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Habenular neurons expressing mu opioid receptors promote negative affect in a projection-specific manner

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Abstract

BACKGROUND—The mu opioid receptor (MOR) is central to hedonic balance, and produces euphoria by engaging reward circuits. MOR signaling may also influence aversion centers, and notably the habenula (Hb), where the receptor is highly dense. Our previous data suggest that the inhibitory activity of MOR in the Hb may limit aversive states. To investigate this hypothesis, we here tested whether neurons expressing MOR in the Hb (Hb-MOR neurons) promote negative affect.

METHODS—Using *Oprm1*-Cre knock-in mice, we combined tracing and optogenetics with behavioral testing to investigate consequences of Hb-MOR neuron stimulation in approach/avoidance (real-time place preference), anxiety-related responses (open field, elevated plus maze and marble burying) and despair-like behavior (tail suspension).

RESULTS—Opto-stimulation of Hb-MOR neurons elicited avoidance behavior, demonstrating that these neurons promote aversive states. Anterograde tracing showed that, in addition to the interpeduncular nucleus (IPN), Hb-MOR neurons project to the dorsal raphe nucleus (DRN). Opto-stimulation of Hb-MOR/IPN terminals triggered avoidance and despair-like responses with no anxiety-related effect, whereas light-activation of Hb-MOR/DRN terminals increased levels of anxiety with no effect on other behaviors, revealing two dissociable pathways controlling negative affect.

CONCLUSION—Together, the data demonstrate that Hb neurons expressing MOR facilitate aversive states via two distinct Hb circuits, contributing to despair-like behavior (Hb-MOR/IPN)

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JB, ED and BLK designed the study; FP performed animal care and genotyping; JB, FA and CT acquired behavioral data; CT and CD contributed to immunohistochemistry and optogenetic experiments; JB, FA, ED and BLK performed the analysis, interpreted data and wrote the manuscript. MAD and ES designed, performed and analyzed electrophysiological experiments. All authors read and approved the submitted version.

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and anxiety (Hb-MOR/DRN). The study supports the notion that inhibition of these neurons by either endogenous or exogenous opioids may relieve negative affect, a mechanism that would have implications for hedonic homeostasis and addiction.

Keywords

opioids; habenula circuitry; avoidance; anxiety; despair-like behavior; optogenetics

INTRODUCTION

The endogenous opioid system regulates hedonic homeostasis, and the mu opioid receptor (MOR) is an essential actor in this process. This receptor mediates the strong euphorogenic properties and abuse potential of medicinal and abused opioid drugs (1, 2). The MOR also contributes to rewarding effects of non-opioid drugs of abuse (3, 4), as well as social and food reward (5, 6), and pharmacological MOR blockade is aversive in both animals and humans, demonstrating that endogenous MOR signaling has rewarding activity (7, 8). Overall, it is well established that MOR activation by opioid drugs or endogenous opioid peptides positively influences the hedonic tone.

Pharmacological experiments have demonstrated that MOR activation is rewarding at several brain sites (9). The positive hedonic effects of MOR activity have been particularly studied within the mesolimbic dopamine circuitry, considered a main reward pathway (10). MOR is also densely expressed in other hedonic hot spots of the brain (9), potentially implicating broader brain circuits in hedonic control. Furthermore, MOR may act at the level of both reward and aversion brain pathways (11) to either facilitate reward processing or limit the impact of aversive stimuli (8). The latter mechanism, however, has been poorly explored.

Intriguingly, MOR is densely expressed in the habenula (Hb) (12, 13), a major aversion center (14). In rodents, this evolutionarily conserved brain structure is divided in a medial (MHb) and a lateral (LHb) subdivision (15) and connects forebrain to midbrain regions, to integrate cognitive with emotional and sensory processing (13, 16). The MOR is expressed predominantly in the MHb, composed of cholinergic and Substance P neurons projecting to the interpeduncular nucleus (IPN), and is also scarcely detected in the lateral part along to the MHb/LHb border (12, 13). Several studies have suggested a role for the MHb in drug withdrawal, which is widely regarded as a strong aversive state. In particular, the MHb showed hyperactivity during nicotine withdrawal (17–19) and the nicotinic system in the MHb was shown to functionally interact with the opioid system in naltrexone-precipitated withdrawal (20–22). Thus, MOR signaling in the habenula likely influences the postulated aversive activity of Hb neurons.

We recently deleted the MOR gene in Chnrb4-positive neurons of the MHb, eliminating about half of habenular MORs, and showed that place aversion conditioning by naloxone administration was less efficient in mutant mice (23). This was the first genetic evidence that the aversive effect of naloxone is mediated, at least in part, by MORs operating at the level of the Hb circuitry. Because MORs are inhibitory receptors, this finding led us

to hypothesize that endogenous MOR signaling normally acts as a brake on Hb neurons expressing the receptor to limit aversive states.

To further test this hypothesis, we focused our attention on this particular neuronal population, thereafter referred to as Hb-MOR neurons, which we expected would encode aversive states. This neuronal population includes mainly MHb neurons and also a few cells in the LHb. We used our newly created *Oprm1*-Cre mice (24) to manipulate Hb-MOR neurons and establish whether these neurons, which normally respond and are inhibited by MOR opioids, indeed encode a negative emotional state. Data from the present study first confirm the original observation, and then demonstrate that opto-activation of Hb-MOR neurons induces aversive states. Moreover, we found that the nature of negative emotional responses differs depending on whether these neurons project to the IPN (Hb-MOR/IPN), the canonical output region of MHb efferences, or to the dorsal raphe nucleus (Hb-MOR/DRN), which we identified as a target site for Hb-MOR cells.

METHODS AND MATERIALS

See Supplementary Information for detailed Methods and Materials

Animals

Conditional *Chnrb4*-MOR mouse line(23) and *Oprm1*-Cre knock-in mouse line(24) were group housed (maximum of five mice per cage) in a temperature- and humidity-controlled animal facility ($21 \pm 2^\circ\text{C}$, $45 \pm 5\%$ humidity) on a 12 h dark/light cycle with food and water available *ad libitum*. All experiments were performed in accordance with the Canadian Council of Animal Care and by the Animal Care Committees and by the Animal Care Committees and with French and European regulations, with authorization APAFYS No.10185–2017061212199805.

Optogenetics and behavior

For optogenetic experiments, adult *Oprm1*^{Cre/Cre} male mice were injected unilaterally with 300 nL of AAV2.EF1a.DIO.ChR2-mCherry (RRID:Addgene_20297) or AAV2.EF1a.DIO.mCherry in the medial subdivision of the habenula (AP: -1.35 , ML: -1.55 , DV $-2.9/2.8/2.7$; volume of injection: 3×100 nL, angle 32°) and implanted above the habenula (AP: -1.35 , DV: -2.5 from dura, ML: -1.55 , angle 32°), the IPN (AP: -3.2 , ML: -0.5 , DV: -4.7 , angle 6°) or the DRN (AP: -4.15 , DV: -3.3 , ML: 0). Behavioral experiments included naloxone place conditioning, real-time place preference, open-field, marble burying, elevated-plus-maze and tail suspension are detailed in Supplementary Information.

Immunohistochemistry for tracing studies

Adult *Oprm1*^{Cre/Cre} male mice were injected unilaterally with 300 nL of AAV2.EF1a.DIO.ChR2-mCherry (RRID: Addgene_20297) as for the optogenetic experiments. A primary anti ds-red antibody was used (1:2000, Takara Bio) for immunostaining in a standard protocol detailed in Supplementary Information.

C-fos immunohistochemistry

Mice were light-stimulated for 3 minutes (at 473 nm, 10 mW, 20 Hz, 10 ms pulse width) in their home cage 90 minutes prior to perfusion for inducing c-fos expression. For each animal (n=5/groups for Figure 1 and Figure 3), equivalent sections were chosen for cell counting using the Allen Brain Atlas as a reference. Immunohistochemistry was performed using a c-fos primary antibody (1:2000, Cell signaling technology) for 4 sections (Hb) and 3 sections (IPN), as described in Supplementary Information.

Ex vivo electrophysiology

Ex vivo electrophysiology was performed on adult (3 months old) *Oprm1^{Cre/Cre}* mice previously infused with either the AAV2.EF1a.DIO.ChR2-mCherry or the AAV2.EF1a.DIO.mCherry viruses, using coordinates of optogenetic experiments. Coronal brain slices (300 μ m) including the Hb were prepared minimum 4 weeks following surgery. Electrophysiological experiments are described in Supplementary Information.

RESULTS

Confirmation that a mu opioid tone modulates aversion in the habenula

Our previous results showed that conditional MOR deletion in the Hb reduces naloxone-induced conditioned place aversion (CPA) in both naïve and morphine-dependent mice (23). We first consolidated the original finding in naïve mice, using an extended conditioning/testing paradigm (Supplementary Figure S1). Conditional *Chnrb4*-MOR mice (lacking MOR in cells expressing the $\beta 4$ subunit of the nicotinic acetylcholine receptor, representing about 50% of habenular MORs) and their control littermates were subjected to conditioned place aversion (CPA) to naloxone. Naloxone (10 mg/kg) induced CPA in both control and *Chnrb4*-MOR mice. The effect was detected along the entire testing procedure (three-way RM ANOVA on tests days 1–16 and 73; Main effect of Treatment, $F_{(1,43)} = 29.094$, **** $p < 0.0001$), and quantitatively depended upon the test day (Treatment x Time interaction effect, $F_{(5,215)} = 9.990$, **** $p < 0.0001$). Aversion to the naloxone-paired compartment was lower in *Chnrb4*-MOR mice compared to their control littermates over the entire procedure (main effect of Genotype, $F_{(1,21)} = 8.550$, $p = 0.0081$). Examined at day 24, 30, 34 and 37 following conditioning, extinction of the naloxone-induced CPA phenotype occurred in both genotypes. However, when a further test session was performed at day 73, only control mice still showed significant place aversion (see statistical details in Supplementary Table S1).

These data show that a long-lasting aversion for the naloxone-paired compartment developed in control mice, which was resistant to extinction, and that in contrast, *Chnrb4*-MOR mice showed a lower aversion that fully reversed upon extinction. This result definitely confirms that the well-described aversive effect of naloxone originates, at least partially, from blocking endogenous MOR signaling in the Hb. Because MOR is an inhibitory receptor, the activity of Hb-MOR neurons should therefore promote aversive states. Testing this hypothesis is the goal of the present study.

Optogenetic stimulation of Hb-MOR neurons induces real-time place avoidance

We first tested the behavioral effect of Hb-MOR neuron activation using optogenetics in a Real-Time Place Test (RTPT) setting. We injected a Cre-dependent AAV2-channelrhodopsin virus in the Hb of *Oprm1*-Cre mice, using stereotaxic coordinates of the MHb to target the majority of Hb-MOR neurons (see Methods), and implanted an optic fiber above the Hb, in order to stimulate cell bodies of Hb-MOR neurons (Figure 1A; 20 Hertz light pulse trains). Hb-MOR/ChR2 mice spent significantly less time in the stimulation side compared to the control group (injected with a Cre-dependent mCherry-expressing AAV; unpaired t test, $t_{(19)} = 2.484$, $*p < 0.05$ Figure 1B–C). The stimulation did not affect the total distance travelled by the mice (unpaired t test, NS; Figure. 1C). Somatic opto-stimulation of Hb-MOR neurons, therefore, is aversive.

To confirm that the opto-stimulation conditions successfully increased neuronal activity, we quantified c-fos expression using immunostaining (Figure 1D–E). We found a significant increase in the number of c-fos+ cells in response to light stimulation in the brain of Hb-MOR/ChR2 mice compared to control animals, both at the level of the fiber optic implant (Hb, unpaired t-test, $t_{(9)} = 3.007$, $**p < 0.01$) and of the main projection site (IPN, unpaired t-test, $t_{(9)} = 3.218$, $**p < 0.01$), confirming efficacy of stimulation parameters. We also performed *ex-vivo* electrophysiological recordings in slices from Hb-MOR/ChR2 mice, at the level of the MHb where Hb-MOR neurons are most dense. Under a loose-cell attached configuration (Figure 1F), MHb neurons were spontaneously active, as previously reported (25). Light pulses at 20Hz (30 s-long trains) increased the firing activity of ChR2-expressing neurons (from 2.8 ± 0.4 Hz in the pre-stimulation period to 24.4 ± 2.3 Hz; $***p < 0.0001$, $n=26$ and 20.5 ± 1.9 Hz; $***p < 0.0001$; $n=26$ during the first and last 5s of stimulation, respectively; Wilcoxon paired signed rank test in both cases). Typical of MHb neurons (25), the intense increase of action potential frequency was followed firstly by a short inhibitory period, then by full recovery of the control firing activity. Experiments using a whole cell voltage clamp configuration also showed large light-elicited currents (Supplementary Figure S2). Neither firing frequency changes nor whole cell excitatory currents were induced by the light pulses in *ex vivo* MOR-MHb neurons from control mice (data not shown). Both c-fos and electrophysiology data therefore confirm that the optogenetic protocol used for behavioral analyses efficiently stimulates Hb-MOR neurons.

Hb-MOR neurons project to the DRN

As most Hb-MOR neurons originate from the MHb subdivision, it was anticipated that the main projection site would be the IPN (16). We tested whether other projection sites could be identified. We injected an anterograde Cre-dependent AAV2-mCherry virus in the Hb of *Oprm1*-Cre animals (see Methods), and amplified the signal by immunostaining after 4 weeks viral expression. As expected, we observed a strong fluorescent signal in both rostral and lateral parts of the IPN. Interestingly, we also identified significant fluorescence in the raphe nucleus, including dorsal raphe (DRN) and median raphe (MRN) nuclei (Figure 2). The DRN is of particular interest in the context of opioid addiction, as we previously showed that the targeted deletion of MORs in the DRN prevents the development of social interaction deficits characterizing mice abstinent from chronic heroin (26). The identification of Hb-MOR neurons projecting to the DRN, therefore, prompted us to investigate the

behavioral consequences of optogenetic stimulation for either Hb-MOR neurons projecting to the IPN (Hb-MOR/IPN) or those projecting to the DRN (Hb-MOR/DRN).

Optogenetic stimulation of Hb-MOR neuron projecting to the IPN produces real-time place avoidance and a despair-like behavior

To test the role Hb-MOR/IPN neurons, we injected the Cre-dependent AAV2 channelrhodopsin virus in the Hb of *Oprm1*-Cre mice as before, and implanted an optic fiber above the IPN (Figure 3A). Light-stimulation produced a strong place avoidance in the RTPT (Figure 3 B–C) (two-way RM ANOVA; significant frequency x virus interaction $F_{(3,66)} = 7.468$, $***p < 0.001$). Hb-MOR/IPN-ChR2 mice spent significantly less time in the light-paired chamber at both the 10 Hz ($**p < 0.01$) and 20 Hz stimulation frequencies tested ($***p < 0.001$) compared to control mice, indicating that the optical stimulation was aversive. The stimulation did not affect total locomotor activity (measured at 20 Hz; unpaired t test, NS).

In the elevated-plus-maze test (Figure 3D), MOR-MHb/IPN-ChR2 and control mice spent the same amount of time in open and closed arms, and in the center. No difference was detected for several other parameters (number of head dips in open arms, number of entries and time in open arms). In the open-field (Figure 3E), Hb-MOR/IPN-ChR2 and control mice spent the same amount of time in the center of the arena (two-way ANOVA, effect of virus, NS). In both groups, the time spent in the center increased similarly as the test progressed corresponding to a decreased level of anxiety (two-way ANOVA, effect of time, $F_{(3,66)} = 7.109$, $***p < 0.001$). No difference was detected between the two groups in the marble burying test (Figure 3F, unpaired t test, NS). Opto-stimulation of the Hb-MOR/IPN pathway therefore had no effect in any of the three anxiety-related tests.

In the tail suspension test (Figure 3G), opto-stimulation significantly increased the mean immobility time for Hb-MOR/IPN-ChR2 mice compared to controls (two-way ANOVA, effect of virus, $*p < 0.05$), suggestive of a despair-like behavior.

We finally verified again whether the optogenetic stimulation modifies neuronal activity (Supplementary Figure S3A). Stimulation of Hb-MOR/IPN neurons significantly increased c-fos activity in the IPN (unpaired t test, $t_{(8)} = 4.926$, $**p < 0.01$), while no modification of c-fos staining was observed at the level of the Hb (unpaired t test, $t_{(8)} = 0.8992$, $p > 0.05$) (Supplementary Fig. S3B). This result suggests that opto-activation at the level of IPN stimulates axonal terminals of Hb-MOR neurons. Altogether, these results demonstrate that selective stimulation of Hb-MOR neurons projecting to the IPN induces avoidance and despair-like behavior without altering levels of anxiety.

Optogenetic activation of habenular Hb-MOR neuron projecting to the DRN increases anxiety-related behavior

To test the role of Hb-MOR/DRN neurons, we injected the Cre-dependent AAV2 channelrhodopsin virus in the Hb of *Oprm1*-Cre mice as before, and implanted an optic fiber above the DRN (Figure 4A). Opto-stimulation of the Hb-MOR/DRN neuron terminals under the same conditions as in previous experiments, did not trigger any significant effect in the RTPT (Figure 4 B–C). Furthermore, no difference was found between MHb-MOR/

DRN-ChR2 and control mice in the time spent on the stimulation-side, at any stimulation light pulse frequency (two-way RM ANOVA, effect of group, NS). Finally, the stimulation did not affect total locomotor activity measured at 20 Hz in MHb-MOR/DRN-ChR2 mice (unpaired t, NS).

The optical stimulation, however, was anxiogenic. In the elevated-plus-maze test (Figure 4D), two-way ANOVA revealed a significant interaction ($F_{(2,45)} = 15.15$, $***p < 0.001$) for the percentage time spent in the two arms. MOR-MHb/IPN-ChR2 mice spent less time in open arms ($**p < 0.01$) and more time in closed arms ($***p < 0.001$) compared to control mice. In addition, the number of entries in the open arms and the number of head dips were reduced in Hb-MOR/DRN-ChR2 compared to controls (unpaired t test, $t_{(15)} = 2.613$, $*p < 0.05$; unpaired t test, $t_{(15)} = 2.204$, $*p < 0.05$, respectively). Finally, the mean time spent in open arms was shorter for Hb-MOR/DRN-ChR2 mice during the 3–6 min stimulation period compared to control mice. This difference became significant during the subsequent OFF 6–9 min period of the test ($*p < 0.05$), suggesting a durable effect of the stimulation of Hb-MOR/DRN neurons on levels of anxiety that may deserve further exploration. In the open-field arena (Figure 4E), Hb-MOR/DRN-ChR2 mice tended to spend less time in the center, although this effect did not reach statistical significance (two-way ANOVA, effect of groups, NS). The time spent in the center increased similarly for the two groups and, as the test progressed, mice habituated to the open field (two-way ANOVA, effect of time, $F_{(3,54)} = 6.745$, $***p < 0.001$). In the marble burying test (Figure 4F), Hb-MOR/DRN-ChR2 mice displayed a higher marble burying score compared to the control group (unpaired t test, $t_{(17)} = 2.76$, $*p < 0.01$), suggesting an increased level of anxiety upon stimulation. The data together concur to demonstrate that opto-stimulation of the Hb-MOR/DRN pathway is anxiogenic, contrasting with the results obtained from the stimulation of the MHb-MOR/IPN pathway.

We examined the effect of the opto-stimulation in the tail suspension test (Figure 4G). MHb-MOR/DRN-ChR2 mice and their controls showed the same time of immobility (two-way ANOVA, effect of groups, NS), suggesting that light stimulation of the DRN projection does not induce despair-like behavior, again contrasting with the effects of the stimulation of MHb-MOR/IPN neurons.

In sum, our data show that selective stimulation of Hb-MOR neurons projecting to the DRN has a strong anxiogenic effect, but does not seem to induce an aversive state that would result in place avoidance or despair-like behavior. The effects of opto-stimulation of this pathway are therefore the mirror image of those elicited by opto-stimulation of the Hb-MOR/IPN pathway, at least under testing conditions of the present study.

DISCUSSION

In sum (Figure 5A), we first confirm that naloxone aversion is reduced when the MOR is deleted from the Hb (23), setting the hypothesis that MOR, an inhibitory G protein coupled receptor, normally inhibits habenula function and thereby tempers the well-described aversive activity of this brain structure. Second, we demonstrate that stimulation of habenular neurons expressing the MOR is aversive, substantiating the notion that MOR

signaling, which is inhibitory by nature (Gi-coupling), may reduce the aversive activity of these Hb-MOR neurons. Third we show that Hb-MOR neurons project to the DRN, in addition to the main IPN projection area, and demonstrate that Hb-MOR neurons projecting to either IPN or DRN modulate distinct emotional responses, uncovering two pathways controlling negative affect under the control of MOR opioids.

Hb-MOR neurons encode an aversive state

While the habenular complex is known to be critical in the emergence of aversive states (14), the specific role of the abundant Hb-MOR neurons was unknown. Hb-MOR neurons form a unique ensemble of habenular neurons that respond to endogenous opioids and exogenous opiate drugs. Elucidating the function of these neurons, therefore, is of high significance to understand mechanisms underlying hedonic homeostasis, as well as dysfunctional circuitries in addiction and mood disorders.

Here, we demonstrate that the activation of Hb-MOR cell bodies, i. e. of the entire population of Hb-MOR neurons, produces an avoidance behavior (Figure 1). This is consistent with other findings demonstrating that Hb activity, and particularly the medial division where MOR is most dense, promotes negative affective states. Thus, early studies showed that habenular lesions induce deficits in avoidance behavior induced by foot-shock (25) and the overexpression of nicotinic $\beta 4$ receptor, enhancing MHb activity, resulted in a strong aversion to nicotine (27). In addition, inhibiting expression of several genes known to promote habenular activity reduced aversive responses: deletion of CB1 receptors in the MHb inhibited expression of aversive memories (28), and knock-down of habenular RSK2, a protein contributing to intracellular signaling in MHb neurons (29) or knockout of GluN3A, an unconventional subunit of the excitatory NMDA receptor (30), both decreased lithium-induced conditioned place aversion. MOR opioids therefore act on aversion-encoding neurons in the Hb.

Questions remain. Hb-MOR neurons are heterogeneous. Most of these neurons are in the MHb, composed of two main neuronal populations, substance P and cholinergic neurons, with regionally distinct distribution and possibly distinct roles (31), and MOR-positive neurons overlap the two neuronal populations (12, 23). Also, the few MOR-positive neurons detected in the LHb remain uncharacterized. Whether these subpopulations of Hb-MOR neurons play distinguishable roles in the aversive responses observed in the present study remains to be clarified, using for example intersectional viral approaches. Another point is that our study suggests the existence of an aversion-reducing opioid tone in the MHb (the naloxone experiment), and demonstrates that Hb-MOR neurons promote a negative affect (the optogenetic experiments). However, physiological mechanisms of MOR stimulation at these neurons are unknown. The origin and exact nature of endogenous opioid peptides that may potentially act at the MOR in this pathway, or the possibility of constitutive MOR activity at this brain site (32, 33), will require future investigations.

Hb-MOR neurons project to the raphe nucleus

Our anterograde tracing experiments (Figure 2) showed that Hb-MOR neurons project to the rostral and lateral part of the interpeduncular nucleus (IPN), consistent with IPN being

the main projection site for the MHb (14), and with our previous finding of highest MOR expression in both the MHb and these IPN subdivisions (12). Interestingly we also found that Hb-MOR neurons project to several raphe structures including the DRN, which is home to serotonergic neurons and was also shown involved in morphine withdrawal (34, 35). In addition, we also observed anterograde fluorescence in the median raphe nucleus, also containing serotonergic neurons and involved in memory consolidation, in interaction with the hippocampus (36). While LHb- connectivity with raphe nuclei is known (37), the possibility of a direct connectivity between MHb neurons and the raphe is less well described (12, 13) but consistent with data from the Allen Brain Atlas connectivity database ([Experiment 300236056](#)).

The Hb-MOR/DRN pathway is of particular interest, as serotonin neurotransmission is key to mood control, negative affect and depressive behaviors (38, 39). The possibility that Hb neurons expressing the MOR and responding to opioid stimulation, could directly influence raphe nuclei functions has implications for Opioids Use Disorders (OUDs). We may speculate that exposure of Hb-MOR/DRN neurons to exogenous opiates will necessarily alter their activity and impact serotonin balance and mood circuits in the brain. Therefore, characterizing both acute and long-term effects of opiates on these neurons will of great interest to understand the negative mood of opioid withdrawal, which is key for therapeutic strategies (40).

Hb-MOR neurons define two dissociable circuits

Another main finding of this work is that Hb-MOR-neurons promote aversive states in a projection-specific manner (Figure 3 and 4). Our data show that opto-activation of Hb-MOR/IPN neurons produces avoidance and a despair-like behavior, whereas a similar manipulation of Hb-MOR/DRN neurons increases levels of anxiety without effect on the other tested behaviors, highlighting two distinct circuits.

The Hb-MOR/IPN finding is consistent with evidence for a role of the MHb, where Hb-MOR neurons are predominant, in a diversity of emotional responses, including fear, anxiety and depressive-like behaviors (41–45). Also, the pathogenesis of depression was correlated with increased IPN metabolism (46–48) and a recent study using a model of chronic stress demonstrated that hyperactivity of the MHb-IPN pathway is linked to depressive behavior, which was reversed by MHb lesion (49). We therefore speculate that activity of Hb-MOR/IPN neurons may generate a negative affect reminiscent of depression-related states. Less is known about a functional connection between the MHb and the DRN. Recent studies in zebrafish and rats observed that alteration of MHb activity modifies both serotonergic immunoreactivity and gene expression levels in the DRN (50, 51). Our own findings suggest that activity of the Hb-MOR/DRN circuit essentially facilitates anxiety states, distinguishable from depressive-like states elicited by the other circuit. However, a strict separation of despair-type and anxiety-related responses is unlikely, as these complex processes often overlap and for example, the silencing of cholinergic habenular inputs to the IPN did not alter basal anxiety level in naïve mice but reduced the exacerbated-anxiety in mice undergoing nicotine withdrawal (17).

Altogether, findings from this study indicate that Hb neurons responding to MOR opioids modulate several aspects of negative affective states, subserved by at least two distinct circuits. Whether Hb-MOR/IPN and Hb-MORDRN indeed represent two distinct cellular populations remains to be determined. Also, our anterograde tracing experiments revealed projections of Hb-MOR neurons to the lateral hypothalamus (not shown), which may also contribute and deserve further investigations.

Conclusion: reward-promoting and aversion-reducing MOR functions

MOR is broadly expressed throughout reward and mood circuits, which regulate all aspects of hedonic homeostasis and are involved in addiction (2). To date, MOR opioids have been mostly associated to reward processes, and the “pro-reward” function of MOR is well-established. This is the first report demonstrating that morphine-responsive output neurons from the habenular complex, recognized as an anti-reward center, promote negative emotional responses and, in addition, via dissociable downstream pathways. Endogenous opioids may therefore alleviate several features of aversive states through this habenular MOR mechanism. In other words, opioids may not only promote reward within the mesocorticolimbic circuitry, as classically described, but also reduce aversive states in the habenula circuitry, and perhaps other circuits (e. g. the extended amygdala) to overall modulate the hedonic balance (Figure 5B) and contribute to allostatic mechanisms underlying addiction (52).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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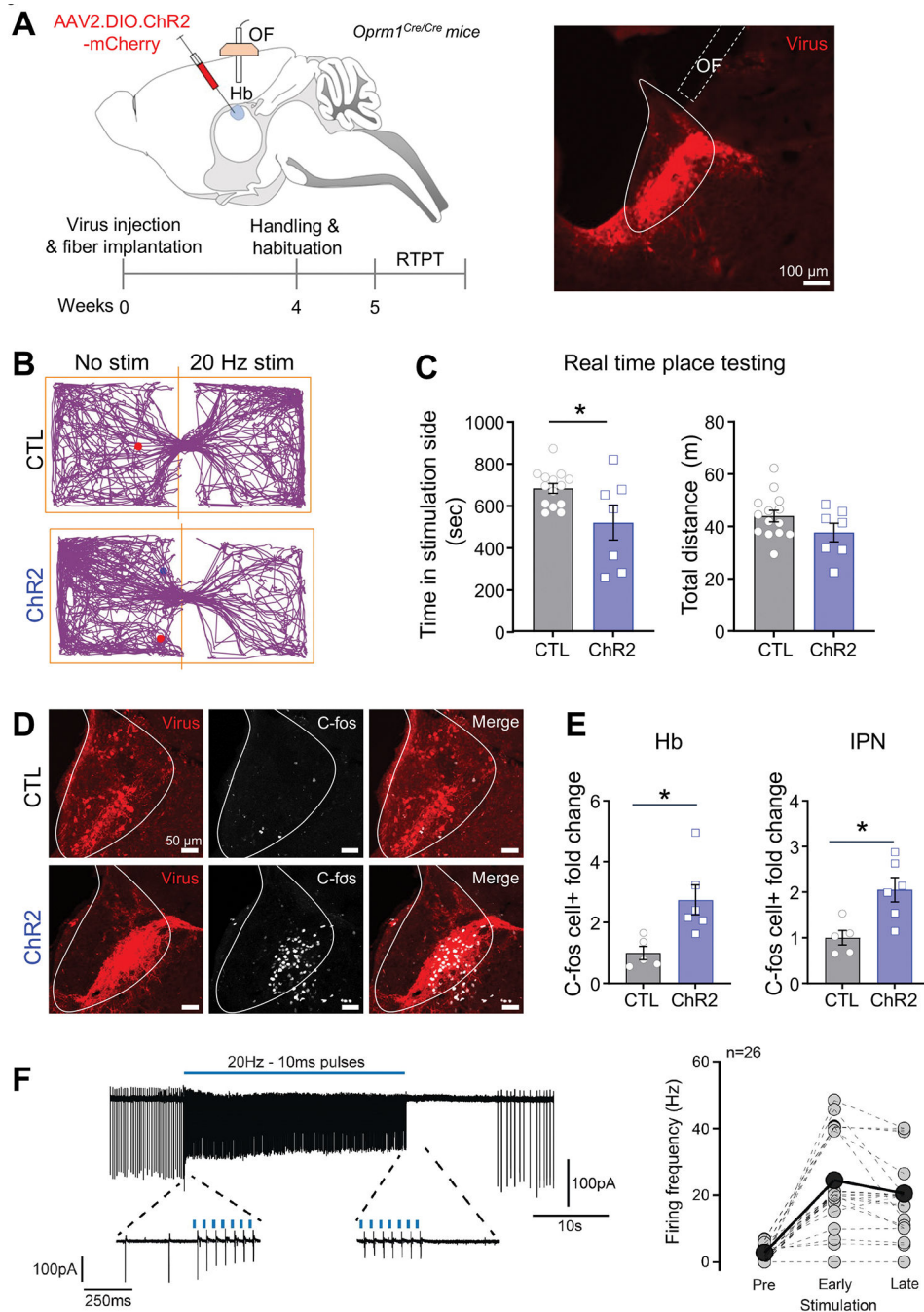


Fig. 1: Opto-stimulation of Hb-MOR neurons induces avoidance behavior.

Oprm1-Cre mice expressing either AAV2.EF1a.DIO.ChR2-mCherry (channelrhodopsin or ChR2) or AAV2.EF1a.DIO.mCherry (control or CTL) were subjected to real-time place preference testing. **A.** Scheme (left) and representative image (right) showing viral expression, with fiber-optic (FO) implantation above the Hb, and timeline for the experimental procedure. **B.** Representative tracking plot of the mice, at 20Hz light pulse frequency (473 nm, 10 mW, 10 ms pulse width). **C.** Light stimulation in Hb-MOR/Hb-ChR2 mice induced significant behavioral avoidance to the light-paired side compared with

control group ($n = 6-12/\text{group}$) without affecting the total distance traveled. $*p < 0.05$. **D.** Confocal imaging of Hb sections demonstrating light-induced neuronal activation using c-fos as a marker of neuronal activity. ChR2-mCherry viral expression (red) and c-Fos immunostaining (white) are shown from representative sections. **A** and **D**: white traces localize the MHb. **E.** Quantification of c-Fos-positive cells upon light stimulation in the Hb and IPN of ChR2 and CTL animals. **F.** Effect of the optostimulation on *ex vivo* MHb-MOR neuron firing. Recordings of MHb-MOR cells were performed in acute slices from *Oprm1*-Cre mice, infected with AAV2.EF1a.DIO.ChR2-mCherry. Left. Representative cell-attached recording showing spontaneous firing of a ChR2-expressing MHb-MOR neuron. A 30 s-long train of blue light pulses (470nm; 10ms, 20Hz; blue line on top) induced a reversible, powerful increase of the firing. Right. Quantification for all recorded cells. The dotted lines link the action potential frequency values of individual neurons during the 5 s preceding stimulation (Pre), the first (Early stimulation) and the last 5 s of the light pulse train. The increase in firing was highly significant in both test periods ($p < 0.001$). Means are represented as $\pm \text{SEM}$.

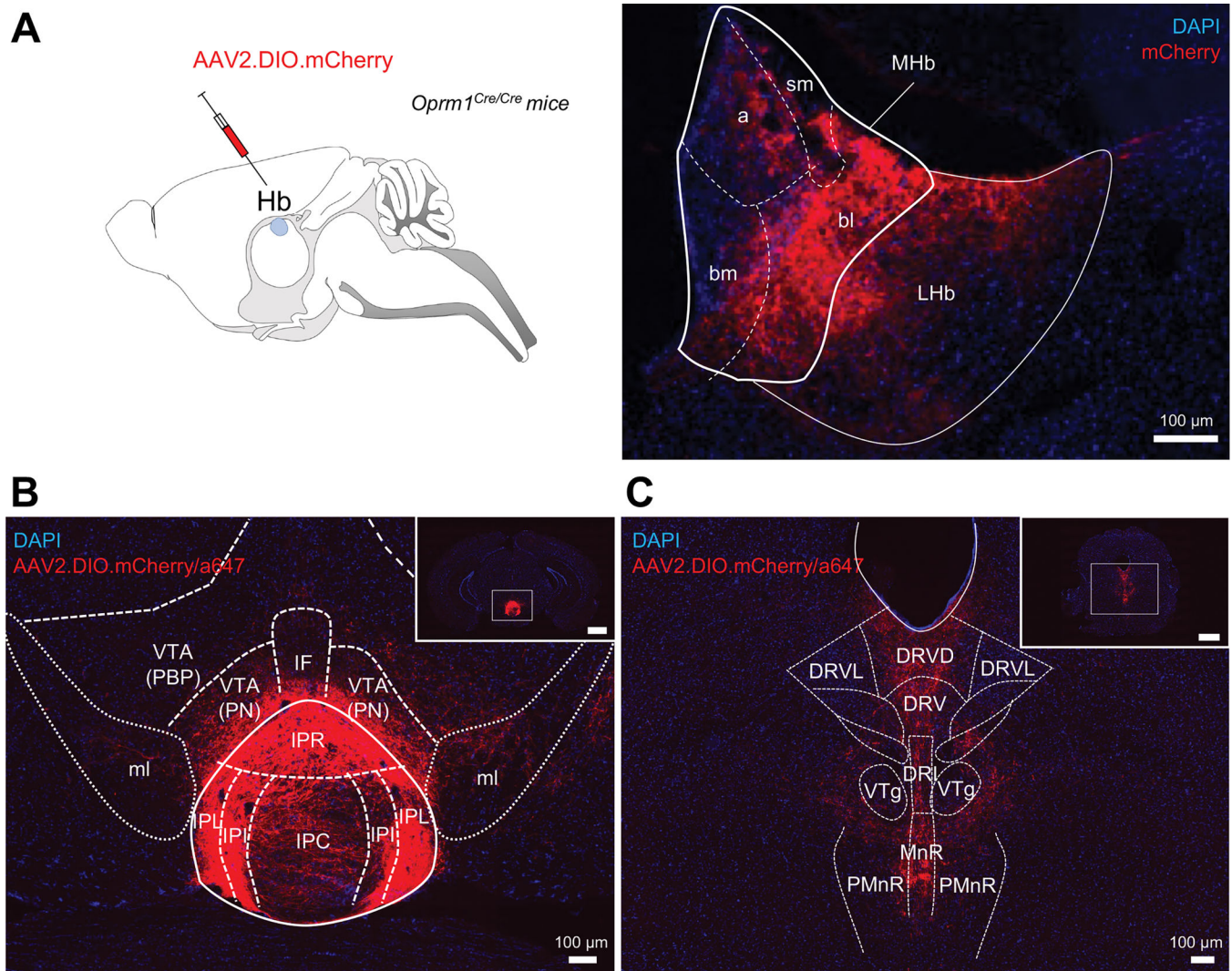


Fig. 2: Identification of projection sites of Hb-MOR neurons.

A. Anterograde tracing using an AAV2-DIO-mCherry virus was performed in the Hb of *Oprm1-Cre* mice, to visualize Hb-MOR projections (n=3). **B, C.** Hb-MOR neurons send dense projections to the rostral and lateral part of the interpeduncular nucleus (B) as well as to raphe regions (C). a= apical, bl= basolateral, bm= basomedial, DRI/DRV/DRD/DRVL: dorsal raphe nucleus interfascicular, ventral, dorsal, ventral lateral, nucleus IPN/IPR/IPL/IPC= interpeduncular nucleus, rostral, lateral, intermediate, Hb= habenula, LHb= lateral habenula, MHb= medial habenula, ml= medial lemniscus, MnR= median raphe nucleus, PBP= parabrachial pigmented area of VTA, PMnR= paramedian raphe nucleus, PN= paranigral nucleus of VTA, sm= stria medullaris, VTA = ventral tegmental area, VTg= ventral tegmental nucleus.

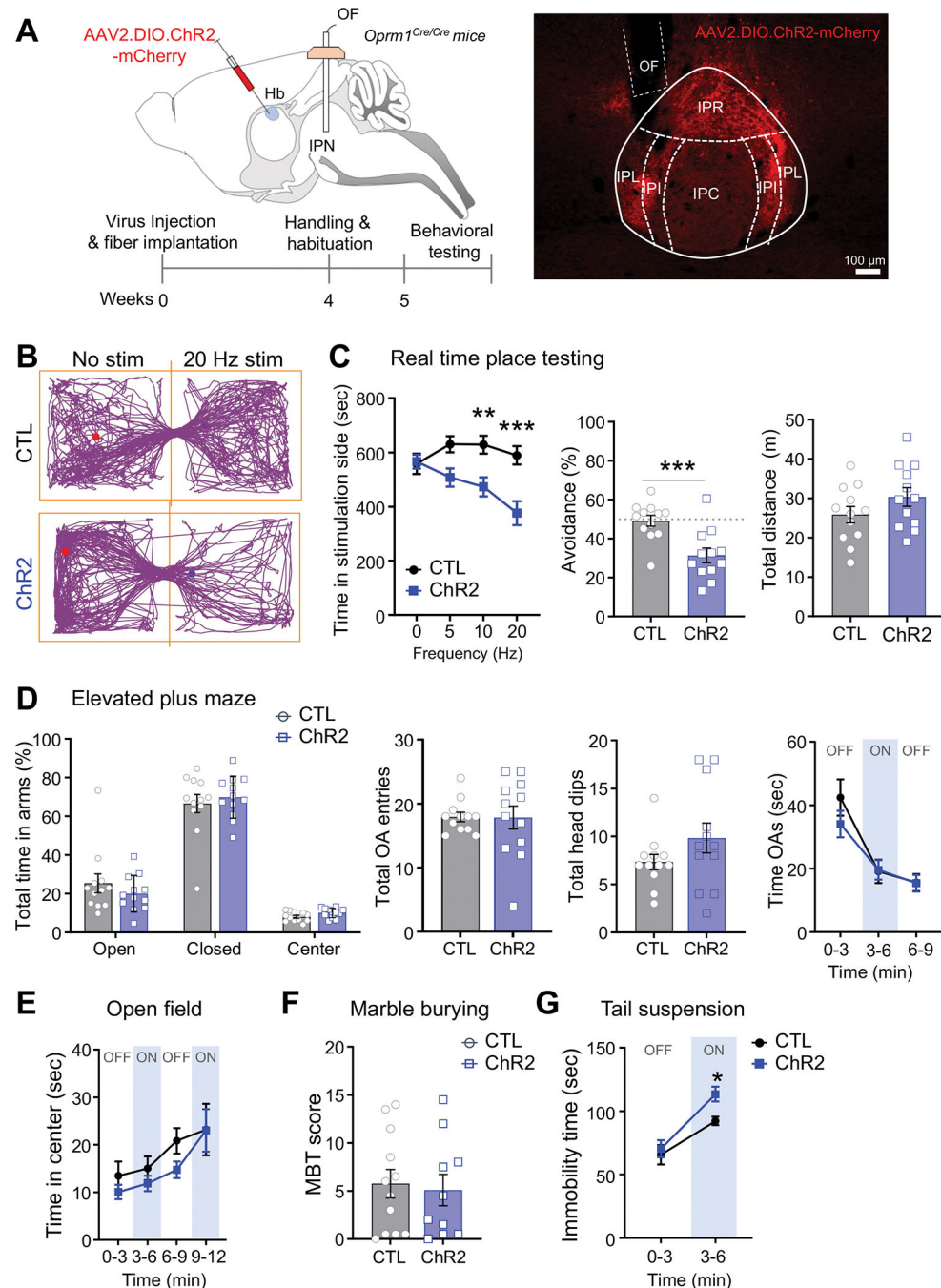


Fig. 3: Optostimulation of Hb-MOR/IPN neurons induces avoidance and a despair-like behavior. *Oprm1-Cre* mice infected with either AAV2.EF1a.DIO.ChR2-mCherry (Hb-MOR/IPN-ChR2) or AAV2.EF1a.DIO.mCherry (CTL) were subjected to several behavioral tests to evaluate consequences of opto-activation on emotional responses. **A.** Scheme (left) and representative image (right) showing viral expression into the MHb, with fiber-optic (FO) implantation above the IPN, and timeline for the experimental procedure. **B.** Real-time place preference testing. Representative tracking plot of the mice at 20Hz stimulation (473 nm, 10 mW, 10 ms pulse width). **C.** (left) Light stimulation in Hb-MOR/IPN-ChR2 mice

induced significant behavioral avoidance to the light-paired side compared with control group (n = 12/groups) at both 10 and 20Hz light stimulation frequencies. At 20Hz, the stimulation triggered avoidance for the light-paired side (middle panel) without affecting the total distance traveled (right; NS). **D.** Elevated plus maze test. Activation of Hb-MOR/IPN neurons did not alter anxiety-level (n = 12/groups) for any of the tested parameters. **E, F.** Open field and marble burying test revealed no effect of the light stimulation in Hb-MOR/IPN-ChR2 (n = 12/groups). **F.** Tail suspension test. Immobility time was significantly increased in Hb-MOR/IPN-ChR2 mice compared to control group (n=9–11/groups). Data are represented as mean values \pm SEM. *p<0.05 **p < 0.01, ***p < 0.001.

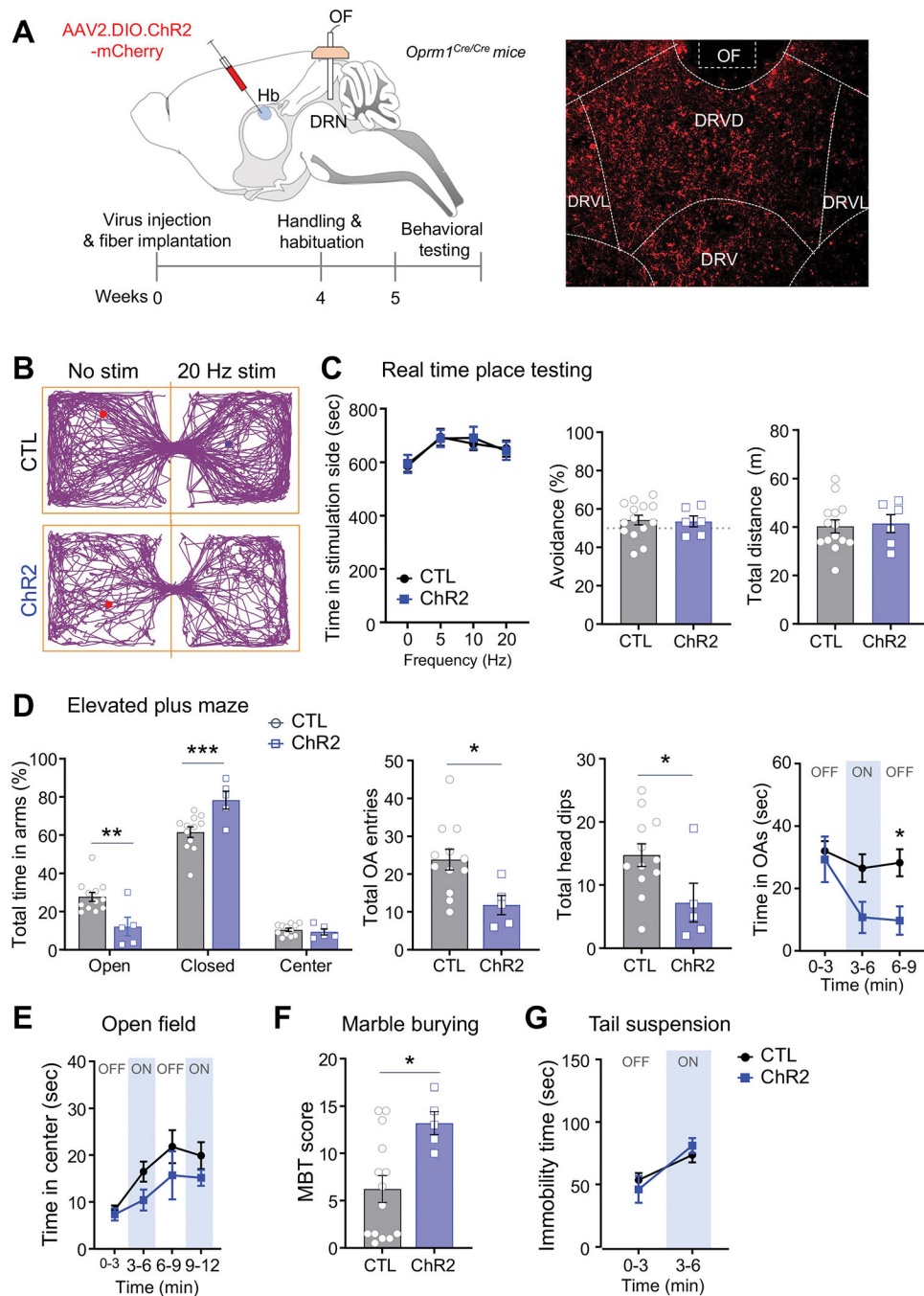


Fig. 4: Optostimulation of Hb-MOR/DRN neurons increases anxiety-related behavior.

Opm1-Cre mice infected with either AAV2.EF1a.DIO.ChR2-mCherry (Hb-MOR/DRN-ChR2) or AAV2.EF1a.DIO.mCherry (CTL) were subjected several behavioral tests to evaluate consequences of opto-activation on emotional responses (as for Hb-MOR/IPN-ChR2 mice in Figure 3). **A.** Diagram (left) and representative image (right) showing viral delivery into the MHb and fiber-optic (FO) implantation above the DRN, as well as a timeline for the experimental procedure. **B.** Real time place preference testing. Representative tracking plot at 20Hz (473 nm, 10 mW, 10 ms pulse width). **C.** (left) Activation of Hb-

MOR/DRN neurons does not produce place avoidance (n=6–14/groups) and (right) further analysis of the 20 Hz stimulation condition indicated no alteration of the total distance traveled. **D.** Elevated plus-maze test. Activation of Hb-MOR/DRN neurons increases levels of anxiety for all the tested parameters (n=5–12/groups). **E.** Open-field testing revealed no effect of the stimulation in Hb-MOR/DRN-ChR2 mice (n=6–14 /group). **F.** Marble burying test. Light-stimulation increased the burying (MBT) score in Hb-MOR/DRN-ChR2 (n=5–14 /group). **G.** Tail suspension test. There was no effect of the light-stimulation in Hb-MOR/DRN-ChR2 mice (n=5–14/group). Data are represented as mean values \pm SEM. *p<0.05 **p < 0.01, ***p < 0.001.

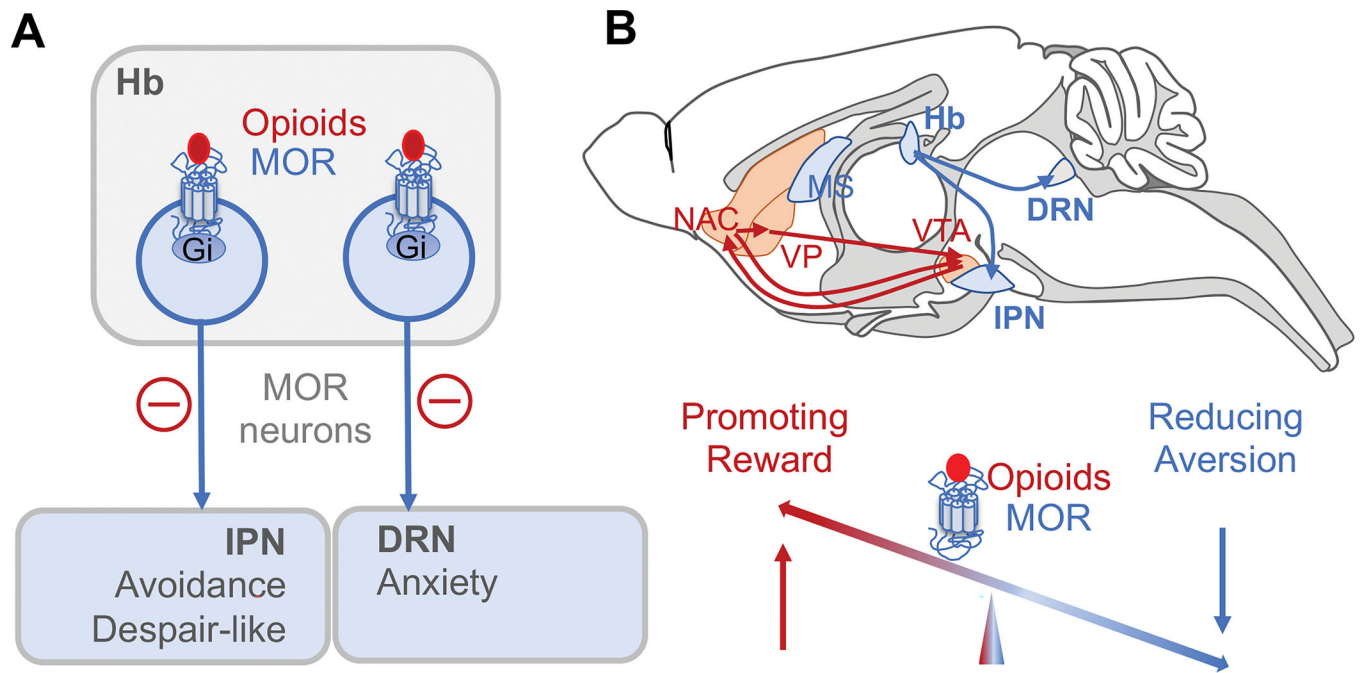


Fig. 5: An aversion-reducing mechanism for MOR signaling.

A. This study demonstrates that (i) blockade of MORs in the Hb is aversive, (ii) stimulation of neurons expressing MOR in the habenula (Hb-MOR neurons) is aversive and (iii) selective stimulation of Hb-MOR/IPN and Hb-MOR/DRN pathways produces avoidance and despair-like behavior for the former, and enhances anxiety for the latter. We propose that endogenous or exogenous MOR signaling in the Hb, which inhibits Hb-MOR neurons (red negative sign), may alleviate several aspects of negative affective states via at least two distinct circuits recruiting either the IPN or the DRN. MOR, mu opioid receptor; MOR neurons, neurons expressing the MOR; Hb, Habenula; IPN, Interpeduncular Nucleus; DRN, Dorsal Raphe Nucleus. **B.** The reward promoting activity of MOR activation is well-established, and particularly well studied in mesolimbic pathways (red). The present study reveals an “aversion-reducing” function of MOR activity (blue) at the level of the Hb. Together, these MOR-mediated mechanisms positively regulate hedonic homeostasis.