) and direct odor (**H**) learning tests in GABA-CRE mice. (**I**) Representative images and quantification of c-FOS positive cells (bottom) in the hippocampus of PV-CRE mice carrying the excitatory DREADD-Gq and treated with saline or CNO. Right, representative images of mCHERRY and c-FOS labelings (separated and merge) in PV-DREADD-Gq mice injected with CNO. Note that around 75% of mCHERRY positive cells are c-FOS positive. (**J,K**) Total liquid consumption during mediated taste (**J**) and direct odor (**K**) learning tests in PV-Cre mice**. ***, $p<0.05$; **, $p<0.01$ (saline vs CNO**,** mCS+ vs mCS-, CS+ vs CS-). For statistical details, see Supplementary Table 2. *ns*, non significant.

Figure	Figure experiment	"n" (per group)	Analysis (post-hoc test reported in figures)	Factors analyzed	D'Agostino & Pearson normality test Passed normality test? (alpha=0.05)	F-ratios	P values
1B	Mediated taste learning in CB1R-KO mice	9-11	Two-way ANOVA repeated measures (Bonferroni)	WT vs KO mCS+ vs mCS-	Yes	Interaction F(1,18)=6.28	0.022
						mCS+ vs mCS- (WT)	0.0002
1C	Mediated odor learning in CB1R-KO mice	14-15	Wilcoxon test	mCS+ vs mCS-	No	WT	0.0103
1D	Direct odor learning in CB1R-KO mice	9-11	Two-way ANOVA repeated measures	WT vs KO CS+ vs CS-	Yes	Interaction F(1,80)=0.14	0.71
						CS+ vs CS- F(1,18)=23.54	0.0001
1E	Direct taste learning in CB1R-KO mice	14-15	Wilcoxon test	CS+ vs CS-	No	WT	0.007
						KO	0.0002
2B	Mediated taste learning in Rimonabant-treated mice	13-14	Wilcoxon test	mCS+ vs mCS-	No	Vehicle	0.001
2C	Direct odor learning in Rimonabant-treated mice	13-14	Two-way ANOVA repeated measures	Veh vs Rimonabant CS+ vs CS-	Yes	Interaction F(1,25)=0.99	0.32
						CS+ vs CS- F(1,25)=42.28	<0.0001
2D	Mediated odor learning in Rimonabant-treated mice	10	Two-way ANOVA repeated measures (Bonferroni)	Veh vs Rimonabant mCS+ vs mCS-	Yes	Interaction F(1,18)=8.249	0.01
						mCS+ vs mCS- (Vehicle)	0.033
2E	Direct taste learning in Rimonabant-treated mice	10	Wilcoxon test	CS+ vs CS-	No	Vehicle	0.002
						Rimonabant	0.002
2F	Mediated odor learning in Rimonabant-treated mice (pre-test)	10	Wilcoxon test	mCS+ vs mCS-	No	Vehicle	0.04
						Rimonabant	0.002
2G	Direct taste learning in Rimonabant-treated mice (pre-test)	10	Wilcoxon test	CS+ vs CS-	No	Vehicle	0.002
						Rimonabant	0.003
2I	Discrimination ratio in Rimonabant-treated mice	10-12	Mann Whitney test	Veh vs Rimonabant	No	Vehicle vs Rimonabant	0.0005
3B (left)	Mediated taste learning in mice with CB1R deletion in hippocampus	11-14	Wilcoxon test	mCS+ vs mCS-	No	Control	0.004
3B (right)	Direct odor learning in mice with CB1R deletion in hippocampus	11-14	Wilcoxon test	CS+ vs CS-	No	Control	0.004
						Deletion	0.018
3C (left)	Mediated odor learning in mice with CB1R deletion in hippocampus	9	Wilcoxon test	mCS+ vs mCS-	No	Control	0.009
3C (right)	Direct taste learning in mice with CB1R deletion in hippocampus	9	Two-way ANOVA repeated measures	Control vs Deletion CS+ vs CS-	Yes	Interaction F(1,16)=0.05	0.81
						CS+ vs CS- F(1,23)=22.99	0.0003
3E (left)	Mediated taste learning in mice with CB1R re-expression in hippocampus	10	Wilcoxon test	mCS+ vs mCS-	No	Rescue	0.017
3E (right)	Direct odor learning in mice with CB1R re-expression in hippocampus	10	Wilcoxon test	CS+ vs CS-	No	Control	0.009
						Rescue	0.035
4A	Mediated taste learning in GABA-CB1R-KO mice	14-16	Wilcoxon test	mCS+ vs mCS-	No	WT	0.0001
4B	Mediated odor learning in GABA-CB1R-KO mice	13-16	Wilcoxon test	mCS+ vs mCS-	No	WT	0.032
4C	Direct odor learning in GABA-CB1R-KO mice	14-16	Wilcoxon test	CS+ vs CS-	No	WT group	0.0002
						KO	0.002
4D	Direct taste learning in GABA-CB1R-KO mice	13-16	Two-way ANOVA repeated measures	WT vs KO CS+ vs CS-	Yes	Interaction F(1,27)=0.054	0.81
						CS+ vs CS- F(1,27)=17.95	0.0002
4E	Mediated taste learning in GABA-CB1R-RS mice	11-12	Wilcoxon test	mCS+ vs mCS-	No	RS	0.003
4F	Mediated odor learning in GABA-CB1R-RS mice	8-9	Two-way ANOVA repeated measures (Bonferroni)	WT vs RS mCS+ vs mCS-	Yes	Interaction F(1,15)=4.55	0.049
						mCS+ vs mCS- (RS)	0.0012
4G	Direct odor learning in GABA-CB1R-RS mice	11-12	Two-way ANOVA repeated measures	WT vs RS CS+ vs CS-	Yes	Interaction F(1,21)=0.49	0.49
						CS+ vs CS- F(1,21)=20.45	0.0002
4H	Direct taste learning in GABA-CB1R-RS mice	8-9	Wilcoxon test	CS+ vs CS-	No	Stop-CB1R	0.027
						RS	0.015
5A	CB1R expression in hippocampus	6-12	One-way ANOVA (Dunnet)	Water vs conditions	Yes	Main Group effect	<0.0001
						Water vs 3 pairings	0.002
5C	LTP in WT and GABA-CB1-KO mice	6-15	Two-way ANOVA (Dunnet)	WT vs KO Water vs conditions	Yes	Interaction F(3,58)=5.79	0.001
						Water vs 3 pairings (WT)	0.029
						Water vs taste (WT)	0.0003
						3 pairings (WT vs KO)	0.024
						Taste (WT vs KO)	<0.0001
5D	I-LTD in WT mice	14-19	One-sample t-test	Each condition	Yes	Naive group	0.0001
						3 pairings group	0.016
5E	I-LTD in WT mice	14-19	Chi-Square test	Water vs conditions		Main effect	0.002
						Naive vs Water	0.01
						Water vs 3 pairings	0.01
6B (top)	Mediated aversion in GABA-CB1R-KO and DIO hM4Di injection	13-15	Three-way ANOVA (Bonferroni)	Treatment vs genotype mCS+ vs mCS-	Yes	Interaction F(1,50)=6.72	0.016
						mCS+ vs mCS- (WT Saline)	0.001
						mCS+ vs mCS- (WT CNO)	0.002
						mCS+ vs mCS- (KO CNO)	<0.0001
6B (bot)	Direct aversion in GABA-CB1R-KO and DIO hM4Di injection	13-15	Three-way ANOVA	Treatment vs genotype CS+ vs CS-	Yes	Interaction F(1,50)=0.15	0.70
						CS+ vs CS- F(1,50)=89.85	<0.0001
6C (top)	Mediated aversion in GABA-CRE and DIO hM3Dq injection	10	Two-way ANOVA repeated measures (Bonferroni)	saline vs CNO mCS+ vs mCS-	Yes	Interaction F(1,18)=6.941	0.016
						mCS+ vs mCS- (Saline)	0.005
6C (bot)	Direct aversion in GABA-CRE and DIO hM3Dq injection	10	Two-way ANOVA repeated measures	saline vs CNO CS+ vs CS-	Yes	Interaction F(1,18)=0.07	0.79
						CS+ vs CS- F(1,18)=41.04	<0.0001
6D (top)	Mediated aversion in PV-CRE and DIO hM3Dq injection	14-15	Two-way ANOVA repeated measures	saline vs CNO mCS+ vs mCS-	Yes	Interaction F(1,27)=0.82	0.37
						mCS+ vs mCS- F(1,27)=22.05	<0.0001
6D (bot)	Direct aversion in PV-CRE and DIO hM3Dq injection	14-15	Wilcoxon test	CS+ vs CS-	No	Saline	0.002
						CNO	0.004

Supplementary Table 1. Statistical analysis. Related to Figure 1-6.

Figure	Figure experiment	"n" (per group)	Analysis (post-hoc test reported in figures)	Factors analyzed	D'Agostino &Pearson normality test Passed normality test? (alpha=0.05)	F-ratios	P values
S2H	Two-choice between odor and water	10	Two-way ANOVA repeated measures	Odor vs Water Sucrose vs Malto	Yes	Interaction F(1,18)=0.40	0.53
						Odor vs Water F(1,18)=4.79	0.042
S2K	Direct conditioning to the light (Vehicle and Rimonabant mice)	10-12	Wilcoxon test	Light vs ITI	No	Vehicle	0.009
						Rimonabant	0.002
S3G	Direct odor learning in HC-CB1R-RS mice	9	Two-way ANOVA repeated measures	WT vs RS CS+ vs CS-	Yes	Interaction F(1,16)=0.05	0.81
						CS+ vs CS- F(1,16)=21.23	0.0003
S3H	Total consumption during mediated aversion test	10	Unpaired t-test	Total consumption	Yes	t(18)=2.46	0.03
S4F	Direct odor learning in GABA-CB1R-KO mice after 6 pairings	10-12	Two-way ANOVA repeated measures	WT vs KO CS+ vs CS-	Yes	Interaction F(1,20)=0.11	0.73
						CS+ vs CS- F(1,20)=18.54	0.0003
S5C	CB1R expression after vehicle or anisomycin in preconditioning	10	Unpaired t-test	Water vs 3 pairings	Yes	t(18)=2.46 (Vehicle)	0.03
S5D	pCREB in hippocampus	5-14	One-way ANOVA (Dunnet)	Water vs conditions	Yes	Main effect	0.0002
						Water vs 3 pairings	0.0001
						Water vs odor	0.0001
S6A	LTP in GABA-CB1R-KO mice	4-5	Unpaired t-test	Saline vs CNO	Yes	t(7)=3.58	0.008
S6E	C-FOS positive cells in GABA-Cre-DREADDGq mice	5	Unpaired t-test	Saline vs CNO	Yes	t(8)=4.502	0.0020
S6F	Mediated taste and direct odor learning in GABA-CRE mice	9	Wilcoxon test	mCS+ vs mCS-	No		0.003
			Unpaired t-test	CS+ vs CS-	Yes	t(8)=4.19	0.003
S6I	C-FOS positive cells in PV-Cre-DREADDGq mice	4-6	Unpaired t-test	Saline vs CNO	Yes	t(8)=2.99	0.017
S6J	Total consumption during mediated test in PV-CRE mice	14-15	Unpaired t-test	Saline vs CNO	Yes	t(27)=3.06	0.004

Supplementary Table 2. Statistical analysis. Related to Figure S2-6.