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Opioids Activate Brain Analgesic Circuits Through Cytochrome P450/Epoxygenase Signaling

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To assess the significance of brain cytochrome P450 (P450) activity in μ opioid analgesic action, we generated a novel mutant mouse with brain neuron-specific reductions in P450 activity; these mice showed highly attenuated morphine antinociception, as compared with controls. Pharmacological inhibition of brain P450 arachidonate epoxygenases also blocked morphine antinociception in mice and rats. These findings show that a neuronal P450 epoxygenase mediates the pain-relieving properties of morphine.

Activation of μ opioid receptors by morphine in the brain stem and spinal cord produces analgesia1, but the relevant post-receptor mechanisms are unknown. In the brain stem, opioid-induced stimulation of descending circuits from the ventrolateral periaqueductal gray (PAG) and rostral ventromedial medulla (RVM) produces powerful inhibition of spinal nociceptive transmission1. In the PAG, μ opioid receptors activate these circuits by increasing a presynaptic, voltage-dependent potassium conductance that inhibits GABA release2. Biochemical3 and electrophysiological2 studies suggest that phospholipase A_2

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AUTHOR CONTRIBUTIONS

J.C., J.N. and J.Y. performed all in vivo studies. C.F., J.G., and W.Y. contributed to the generation of the Null mouse. C.F. performed most of the Null mouse characterization, with additional help from J.G., M.B., and J.C. P.A., J.M., and A.S. assisted with histochemistry and microscopy. M.V., J.C., B.K., J.B., O.Z. and R.L. performed drug, receptor and enzyme assays. Z.S., S.Z., and M.W. synthesized MW06-25. S.O.Z. provided the CamKIIα-Cre mouse. J.C., C.F., X.D. and L.H. wrote the manuscript. X.D. and L.H. supervised the overall design and performance of the project.

(PLA_2), and its product arachidonic acid, are important in such analgesic signaling. Presently, the arachidonate epoxygenase pathway (Suppl. Fig.1), which uses P450s to produce up to four distinct epoxyeicosatrienoic acid isomers (EETs)4, was evaluated as a brain μ opioid analgesic transduction mechanism.

To circumvent the complexities of over 120 distinct mouse P450 genes (http://drnelson.utmem.edu/CytochromeP450.html) and the overlapping functions of multiple P450s, we generated a brain neuron-specific transgenic mouse (brain-*Cpr*-null, Null). In these mice, the floxed *Cpr* gene (encoding cytochrome P450 reductase [CPR], the enzyme required for all microsomal P450 activity) was targeted for deletion in Cre-expressing CNS neurons (via CamKIIα-Cre, see Suppl. Methods). Consistent with the pattern of CamKIIα-Cre expression5, immunohistochemistry confirmed the loss of neuronal CPR in the cerebral cortex (Fig. 1), hippocampus, and hypothalamus (Suppl. Fig. 2) of Null, but not control mice. However, CamKIIα-Cre expression is not limited to the forebrain5. Careful inspection of the brain stem of Null mice revealed a discrete population of neurons in the ventrolateral PAG that lacked anti-CPR immunolabeling; CPR was abundantly expressed in the same-sized neurons from the same brain area of control mice (Fig. 1). As compared with controls, Null mouse brains showed reductions in CPR protein levels (Suppl. Fig. 3a,b), CPR activity (Suppl. Fig. 3c), and P450 activity (Suppl. Table 1). CPR protein levels in brain stem homogenates were not different between genotypes (Suppl. Fig. 3a,b), consistent with the highly localized loss of CPR seen in the Null PAG (Fig. 1). Null mice showed good health, normal behavior, and brain morphology (see Suppl. Methods). All animal experiments were approved by the Institutional Animal Care and Use Committees of either Albany Medical College or the Wadsworth Center.

We used Null mice to probe the significance of neuronal P450s in morphine antinociception. Systemically-administered morphine increased thermal nociceptive latencies in control mice; this effect was abolished in Null mice 40 and 60 min after drug (Fig. 2a). ANOVA (between groups #1: drug; between groups #2: genotype; within [repeated measures]: time) found main effects of genotype ($F_{1,15}$=7.4, P<0.02), drug ($F_{1,15}$=17.5, P<0.01), and time ($F_{3,45}$=10.4, P<0.01), significant drug by genotype ($F_{1,15}$=7.4, P<0.02), time by genotype ($F_{3,45}$=3.8, P<0.02), time by drug ($F_{3,45}$=6.0, P<0.01), and three-way ($F_{3,45}$=3.8, P<0.02) interactions. Further studies documented that this deficit occurred in Null mice of both sexes, and after varying doses of morphine (Fig. 2b). ANOVA (between groups #1: dose; between groups #2: genotype) found significant main effects of dose ($F_{2,39}$=15.2, P<0.01) and genotype ($F_{1,39}$=16.2, P<0.01) and a significant two-way interaction ($F_{2,39}$=4.0, P<0.05). Null mice also showed deficient antinociceptive responses to morphine assessed in a mechanical nociceptive test (Fig. 2c). ANOVA (between groups #1: drug; between groups #2: genotype; within [repeated measures]: time) found significant main effects of drug ($F_{1,15}$=26.8, P<0.01), genotype ($F_{1,15}$=8.8, P<0.01), time ($F_{1,15}$=17.0, P<0.01), and significant genotype by drug ($F_{1,15}$=7.0, P<0.02), time by drug ($F_{1,15}$=14.2, P<0.01), time by genotype ($F_{1,15}$=7.0, P<0.02), and three-way interactions ($F_{1,15}$=6.5, P<0.05). Baseline (pre-drug) nociceptive latencies and those following saline treatment did not differ between genotypes (Figs. 2a–c). Baseline latencies were also not different between genotypes following exposure to milder thermal (48°C vs. 55°C) or mechanical (400 g vs. 600 g)

stimuli (data not shown). Thus, Null mice respond normally to painful stimuli, but are highly resistant to the antinociceptive properties of morphine. Furthermore, the lack of genotype differences in either opioid receptor properties or brain morphine levels (Suppl. Fig. 4) indicates that these variables do not explain the defective morphine antinociception in Null mice. The absence of genotype differences in brain morphine levels is consistent with the known metabolism of morphine by glucuronidation, a non-P450 pathway. Presently, it was not necessary to measure brain levels of the active metabolite morphine-6-glucuronide, since this metabolite is not formed in mice6. In complementary pharmacological experiments in control mice, we found that treatment with the P450 blocker MW06-25 attenuated morphine antinociception (Fig. 3a), consistent with the findings in Null mice. ANOVA (between groups: drug; within groups [repeated measures]: time) found significant main effects of drug ($F_{5,84}$=6.7, P<0.01), and time ($F_{4,84}$=11.0, P<0.01) and a significant drug by time interaction ($F_{20,84}$=2.9, P<0.01). MW06-25 lacked activity on other arachidonate metabolic pathways (Suppl. Table 2), confirming a P450 target for this drug (Suppl. Fig. 5). Note that systemic morphine antinociception was not completely abolished in Null mice (Fig. 2b) or by P450 inhibitors in control subjects (Fig. 3a). Explanations for this residual morphine effect might include incomplete attenuation of supraspinal P450 activity, spinal sites for opioid action, or alternative, non-epoxygenase opioid transduction mechanisms2. In further studies in rats, we found that CNS administration of several P450 inhibitors (Suppl. Fig. 5) also blocked the antinociceptive effects of *centrally*-administered morphine (Fig. 3b), confirming a supraspinal localization (e.g. PAG, RVM) for the opioid-P450 interaction. ANOVA (between groups: drug; within groups [repeated measures]: time) found a significant main effect of time ($F_{3,81}$=16.3, P<0.01) and a significant drug by time interaction ($F_{12,81}$=2.3, P<0.02). The CPR-deficient ventrolateral PAG neurons identified in Null mice (Fig. 1) may normally contain the analgesia-relevant P450s, but this requires further study. P450s can metabolize arachidonic acid by hydroxylation as well as by epoxidation. However, we found that treatment with MS-PPOH (which inhibits the latter, but not the former reaction7) blocked morphine antinociception in both control mice (Fig. 3a) and rats (Fig. 3b), strongly supporting a P450 epoxygenase mechanism in morphine action.

Mu opioid receptors couple with G proteins to stimulate many intracellular pathways8, but epoxygenase-mediated μ signaling has not been previously proposed. Consistent with this model (Suppl. Fig. 1), μ activation can increase arachidonate synthesis through stimulation of $PLA_2$2,3. In brain, this could occur via sequential activation of PLCγ, inositol-1,4,5-triphosphate receptors9,10, and calcium-dependent PLA_2. Alternatively, arachidonate synthesis could be increased via diacylglycerol metabolism. Other consistent findings include the potentiation of μ agonist action by COX inhibitors11, and the enhanced morphine antinociception in 12-LOX knockout mice12. Both observations could be explained via shunting of arachidonate from the COX or 12-LOX pathways, respectively, to the epoxygenase pathway. A further essential finding in support of the epoxygenase model is the recently-described antinociceptive activity of the epoxygenase product 14,15-EET following PAG administration13. Although EETs have numerous biological activites4, the mechanisms by which this or other EET isomers might activate analgesic circuits remain to be established. EETs may act on EET receptors, or directly on ion channels4. However, EET

metabolites are numerous4 and could also be active. A voltage-gated potassium channel may be essential for μ agonist action in the PAG2, but the effects of EETs on these channels have not yet been determined.

Previously, a P450 inhibitor was reported to block μ opioid action in vitro2. Because P450 enzymes were thought to be important within the 12-LOX metabolic pathway, this and other findings led the authors to propose a μ opioid-12-LOX mechanism for analgesic signaling2,11. Although P450 activity may not be required for the synthesis of 12-LOX metabolites14, both 12-LOX and P450 epoxygenase enzymes could be important in μ opioid action. To further address this, additional studies are needed to identify the arachidonate metabolite(s) which are released by morphine and are required for its analgesic actions.

The present results show an essential role for brain P450 epoxygenases in the pain-relieving properties of μ opioids, but additional studies are needed to fully understand the significance of these findings. For example, other brain-acting analgesics (e.g. delta and kappa opioids, cannabinoids15) may require P450 activity. In the case of morphine, it is not known whether P450s play a specific role in μ-activated analgesic circuits, or a more general role in opioid receptor transduction mechanisms. For example, P450s may not be required for morphine's therapy-limiting side effects, such as respiratory depression, tolerance, or rewarding effects leading to addiction. Identification of the analgesia-relevant brain P450s could lead to new treatments for pain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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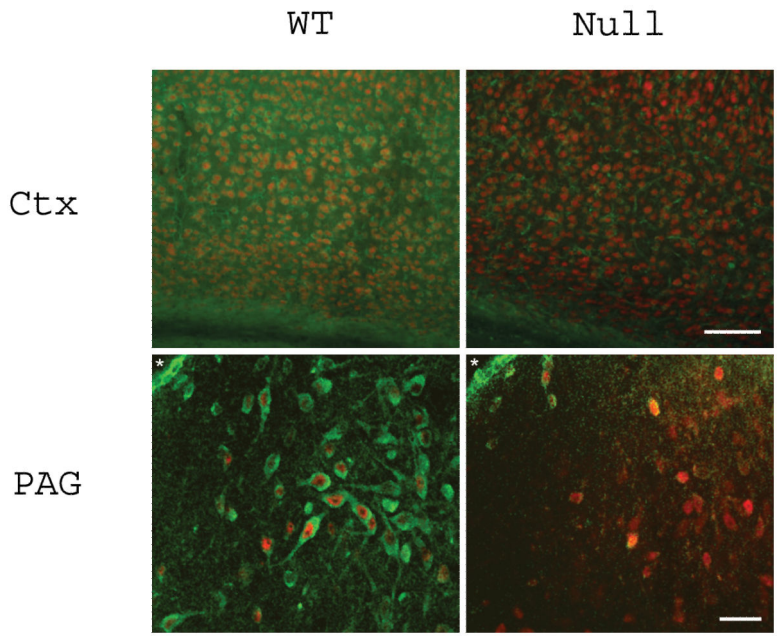
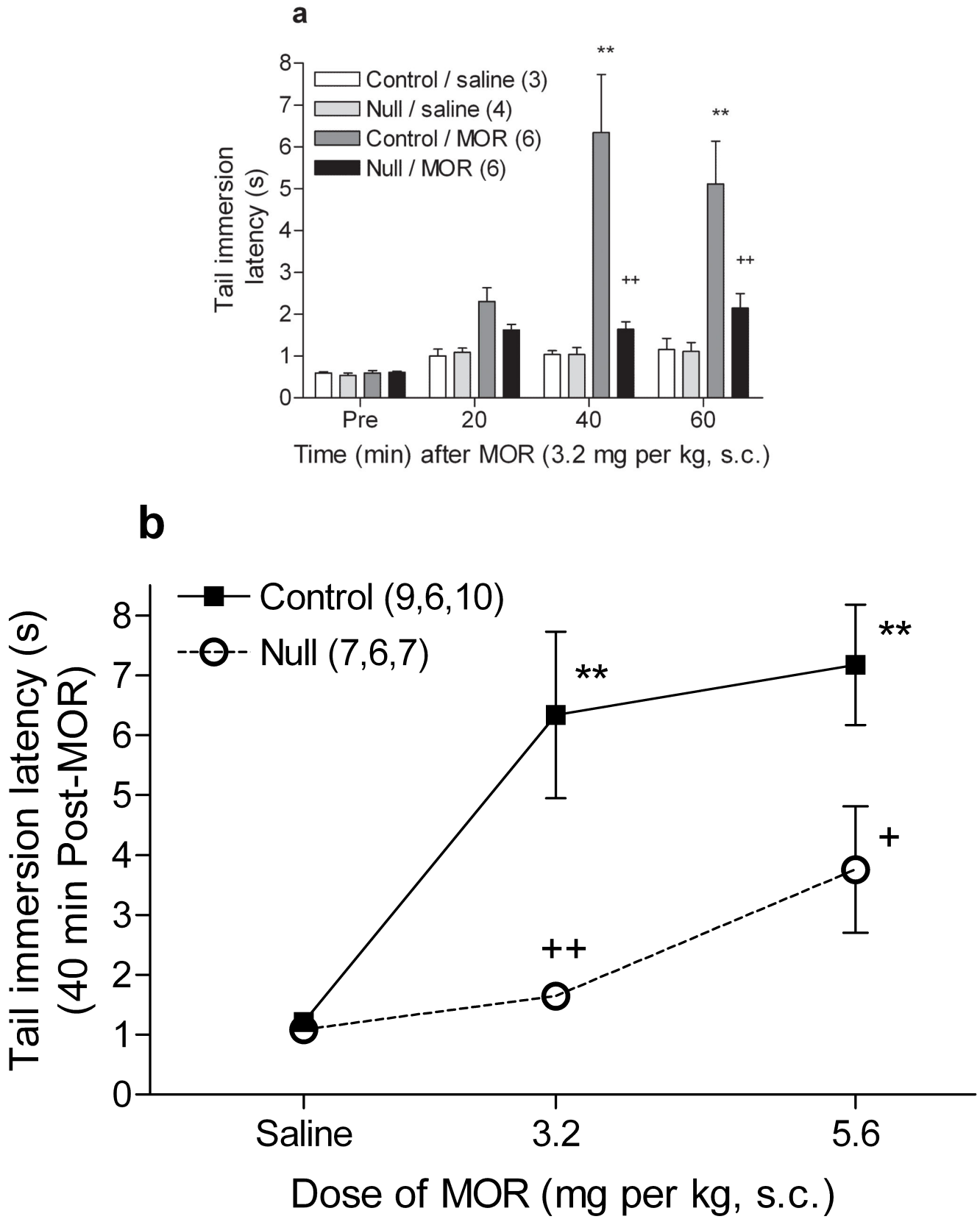


Figure 1.

Double immunolabeling showing expression of neuronal CPR in cerebral cortex (Ctx**,** top) and PAG (bottom) in control (WT, left) and Null (right) mice. Coronal, free-floating sections were co-incubated with a polyclonal antibody against CPR (green) and a monoclonal antibody to neuronal marker NeuN (red). **Top**: Merged images show expression of CPR in control, but not in Null cortical neurons (corpus callosum and Ctx layer VI shown near bottom, scale bar: 200 μm). **Bottom**: Confocal merged images of ventrolateral PAG (−4.2 to −4.6 mm from bregma) showing large neurons with cytoplasmic CPR expression in control mice (left). A field of similar-sized neurons from Null mice (right) shows the absence of CPR expression. Typical results, representative of at least four mice in each group, are shown. *Upper left corner of both images shows the cerebral aqueduct (scale bar: 50 μm).



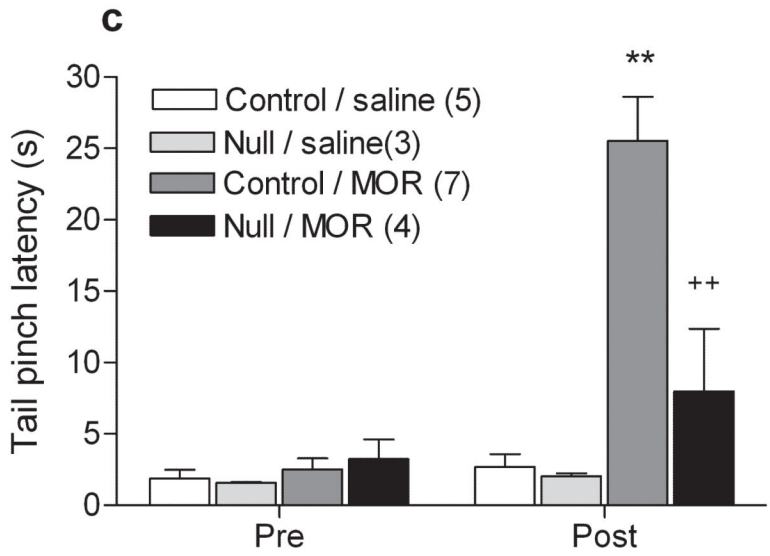


Figure 2.

Morphine antinociception in brain CPR-deficient (Null) and control mice. **(a)** Females were tested for baseline nociceptive responses (Pre), received saline or morphine (MOR, 3.2 mg/kg, s.c.) and were re-tested. Ordinate shows latencies (s, mean ± SEM) for the n values in parentheses. **,^{++}P<0.01 vs. control/saline, control/MOR, respectively, at the same time. **(b)** Dose-response curves were constructed by testing males and females as in (a) following saline or morphine (5.6 mg/kg, s.c.). Latencies (ordinate, s, mean ± SEM; 40 min after drug) did not differ between sexes by ANOVA, and were pooled. Data from the 3.2 mg/kg morphine treatment groups (40 min) are re-drawn from (a). **P<0.01 vs. saline within genotype; $^{+,++}P$<0.05, 0.01, respectively vs. control at the same dose. **(c)** Tail-pinch latencies (ordinate, s, mean ± SEM) were measured before (Pre) and 41 min after (Post) morphine (5.6 mg/kg, s.c.) in males. **,^{++}P<0.01 vs. control/ saline and control/MOR, respectively.

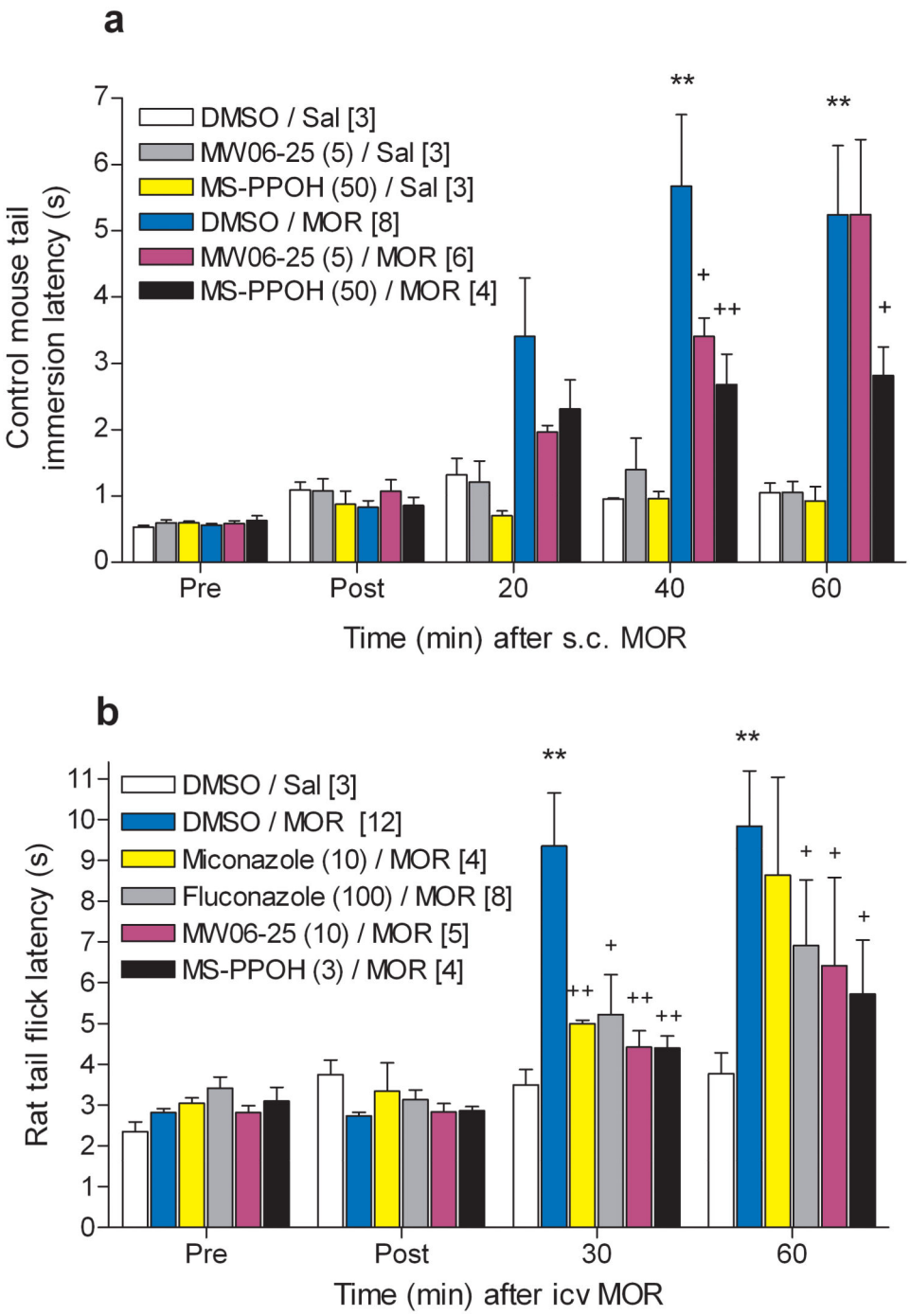


Figure 3.

Effects of P450 and epoxygenase inhibitors on morphine antinociception. (**a**) Control mice were baseline (Pre) tested for nociceptive latencies (ordinate, s, mean ± SEM), re-tested 15 min after intracerebroventricular (icv) infusion (Post) of inhibitors or vehicle (DMSO), and at the time points (abscissa) following morphine administration (MOR, 5.6 mg/kg, s.c.). For each group, n values are in brackets; doses of inhibitors (nmol, icv) are in parentheses. $^{**}P<$ 0.01 vs. DMSO/Sal; $^{+,++}P < 0.05$, 0.01 vs. DMSO/MOR at the same time. (**b**) Rats were

tested for tail flick nociceptive responses exactly as in (a) except that icv morphine (MOR, 20 μg) was administered immediately following post-icv testing. When tested in the absence of morphine, none of these inhibitor treatments modified nociceptive latencies (data not shown). Significant differences are labeled as in (a). Results were nearly identical on the hot plate test (data not shown).