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Habenular CB₁ Receptors Control the Expression of Aversive Memories

Highlights

- CB₁R are presynaptically expressed on MHb neurons projecting to the IPN
- MHb-CB₁R deletion inhibits the expression of aversive memories
- MHb-CB₁R control aversive memories via cholinergic transmission in the IPN
- MHb-CB₁ controls optogenetically evoked cholinergic transmissions in the IPN

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In Brief

Soria-Gómez et al. show that CB₁ receptors expressed at presynaptic terminals of medial habenula neurons projecting to the interpeduncular nucleus specifically control the expression of aversive memories. This function requires negative control of cholinergic, but not glutamatergic, neurotransmission.



Habenular CB₁ Receptors Control the Expression of Aversive Memories

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SUMMARY

Expression of aversive memories is key for survival, but the underlying brain mechanisms are not fully understood. Medial habenular (MHb) axons corelease glutamate and acetylcholine onto target postsynaptic interpeduncular (IPN) neurons, but their role in aversive memories has not been addressed so far. We found that cannabinoid type 1 receptors (CB₁R), key regulators of aversive responses, are present at presynaptic terminals of MHb neurons in the IPN. Conditional deletion of CB₁R from MHb neurons reduces fear-conditioned freezing and abolishes conditioned odor aversion in mice, without affecting neutral or appetitively motivated memories. Interestingly, local inhibition of nicotinic, but not glutamatergic receptors in the target region IPN before retrieval, rescues these phenotypes. Finally, optogenetic electrophysiological recordings of MHb-to-IPN circuitry revealed that blockade of CB₁R specifically enhances cholinergic, but not glutamatergic, neurotransmission. Thus, presynaptic CB₁R control expression of aversive memories by selectively modulating cholinergic transmission at MHb synapses in the IPN.

INTRODUCTION

The habenular complex is divided into the lateral (LHb) and the medial portion (MHb) (Hikosaka, 2010) (Figures 1A and 1B), and, by linking the limbic forebrain and the midbrain-extrapyramidal motor system, it regulates physiological and pathological behaviors (Sandyk, 1991). Whereas the functions of the LHb have been the focus of intense research (Geisler and Trimble, 2008; Hikosaka, 2010), less is known regarding the roles of the MHb (Viswanath et al., 2013). Recent studies, however, revealed that the MHb modulates locomotion and emotional responses (Agetsuma et al., 2010; Hsu et al., 2014; Kobayashi et al.,

2013; Yamaguchi et al., 2013). The main target of MHb neurons is the mesencephalic interpeduncular nucleus (IPN) (Contestabile et al., 1987) (Figure 1B). Selective modulation of this circuit in zebrafish impacts on aversive responses (Agetsuma et al., 2010). However, whether similar functions exist in the mammalian brain is unknown.

Choline acetyl transferase (ChAT)-containing MHb neurons (Contestabile et al., 1987) can corelease both acetylcholine and glutamate onto target IPN neurons (Hu et al., 2012; Ren et al., 2011). Nevertheless, the behavioral implications of this corelease have not been investigated yet.

The CB₁R are present in habenula neurons, but their subregional location and their functions are unknown (Marsicano and Kuner, 2008; Marsicano and Lutz, 1999; Matsuda et al., 1993). CB₁R and their endogenous ligands form the endocannabinoid system (ECS) (Piomelli, 2003), which, at synaptic level, retrogradely decreases the release of several neurotransmitters (Castillo et al., 2012; Degroot et al., 2006; Kano et al., 2009; Marsicano and Lutz, 2006). Many functions of the MHb, such as the regulation of aversive memories, are also under the control of the ECS (Busquets-Garcia et al., 2015; Marsicano et al., 2002; Morena and Campolongo, 2014; Riebe et al., 2012). This suggests that a control of the activity of the MHb-to-IPN circuit by presynaptic CB₁R might regulate aversive memories.

In this study, we analyzed whether CB₁R in MHb neurons (hereafter called MHb-CB₁R) regulate aversive responses. The results reveal that endocannabinoid control of MHb-to-IPN cholinergic, but not glutamatergic, neurotransmission exerts a necessary role in the expression of aversive memories.

RESULTS

Specific Deletion of the CB₁ Gene in the MHb

CB₁R are widely expressed in different brain regions (Herkenham et al., 1990; Marsicano and Kuner, 2008), including the habenula (Marsicano and Lutz, 1999; Matsuda et al., 1993). However, the exact localization of habenular CB₁R mRNA was not addressed. By fluorescent in situ hybridization (FISH), we found that CB₁R mRNA is present in the MHb and in the adjacent paraventricular thalamic nucleus (PVT), whereas no expression was observed in the LHb (Figure 1A and see

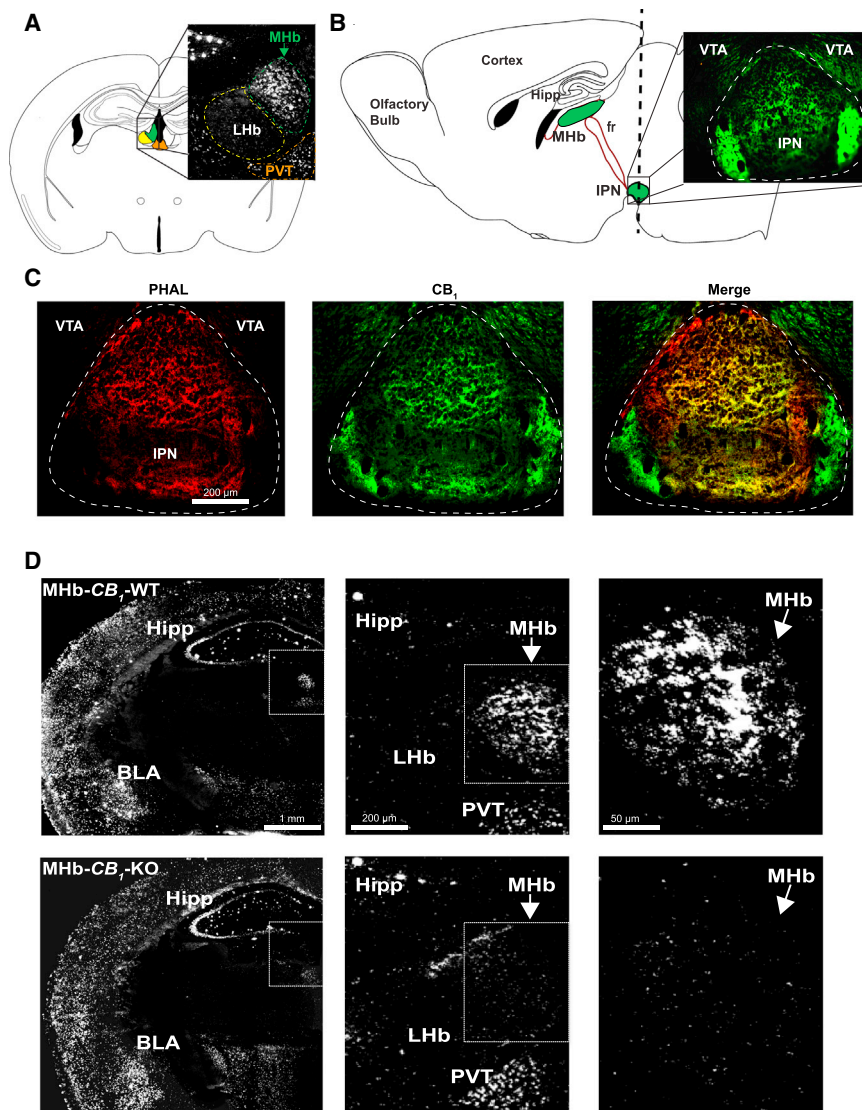


Figure 1. CB₁R are Expressed at Presynaptic Terminals of MHb Neurons Projecting to the IPN

(A) Schematic drawing showing the localization of the habenular complex and adjacent structures in a coronal view of the mouse brain. (Inset) Fluorescent in situ hybridization (FISH) micrograph showing the expression of CB₁R mRNA in the region. Green dotted line, MHb; yellow dotted line, LHb (lateral habenula); orange dotted line, PVT (paraventricular thalamus).

(B) Schematic representation of the MHb projection toward the IPN through the *fasciculus retroflexus* (fr) in a sagittal view of the mouse brain. (Inset) Fluorescent immunohistochemistry coronal micrograph (vertical dashed line) showing the expression of CB₁ protein in the IPN. Hipp, hippocampus.

(C) Representative epifluorescent micrograph images from coronal sections showing the presence of the anterograde tracer *phaseolus vulgaris-leucoagglutinin* (PHAL; left panel, red) previously injected into the MHb (Figure S1; n = 3), and the CB₁ receptor immunoreactivity (middle panel, green) in the IPN. Note the strong colocalization (right panels, yellow) in the middle part of the IPN. VTA, ventral tegmental area.

(D) Representative FISH micrographs showing the expression of CB₁ receptor mRNA in control MHb-CB₁-WT (top panel) and mutant MHb-CB₁-KO mice (bottom panel). BLA, basolateral amygdala. See also Figure S1.

ble S2), but not in adjacent areas (e.g., PVT; Figures 1D and S1A; Table S2).

Role of MHb-CB₁R in Aversive Conditioning

MHb-CB₁-KO mice displayed a strongly reduced freezing response in cued and contextual fear conditioning experiments as compared to MHb-CB₁-WT littermates

(Figures 2A–2D; Table S1). Importantly, the mutation did not affect potentially confounding related behaviors, such as pain (shock) sensitivity, unconditioned freezing, locomotion, and anxiety-like behavior (Figures S2A–S2J; Table S2).

To test whether this function of MHb-CB₁R extends to other aversive memory tasks, we evaluated conditioned odor aversion (COA) (Chapuis et al., 2007; Desgranges et al., 2009). Whereas MHb-CB₁-WT mice displayed a clear odor aversion, MHb-CB₁-KO littermates failed to express COA (Figure 2E; Table S1). Control analyses revealed that liquid ingestion, odor perception, and neophobia were not affected by the mutation (Figure S2K; Table S2).

MHb-CB₁-KO mice did not display any alteration in a sucrose-conditioned odor preference task (appetitive memory; Pinhas et al., 2012; Figure S2L; Table S2). In addition, the same mice displayed normal novel object recognition memory (“neutral” memory; Puighermanal et al., 2009; Figures S2M and S2N; Table S2), while maintaining the expected phenotype

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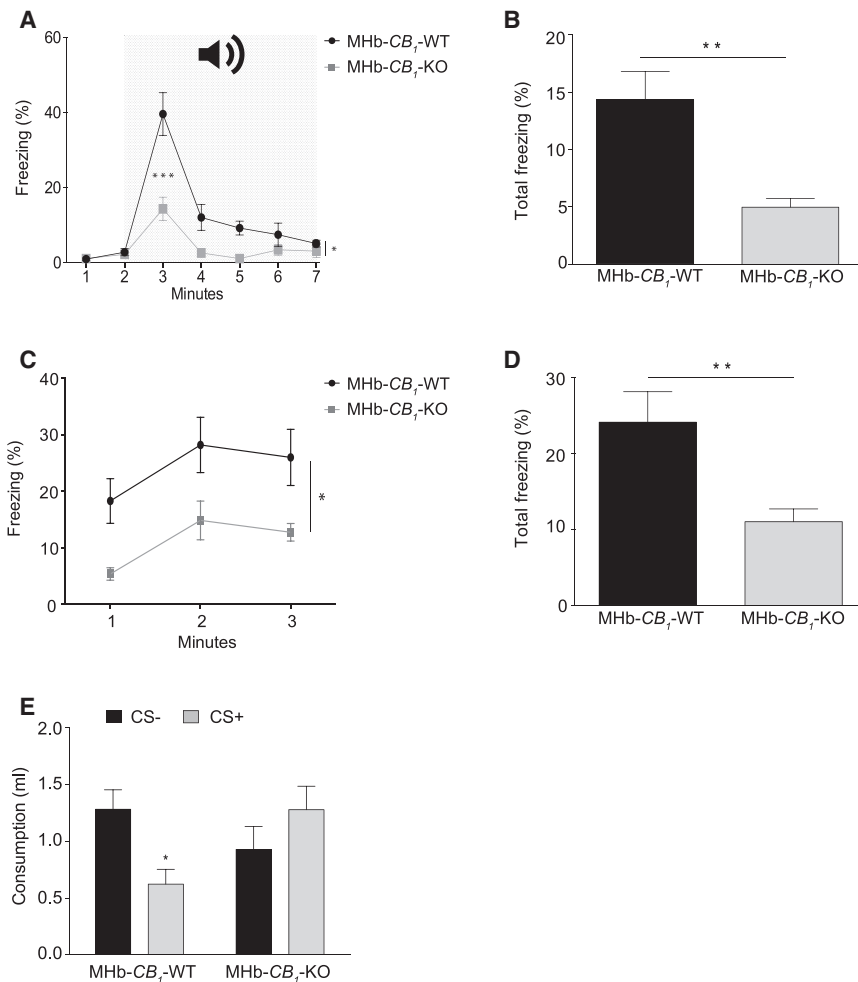


Figure 2. MHB-CB₁R Are Necessary for Aversive Memories

(A and B) Time course (A) and total (B) freezing responses of MHB-CB₁-KO mice (n = 6) and MHB-CB₁-WT controls (n = 7) undergoing cued fear conditioning.

(C and D) Time course (C) and total (D) freezing responses of MHB-CB₁-KO mice (n = 11) and MHB-CB₁-WT controls (n = 9) undergoing contextual fear conditioning.

(E) Odorized water intakes of MHB-CB₁-WT (n = 8) and MHB-CB₁-KO mice (n = 10) undergoing conditioned odor aversion experiments. CS+, conditioned odor; CS-, neutral odor. Data, means ± SEM. *p < 0.05; **p < 0.01, ***p < 0.001. For details of statistical analyses, see Table S1. See also Figure S2.

MHB-CB₁R-Dependent Decrease of Cholinergic Transmission in the IPN Mediates Expression of Aversive Memories

MHB neurons can corelease glutamate and acetylcholine onto IPN neurons (Hu et al., 2012; Ren et al., 2011). Thus, the phenotype of MHB-CB₁-KO mice might be due to the lack of CB₁R-dependent negative control of release, eventually leading to an excess of either or both neurotransmitters in the IPN. To address this issue, we adopted intra-IPN pharmacological approaches aimed at reducing this potential excess of neurotransmitter release (see Bellocchio et al., 2010; Soria-Gómez et al., 2014a).

in a fear-conditioning test (Figure S2O; Table S2). Finally, MHB-CB₁R deletion did not alter sucrose preference (Figure S2P; Table S2).

Thus, MHB-CB₁R signaling is necessary for aversive conditioning based on different sensory modalities, but they are dispensable for appetitive or “neutral” memory and sucrose preference.

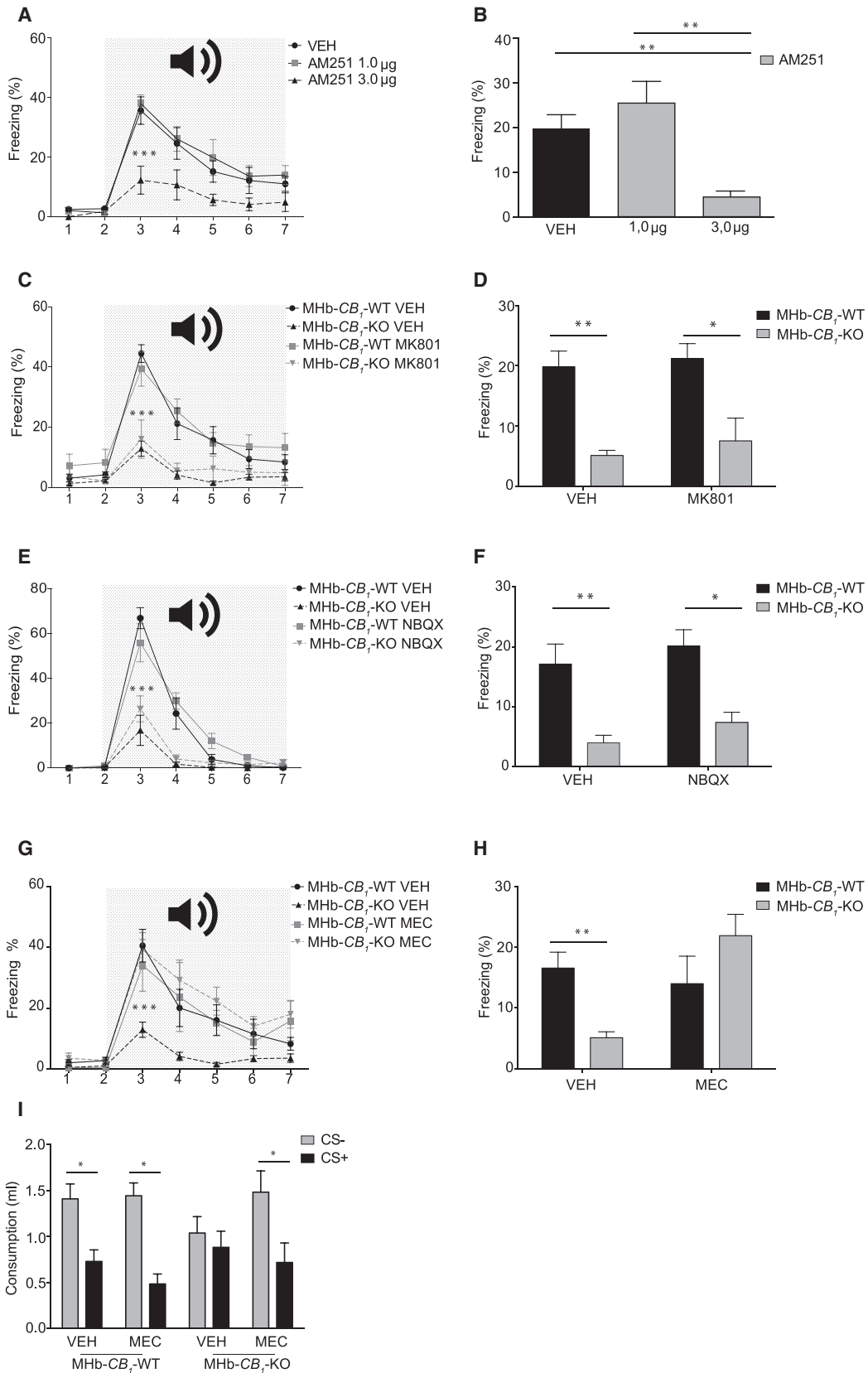
MHB-CB₁R in the IPN Control the Expression of Conditioned Freezing

The main mechanism of action of the ECS in the brain is the negative control of presynaptic neurotransmitter release (Castillo et al., 2012; Degroot et al., 2006; Kano et al., 2009; Marsicano and Lutz, 2006; Piomelli, 2003). Thus, MHB-CB₁R might modulate aversive memories by the presynaptic regulation of neurotransmission in the IPN.

Intra-IPN injections of the CB₁R antagonist AM251 in wild-type C57BL/6-N mice before retrieval of cued fear memory produced a dose-dependent reduction of freezing to levels very similar to the ones of MHB-CB₁-KO mice (Figures 3A, 3B, S3A, and S3B; Tables S1 and S2), indicating that presynaptic MHB-CB₁R acutely control expression of aversive memories in the IPN.

First, we performed dose-response experiments injecting the NMDA receptor antagonist MK801, the AMPA receptor antagonist NBQX, or the nicotinic receptor antagonist mecamylamine (MEC) into the IPN before retrieval test of cue-conditioned C57BL/6-N mice. Higher doses (3 μg of MK801 or MEC, 0.1 μg of NBQX, respectively) reduced conditioned freezing (Figures S3C–S3H; Table S2). However, lower doses (1 μg of MK801 or MEC, and 0.05 μg of NBQX, respectively) were devoid of any effect (Figures S3C–S3H; Table S2), and were chosen as “subeffective” doses for further experiments. The intra-IPN injection of 1 μg MK801 or 0.05 μg NBQX before retrieval test did not alter tone-induced freezing of either conditioned MHB-CB₁-WT or MHB-CB₁-KO mice (Figures 3C–3F; Table S1). Conversely, intra-IPN infusion of 1 μg MEC had no effect on MHB-CB₁-WT controls, but it fully rescued the freezing behavior of the mutants (Figures 3G and 3H; Table S1), without altering locomotion (Figures S3I–S3K; Table S2) or unconditioned freezing before tone presentation (Figure 3G). Furthermore, the same treatment with MEC before COA test also rescued the conditioned aversion of MHB-CB₁-KO mice (Figure 3I; Table S1).

These data indicate that CB₁R-dependent control of cholinergic, but not glutamatergic, neurotransmission in the



(legend on next page)

MHb-to-IPN circuit mediates the expression of aversive memories, independently of the sensory modalities involved.

CB₁R in the IPN Selectively Control Cholinergic Transmission

Cholinergic/glutamatergic afferents from ventral MHb neurons target mainly the medial part of the IPN, whereas dorsal MHb neurons express Substance P and mainly project to the lateral portions of the IPN (Eckenrode et al., 1987; Hsu et al., 2014; Ren et al., 2011). Immunohistochemistry revealed that MHb-CB₁R deletion increased the number of cells expressing the marker of neuronal activity c-Fos in the medial IPN, with no effect in the lateral portion (Figures S4A and S4C; Table S2). Thus, MHb-CB₁R appear to exert a specific presynaptic control of the activity of MHb cholinergic neurons in the medial IPN. To directly test this possibility, we performed electrophysiological recordings in IPN slices derived from mutant mice expressing the light-gated cation channel ChannelRhodopsin-2 (ChR2), exclusively in cholinergic neurons (ChAT-ChR2-EYFP mice; Figures S4D–S4F), which have been used to study the MHb-to-IPN cholinergic pathway (Hu et al., 2012; Ren et al., 2011). Light-induced stimulation of cholinergic terminals in these slices can simultaneously evoke both acetylcholine and glutamate release onto IPN neurons (Hu et al., 2012; Ren et al., 2011; Figure 4). The application of the CB₁R antagonist AM251 was not able to significantly alter light-evoked presynaptic glutamatergic release in the IPN (Figure 4A–4D; Table S1). In contrast, the same treatment increased cholinergic neurotransmission evoked by photostimulation (Figures 4E–4H; Table S1). Thus, presynaptic CB₁R control cholinergic, but not glutamatergic, neurotransmission in the MHb-to-IPN pathway.

DISCUSSION

These data show that the expression of aversive memories is under the control of CB₁R in MHb neurons synapsing onto IPN cells. Genetic deletion of MHb-CB₁R and pharmacological inhibition of CB₁R activity in the IPN both result in reduced aversive acquired responses, without altering appetitive or “neutral” memories. Thus, the ECS specifically controls the MHb-to-IPN circuit to promote aversive memory expression. Our data indicate a specific role of MHb-CB₁R in expression of aversive memories, but a role of these receptors in other phases of learning

(e.g., acquisition or consolidation) cannot be currently excluded. However, MHb-CB₁R are clearly necessary for the expression of acquired aversive memories under different sensory modalities.

This physiological function of CB₁R is surprising, because large evidence indicates that the global genetic deletion or systemic pharmacological blockade of CB₁R during aversive memory retrieval generally results in increased expression (or decreased extinction) of conditioned freezing (Busquets-Garcia et al., 2015; Marsicano et al., 2002; Morena and Campolongo, 2014; Riebe et al., 2012). Thus, the present data reveal potentially opposite consequences of general endocannabinoid actions as compared to their regulation of specific circuits. Interestingly, other recent studies revealed that the functions of CB₁R depend on the cell type(s) and the circuit(s) on which they are activated (Busquets-Garcia et al., 2015). For instance, despite a general hyperphagic role of the ECS (Bellocchio et al., 2010; Di Marzo et al., 2001), certain populations of CB₁R in specific neuronal types and/or in specific circuits exert opposite functions, either promoting or inhibiting food intake (Bellocchio et al., 2010; Busquets-Garcia et al., 2015; Soria-Gómez et al., 2014a, 2014b). The present data suggest that, in front of a general inhibitory function of the ECS on the expression of aversive memories (Busquets-Garcia et al., 2015; Marsicano et al., 2002; Morena and Campolongo, 2014; Riebe et al., 2012), MHb-CB₁R exert the opposite promoting function. This “polymodal” regulation of behavior by CB₁R represents an example of the emerging self-regulatory and fine-tuned control of brain processes.

From the mechanistic point of view, our data consistently indicate that MHb-CB₁R mediate the expression of aversive memories via selective presynaptic modulation of cholinergic, but not glutamatergic transmission in the IPN. The behavioral phenotypes of MHb-CB₁-KO mice are fully rescued by partial inhibition of nicotinic receptors in the IPN, but not by the same approach targeting NMDA or AMPA glutamate receptors. Considering the impact of CB₁R on glutamatergic transmission in other brain regions (Busquets-Garcia et al., 2015), it is still possible that MHb-CB₁R control other behaviors by modulating glutamate release in the IPN. Consistent with the behavioral results, modulation of IPN acetylcholine, but not of glutamate neurotransmission, is under the control of CB₁R in electrophysiological settings. Corelease of glutamate by neurons that were generally thought to use only modulatory neurotransmitters such as acetylcholine, serotonin, or dopamine has been recently

Figure 3. CB₁R Mediate the Expression of Aversive Memories by Modulating Cholinergic, but Not Glutamatergic, Activity in the IPN

(A and B) Time course (A) and total (B) freezing responses of C57Bl/6N mice undergoing cued fear conditioning after pretest intra-IPN infusion of the CB₁ receptor antagonist AM251 1 μg (n = 5), AM251 3 μg (n = 6), or vehicle (VEH, n = 15).

(C and D) Time course (C) and total (D) freezing responses of MHb-CB₁-WT and MHb-CB₁-KO mice undergoing cued fear conditioning after pretest intra-IPN infusion of vehicle (VEH) or the glutamatergic NMDA receptor antagonist MK801 (1 μg). MHb-CB₁-WT VEH, n = 6; MHb-CB₁-WT MK801, n = 8; MHb-CB₁-KO VEH, n = 11; MHb-CB₁-KO MK801, n = 8.

(E and F) Time course (E) and total (F) freezing responses of MHb-CB₁-WT and MHb-CB₁-KO mice undergoing cued fear conditioning after pretest intra-IPN infusion of vehicle (VEH) or the AMPA receptor antagonist NBQX (0.05 μg). MHb-CB₁-WT VEH, n = 6; MHb-CB₁-WT NBQX, n = 6; MHb-CB₁-KO VEH, n = 6; MHb-CB₁-KO NBQX, n = 9.

(G and H) Time course (G) and total (H) freezing responses of MHb-CB₁-WT and MHb-CB₁-KO mice undergoing cued fear conditioning after pretest intra-IPN infusion of vehicle (VEH) or the nicotinic receptor antagonist mecamylamine (MEC, 1 μg). MHb-CB₁-WT VEH, n = 10; MHb-CB₁-WT MEC, n = 6; MHb-CB₁-KO VEH, n = 11; MHb-CB₁-KO MEC, n = 9.

(I) Odorized water intakes of MHb-CB₁-WT and MHb-CB₁-KO mice undergoing conditioned odor aversion (n = 7–13 per group) after pretest intra-IPN infusion of VEH or MEC (1 μg). CS+, conditioned odor; CS-, neutral odor. Data, means ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001. For details of statistical analyses, see Table S1. See also Figure S3.

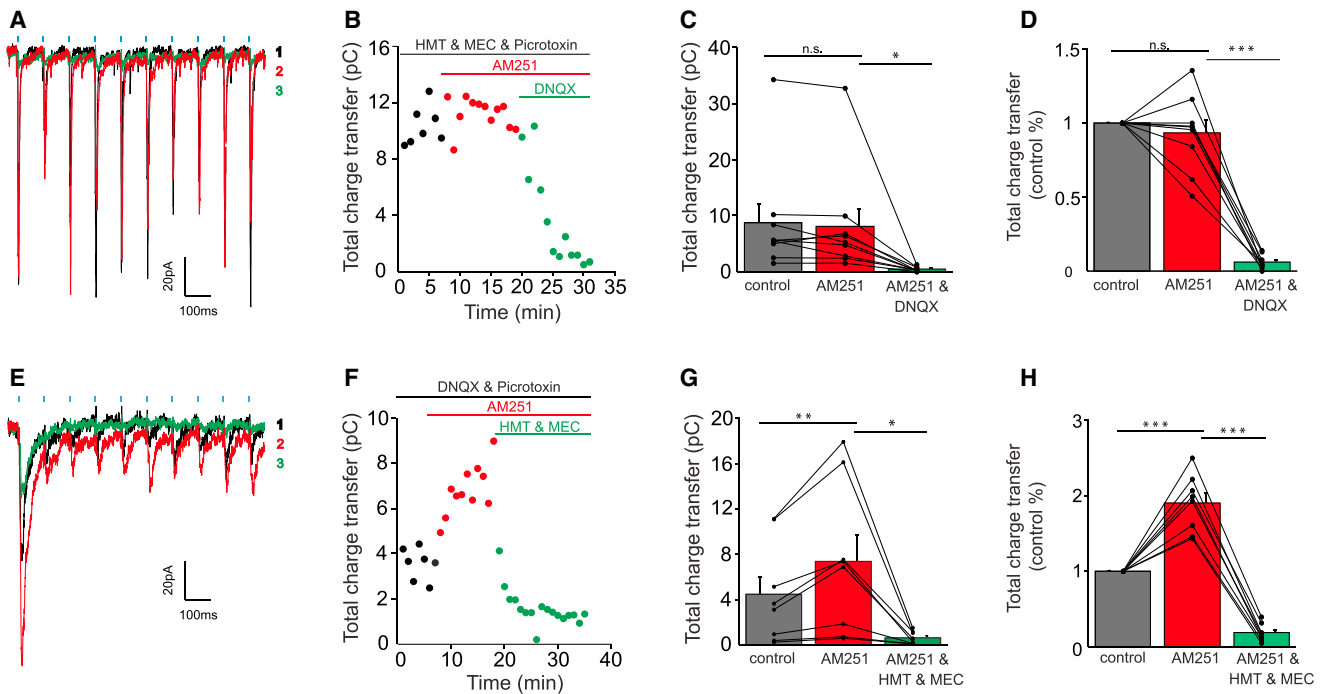


Figure 4. CB₁R Activity Specifically Regulates Cholinergic Transmission in the MHb-to-IPN Circuit

(A–D) The CB₁R antagonist AM251 does not affect glutamatergic transmission from the MHb to the IPN. IPN neurons in brain slices were recorded in the whole-cell voltage-clamp mode. EPSCs were evoked by a train of light stimulation (5 ms, 10 Hz; blue bar) of MHb axonal terminals of ChAT-ChR2-EYFP mice.

(A) Light-evoked glutamatergic EPSCs traces from a representative IPN neuron before (black trace and symbols) and during (red traces and symbols) the application of AM251 (10 μM). Each trace represents the average of three consecutive sweeps. Glutamatergic currents were isolated under constant perfusion of nicotinic acetylcholine receptors (nAChR) blockers hexamethonium (HMT, 50 μM) and mecamylamine (MEC, 10 μM) and GABA_A receptor blocker picrotoxin (50 μM). The EPSCs were completely abolished by AMPA-type glutamate receptor blocker DNQX (10 μM, green traces and symbols).

(B) Plots depicting the total charge transfer across all EPSCs versus time for the same cell as in (A). Colors indicate the different treatments as in (A).

(C) Summary data of the effect of AM251 on glutamatergic EPSCs (n = 9 cells).

(D) Normalized data of the effect of AM251 on glutamatergic EPSCs (same cells as in (C)).

(E–H) The CB₁ receptor antagonist AM251 blocks cholinergic transmission from the MHb to the IPN. Cholinergic EPSCs were elicited by a train of light stimulation of MHb terminals (5 ms, 10 Hz; blue bar) and isolated by DNQX and picrotoxin (black traces and symbols).

(E) Light-evoked cholinergic EPSCs traces from a representative IPN neuron before (black trace and symbols) and during (red traces and symbols) the application of AM251 (10 μM). AM251 application enhanced the EPSCs of an IPN neuron. These EPSCs were completely blocked by nicotinic blockers HMT (50 μM) and MEC (10 μM, green traces and symbols), demonstrating their cholinergic nature. Traces represent the average of three consecutive sweeps.

(F) Plots depicting the total charge transfer across all EPSCs versus time for the same cell as in (E). Colors indicate the different treatments as in (E).

(G) Summary data of the AM251 effect on cholinergic EPSCs (n = 8 cells).

(H) Normalized data of the effect of AM251 on cholinergic EPSCs (same cells as in (G)). ***p < 0.001; **p < 0.01; *p < 0.05; n.s., not significant. For details of statistical analyses, see Table S1. See also Figure S4.

demonstrated (Ren et al., 2011; Stuber et al., 2010; Tecuapetla et al., 2010; Varga et al., 2009) and could involve specific anatomical microdomains in single axons (Zhang et al., 2015). The present data provide an example of the behavioral relevance of these unconventional synaptic events. Similar to our results, recent data show that CB₁R activity can determine the identity of the neuromodulators used by single hypothalamic neurons (Koch et al., 2015). However, the mechanistic explanation of this neurotransmitter-specific regulation is not currently known. Frequency-dependent neuronal activity could determine cellular functions of MHb-CB₁R, as previously shown in GABAergic hippocampal neurons (Földy et al., 2006).

Presynaptic control of cholinergic transmission by MHb-CB₁R is consistent with the location of CB₁R mRNA in the ventral part of MHb and of CB₁R protein in the medial part of the IPN, known

to receive cholinergic MHb inputs (Eckenrode et al., 1987; Hsu et al., 2014; Ren et al., 2011). CB₁R are also present in the dorsal part of the MHb and at its main target region, the lateral portion of IPN. Nevertheless, our data clearly suggest that this subpopulation of dorsal MHb-CB₁R does not participate in the expression of aversive memories. Previous studies showed that afferents to the dorsal or ventral parts of the MHb differentially regulate immediate postshock freezing, in apparent contradiction to our present results (Yamaguchi et al., 2013). However, by focusing on the CB₁R-dependent presynaptic control of efferent MHb projections onto long-term fear memory expression, our study addressed different aspects. As dorsal and ventral MHb afferents and efferents also likely contain presynaptic CB₁R, however, their specific behavioral role(s) will be an interesting issue for future studies.

The present data are also in agreement with the known ability of the ECS to modulate cholinergic transmission in other brain regions (Degroot et al., 2006). In particular, our data clearly indicate a presynaptic control of cholinergic release at MHb-to-IPN synapses by MHb-CB₁R during expression of aversive memories. Nicotinic receptors play a key role in these mechanisms, but the exact cellular targets of acetylcholine in this context are still to be determined. Indeed, nicotinic receptors are present at postsynaptic sites in the IPN, but also likely at presynaptic level (autoreceptors) or even on glial cells (Covernton and Lester, 2002; Liu et al., 2015). Thus, it will be interesting to address the “post-release” mechanisms linking CB₁R-dependent control of cholinergic transmission in the IPN to the expression of aversive memories.

Altogether, this study shows that CB₁ receptors control the expression of aversive memories through the modulation of MHb-to-IPN cholinergic, but not glutamatergic, neurotransmission. Both the MHb and the ECS have been implicated in the pathogenesis of diseases linked to emotional processing such as depression, anxiety disorders or drug addiction (Hsu et al., 2014; Morena and Campolongo, 2014; Sandyk, 1991). Considering that the control of aversive memories is an integral part of these disorders, the present data suggest that MHb-CB₁R might represent a potential therapeutic target to tackle important brain diseases.

EXPERIMENTAL PROCEDURES

Methods, materials, and any associated references are described in detail in [Supplemental Experimental Procedures](#).

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, two tables, and Supplemental Experimental Procedures and can be found with this article at <http://dx.doi.org/10.1016/j.neuron.2015.08.035>.

AUTHOR CONTRIBUTIONS

E.S.-G., G.F., M.L., and G.M. designed research; E.S.-G., A.B.-G., A.M., F.H., A.C., L.R., I.L., L.A., T.W., D.V., and P.V. performed research; E.S.-G., G.F., M.L., and G.M. supervised research; E.S.-G., A.B.-G., A.M., F.H., G.F., M.L., and G.M. analyzed data; E.S.-G. and G.M. wrote the manuscript. All authors edited the manuscript.

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