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Mu opioid receptors-expressing neurons in the dorsal raphe nucleus are involved in reward processing and affective behaviors

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Abstract

BACKGROUND: Mu opioid receptors (MORs) are key for reward processing, mostly studied in dopaminergic pathways. MORs are also expressed in the dorsal raphe nucleus (DRN), central for the modulation of reward and mood, but MOR function in the DRN remains underexplored. Here, we investigated whether MOR-expressing neurons of the DRN (DRN-MOR neurons) participate to reward and emotional responses.

METHODS: We characterized DRN-MOR neurons anatomically using immunohistochemistry, and functionally using fiber photometry in responses to morphine and rewarding/aversive stimuli. We tested the effect of opioid uncaging on the DRN on place conditioning. We examined the effect of DRN-MOR neuron opto-stimulation on positive reinforcement and mood-related behaviors. We mapped their projections, and selected DRN-MOR neurons projecting to the lateral hypothalamus (DRN/LH-MOR) for a similar optogenetic experimentation.

RESULTS: DRN-MOR neurons form a heterogeneous neuronal population, essentially composed of GABAergic and glutamatergic neurons. Calcium activity of DRN-MOR neurons was inhibited by rewarding stimuli and morphine. Local photo-uncaging of oxymorphone in DRN produced conditioned place preference. DRN-MOR neuron opto-stimulation triggered real-time place preference and was self-administered, promoted social preference; reduced anxiety and passive

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coping. Finally, specific opto-stimulation of DRN/LH-MOR neurons recapitulated reinforcing effects of total DRN-MOR neuron stimulation.

CONCLUSIONS: Our data show that DRN-MOR neurons respond to rewarding stimuli, and that their opto-activation has reinforcing effects and promotes positive emotional responses, an activity partially mediated by their projections to the LH. Our study also suggests a complex regulation of DRN activity by MOR opioids, involving mixed inhibition/activation mechanisms that fine-tune DRN function.

Keywords

opioid circuitry; dorsal raphe nucleus; positive affect; mouse behavior; optogenetics; fiber photometry

INTRODUCTION

Mu opioid receptors (MORs), are the primary targets of opioid drugs (such as morphine and heroin), and have been extensively studied in circuits related to pain-relief and drug reward. MORs are also important endogenous regulators of hedonic homeostasis (1) and mood (2). The dorsal raphe nucleus (DRN) is the main producer of serotonin in the brain, a key center for reward processing and emotional responses (3, 4), and includes a significant number of cells (about 30%) expressing MORs (5, 6). In addition, chronic exposure to opioids modulates DRN activity (7) and alters mood states (8). Therefore, the DRN might be a region of particular interest to understand MOR-regulated hedonic homeostasis and affective processes.

There is cellular and molecular evidence for opioid-mediated modulation of serotonin in the DRN. MOR activation in the DRN blocks the tonic inhibitory activity of local GABAergic neurons onto serotonin neurons, which triggers serotonin release (9–11). Further, during opioid withdrawal, serotonin levels drop below baseline, because of increased inhibitory GABAergic tone on serotonin neurons (12, 13). Finally, serotonin turnover in the DRN remains altered up to four weeks after the termination of a chronic morphine exposure (7). Based on current knowledge, the consensus is that MORs in the DRN are mainly expressed in GABAergic interneurons, and that opioid-induced MOR activation triggers serotonin release through a disinhibition mechanism (2), similar to the dopamine release disinhibition mechanism in the ventral tegmental area (VTA, (14)).

Because this opioid/serotonin interaction has strong implications for reward and mood processing, as well as pathological states including addiction and depression, we here adopted a circuit approach to investigate mechanisms underlying MOR-regulated behaviors at the level of the DRN. To this aim, we took advantage of a MOR-Cre line created in our laboratory (15) to study DRN neurons that express MORs (DRN-MOR neurons), and circuits in which these neurons are embedded. We addressed four questions: i) What are neuronal cell types that express MORs in the DRN and where do they project? ii) Do DRN-MOR neurons respond to stimuli of positive and/or negative valence? iii) Does stimulation of these neurons modify behavior? iv) How do specific projections contribute to these behaviors? Our data show that DRN-MOR neurons form a heterogenous cell population,

whose output activity has rewarding, prosocial, anxiolytic and antidepressant-like effects, partly mediated by DRN-MOR neurons projecting to the lateral hypothalamus (LH).

METHODS AND MATERIALS

See Supplementary Information for detailed Methods and Materials

Animals

Adult male mice (20-45g) used for experiments were group housed (maximum of five mice per cage) in a temperature- and humidity- controlled animal facility (21±4°C, 45± 10% humidity) on a 12h dark/light cycle. Wildtype (C57BL/6N) mice (conditioned place preference (CPP)) and MOR-Cre knock-in mice (for all other experiments) (15) were given access to food and water *ad libitum*, unless otherwise noted. All mice were monitored for health status daily for the entirety of the study. All experiments were performed in accordance to ethical guidelines (Canadian Council of animal Care, Animal Care Committees).

Drugs

Morphine sulfate (NIH, NIDA Drug Supply Program) and naloxone (Sigma) was prepared in saline (0.98% sodium chloride) and injected intraperitoneally (i.p.) at a volume of 10mL/kg.

Immunohistochemistry

MOR-Cre male mice were injected with 500nL of AAV2-hSyn-DIO-mCherry in the DRN. For neuronal characterization, immunostaining was performed on DRN slices using the following antibodies: anti-TH, anti-TPH2, anti-GABA, anti-VGLUT3, and imaged using a confocal microscope. For anterograde tracing, immunostaining was performed on brain slices (4 slices per mice; n=3) using an anti-ds-red antibody and imaged with a slide scanner.

Fiber Photometry experiments

MOR-Cre male mice were injected with 500nL of AAV1-hsyn-flex-GCAMP6m-WPRE-SV40 in the DRN and implanted with an optic fiber (OF) above the DRN. Calcium signaling of DRN-MOR neurons was recorded during behavioral tests including morphine injection (n=5/5), three-tastes test (n=8) (16) and sucrose self-administration (n=6).

Oxymorphone uncaging conditioned place preference

Male mice were implanted with an OF above the DRN, and underwent a CPP procedure using photo-activable oxymorphone (PhOX) (17) in the DRN (n=6; see Suppl Information for details).

Optogenetic experiments

MOR-Cre male mice were injected with 500nL of either AAV2-hsyn-DIO-ChR2-mCherry (ChR2 mice) or AAV2-hSyn-DIO-mCherry (CTL mice) in the DRN and implanted with an OF either above the DRN (n=10/15) or the lateral hypothalamus (LH; n=18/13). Behavioral

experiments included real time place testing (RTPT), 3-chamber social preference test (SPT), marble burying test (MBT), tail suspension test (TST) and opto-intracranial self-stimulation (oICSS).

Statistical analysis

See Suppl Information and Suppl Table 1.

RESULTS

DRN-MOR neurons form a heterogeneous cell population

We first characterized neuronal cell types expressing MORs in the DRN. We labelled MOR-neurons by injecting a Cre-dependent mCherry reporter virus in the DRN of MOR-Cre mice (Figure 1A; complete viral expression pattern displayed in Suppl Figure S1), and used immunohistochemistry to label the four main known neuronal types of the DRN (Figure 1B): serotonergic, GABAergic, glutamatergic and dopaminergic (3, 4) (Suppl Figure S2). Neurons showing colocalization between mCherry and the antibody were counted using confocal microscopy. As expected from the literature (2, 6), only a small proportion (6%) of DRN-MOR neurons colocalized with TPH2, the marker of serotonergic neurons (Figure 1C, G), and a larger proportion (65%) colocalized with GABA (Figure 1D, G), the marker for GABAergic neurons. Strikingly, a large proportion (55%) of mCherry expression colocalized with VGLUT3 (Figure 1E, G), revealing that MOR-neurons in the DRN are also glutamatergic. No colocalization between mCherry and TH was detected (Figure 1F), suggesting that dopaminergic DRN neurons do not express MOR. Of note, a similar distribution of mCherry labeled MOR-neurons was found across distinct DRN subregions (not shown).

We also mapped the projections of DRN-MOR neurons that were labelled by the anterograde virus (Figure 1A). Several brain regions known to play a central role in reward and mood processing were detected (Figure 1E). There was fluorescent signal in the nucleus accumbens (preferentially in the shell), as well as other hedonic and social hotspots including the septum (18), several nuclei of the hypothalamus (19, 20), and the VTA (21, 22). We also found projections to brain regions related to negative affect and anxiety, including inputs to bed nuclei of the stria terminalis (23), the habenular nuclei (medial and lateral) (24), and both basolateral and central extended amygdala (25).

DRN-MOR neurons are inhibited by morphine and rewarding stimuli

To examine the activity of DRN-MOR neurons, we injected a Cre-dependent virus expressing the calcium indicator GCaMP6m in the DRN of MOR-Cre mice and implanted an OF 100μm above the injection site (Figure 2A). We first tested the response of DRN-MOR neurons to a systemic morphine injection: we recorded the baseline activity of DRN-MOR neurons for 10 min as animals explored the open field arena. After this habituation period, mice were injected i.p. with either saline or morphine (10mg/kg) (26), and placed back in the open field for 25 min, where locomotion and neuronal activity were monitored. As expected, the morphine challenge induced a significant increase of locomotion 15 min after injection (Suppl Figure S3; two-way RM ANOVA, Time x Treatment effect, $p<0.001$).

Analysis of the fluorescent signal (averaged over 5 min periods) demonstrated that systemic morphine decreased DRN-MOR neuron activity from 5 ($p<0.001$) to 15 min ($p<0.05$) after injection, with a return to baseline at 20 min (Figure 2B). During this time period, saline-treated mice did not show any significant fluctuation of the calcium compared to their baseline activity (Figure 2B; two-way RM ANOVA, Time x Treatment effect, $p<0.01$). These results demonstrate that MOR activation by morphine reduces the activity of DRN-MOR neurons, as expected for an inhibitory Gi/o coupled receptor.

In another cohort, we investigated the effect of rewarding and aversive stimuli on the calcium-sensor signal by presenting water, sucrose or quinine to water-restricted mice (Figure 2C) (16). When CGaMP activity was time-locked to the onset of each drinking event (Figure 2D, E), we observed a robust increase of the fluorescent signal at the time mice approached the liquid (two-way RM ANOVA, Time effect, $p<0.01$), and the effect was comparable for the three stimuli (two-way RM ANOVA, Time x Taste effect, n.s.). When the mice consumed the liquid, the fluorescent signal dropped below baseline for the two rewarding stimuli (water and sucrose), (two-way RM ANOVA, Time effect, $p<0.01$). In contrast, the fluorescent signal went back to baseline for the aversive stimulus (quinine) (two-way RM ANOVA, Taste effect, $p<0.05$). This result shows that DRN-MOR neurons are activated at the onset of drinking, and this activation is followed by an inhibition of DRN-MOR neuron activity. The latter inhibition goes below baseline only when the stimulus is rewarding, and is time-locked with the licking behavior. Interestingly, the calcium activity of DRN-MOR neurons was also observed in a sucrose self-administration experiment showing a significant increase of the calcium signal upon activation of the cue associated with sucrose delivery (cue light on, one-way ANOVA, Event effect, $p<0.0001$), as well as at the onset of drinking (Suppl Figure S4), suggesting that DRN-MOR neurons activation represents reward anticipation.

We next determined whether endogenous opioids are involved in the time-locked activity of DRN-MOR neurons during reward approach and consumption. We thus recorded DRN-MOR neuron activity in response to sucrose in mice that were administered either with saline or naloxone (a MOR antagonist) 30 min prior to the trial (Figure 2F). As previously observed, approach to the sucrose elicited an elevation of the fluorescent signal (Figure 2G, H; two-way RM ANOVA, Time x Treatment effect, $p<0.05$), which was not affected by naloxone (n.s.). For saline-treated animals, the signal then decreased below baseline upon sucrose consumption as observed previously. For naloxone-treated animals however, the signal rapidly reversed to baseline, less than 10 sec after consumption had started (Figure 2H; $p<0.05$). This result suggests that the second phase of the response associated with reward consumption required endogenous MOR signaling in DRN neurons.

MOR activation in the DRN produces a conditioned place preference

We next tested whether the local application of MOR agonist in the DRN has reinforcing properties. Rather than implanting highly invasive cannulas for infusion of a MOR agonist, we used PhOX, a newly developed photocaged derivative of the MOR agonist oxymorphone that can be locally photoactivated after systemic administration (17). Mice were implanted with an OF in the DRN, and the effect of PhOX photo-uncaging was evaluated using a

place-conditioning (CPP) paradigm (Figure 3A). After two conditioning sessions in each condition, mice showed increased preference for the PhOX paired-chamber (Figure 3B, two-way ANOVA, Session x Treatment effect, $p<0.001$) compared to the vehicle-paired chamber ($p<0.05$) and compared to the pre-test ($p<0.01$). Thus, the pharmacological activation of MOR locally in the DRN produces a strong place preference.

Opto-stimulation of total DRN-MOR neurons has reinforcing effects and promotes positive emotional responses

We next tested whether manipulating the activity of the entire DRN-MOR neuron population would influence reward- and mood-related behaviors. MOR-Cre mice were injected with a Cre-dependent virus expressing the light-sensitive protein ChR2 mice in the DRN, and subsequently implanted with an OF at the same site (Figure 4A). Four weeks after surgery, one behavioral experiment was performed every week (Figure 4B). We first tested the consequences of DRN-MOR neuron opto-activation using a RTPT (Figure 4C). ChR2 mice, but not CTL mice, expressed a strong preference for the stimulation-paired chamber, (two-way RM ANOVA, Virus x Laser effect, $p<0.0001$). The preference was observed when the LS was delivered at 20 Hz ($p<0.0001$), a preference that persisted during the second off session ($p<0.05$). Then, LS- and control-paired chambers were switched and again, mice spent more time in the LS-paired chamber when delivered at 1Hz ($p<0.0001$).

To further test the reinforcing properties of DRN-MOR neuron activation, ChR2 mice were trained using an oICSS paradigm (Figure 4D). Mice readily learned to self-administer the LS, performing 72 ± 9 active NP during the first operant session. Animals learned to discriminate significantly the active from the inactive NP at the third session (two-way RM ANOVA, nose poke effect, $p<0.01$). The number of active responses increased during subsequent operant sessions, reaching up to 197 ± 26 active NP at the fifth session (two-way RM ANOVA, Session effect, $p<0.0001$). In accordance with the RTPT results, these data suggest that DRN-MOR neuron opto-activation has reinforcing properties.

We also tested whether systemic morphine would occlude DRN–MOR neurons self-stimulation, as previously shown for VTA dopaminergic neurons (27). We observed that i.p. morphine injection 15 min prior to the operant session, but not saline (Figure 4D, session 9), decreased self-stimulation in a dose-dependent fashion, ultimately suppressing the behavior at the highest dose of morphine (Figure 4D, sessions 10-12, two-way RM ANOVA, Nose poke x Session effect, $p<0.0001$). Mice returned to their baseline active responding during a final operant session (Figure 4D, session 13). This result may suggest that the morphine reward supersedes the LS reward, or alternatively, that morphine is able to block reinforcing effects of the ChR2-mediated stimulation, possibly by activating DRN-MORs and thus inhibiting DRN-MOR neurons responsible for the oICSS.

Implication of the DRN in regulating sociability and mood is well established, therefore we also investigated if the opto-activation of DRN-MOR neurons influences these behaviors. Mice were first tested in the SPT: after habituation, animals could freely explore a three-chamber apparatus with one chamber containing a conspecific mouse. Mice expressing ChR2 in DRN-MOR neurons spent more time in the social-paired chamber upon LS (Figure 4E; two-way RM ANOVA, Laser x Virus effect, $p<0.05$), suggesting that the opto-activation

of DRN-MOR neurons enhances preference for the social chamber. We next assessed whether opto-activation of DRN-MOR neurons modulates expression of anxiety levels using the MBT. ChR2 mice buried less marbles than the CTL (Figure 4F; unpaired t-test, $p<0.05$) indicating a possible anxiolytic-like effect of DRN-MOR neuron opto-activation. Finally, we used the TST to measure passive coping in a stressful situation. ChR2 mice spent less time immobile than CTL mice during and after LS (Figure 4G; two-way RM ANOVA, Time x Virus effect, ***$p<0.001$ and *$p<0.5$ respectively), suggesting that the opto-stimulation favors active escape responses. Altogether, results from these three paradigms converge to support the notion that the activation of DRN-MOR neurons promotes positive emotional responses. Importantly, DRN-MOR neuron opto-activation did not affect locomotion neither in the RTPP nor in the SPT (Suppl Figure S5A, C).

Opto-activation of DRN/LH-MOR neurons has reinforcing effects and reduces passive coping

Intrigued by the fact that (i) on one hand, exogenous and endogenous opioids inhibit MOR-neuron activity (fiber photometry) and produce CPP (PhOX uncaging), consistent with the serotonin disinhibition hypothesis and (ii) on the other hand, activation of DRN-MOR neurons positively -rather than negatively- influence affective states (optogenetics), we reasoned that different DRN-MOR neuron populations mediate these seemingly opposing activities. To tease apart distinct functions of DRN-MOR neurons, we chose to focus on a selected DRN-MOR neuron projection site. Among the several possibilities (Figure 1E), we selected the LH because this brain area is associated with motivated behaviors (19, 20), and micro-infusion of MOR agonists at this site demonstrated a role for MOR signaling in reward (28) as well as morphine withdrawal (20). We injected the Cre-dependent ChR2 virus in the DRN and implanted an OF above the LH of MOR-Cre mice (Figure 5A), and assessed effects of the opto-stimulation of DRN/LH-MOR neurons four weeks after surgery (Figure 5B). In the RTPT, opto-stimulation elicited a preference for the stimulation-paired chamber (Figure 5C, two-way RM ANOVA, Laser x Virus effect, $p<0.001$) when the LS was delivered at 20 Hz ($p<0.01$), reversed to baseline during the off period, and had no effect at 1 Hz (n.s.) indicating that DRN/LH-MOR neurons activation has reinforcing effects, but only under the 20 Hz LS conditions.

In the oICSS paradigm, mice learned to self-stimulate their DRN/LH-MOR neurons (Figure 5D), performing 46 ± 13 active responses during the first operant session and discriminating the active from the inactive NP from the second session onwards (two-way RM ANOVA, nose poke effect, $p<0.01$). The number of active NP increased in subsequent operant sessions reaching 107 ± 13 NP (two-way RM ANOVA, Session effect, $p<0.001$). Systemic morphine injection prior to the operant session inhibited the self-stimulation behavior (Figure 5D, session 12, two-way RM ANOVA, Nosepoke x session effect, $p<0.0001$), as was observed for the total DRN-MOR neuron population. Of note, the number of active responses to obtain an opto-stimulation was lower, as mice receiving DRN/LH-MOR neuron stimulation performed 81 ± 13 active NP during acquisition (sessions 1-8) whereas mice receiving total DRN-MOR neuron stimulation performed in average 135 ± 12 active NP (Suppl Figure S6). These data indicate that the subpopulation of DRN-MOR/LH neurons partially mediate the reinforcing effect elicited by DRN-MOR neuron activation.

The SPT (Figure 5E, two-way RM ANOVA, Laser x Virus effect, p=0.3476) and the MBT (Figure 5F, unpaired t-test, p=0.7889) did not reveal any detectable effect of LS in ChR2 mice compared to the CTL. However, the TST showed reduced immobility in ChR2 mice (Figure 5G, two-way RM ANOVA, Time x Virus effect, $p<0.01$) during ($p<0.001$) and after LS ($p<0.05$), to an extend similar to opto-stimulation of total DRN-MOR neurons (Figure 4G). These data suggest that DRN/LH-MOR neurons are not involved in pro-social and anxiolytic effects seen for the opto-stimulation of total DRN-MOR neurons, but likely account for their effects on active coping responses. DRN/LH-MOR neuron opto-activation also did not affect locomotion neither in the RTPP nor in the SPT (Suppl Figure S5B, D).

DISCUSSION

The present study is focused on the role of DRN neurons that express MORs. In sum, we first characterized DRN-MOR neurons and found heterogeneity in this neuronal population (essentially GABAergic and glutamatergic) and also identified several projections sites, including the LH. Second, we observed reduced calcium activity of DRN-MOR neurons in response to systemic morphine injection and to reward consumption, the latter being naloxone-reversible. Third, we showed that local MOR activation by ligand uncaging produces CPP. Fourth, we found that opto-stimulation of the entire DRN-MOR neuron population has reinforcing effects, promotes social preference and active coping, and reduces levels of anxiety. Fifth, opto-stimulation of projecting DRN/LH-MOR neurons recapitulated some of the effects observed for total DRN-MOR neurons, including reinforcing effects and active coping.

The data together demonstrate that neurons expressing MORs are involved in reward and mood processing within the DRN circuitry. A large body of literature has long demonstrated the key role of the DRN in reward (21, 22, 29, 30), social behaviors (31, 32) and negative affect (33, 34), and novelty of this study is the demonstration that many DRN-regulated behaviors engage neurons that also express MORs, and can therefore be modulated by endogenous and/or exogenous opioids.

Rewarding stimuli and MOR opioids inhibit DRN-MOR neurons

The fiber photometry results show that (i) systemic morphine injection reduces calcium currents in DRN-MOR neurons and (ii) in the water/sucrose/quinine and sucrose self-administration test, the calcium signal is reduced under baseline upon reward consumption. Thus, both exogenous and endogenous activation of the receptor reduce the activity of DRN-MOR neurons, which is in accordance with the inhibitory nature of the MORs (35, 36). These findings are also consistent with the current thinking that MORs, in the DRN, are essentially expressed in local GABAergic interneurons and that MOR agonists likely disinhibit the activity of serotonergic DRN neurons, thereby facilitating reward and promoting positive emotional responses (2, 9, 29, 37–39). Our results also extend the study of Li *et al.* showing inhibition of GABAergic DRN neurons during reward consumption (40), and designate MOR signaling as one candidate mechanism for the proposed reward-induced disinhibition mechanism. This interpretation is further in line with our finding that local activation of MORs in the DRN using PhOX (41) produces a CPP. For this experiment,

and although we cannot rule out an implication of MORs expressed at terminals of afferent neurons notably from the VTA (42), the most plausible interpretation is that local MORs expressed in DRN-cells are driving the reinforcing properties of PhOX upon uncaging.

Heterogeneity and complexity of DRN-MOR neuron function

Optogenetic data, however, suggest a more complex picture of DRN-MOR neuron function, as their opto-activation has strong reinforcing effects and promotes positive emotional responses.

One explanation may arise from the detailed observation of fiber photometry experiments that, in fact, shows a bimodal response of DRN-MOR neurons. In the latter experiments, calcium activity increases while mice approach the salient stimulus and peaks at the onset of drinking, after which the calcium signal drops below baseline upon reward consumption (see above). We can therefore hypothesize that the initial DRN-MOR neurons activation is associated with reward anticipation and seeking whereas the subsequent DRN-MOR neuron inhibition is associated with reward consumption. It is possible that, in the oICSS experiment, opto-stimulation of DRN-MOR neurons engages a similar reward seeking behavior. Follow-up studies will investigate how DRN-MOR neurons inhibition and activation may encode different aspect of reward anticipation, seeking and experience, and identify the distinct DRN-MOR subpopulations involved in each of these aspects.

A second explanation for the complexity of our data is the somehow unexpected finding that DRN-MOR neurons are both GABAergic and glutamatergic with equal abundancy. There is ample evidence for the very distinct roles of DRN neuron subpopulations. Relevant to our study, previous work showed that opto-stimulation of GABAergic cell bodies of the DRN fails to induce nosepoking for the LS in a oICSS paradigm (30) and also the calcium activity of these cells does not increase upon sucrose drinking (40). Other studies show that positive reinforcement in the DRN is primarily driven by its glutamatergic projections, notably to the VTA (21, 22, 30). Further, the opto-stimulation of neurons expressing VGLUT3, representing approximately 10% DRN neurons (43, 44), was shown to have reinforcing properties (21, 30). Finally, the DRN also includes peptidergic cell types involved in reinforcement learning and emotional responses (45). Intersectional genetic approaches (46) may allow identifying the specific roles of DRN-MOR neuron subpopulations in reinforcement and mood-regulating processes. Another approach is to target DRN-MOR neuron sub-populations via opto-stimulation of the projection sites, which we performed as a second step.

Several effects of DRN-MOR neurons opto-activation functionally map onto their projections to LH

We here show that opto-stimulation of DRN-MOR/LH projection neurons has reinforcing properties (RTPT and oICSS) and reduces passive coping behavior (TST). This finding is novel, as although projections from LH and other hypothalamic nuclei to the DRN were shown to modulate reward and affective behaviors (47), the reverse (projection from DRN to hypothalamus) remained unexplored and deserves further studies.

The specific opto-activation of DRN-MOR neuron terminals in the LH, recapitulated several behaviors obtained with the opto-stimulation of all DRN-MOR neurons (RTPT, oICSS and TST), but not others (social interactions and anxiety-related behaviors). In addition, stimulation of DRN-MOR neurons at 1Hz was reinforcing, but not of DRN/LH-MOR neurons. Further, the lower level of oICSS from DRN/LH-MOR neurons, compared to DRN-MOR neurons, and the fact that a higher dose of morphine was required for occluding LS self-administration suggests that the self-stimulation behavior is less potent when driven by the subpopulation of DRN/LH-MOR neurons alone, and might engage different neuronal population. Thus, although DRN/LH-MOR neurons account for a substantial part of DRN-MOR neuron functions, other types of DRN-MOR neuron projections are expected to be involved in the modulation of reward- and mood-related behaviors: DRN-MOR neurons projecting to the VTA could play a role in reward processing (21, 22, 30) and social behavior (48), and other projections, such as the BNST (23) or the amygdala (25, 49, 50) might govern expression of anxiety and active coping responses.

Conclusion

Our study demonstrates that DRN neurons that respond to exogenous and endogenous opioids contribute to facilitate reward and promote positive emotional responses, via complex circuit mechanisms that likely involve both local and projecting neurons. Questions remain about the respective importance of inhibitory and excitatory DRN-MOR neurons in modulating behavioral outcomes, and how these neurons adapt under chronic opioid exposure. The high complexity of mechanisms underlying opioid-regulated DRN function likely participates to the highly variable subjective experience and liking of opioids (51–54). Also, understanding mechanisms of morphine activity on DRN neurons may contribute to unravel mood-altering effects of opioids, and contribute to understanding the complex co-morbidity between OUDs and mood disorders (55).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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LW designed and performed experiments, collected and analyzed data, wrote the manuscript; EC and MF assisted for immunohistochemistry and microscopy data acquisition; DAJ and SPM performed the PhOX CPP and MRB provided the resources for it; VM and SBH helped with setting up experiments and data analyses for fiber photometry and oICSS respectively. ED and BLK designed experiments, discussed results, wrote the manuscript and provided resources. All authors read and approved the submitted version.

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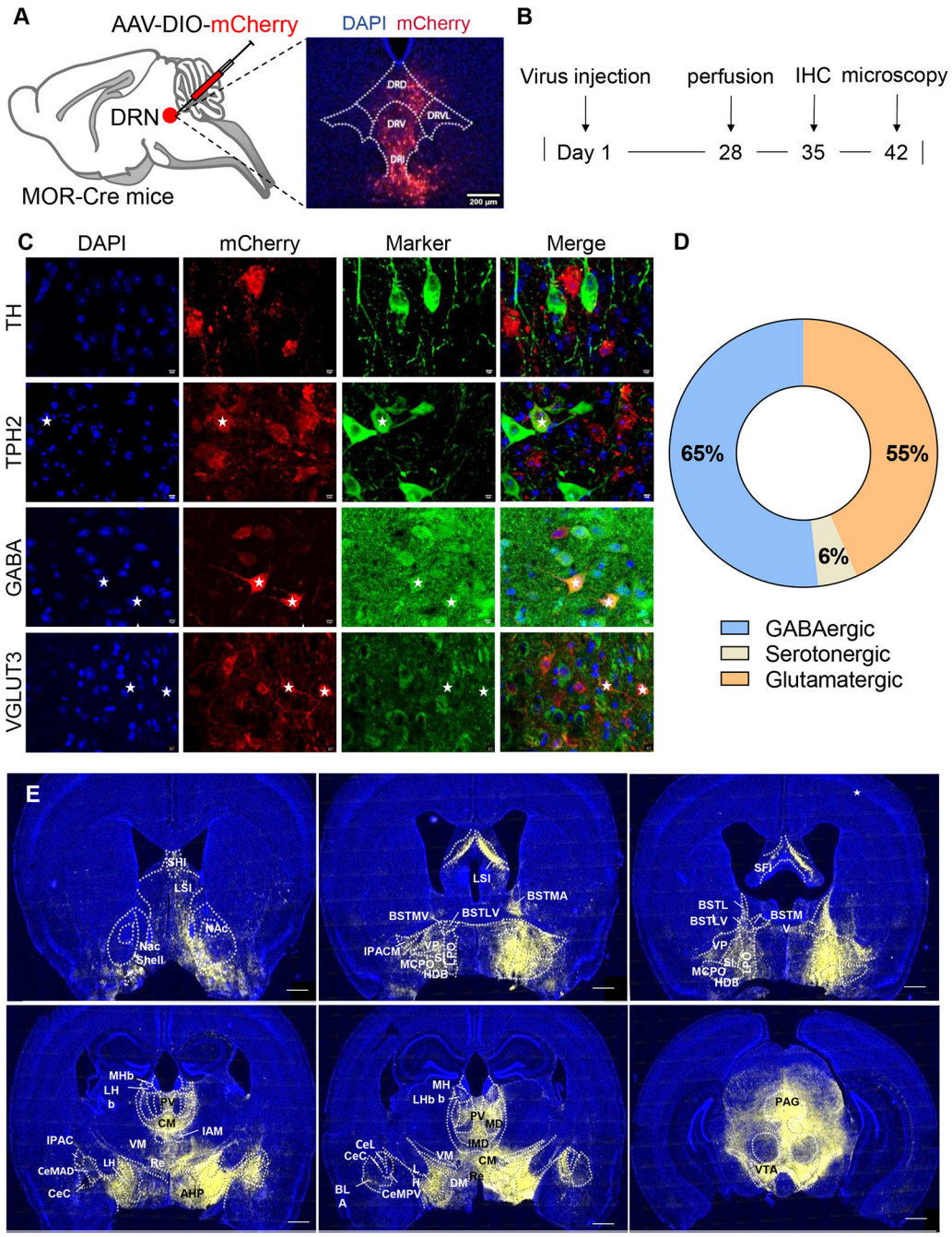

Figure 1. DRN-MOR neurons are heterogeneous and innervate brain regions related to reward/aversion processing and affective responses.

(A) Schematic of viral injection of the Cre-dependent virus expressing mCherry in the DRN of MOR-Cre mice used for immunohistochemistry and anterograde tracing (n=3), with an example pattern of viral expression in the DRN (scale bar, 200μm). **DRD**: dorsal raphe nucleus, dorsal part; **DRI**: dorsal raphe nucleus, interfascicular part; **DRV**: dorsal raphe nucleus, ventral part; DRVL: dorsal raphe nucleus, ventrolateral part. **(B)** Schematic of the experimental timeline. **(C)** Representative images of mCherry and neuronal subtypes

marked with TH; TPH2; GABA; and VGLUT3 antibodies in DRN brain slices; cell bodies showing colocalization between mCherry and antibodies fluorescence are highlighted with a white star (scale: 5μm). (**D**) Proportion of DRN-MOR neurons labelled with mCherry colocalizing with neuronal markers. **(E)** Representative images of DRN-MOR projections labelled with mCherry fluorescence and artificially displayed in yellow (n=3, scale bar: 200μm). DRN-MOR neurons notably send projections along the rostro-caudal axis: the nucleus accumbens core (NAc) and shell (NAc shell) and the septum (LSI: lateral septal nucleus; SHI: septohippocampal nucleus), the bed nucleus of the stria terminalis (BST; BSTLI: BST lateral division, intermediate part; BSTLV: BST lateral division, ventral part; BSTMA: BST medial division, anterior part; BSTMV: BST medial division, ventral part), the nucleus of the horizontal limb in the diagonal band (HDB), the lateral preoptic area (LPO), the interstitial nucleus of the posterior limb of the anterior commissure (IPAC), the magnocellular preoptic nucleus (MCPO), the substantia innominata (SI) and the ventral pallidum (VP); the amygdalar formation (BLA: basolateral amygdaloid nucleus; Ce: central amygdaloid nucleus; CeC: Ce, capsular part; CeL: Ce, lateral part; CeMAD: Ce, medial division, anterodorsal part; CeMPV: Ce, medial posteroventral part), the habenula (MHb: medial habenula; LHb: Lateral habenula), the hypothalamus (LH: lateral hypothalamis area) and the thalamus (IAM: interanteromedial thalamic nucleus; IMD: intermediodorsal thalamic nucleus, MD: mediodorsal thalamic nucleus; PV: paraventricular thalamic nucleus; Re : reuniens thalamic nucleus ; VM: ventromedial thalamic nucleus) and the ventral Pallidum (the VP). (M) the ventral tegmental area (VTA) and the periaqueductal gray (PAG).

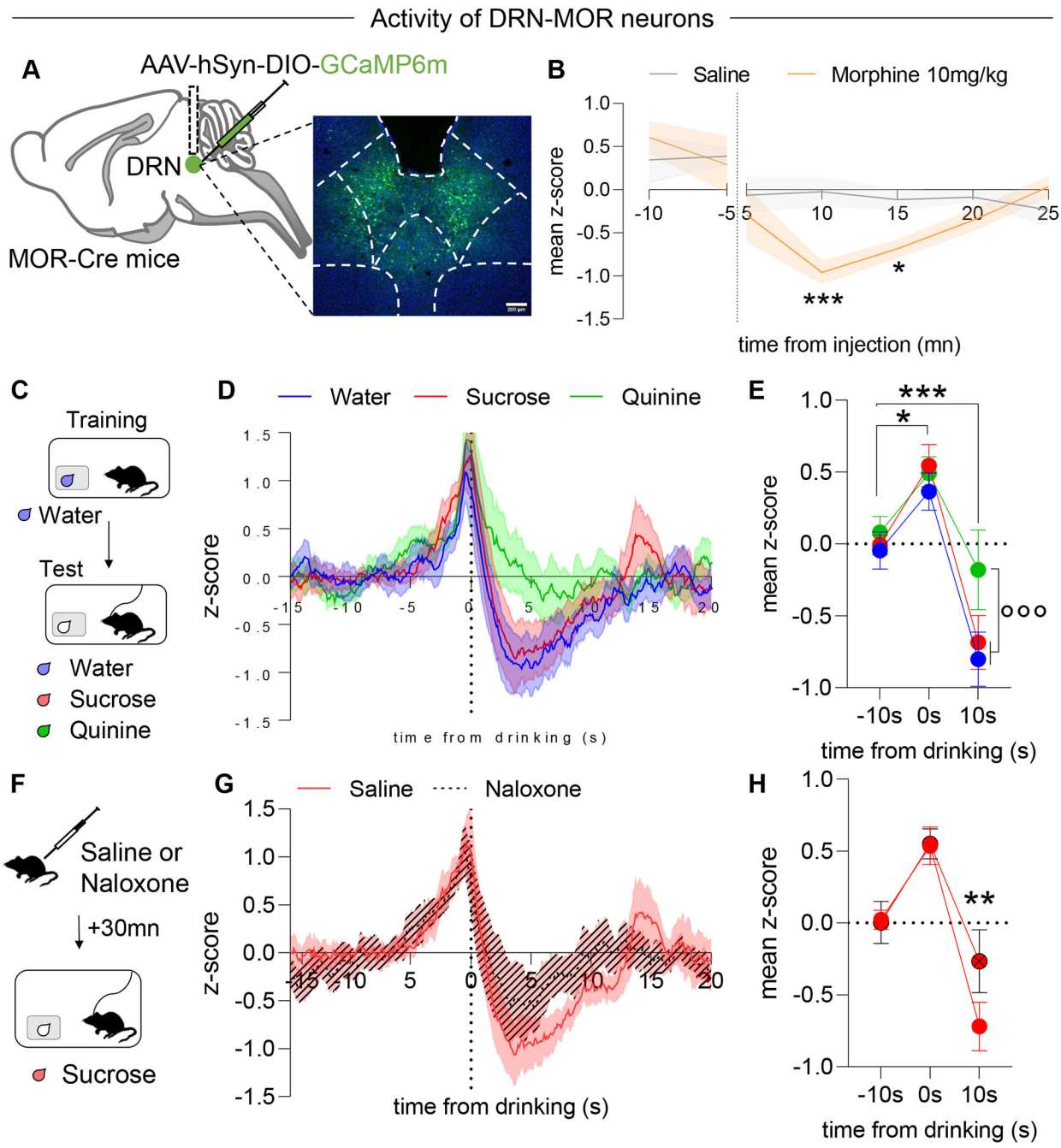

Figure 2. The activity of DRN-MOR neurons is modulated by systemic morphine, approach to a salient stimulus and reward consumption.

(A) Schematic of viral injection of the GCaMP expressing Cre-dependent virus and optic fiber (OF) implantation in the DRN of MOR-Cre mice to record activity of DRN-MOR neurons, and a representative pattern of viral expression in the DRN (scale bar, 200μm). **(B)** Time courses of average GCaMP6m transient z-scores event-locked to saline (n=5) or 10mg/kg morphine (n=5) injection (from −10min to +30min) demonstrating an effect of morphine on DRN-MOR neurons calcium activity at 10 and 15min after injection. **(C)**

Schematic of the three-tastes test: the activity of DRN-MOR neurons was recorded while the mouse was presented three liquids: water, sweet water (sucrose 5%) or bitter water (quinine 1mM). **(D)** Time courses of average GCaMP6m transient events-locked to drinking episodes (15s before and 20s after each drinking event) (n=8). **(E)** Quantification and comparison of the fluorescence average z-scores in DRN-MOR neurons before (−10s), during (0s) and after drinking (+10s) for the three tastes (n=8), demonstrating that calcium signal increased while approaching the cup, and decreased below baseline while consuming the liquid. In addition, it also reveals that calcium activity is significantly different while mice are exposed to quinine compared to water and sucrose. **(F)** Schematic of the sucrose drinking test after systemic naloxone (1mg/kg) or saline. **(G)** Time courses of average GCaMP6m transient events locked to each sucrose drinking event (n=9). **(H)** Quantification and comparison of the fluorescence average z-scores in DRN-MOR neurons before (−10s), during (0s) and after drinking sucrose (+10s) with prior treatment with saline or naloxone (n=9), demonstrating a significant effect of naloxone treatment 10s after drinking. Data are represented as mean ± SEM. *: $p<0.05$; **: $p<0.01$; ***/°°°: $p<0.001$.

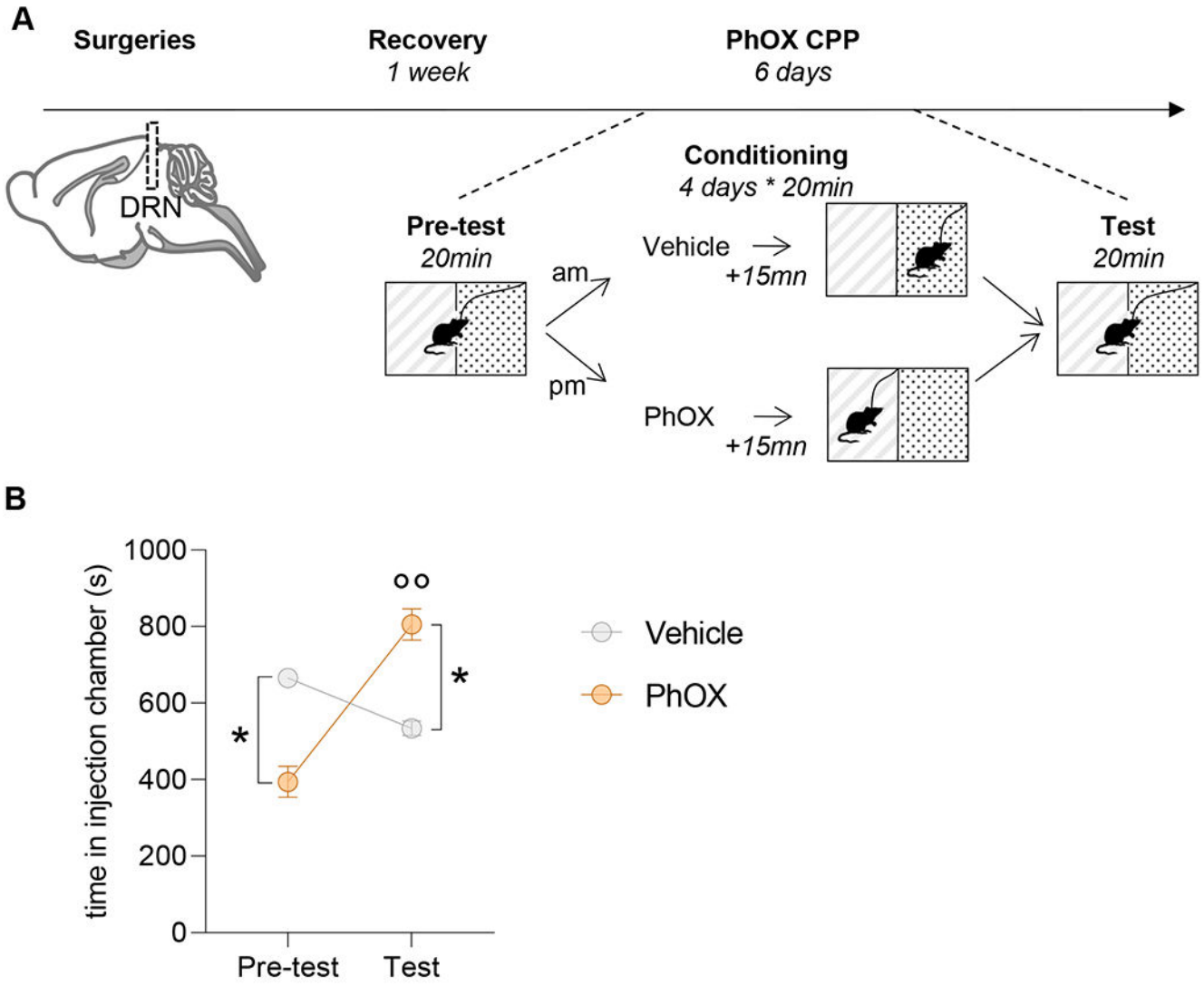

Figure 3. Local MOR activation in the DRN by opioid uncaging produces a conditioned place preference.

(A) Schematic of the experimental timeline. **(B)** PhOX photoactivation in the DRN induces a strong preference for the PhOX-paired chamber (n=6) during the test compared to the pre-test ($p<0.01$) and to the vehicle-paired chamber ($p<0.05$). Data are represented as mean ± SEM. *: $p<0.05$; ***: $p<0.01$.

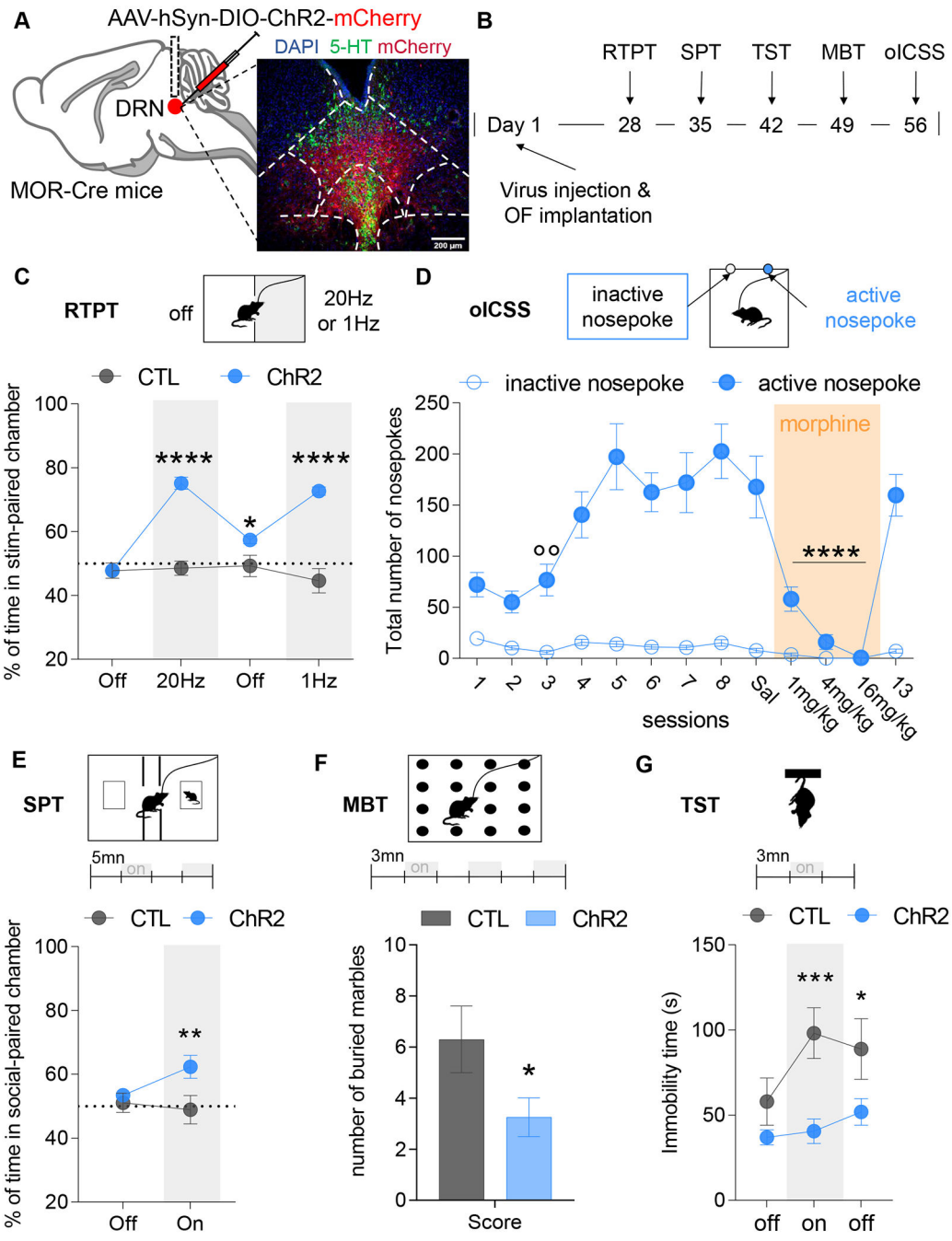

Figure 4. Opto-activation of DRN-MOR neurons has reinforcing effect and promotes positive emotional responses.

(A) Schematic of viral injection of the Cre-dependent ChR2-mCherry expressing virus and optic fiber (OF) implantation in the DRN of MOR-Cre mice, and a representative pattern of viral expression in the DRN (scale bar, 200µm). **(B)** Schematic of the experimental timeline. **(C)** Real time place testing (RTPT): laser stimulation induced a preference for the stimulation-paired chamber at 20Hz and at 1Hz in ChR2 mice (n=10/15). **(D)** Opto-intracranial self-stimulation (oICSS): ChR2 mice discriminated the active from the inactive

nosepoke at session 2 (°°: $p<0.01$), and morphine injection prior to the session inhibits self-stimulation behavior (n=14). **(E)** social preference test (SPT): laser stimulation significantly increased the time spent in the social-paired chamber in ChR2 mice (n=10/14). **(F)** Marble burying test (MBT): laser stimulation decreased the number of buried marbles by ChR2 mice (n=10/14). **(G)** Tail suspension test (TST): laser stimulation decreased immobility time of ChR2 mice (n=7/13). Data are represented as mean ± SEM. *: $p<0.05$; **/°°: $p<0.01$; ***: $p<0.001$.

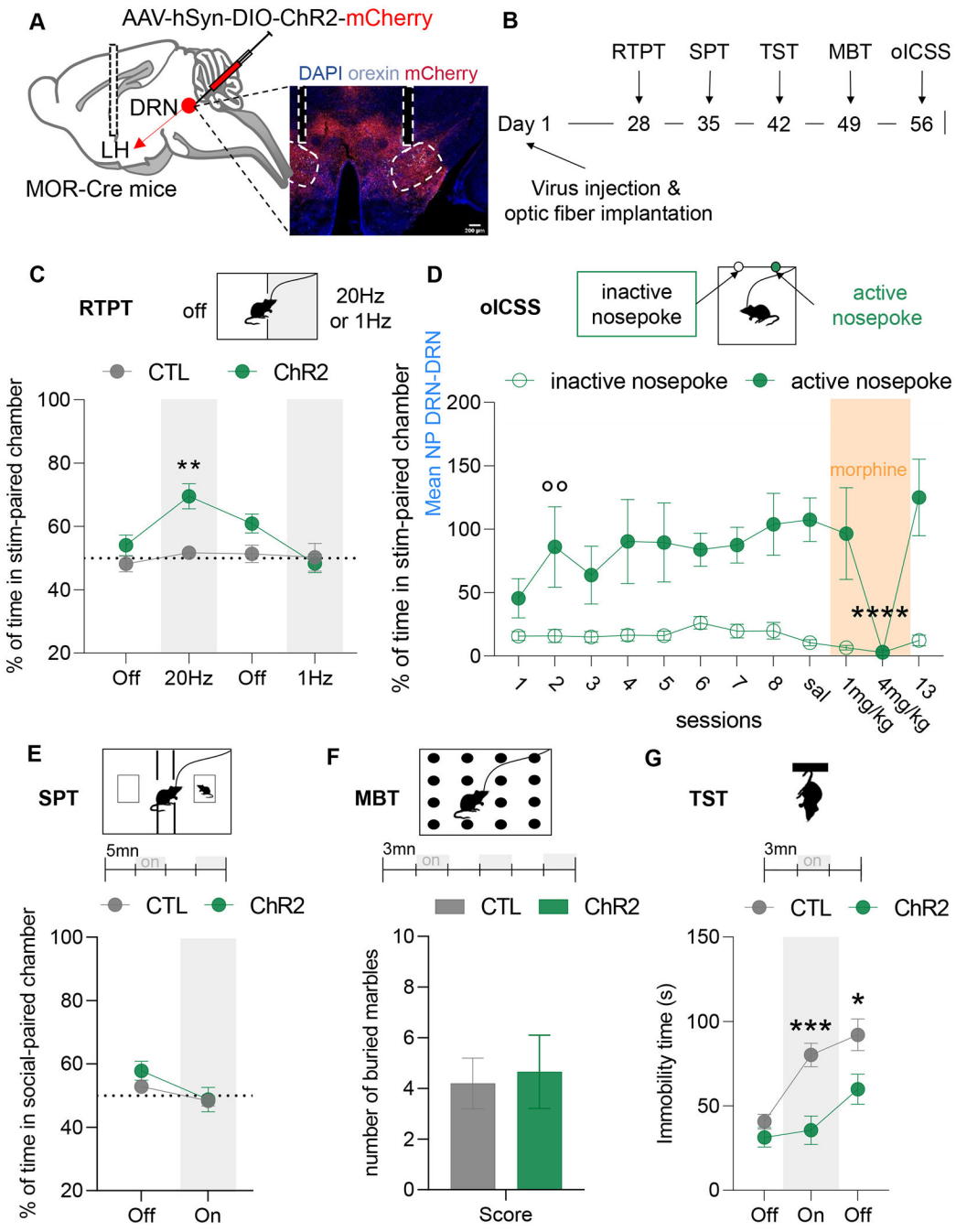

Figure 5. Opto-activation of DRN-MOR neuron projections to the LH has reinforcing properties is rewarding and decreases immobility time in the tail suspension test.

(A) Schematic of viral injection of the Cre-dependent ChR2-mCherry expressing virus in the DRN and optic fiber (OF) implantation in the LH of MOR-Cre mice, and a representative pattern of viral expression and optic fiber implantation in the LH (scale bar, 200μm). **(B)** Schematic of the experimental timeline. **(C)** Real time place testing (RTPT): laser stimulation induced a preference for the stimulation-paired chamber at 20Hz but not at 1Hz in ChR2 mice (n=18/13). **(D)** Opto Intracranial Self-Stimulation (oICSS): ChR2 mice

discriminated the active from the inactive nosepoke at session 2 (°°: $p<0.01$), and morphine injection prior to the session inhibits self-stimulation behavior (n=13). **(E)** Social preference test (SPT): laser stimulation did not increase the time spent in social-paired chamber (n=18/13). **(F)** Marbles burying test (MBT): laser stimulation did not decrease the number of buried marbles (n=18/13). **(G)** Tail suspension test (TST): laser stimulation decreased immobility time of ChR2 mice. Data are represented as mean ± SEM. *: $p<0.05$; **/°°: $p<0.01$; ***: $p<0.001$.