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NOCICEPTION INCREASES DURING OPIOID INFUSION IN OPIOID RECEPTOR TRIPLE KNOCK-OUT MICE

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Abstract—Opioids are extensively used analgesics yet can paradoxically increase pain sensitivity in humans and rodents. This hyperalgesia is extensively conceptualized to be a consequence of opioid receptor activity, perhaps providing an adaptive response to analgesia, and to utilize *N*-methyl-D-aspartate (NMDA) receptors. These assumptions were tested here in opioid receptor triple knock-out (KO) mice lacking all three genes encoding opioid receptors (μ, δ, and κ) by comparing their thermal nociceptive responses to the opioids morphine and oxymorphone with those of B6129F$_1$ controls. Injecting acute opioid bolus doses in controls caused maximal analgesia that was completely abolished in KO mice, confirming the functional consequence of the KO mouse opioid receptor deficiency. Continuous opioid infusion by osmotic pump in control mice also initially caused several consecutive days of analgesia that was shortly thereafter followed by several consecutive days of hyperalgesia. In contrast, continuously infusing KO mice with opioids caused no detectable analgesic response, but only immediate and steady declines in nociceptive thresholds culminating in several days of unremitting hyperalgesia. Finally, injecting the non-competitive NMDA receptor antagonist MK-801 during opioid infusion markedly reversed hyperalgesia in control but not KO mice. These data demonstrate that sustained morphine and oxymorphone delivery causes hyperalgesia independently of prior or concurrent opioid or NMDA receptor activity or opioid analgesia, indicating the contribution of mechanisms outside of current conceptions, and are inconsistent with proposals of hyperalgesia as a causative factor of opioid analgesic tolerance.

Key words: hyperalgesia, morphine, oxymorphone, knock-out mice, nociception, NMDA.

Opioids continue to be among the most efficacious and widely used analgesics for moderate to severe pain. Their clinical use, however, can be complicated by unwanted side-effects, including a paradoxical ability to enhance pain sensitivity (i.e. hyperalgesia) (Arner et al., 1988; De Conno, 1991). These observations are confirmed in laboratory studies where sustained opioid delivery via continuous infusion or repeated injection reduces sensory thresholds in rodents, resulting in hypersensitivity to tactile and thermal stimuli (Mao et al., 1994, 2002; Vanderagh et al., 2001; Kest et al., 2002; Xie et al., 2005; Gardell et al., 2006; Juni et al., 2006).

An array of mechanisms is proposed to underlie opioid-induced hyperalgesia. For example, morphine and other opioids has been demonstrated to directly activate a subpopulation of opioid receptors coupled to an excitatory (i.e. G_s) effector mechanism, distinct from those (i.e. $G_{i/o}$-coupled) mediating analgesia, to prolong the action potential of dorsal root ganglion neurons (Crain and Shen, 2000). Other findings are consistent with the hypothesis that hyperalgesia results from an opioid receptor-mediated opponent-process which acts as an adaptive foil to opioid analgesia (Simonnet and Rivat, 2003). Hyperalgesia is again characterized as an adaptive response in a proposed signal transduction theory of pain processing whereby the nociceptive threshold is dynamic, employing a moving weighted average dependent on recent past sensory input (Xu et al., 2003). Accordingly, morphine stimulation of opioid receptors causes analgesia that is logically followed by hyperalgesia. A series of studies also describes a system-wide mechanism integrating spinopetal projections from the rostro-ventral medulla with spinal alterations that modulate primary afferent activity (Ossipov et al., 2004). Despite their diversity, these accounts unanimously agree that hyperalgesia is a consequence of opioid receptor-mediated mechanisms. Recently, this supposition was specifically tested by spinally infusing rats for several days with oxymorphone enantiomers (Gardell et al., 2006). Only the levorotatory isomer caused thermal and tactile hypersensitivity, indicating that oxymorphone produces hyperalgesia by specifically interacting with opiate receptors.

N-methyl-D-aspartate (NMDA) receptor activity contributes to central hyperactive states associated with increased nociception (Mao et al., 1995) and NMDA antagonists are widely reported to attenuate hyperalgesia caused by assorted opioids in rodents and humans (see review: Xu et al., 2003). Mechanistically, it has been suggested that hyperalgesia might result from increased activity at pre-synaptic NMDA receptors localized to central primary afferent terminals (Liu et al., 1994, 1997), where they may be anatomically linked with opioid receptors, causing spinal sensitization and increased nociceptive in-

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Abbreviations: BL, baseline; KO, opioid receptor triple knock-out; MK801, (+)-5-methyl-10,11-dihydro-5*H*-dibenzo(a,d)cyclohepten-5,10-imine hydrogen maleate; M3G, morphine-3β-glucuronide; NMDA, *N*-methyl-D-aspartate.

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put (Ossipov et al., 2004). NMDA antagonists including the non-competitive MK-801 also potentiate analgesia, shifting the morphine dose-response curve leftward (Kozela et al., 2001; Nemmani et al., 2004). Accordingly, NMDA antagonists might attenuate hyperalgesia only indirectly, by increasing the latent morphine analgesia obfuscated by—but concurrent with—increased nociception (Juni et al., 2006).

Here, we tested the assumptions that prior or concurrent opioid analgesia and/or receptor activity is critical to opioid hyperalgesia using opioid receptor triple knock-out (KO) mice lacking all three opioid receptor genes (Clarke et al., 2002; Cox et al., 2005). To confirm the functional consequence of opioid receptor KO, KO and B6129F$_1$ control mice were injected with acute bolus doses of morphine and oxymorphone and assayed for analgesia. Both strains were also assayed daily for nociception while continuously infused with morphine and oxymorphone for 7 and 10 days, respectively. Finally, we addressed the possibility that NMDA receptors contribute to opioid hyperalgesia by interacting with opioid receptors or opioid analgesia by injecting MK-801 and assaying nociception in hyperalgesic KO mice during morphine and oxymorphone infusion.

EXPERIMENTAL PROCEDURES

Subjects

KO mice were generated in the Pintar laboratory by cross-breeding mice singly deficient in the genes coding for μ, δ, and κ receptors using standard homologous recombination techniques (Clarke et al., 2002; Cox et al., 2005). Accordingly, B6129F$_1$ mice were bred and served as controls. The combinatorial mice are devoid of brain or spinal cord [^{3}H]naloxone receptor labeling, indicating the complete absence of any μ, δ, and κ opioid receptor subtype, and lack gross behavioral or anatomical alterations (Clarke, 2002; Cox et al., 2005). Subjects were housed four to a cage and maintained on a 12-h light/dark cycle in a temperature-controlled environment with unrestricted food and water and tested as adults. To eliminate any interaction of sex with nociceptive sensitivity and morphine analgesia (Kest et al., 2000), only males were tested. For all conditions, naïve groups of $n>6$/strain were used. All protocols were carried out in accordands with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering.

Drug doses and delivery

Morphine (gift of NIDA Drug Supply Program, Bethesda, MD, USA) and oxymorphone (gift of Dr. C. E. Inturrisi, New York, NY, USA) were used as opioid test probes since both were used in previous studies specifically assaying the contribution of opioid receptor activity and analgesia to opioid-induced hyperalgesia (Gardell et al., 2006; Juni et al., 2006). Both drugs and the non-competitive NMDA receptor antagonist MK-801 (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in physiological saline (0.9% NaCl). Acute bolus doses of opioid (10 mg/kg) and MK-801 (0.05 mg/kg) were injected s.c. Osmotic pumps (Model 2001; Durect Corporation, Mountain View, CA, USA) filled with morphine and oxymorphone were implanted s.c. under oxygen/isoflurane inhalant anesthesia through a small dorsal midline incision to provide for their continuous infusion at cumulative daily doses of 40.0 and 20.0 mg/kg, respectively.

Nociceptive assay

The tail-withdrawal test of D'Amour and Smith (1941) was chosen for its stability in the context of repeated testing (Kest et al., 2002; Juni et al., 2006). Tails were immersed in water maintained by an immersion circulator pump (Fisher Isotemp Model 71; Fisher Scientific, Hampton, NH, USA) at 47.3 °C$\pm$0.2 °C, which elicits pre-opioid baseline (BL) latencies between 7 and 9 s, minimizing possible floor effects during hyperalgesia. Latency to withdrawal was recorded twice at 20 s intervals and averaged. A cutoff latency of 30 s was employed to prevent tissue damage. Nociception was tested near mid-photophase to reduce circadian effects on nociception (Kavaliers and Hirst, 1983).

After obtaining BL latencies, mice were injected with an acute opioid bolus dose and retested for 120 min or implanted with opioid filled pumps and retested every subsequent 24 h during infusion. MK-801 was tested in separate infusion groups at intervals determined in the above study to cause morphine and oxymorphone hyperalgesia (days 6 and 9, respectively) in control and KO mice. In these groups, latencies were assayed prior to infusion (BL) and MK-801 injection to confirm manifest hyperalgesia, and for 120 min after MK-801.

Data analysis

Withdrawal latencies were analyzed using two-way (strain$\times$time) repeated-measures ANOVA followed by Tukey-Kramer tests post hoc. Differences that exceeded an $\alpha=0.05$ criterion were considered significant.

RESULTS

Opioid analgesia is abolished in opioid KO mice

Acute morphine and oxymorphone bolus doses produced large significant main effects of strain (morphine: $F_{(1,14)}=406.4$; oxymorphone: $F_{(1,11)}=20443.1$), time (morphine: $F_{(4,56)}=277.7$; oxymorphone: $F_{(4,44)}=736.3$), and their interaction (morphine: $F_{(4,56)}=263.8$; oxymorphone: $F_{(4,44)}=741.5$; $P=<0.0001$ for each analysis). The striking effect of triple opioid receptor KO on morphine and oxymorphone analgesia is shown in Fig. 1A and B, respectively. In control mice, morphine increased withdrawal latencies approximately two- to threefold from BL (time 0) values for 90 min, whereas oxymorphone elevated latencies to maximally allowable values during the entire 120 min testing period. Both drugs were without even minimal effect on KO latencies which remained completely unchanged from BL values. There were no BL differences between KO and control mice prior to either opioid injection.

Increased nociception during opioid infusion in mice lacking opioid receptors

Morphine infusion in control and KO mice caused significant latency alterations related to strain ($F_{(1,12)}=418.7$; $P<0.0001$), time ($F_{(7,84)}=178.2$; $P<0.0001$), and their interaction ($F_{(7,84)}=83.5$; $P<0.0001$). For control mice, latencies were increased relative to BL (day 0) values on the first 2 days, then followed by decreases on days 4–7 (Fig. 2A), demonstrating significant analgesia followed by hyperalgesia, respectively. In KO mice, only significant latency de-