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Original Reports

Opposing effects of mu opioid receptors on dopamine D1 and D2 receptor expressing neurons in opioid mediated antinociception

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ABSTRACT

There is extensive interaction between systems involved in pain processing and motivation, where the aberrant functioning of salience circuits likely contributes to chronic pain, as well as increased susceptibility to opioid misuse and opioid use disorder. This study asks to what extent mu opioid receptors (MORs) in dopamine D1 receptor (D1R), D2 receptor (D2R) or adenosine A2a receptor (A2aR) expressing neurons contribute to the expression of pain and opioid antinociception. We ablated MORs in dopamine receptor expressing neurons by breeding D1R, D2R or A2aR-cre with MOR^{loxP} mice, which was confirmed by RNAscope multiplex fluorescent *in situ* hybridization. To determine the role of these MORs in nociception, we assessed the nociceptive responses in the hot plate and formalin tests with and without treatment with oxycodone (3 mg/kg, i.p.). Pain-like behavior in a thermal assay, mechanical thresholds following nerve injury, and the formalin test were not altered by genotype. However, oxycodone-induced antinociception in the formalin test was differentially altered. Opioid antinociception was attenuated in mice that lacked MORs in D1R neurons, but was enhanced when MORs were ablated in either the D2R and A2aR neurons. In contrast, there was no effect of genotype on oxycodone-induced antinociception in the thermal nociceptive test. Together, these data show that MORs in D1R expressing neurons is necessary for opioid-induced antinociception in a model of tonic inflammatory pain, but not acute thermal pain. Whereas, MOR in D2R and A2aR expressing neurons had a tonic inhibitory tone on opioid-mediated antinociception in the formalin test.

Perspective: This article presents evidence that mu opioid receptors in dopamine receptor containing neurons differentially modulate opioid antinociception in the formalin test but not threshold evoked phasic pain. The endogenous opioid system in these neuronal populations does not appear to modulate various pain behaviors.

Introduction

The mesolimbic dopamine circuitry is a primary network responsible for the processing of motivated behaviors and drug reward. The dopamine neurons originating in the ventral tegmental area (VTA) send projections to many brain regions, but their projection onto medium spiny neurons within the nucleus accumbens (NAc) mediates reward processing, motivation, and reinforcement learning. Thus, this subcortical ventral striatal structure is known for its involvement in motivational salience, especially reward and positive affect, as well as motivated behavior for pain avoidance. An acute noxious stimulus increases extracellular dopamine release in the NAc as detected by fast scanning cyclic voltammetry,[1] decreases signal in functional MRI studies [2] and reduces dopamine neuronal firing. [3] Chemogenetic activation of VTA dopamine neurons reduces nociceptive behavior and is opioid sparing in a model of persistent inflammatory pain. [4]

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Mu opioid receptors (MORs) are densely expressed on subpopulations of NAc GABAergic medium spiny neurons (MSNs) [5] and morphine injection into the NAc reduces formalin-induced nocifensive behaviors [6]. Interestingly, MOR expression on dopamine D1 receptor (D1R) expressing MSNs is sufficient to induce dopamine release *via* a circuit projecting from the NAc. Dopamine D1R MSNs project to the VTA and are sufficient to induce the rewarding effects of opioids as measured both by place preference and self-administration.[7] Indeed, MORs in different striatal MSNs play specific roles in reward-associated behavior.[8] However, while MORs are expressed on multiple neuronal types within the striatum,[7] it is unknown to what extent they contribute to opioid analgesia or endogenous regulation of nociception in acute, tonic, and chronic pain states. Using Dlx5/6-cre mice to genetically delete MORs from all forebrain GABA neurons including the striatum, Kieffer and colleagues [9] reported that heroin-induced neuronal activity (*via* c-fos) was modified, but there was no genotype effect of opioid antinociception in acute pain assays, where similar results were reported by others.[10] The conclusions of this study are surprising given the previous studies that show the importance of striatal circuits in opioid reward and analgesia.

The expression of MORs in the striatum is compartmentalized into striosomes (patches) where they are present on GABAergic direct (D1R) and indirect (D2R) pathways. While there is evidence that receptors on these neurons are important for action-outcome evaluation and reward learning, our understanding of how they may modulate nociception is unknown. In the present study, we asked to what extent MORs expressed in dopamine D1R, D2R and adenosine A2a receptor (A2aR) expressing neurons contribute to the expression of nociception in acute, tonic, and chronic pain states, as well as their contribution to opioid-mediated antinociception. We used a breeding strategy using floxed MOR, in which exons two and three of the MOR gene are flanked by LoxP sites with 3 different cre-recombinase mice (D1Rcre, D2Rcre and A2aRcre). We previously used this strategy and verified these deletions using RNAScope *in situ* mRNA hybridization.[8]

Methods

Animals

All procedures were performed on male and female adult mice, authorized by the Institutional Animal Care and Use Committee (IACUC) and are in compliance with the Policies on the Use of Animals in Research and were compliant with ARRIVE guidelines. All animals were bred in an on-site breeding colony and genotyped with Transnetyx prior to transferring to vivarium rooms for housing and experimentation. Mice were allowed to habituate to their new housing environment a minimum of 7 days prior to handling. The experimental genotype groups were created by breeding a flMOR mouse (loxP sites flanking exons 2–3 of the *oprm1* gene on a 50:50 C57BL/6J:129 Sv background, stock #030074, The Jackson Laboratory) with a cre-driver mice in order to express a cre-recombinase genotype (D1R-cre; B6. FVB(Cg)-Tg (Drd1-cre)EY262Gsat/Mmucd, stock #030989-UCD, 100% C57BL/6, D2R-cre; B6. FVB(Cg)-Tg(Drd2-cre)ER44Gsat/Mmucd, stock #032108-UCD, 100% C57BL/6, 100% C57BL/6J, or A2aR-cre; B6. FVB(Cg)-Tg (Adora2a-cre)KG139Gsat/Mmucd, stock #036158-UCD, 100% C57BL/6). For controls, cre- littermates were utilized. Mice were utilized between age 8–32 weeks and body weight 20–36g. All animals were group-housed (2–4 per cage) for the duration of the experiment on a 12hr/12hr reverse light-dark cycle with access to food and water *ad libitum*. Experiments were all performed during the dark phase of the reverse light-dark cycle between the hours of 10am-3pm. Experimenters were blind to genotype when conducting the behavioral testing and data analysis. No animals were excluded from data analysis.

Drugs

The Schedule II drug oxycodone was obtained from the NIDA Drug Supply Program (RTI). Oxycodone hydrochloride was used at dosage of a 3.0 mg/kg in a solution with pH 7.4 buffered 0.01 M PBS. This dose was chosen based on pilot studies to obtain a submaximal analgesic response in the formalin test. Animals were injected with 0.1 mL/10g body weight of a 0.3 mg/mL oxycodone solution or 0.1 M phosphate buffered saline (PBS) vehicle. Formalin (2.5% and 5%) was made from 4% paraformaldehyde in 0.1 M phosphate buffer, where 10% formalin is equivalent to 4% paraformaldehyde.

Formalin test

Plexiglass boxes (28 × 28 × 18 cm) on top of an angled 45° mirror was used for the formalin test. Mice were placed into the testing boxes for 10 min to habituate to the testing environment prior to further experimentation. Following habituation, mice were wrapped in a towel and gently restrained prior to injecting 30 μL of either 2.5 or 5% formalin subcutaneously in the plantar surface of their left hind paw. Animals were immediately placed back into boxes and observed for nocifensive behavior (shaking, biting, and licking of the injected paw) for 50 min. Time spent exhibiting these behaviors was recorded by an observer blind to genotype and treatment. To assess opioid-induced antinociception, mice were injected with vehicle (PBS) or oxycodone (3 mg/kg, i.p.) following the 10 min of habituation and 10 min prior to the formalin injection. Immediately after the formalin testing, mice were anesthetized with isoflurane and euthanized to collect brains that were subsequently used for *ex vivo* experimentation for RNAScope mRNA.

Hot plate test

Mice were habituated to the behavioral testing room and to the hot plate device (at ambient temperature) the day prior to experimentation for 1 h and 5 min, respectively. The hot plate test (52 ± 0.5 °C) was used to measure response latencies.[11,12] The surface temperature was continuously monitored with a digital thermometer. The time between the placement of the animal on the hot plate and the occurrence of a nociceptive response (hind paw licking, jumping, side of cage climbing, and hind paw flicking) was recorded as the response latency. Baselines for each animal were determined twice not less than 10 min apart, ensuring enough time for recovery of skin temperature after the stimulus. The average baseline was tripled and set as the cutoff (maximum time for each animal spent on the hot plate). To assess opioid-induced antinociception, mice were injected with vehicle (saline) or oxycodone (3 mg/kg, i.p.) and thermal thresholds were tested every 10 min for 50 min.

Mechanical nociceptive thresholds

Mechanical thresholds were assessed by using a graded series of von Frey filaments. For three days prior to initiating any experimental procedure, mice were habituated to the environment and handler. Mice were habituated for 30 min to Plexiglass boxes (9 cm x 10 cm x 17.5 cm) on an elevated metal grid. Von Frey filaments (Touch-Test; North Coast Medical, Inc, San Jose, CA) were used in an up-down method to determine mechanical withdrawal threshold to assess the occurrence of changes in mechanical withdrawal thresholds. The 50% paw withdrawal threshold (PWT) was obtained by analyzing the data using methods described by Chaplan and colleagues.[13]

Surgery

Mice were anesthetized with isoflurane (2% maintenance), shaved on their left hind limb and the skin cleaned with alcohol swabs and io-

dine prior to incision. After dissecting through the muscle to expose the main branch of the sciatic nerve, we placed a 2 mm long PE-20 polyethylene cuff around the entire sciatic nerve proximal to bifurcation, such that the nerve was firmly wrapped by the tubing as previously described.[14,15] Mice received eye gel, supportive lactated ringers (2 mL s.c.) and acetaminophen (32 mg/kg, orally) prior to and daily for 72 h postoperatively. Mice recovered for two weeks prior to further experimentation and handling. Sham surgeries consisted of similar exposure to isoflurane anesthesia and skin incision, but no muscle damage or manipulation of the nerve.

In situ hybridization

We used RNAscope (Advanced Cell Diagnostics Inc., Newark, CA) for *in situ* hybridization to colocalize mRNAs for *penk* and *oprm1* and *pdyn* and *oprm1*. Brains were collected following euthanasia, frozen in 2-methyl butane, and stored at −80 °C until sectioning. Coronal sections of the dorsal striatum were collected with a cryostat at 16 μm at −15 °C and mounted on poly-lysine-coated slides (Cat. #63410–02).

In situ hybridization was conducted using the RNAscope fresh-frozen V2 protocol. Briefly, sections were incubated in freshly prepared 4% paraformaldehyde in phosphate-buffered saline for 1 hr, followed by sequential dehydration in 50% EtOH, 70% EtOH, and 100% EtOH for 5 min each. Sections were then incubated with the necessary reagents from the Multiplex Fluorescent Reagent Kit V2 (ACD #323110) in a HybEZ oven. Probes were as follows: *oprm1* (Cat#315841) channel 1, *penk* (Cat#318761-C2) channel 2, and *pdyn* (Cat# 318771-C3) channel 3. *Penk* and *pdyn* were run on separate sections with *oprm1*. Opal dyes 520 and 650 were paired with *penk/pdyn* and *oprm1*, respectively (SKU FP1487001KT and FP1496001KT, respectively). To stain cell nuclei, DAPI was used and washed off after 2 min. ProLong Diamond Antifade Mountant (Molecular Probes P36966) was added before cover-slipping. Visualization was carried out using a Zeiss LSM800 microscope, and images were processed with the Zen software. Cell nuclei in each field of view were identified *via* DAPI staining.

Experimental design and data analysis

Experiment 1. Testing the effect of genotype in the hot plate test with and without oxycodone.

Following establishing baselines, mice were given oxycodone (3 mg/kg, i.p.) or vehicle (saline, i.p.). Thermal thresholds were determined at 10 min after the administration of oxycodone for each subject. Following experimentation mice were euthanized with isoflurane. Data are expressed as mean +/- SEM for all groups with Ns of 7–11 per group based on effect size analysis. Raw data were analyzed by a repeated measures 2-way ANOVA followed by the Sidak's *post hoc* analysis for comparing treatment and genotype. Data were also analyzed following transformation to Percent Maximum possible effect (%MPE) defined as (post drug latency – baseline latency)/baseline latency *100. A repeated measures 2-way ANOVA was used to analyze the time course data obtained from the hot plate testing using Prizm v10 (GraphPad 10.0 software). A p-value less than 0.05 was considered significant.

Experiment 2. Testing genotype in formalin with and without oxycodone.

Mice were divided equally into vehicle or oxycodone groups, balancing for sex and genotype. Animals then underwent formalin testing as described above, with the drug group first receiving a 3mg/kg of body weight dose of oxycodone (i.p.). Following formalin testing, we analyzed genotype effects on basal nociception (no-drug) as well as oxycodone-induced analgesia (drug). Data are expressed as mean +/- SEM for all groups Ns of 7–31 per group based on effect size analysis. A repeated measures two-way ANOVA was used to analyze the time course data using Prizm v10 (GraphPad 10.0 software) followed by Sidak's multiple comparison *post hoc* analysis for comparing treatment and genotype. A p-value less than 0.05 was considered significant. Area under the curve (AUC) data was calculated for the entire 50 min of the test, or broken into the first phase (0–10 min) and the second phase (20–50 min). The AUC was calculated by summing the total time spent showing a nocifensive response, and analyzed by an unpaired Student t-test. Heat maps of the time spent performing nocifensive behavior for each individual animal as well as the mean of each genotype group are presented, where the stronger, more intense color denotes greater pain-like behavior and white as no pain-like behavior.

Experiment 3. Determine the effect of genotype on mechanical withdrawal thresholds.

Following establishing baseline mechanical thresholds, mice randomly assigned to a surgical group (with each cage getting the same surgery). Data are expressed as mean +/- SEM for all groups Ns of 5–7 per group based on effect size analysis. Mechanical withdrawal thresholds were analyzed by a 2-way ANOVA followed by the Sidak's *post hoc* analysis for comparing treatment and genotype using Prizm v10 (GraphPad 10.0 software). A p-value less than 0.05 was considered significant.

Results

To determine the role of MORs in dopamine D1R and A2aR-expressing neurons in opioid-induced antinociception, we tested the effects of oxycodone (3.0 mg/kg, i.p.) in D1R cre +/- flMOR/flMOR and A2aR cre +/- flMOR/flMOR mice in the hot plate test. Ablation of MORs from D1R or A2aR-expressing neurons did not affect baseline nociceptive thresholds denoted as time zero (Fig. 1a,b). Statistical analysis by an unpaired student t-test showed no significant effect of cre for the A2aR ($t = 1.452$, $df = 17$, $p = 0.1648$) or D1R ($t = 1.113$, $df = 14$, $p = 0.2846$).

Opioid-mediated antinociception was assessed every 10 min for the first 20 min and every 15 min for 30 min following oxycodone injection in the A2aR cre + flMOR/flMOR and wild-type littermate mice (Cre-) as well as the D1R cre + flMOR/flMOR and wild-type littermate (Cre-) mice. Oxycodone increased thermal nociceptive thresholds in both cohorts (Fig. 1a,b). Statistical analysis of the A2aR mice by repeated measures 2-way ANOVA revealed a significant effect of time ($F_{(2.760,44.15)} = 25.84$, ****$p < 0.0001$), but no effect of genotype ($F_{(1,16)} = 2.199$, $p = 0.1575$) and there was no interaction between time and genotype observed ($F_{(4,64)} = 0.2083$, $p = 0.9329$). Statistical analysis of the D1R mice by repeated measures 2-way ANOVA revealed a significant effect of time ($F_{(3.262,42.41)} = 48.08$, ****$p < 0.0001$), but no effect of genotype ($F_{(1,13)} = 0.2101$, $p = 0.6542$) and there was no interaction between time and genotype observed ($F_{(4,52)} = 0.2457$, $p = 0.9110$). Data was converted to percent maximum possible effect and presented in Fig. 1 (right column). Again, data show there was no significant effect of genotype on oxycodone-induced antinociception.

Given there was no effect of baseline nociceptive thresholds in an acute pain model, we asked to what extent sensory mechanical thresholds may be altered in a model of neuropathic pain. Mechanical withdrawal thresholds prior to and following nerve injury *via* CCI were determined for both D1R-cre/floxed MOR and respective cre- littermates as well as A2aR-cre/floxed MOR and respective cre- littermates. The timeline of the experiment is presented in Fig. 2a. Withdrawal thresholds were significantly lower in CCI mice in both the D1R (Fig. 2b) and the A2aR cohorts (Fig. 2c). However, there was no effect of genotype on baseline mechanical thresholds in the absence of injury or in the presence of neuropathic pain. Statistical analysis of the D1R mice by a 2-way ANOVA revealed a significant effect of surgery ($F_{(1,20)} = 30.68$, ****$p < 0.0001$), but no effect of genotype ($F_{(1,20)} = 3.524$, $p = 0.0752$) and there was no interaction between time and genotype observed

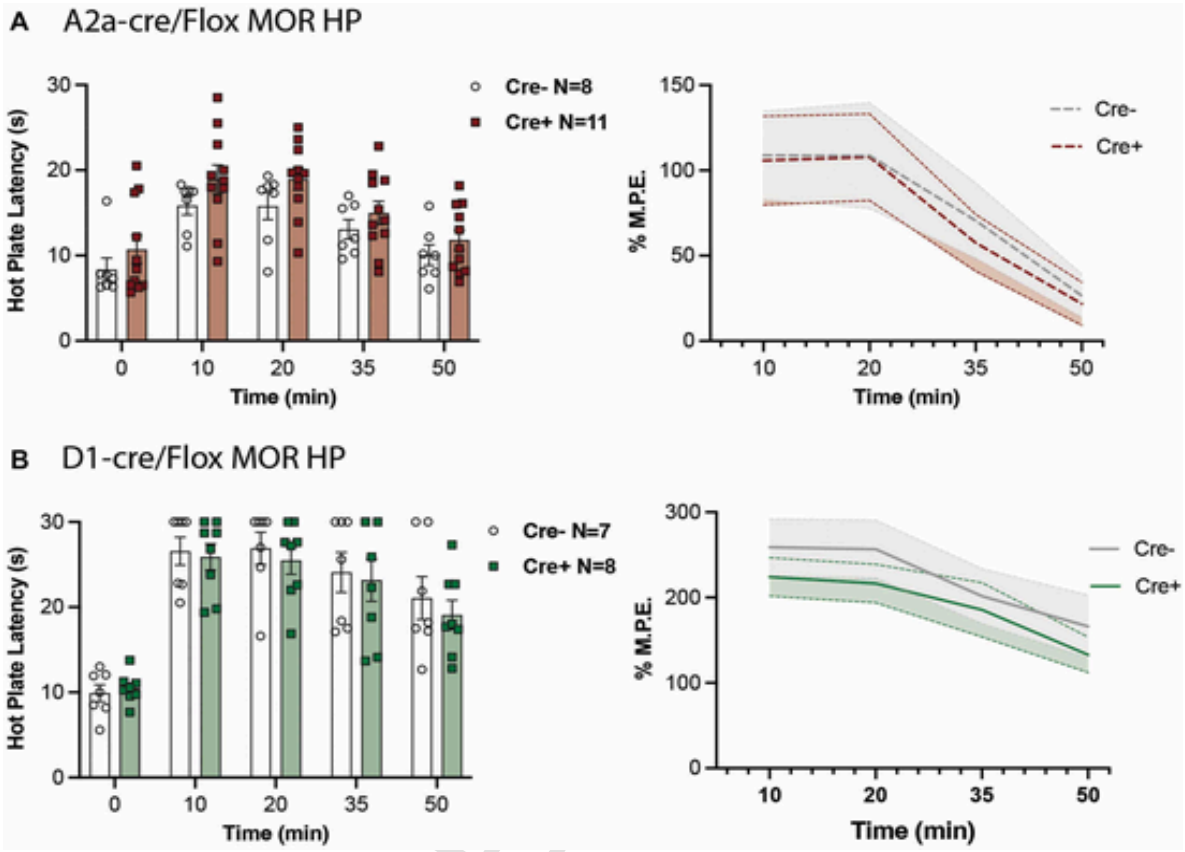

Fig. 1. Ablation of MORs from D1R or A2aR expressing neurons had no effect on baseline thermal nociception or oxycodone-induced thermal antinociception. There was no effect of genotype (time point 0) on thermal nociceptive thresholds (a,b). Thermal nociceptive thresholds were determined for 50 min following oxycodone injection (0.3 mg/kg, i.p.) in the hot plate test. Oxycodone increased thresholds above baseline values in both the A2aR-cre (a) and D1R-cre Floxed MOR mice (b) but there was no effect of genotype for either cohort. Thermal threshold latencies were transformed to percent maximum possible effect (%MPE) (right columns). Again, there was no effect of genotype in opioid-mediated antinociception. Data are expressed as mean +/- SEM for N = 7–11 per group.

($F_{(1,20)}$ = 0.0205, p = 0.8879). Statistical analysis of the A2aR mice by a 2-way ANOVA revealed a significant effect of surgery ($F_{(1,20)}$ = 11.63, **p = 0.0028), but no effect of genotype ($F_{(1,20)}$ = 0.3464, p = 0.5628) and there was no interaction between time and genotype observed ($F_{(1,20)}$ = 1.91e-009, p > 0.9999).

We next asked if opioid antinociception may be modulated by ablation of MORs in the D1R neurons (Fig. 3a). Both cre+ and cre- mice from each cohort were injected with oxycodone (3 mg/kg, i.p.) 10 min prior to injection of 5% formalin and nocifensive behavior was determined. In the A2aR cohorts, the ablation of MORs from this population of neurons (cre+ mice) enhanced oxycodone antinociception compared to the cre- mice (Fig. 3b). A 2-way repeated measures ANOVA revealed an effect of time (F (9, 170) = 7.828, ****p < 0.0001), and an effect of genotype ($F_{(1, 19)}$ = 2.778, *p = 0.0484) but there was no interaction ($F_{(9, 170)}$ = 1.532, p = 0.1402). Calculating the area under the curve for the entire 50-minute test or broken into phase 1 and 2 revealed that there was a significant effect only in the second phase of the test (t = 2.116, df = 19, *p < 0.0478) (Fig. 3c). A heat map of the individual pain-like behavior per animal that provides a visualization of male and female mice per genotype (Fig. 3d) or combined by genotype (Fig. 3e) is presented. The ablation of MORs from D2R expressing neurons exhibited a similar behavioral phenotype to the A2aR cohorts, where opioid antinociception was enhanced when MORs were absent from these neurons (Fig. 3f-i). The ablation of MORs from D2R neurons significantly enhanced opioid antinociception (Fig. 3f,i). A 2-way repeated measures ANOVA revealed an effect of time ($F_{(9, 189)}$ = 8.720 = 4.919, ****p < 0.0001), an effect of genotype ($F_{(1, 21)}$ = 8.249, **p = 0.0091) and an interaction ($F_{(9, 189)}$ = 2.079, *p = 0.0332). Calculating the area under the curve for the entire 50-minute test or broken into phase 1 and 2 revealed that there was a significant effect in the second phase of the test (t = 2.210, df = 21, *p < 0.0383) (Fig. 3c). A heat map of the individual pain-like behavior per animal that provides a visualization of male and female mice per genotype (Fig. 3h) or combined by genotype (Fig. 3i) is presented.

In contrast to A2aR and D2R cohorts, when MOR was ablated from D1R neurons (Fig. 4a), opioid antinociception was attenuated (Fig. 4b). A 2-way repeated measures ANOVA of the time course data revealed an effect of time (F (4.098, 81.95) = 4.919, **p = 0.0012), and an effect of genotype ($F_{(1, 20)}$ = 4.915, *p = 0.0384) but there was no interaction ($F_{(9, 180)}$ = 0.6187, p = 0.7801). Calculating the area under the curve for the entire 50-minute test or broken into phase 1 and 2 revealed that there was a significant effect over the 50 min but not for one specific phase of the pain (t = 2.412, df = 20, *p < 0.0256) and a significant effect in the second phase (t = 2.184, df = 20, *p < 0.0414) (Fig. 4c). A heat map of the individual pain-like behavior per animal that provides a visualization of male and female mice per genotype (Fig. 4d) or combined by genotype (Fig. 4e) is presented.

To determine to what extent the changes in opioid antinociception may be driven solely by engagement of MORs in formalin-induced pain, we examined the potential effects of genotype where MOR was ablated from D1R, A2aR or D2R neurons in both 2.5% (Fig. 5, left column, a,c,e) and for the D1R and A2aR cohorts with 5% formalin (Fig. 5, right column, b,d) injected subcutaneously into the plantar surface of the mouse hind paw. Formalin-induced biphasic nocifensive behaviors

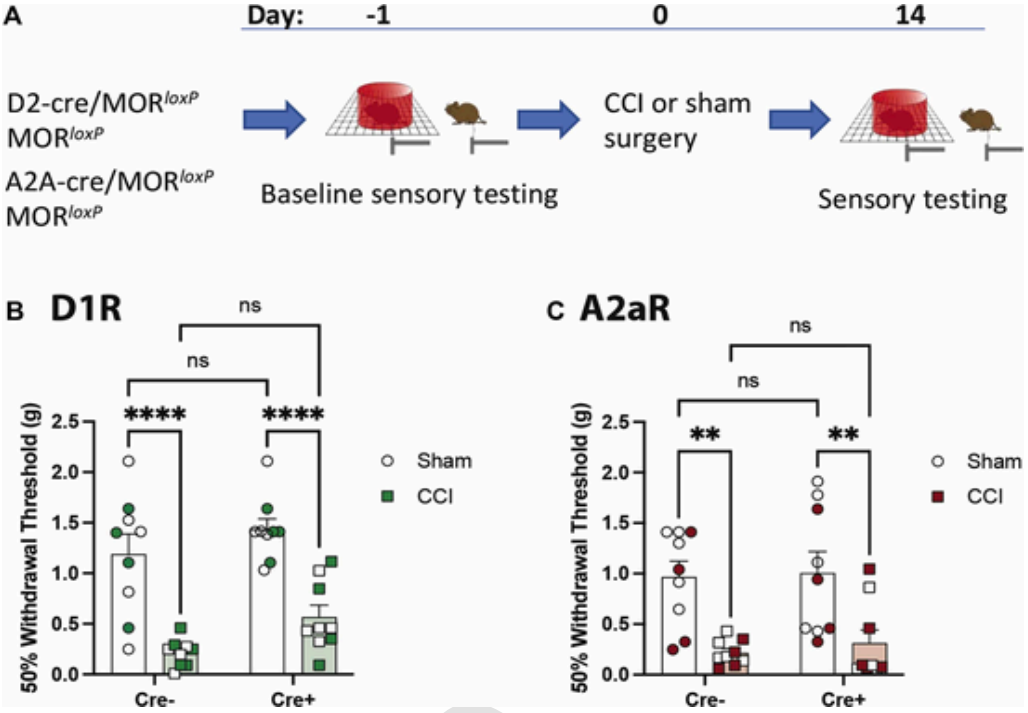

Fig. 2. Ablation of MORs from either D1R or A2aR had no effect on baseline nociceptive thresholds in a model of neuropathic pain. Mechanical withdrawal thresholds were determined 14 days following either sham or chronic constriction injury (CCI) of the sciatic nerve to induce neuropathic pain. (a) Flow chart of experimental design. (b) There was no effect of genotype in mechanical withdrawal thresholds in either sham or CCI D1R cohorts. However, there was an effect of surgery where CCI mice showed a reduced mechanical withdrawal threshold independent of genotype. Statistical analysis of the D1R mice by a 2-way ANOVA revealed a significant effect of surgery ($F_{(1,20)}$ = 30.68, ****p < 0.0001), but no effect of genotype ($F_{(1,20)}$ = 3.524, p = 0.0752) and there was no interaction between time and genotype observed ($F_{(1,20)}$ = 0.0205, p = 0.8879). (c) There was no effect of genotype in mechanical withdrawal thresholds in either sham or CCI A2aR cohorts. However, there was an effect of surgery where CCI mice showed a reduced mechanical withdrawal threshold independent of genotype. Statistical analysis of the D1R mice by a 2-way ANOVA revealed a significant effect of surgery ($F_{(1,20)}$ = 30.68, ****p < 0.0001), but no effect of genotype ($F_{(1,20)}$ = 3.524, p = 0.0752) and there was no interaction between time and genotype observed ($F_{(1,20)}$ = 0.0205, p = 0.8879). Statistical analysis of the A2aR mice by a 2-way ANOVA revealed a significant effect of surgery ($F_{(1,20)}$ = 11.63, **p = 0.0028), but no effect of genotype ($F_{(1,20)}$ = 0.3464, p = 0.5628) and there was no interaction between time and genotype observed ($F_{(1,20)}$ = 1.91e-009, p > 0.9999). Data are expressed as mean +/- SEM for N = 9 per group with equal numbers of male and female mice per condition; white symbols are females and color symbols are males.

characterized by licking and flinching of the injected hind paw in all groups. However, there was no effect of genotype for any of the cohorts tested in either the 2.5 or 5% formalin tests. A 2-way repeated measures ANOVA revealed no effect of genotype for the 2.5% formalin (D1R cohorts: genotype, $F_{(1, 24)}$ = 0.0094, p = 0.9234), A2aR cohorts: genotype, $F_{(1, 18)}$ = 2.135, p = 0.1612, D2R cohorts: genotype, F(1, 52) = 3.918, p = 0.0531) and the 5% formalin (D1R cohorts: genotype, $F_{(1, 13)}$ = 0.0002, p = 0.9887), A2aR cohorts: genotype, $F_{(1, 5)}$ = 0.01289, p = 0.9140).

Validation of the selectivity and extent of MOR knockdown in striatal subpopulations was conducted on the D1cre + flMOR transgenic line. Previous studies from our lab show the effectiveness of this breeding strategy in all the transgenic mice lines used in this study and characterized and validated the mouse colonies with selective knockdown.[8] Fig. 6 presents representative images of *oprm1* localization in either *pdyn* (D1R) or *penk* (D2R) neuronal populations. Cells labeled for *pdyn* expressed very low *oprm1* (Fig. 6a) compared to that expressed in *penk* (Fig. 6b) in the striatum. Fig. 6c demonstrates *oprm1* expression co-localized with *pdyn* neurons, where only about 12% of neurons appeared to have co-labeling for *pdyn* and *oprm1* in the D1-cre + flMOR mice compared to 40% of co-localization in the D1cre-flMOR mice (Fig. 6d).

Discussion

In this study we report the differential effects of MORs on D1 and D2 dopamine receptor expressing neurons (D1 *vs* D2 MORs) on opioid antinociception. Our results demonstrate that ablation of MORs from D1R expressing neurons enhanced opioid-induced antinociception, while ablation in D2R expressing neurons attenuated opioid-induced antinociception. This effect was only evident in a model of tonic inflammatory (formalin) pain and not in a phasic thermal pain test. While we cannot be confident in the circuitry underlying these effects, the reproducible effects of enhanced opioid antinociception when MOR is deleted from D2R and A2a expressing neurons suggests that this effect is likely mediated by striatal circuits, as A2a receptors are co-localized with D2R and enkephalin expressing GABAergic medium spiny neurons.[16–18]

There is strong evidence that the formalin test engages in cortical structures for the expression of nocifensive and aversive behaviors. It is well accepted that the second phase of the formalin test is driven by activation and peripheral sensitization of C-fibers based many studies including electrophysiological recordings of C-fibers in awake and anesthetized animals.[19,20] However, the second phase of the formalin test is also thought to encompass a 'emotional, bothersome' component of the pain experience. Lesions or inhibition of the anterior cingulate cortex, amygdala, or medial thalamic nuclei reduce behaviors in Phase 2 [21–24] with no change in Phase 1, whereas lesions of the entorhinal cortex significantly increases the second phase with no change in the first phase

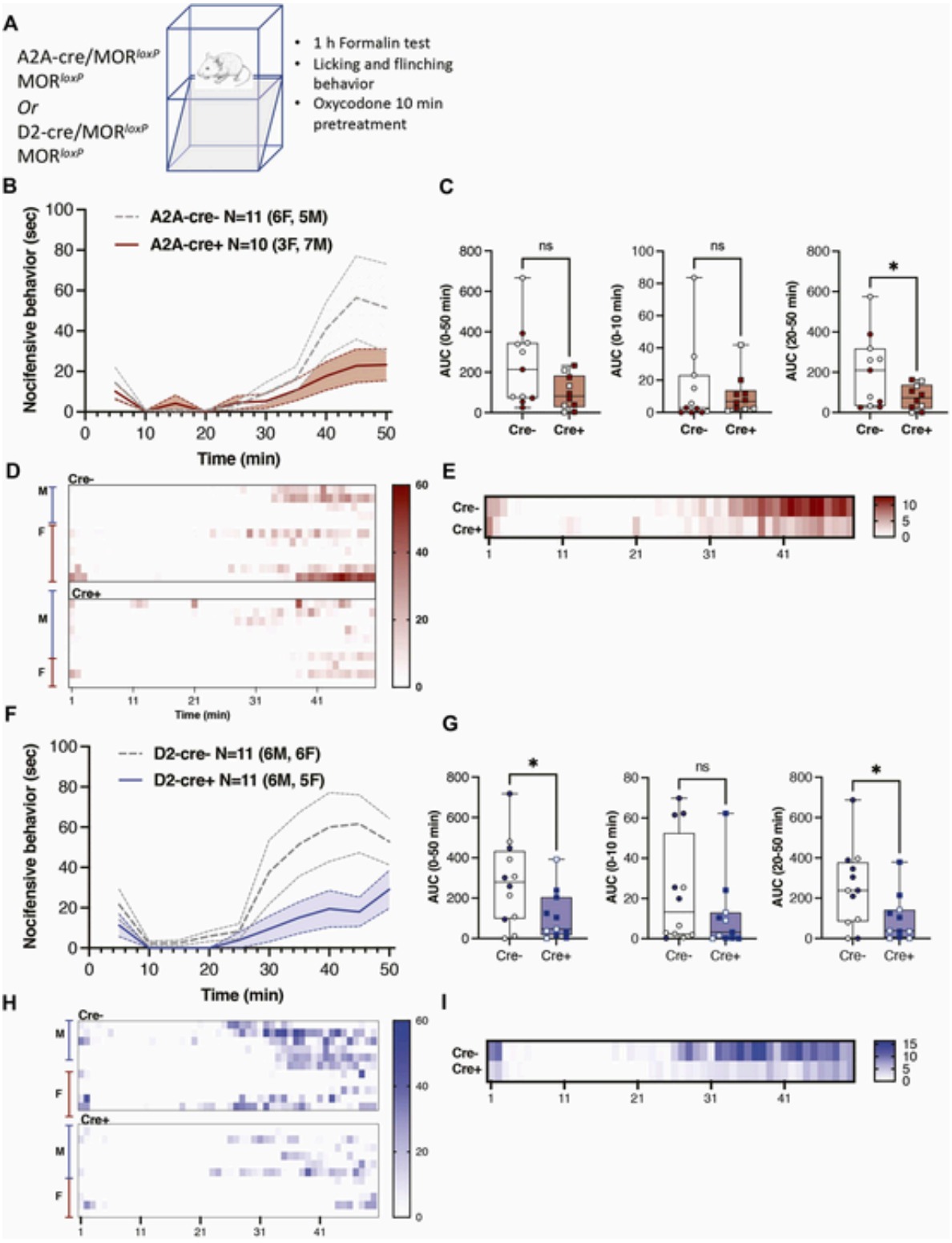

Fig. 3. Opioid-induced antinociception is enhanced in mice where MOR was ablated from A2aR or D2R neurons. (a) Cartoon of behavioral test (b) Time course of nocifensive behaviors (licking, flinching) produced by 5% formalin is presented beginning 10 min following oxycodone (3 mg/kg, i.p.) injection for both A2aR cohorts. Opioid induced antinociception was enhanced in the cre+ animals that had ablation of MORs in these neurons. A 2-way ANOVA revealed an effect of genotype ($F_{(1, 19)} = 2.778$, *p = 0.0484). (c) Area under the curve (AUC) from data presented in panel b for the entire 50 min or divided into the first or second phase of the formalin test is presented. An effect of genotype was detected in the AUC of the 2nd phase of the formalin test showing that ablation of MOR from A2aR neurons enhanced opioid antinociception (unpaired student t-test: t = 2.116, df = 19, *p < 0.0478). (d) A heat map of each individual animal with more intense color repre-

Fig. 3.—*continued*
senting more pain-like behavior in one-minute bins for male and female mice for each genotype. (e) A heatmap with more intense color representing more pain-like behavior in one-minute bins collapsed by sex to show genotype differences. (f) Time course of nocifensive behaviors (licking, flinching) produced by 5% formalin is presented beginning 10 min following oxycodone (3 mg/kg, i.p.) injection for both D2R cohorts. Opioid induced antinociception was enhanced in the cre+ animals that had ablation of MORs in these neurons. A 2-way ANOVA revealed an effect of genotype ($F_{(1, 21)} = 8.249$, $^{**}p = 0.0091$). (g) Area under the curve (AUC) from data presented in panel f for the entire 50 min or divided into the first or second phase of the formalin test is presented. An effect of genotype was detected in the AUC of the overall time course and the 2nd phase of the formalin test showing that ablation of MOR from D2R neurons enhanced opioid antinociception (unpaired student t-test: $t = 2.210$, $df = 21$, $^{*}p < 0.0383$). (h) A heat map of each individual animal with more intense color representing more pain-like behavior in one-minute bins for male and female mice for each genotype. (i) A heatmap with more intense color representing more pain-like behavior in one-minute bins collapsed by sex to show genotype differences. Data are expressed as mean +/- SEM for N = 10–12 per group.

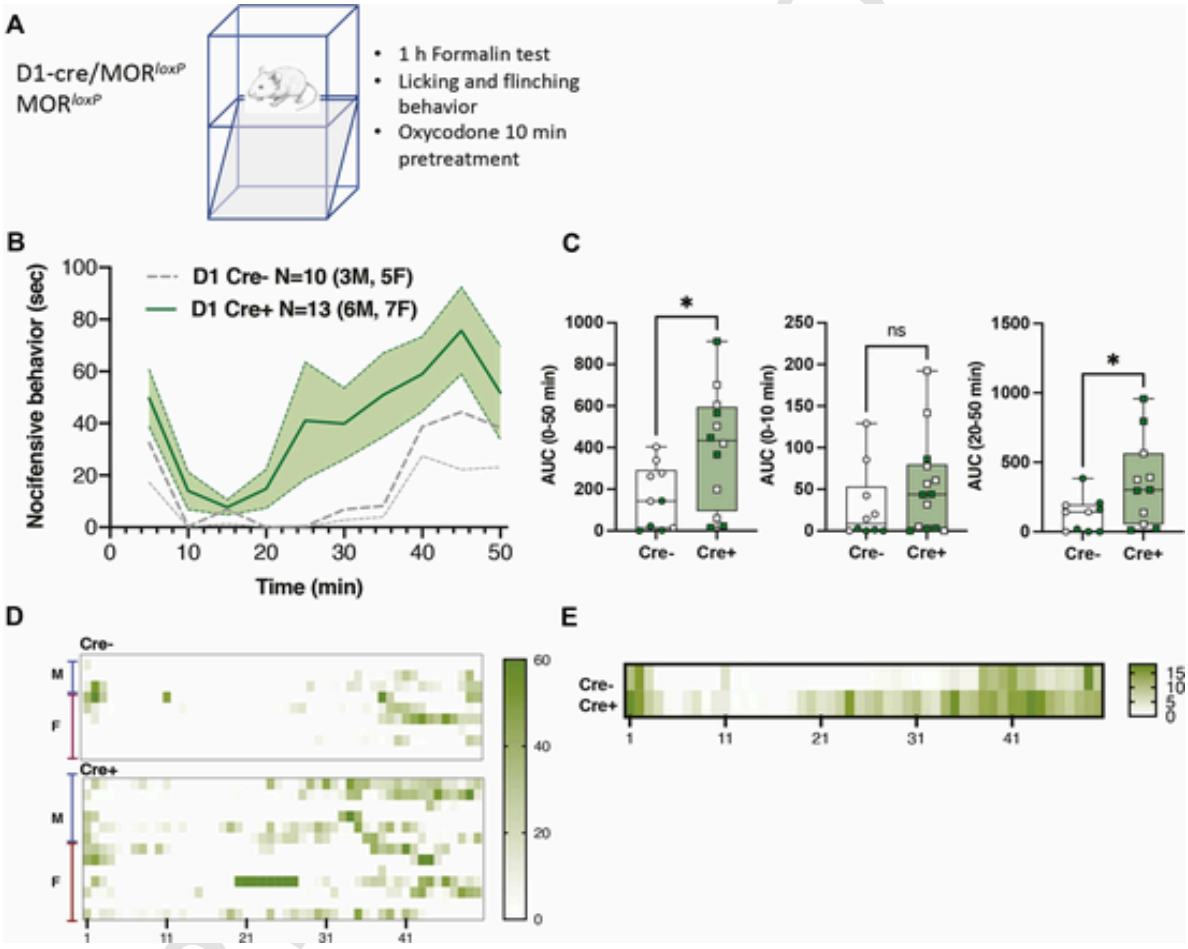

Fig. 4. Opioid-induced antinociception is reduced in mice where MOR was ablated from D1R neurons. (a) Cartoon of behavioral test (b) Time course of nocifensive behaviors (licking, flinching) produced by 5% formalin is presented beginning 10 min following oxycodone (3 mg/kg, i.p.) injection for both D1R cohorts. Opioid induced antinociception was reduced in the cre+ animals that had ablation of MORs in these neurons. A 2-way ANOVA revealed an effect of genotype ($F_{(1, 20)} = 4.915$, $^{*}p = 0.0384$). (c) Area under the curve (AUC) from data presented in panel b for the entire 50 min or divided into the first or second phase of the formalin test is presented. An effect of genotype was detected in the AUC of the full 50 min showing antinociception was reduced in mice that lacked MORs in D1R neurons (unpaired student t-test: $t = 2.412$, $df = 20$, $^{*}p < 0.0256$) as well as for the second phase (unpaired student t-test: $t = 2.184$, $df = 20$, $^{*}p < 0.0417$). (d) A heat map of each individual animal with more intense color representing more pain-like behavior in one-minute bins for male and female mice for each genotype. (e) A heatmap with more intense color representing more pain-like behavior in one-minute bins collapsed by sex to show genotype differences.

of the formalin test.[25] These studies support the idea that Phase 2 includes affective-motivational processing not present in the purely sensory experience. There is also evidence that dopaminergic and opioids interact in the nucleus accumbens to suppress formalin induced pain.[26]

The MOR is expressed into striosomes within the dorsal and ventral striatum, where they are expressed on a subpopulation of medium spiny neurons in the canonical direct (D1R) and indirect (D2R/A2aR) pathways. The striosomes are defined by the dense expression of MORs forming a patch-like compartment. Much is known about the striosome circuit dynamics and their engagement in action-outcome evaluation thereby influencing reinforcement learning,[27] affective and motivational processing [28,29] and aversive learning that drives negative reinforcement.[30,31] However, there have been few studies to examine the sufficiency or necessity of MORs in these circuits in opioid mediated analgesia. Ablation of MORs from all GABAergic forebrain neurons (including the striatum) had no effect on opioid antinociception in acute thermal tests.[9] Similarly, mice that had a conditional bacterial artificial chromosome rescue strategy to express MOR in a subpopulation of striatal direct-pathway neurons enriched in the striasome [7] showed no opioid antinociception in acute thermal nociceptive tests. These studies are

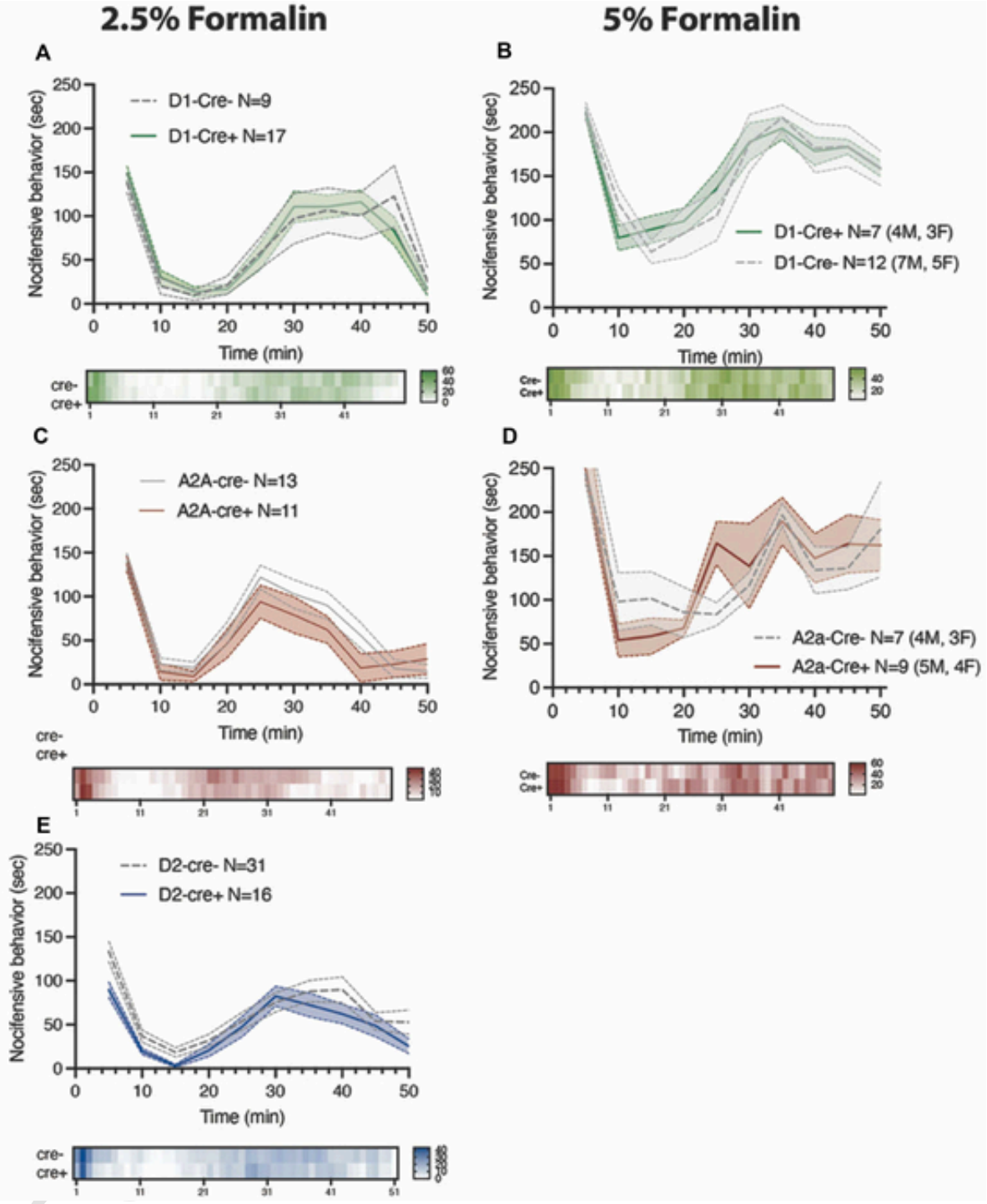

Fig. 5. Ablation of MORs from D1R, D2R or A2aR neuronal populations had no effect on formalin-induced nocifensive pain-like behavior. Intraplantar injection of either 2.5% (left column) or 5% (right column) formalin-induced a biphasic nocifensive (licking, flinching) response in all mice independent of genotype. In the 2.5% formalin test, (a) D1R cohorts show no effect of genotype in a 2-way ANOVA ($F_{(1,\ 24)} = 0.0094$, $p = 0.9234$), (c) nor A2aR cohorts ($F_{(1,\ 18)} = 2.135$, $p = 0.1612$), nor D2R cohorts ($F(1,\ 52) = 3.918$, $p = 0.0531$). In the 5% formalin test, (b) D1R cohorts show no effect of genotype in a 2-way ANOVA ($F_{(1,\ 13)} = 0.0002$, $p = 0.9887$), (d) nor the A2aR cohorts ($F_{(1,\ 5)} = 0.01289$, $p = 0.9140$). Data are expressed as mean +/- SEM for N = 7–31 per group.

consistent with our results showing opioid antinociception in the hot plate test was intact when MOR was ablated from either D1R or D2R expressing neurons.

Our primary finding was that opioid antinociception was modified by specific ablation of MORs in dopamine receptor expressing neurons, with opposing effects depending on which neuronal population was targeted. This is perhaps not surprising given that these outputs from the striatum are organized in two pathways that have opposing effects on movement, reinforcement, and reward-related behaviors [8,32–35] *via* striatonigral and striatopallidal neurons [36,37]. Formalin-induced nociceptive behavior was shown to modify ventral striatal D2R MSN neuronal activity and optogenetic inhibition of ventral striatal D2R MSNs in-

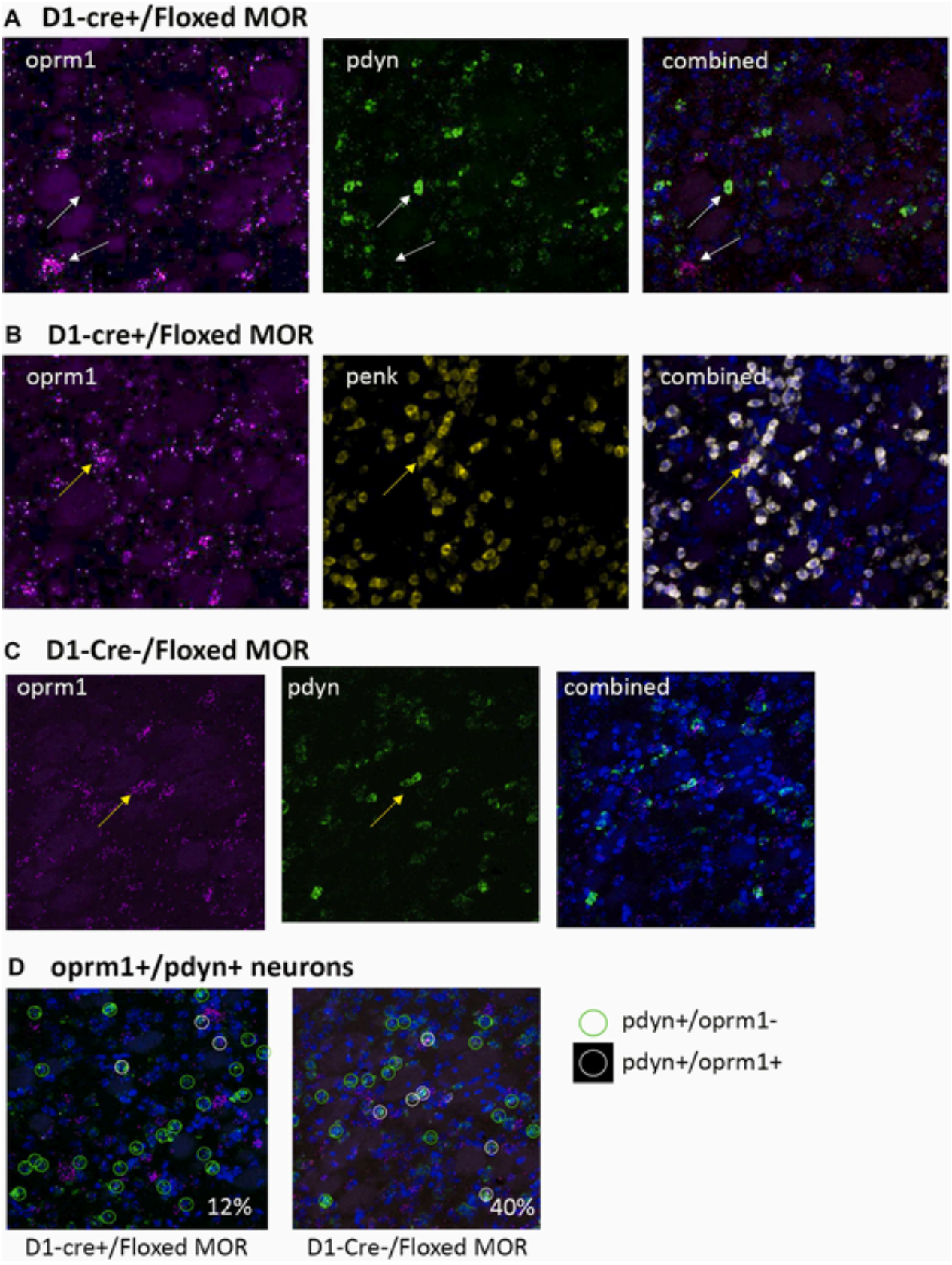
A D1-cre+/Floxed MOR
oprm1
pdyn
combined
B D1-cre+/Floxed MOR
oprm1
penk
combined
C D1-Cre-/Floxed MOR
oprm1
pdyn
combined
D oprm1+/pdyn+ neurons
12%
40%
pdyn+/oprm1-
pdyn+/oprm1+
D1-cre+/Floxed MOR
D1-Cre-/Floxed MOR

Fig. 6. Confirmation of transcript reduction by RNAScope multiplex *in situ* hybridization. *In situ* hybridization on brain sections from D1cre + /flMOR mice in the dorsal striatum. Cell nuclei were stained with DAPI (blue). Antisense probes to localize *oprm1* (a, b, c, left panels), *pdyn* (a, c, middle panel), and *penk* (b, middle panel) mRNA were visualized. Overlay (combined) channels are the right panels in both a, b and c. Puncta for *oprm1* and *pdyn* were less colocalized (a) than those for *oprm1* and *penk* (b) in D1cre + /flMOR mice or D1cre-/flMOR mice (c) demonstrating reduction of opioid receptor transcript in the *pdyn* (D1 receptor) expressing cells. (d) Co-localization of *oprm1* with *pdyn* in D1cre + /flMOR (~12%) and D1cre-/FlMOR (~40%) mice was quantified, where green circles show *pdyn* + neurons without *oprm1* expression, and white circles show co-localization of both transcripts. Arrows indicate examples of colocalized and non-colocalized mRNA in the same cell.

creased formalin induced pain behavior.[38] This data is consistent with our study where we are removing the inhibitory tone of MOR activation of D2 neurons and showing enhanced opioid analgesia. We suggest that the imbalance with a loss of opioid tone on the D2 MSNs to now have a stronger effect on the D1R population that leads to greater opioid mediated antinociception. It remains surprising though that endogenous activation of MORs in the absence of exogenous opioids had no effect on pain-like behavior produced by either 2.5 or 5% formalin.

There is precedence that MORs in the striatum may modulate nociception. Previous studies showed that morphine microinjected into the head of the caudate inhibited nociceptive responses of the parafascicular nucleus of the thalamus.[39] Microinjection of morphine into the nucleus accumbens caused release of enkephalin into the amygdala.[40,41] Although, microinjection of an opioid antagonist into the nucleus accumbens did not attenuate systemic morphine antinociception in the formalin test,[42] yet others reported that microinjection of morphine into the nucleus accumbens reduced formalin nocifensive behaviors.[6] Further, the dysfunction of dopaminergic neurons in the VTA contributes to excessive pain-like behavior [43] and activation of VTA dopamine neurons reverses pathological allodynia caused by nerve injury or bone pain.[44] These latter studies emphasize the engagement of dopamine in nociception. However, it remains unclear if a striatal mechanism is responsible for the loss of opioid analgesia when MOR is ablated from D1R neurons. To our knowledge there has not been a thorough investigation of colocalization of dopamine receptor and MOR expression in the central nervous system, although they are known to be colocalized in the striatum, cortex and periaqueductal gray. Dopamine projects to many brain regions that modulate nociception including the central amygdala. Bilateral neurotoxic lesions central amygdala, (but not lesions of the basolateral nucleus) reliably attenuated morphine antinociception in the 2nd phase of the formalin test [24] as well as opioid antinociception in the tail flick thermal test.[24] Given we have differential effects of MOR ablation on pain modality in our study, it is unlikely that the effects of MOR ablation on opioid antinociception is mediated by this brain region.

A limitation of this work is our utilization of a breeding strategy to ablate MORs from D1R, A2aR or D2R neuronal populations and thus cannot inform specific circuits responsible for the changes in opioid mediated antinociception. Additionally, it remains unclear if ablation of MORs from these populations would modify the affective component of pain. Formalin-induced conditioned place avoidance engages the rostral anterior cingulate cortex [45] that projects to the nucleus accumbens to modulate affective and motivation aspects of pain.[46] We hope to address some of the limitations of our current work by specific circuit manipulation.

In conclusion, our data demonstrates that MORs have opposing effects on D1R and D2R neuronal populations that have opposing effects on opioid analgesia. Although this effect does not appear to translate to acute sensory noxious stimuli. Our data also suggests that endogenous activity at MORs on dopamine receptor expressing neurons has little if any effect on tonic nociception as there was no effect of ablation on baseline nociception in the formalin test, hot plate test or mechanical sensitivity in a model of neuropathic pain.

Disclosures

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Declaration of Competing Interest

The authors of this work certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this article. The authors also conform that generative AI was not used in the scientific writing of this manuscript.

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Data availability

The data that support the findings of this study are available on request from the corresponding author (CMC).

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