# ■ PAIN MEDICINE

# *Morphine-6β-glucuronide Rapidly Increases Pain Sensitivity Independently of Opioid Receptor Activity in Mice and Humans*

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*Background:* Previous data indicate that morphine-6β-glucuronide (M6G), a morphine metabolite with analgesic properties, can paradoxically increase pain sensitivity in mice and humans. The authors tested mice and humans for M6G hyperalgesia and assessed the contribution of *N*-methyl-D-aspartate receptor activity in mice.

*Methods:* Nociception after acute injection (10 mg/kg) and chronic infusion (1.6 mg/kg per 24 h) of M6G or saline was assayed using the tail-withdrawal test in CD-1 mice implanted with pellets containing the opioid antagonist naltrexone or placebo and in knockout mice lacking μ-, κ-, and δ-opioid receptors and their B6129F$_1$ controls. In volunteers, responses to heat pain were tested after a M6G (0.4 mg/kg) injection in the presence of a continuous high naloxone (0.04-mg/kg bolus followed by 0.04 mg/kg per hour) or saline background infusion.

*Results:* Acute M6G injection evoked analgesia in CD-1 mice implanted with placebo pellets and B6129F$_1$ control mice, whereas it caused hyperalgesia in CD-1 mice treated concurrently with naltrexone and in knockout mice. Continuous M6G infusion produced hyperalgesia within 24 h, lasting for a minimum of 6 days, in both placebo- and naltrexone-pelleted mice. The *N*-methyl-D-aspartate receptor antagonist MK-801 (0.05 mg/kg) blocked and reversed hyperalgesia after the acute injection and continuous infusion of M6G, respectively. In humans, M6G increased heat pain sensitivity for at least 6 h independently of simultaneous naloxone infusion.

*Conclusions:* These data indicate that M6G causes hyperalgesia independent of previous or concurrent opioid receptor activity or analgesia. In mice, a causal role for the *N*-methyl-D-aspartate receptor is also indicated.

IN contemporary clinical medicine, μ opioids such as morphine are the first choice for treating severe acute and chronic pain.[1] However, chronic opioid use is associated with several unwanted side effects, including a paradoxical increase in pain sensitivity. This opioid-induced hyperalgesia has been reported in preclinical studies with rodents and humans and described in the clinical literature.[1-4] Although it is widely postulated that activating opioid receptors or opioid analgesia is critical initial prerequisites for opioid-induced hyperalgesia,[5-9] contrary results have been recently reported. For example, infusing the μ opioids morphine and oxymorphone evoked hyperalgesic responses within 48 h in opioid receptor triple knockout (TrKO) mice completely devoid of μ-, κ-, and δ-opioid receptors.[10] Hyperalgesia during continuous morphine infusion is also observed in outbred CD-1 mice implanted with pellets containing naltrexone, a general opioid receptor antagonist.[11,12] *N*-methyl-D-aspartate (NMDA) receptor antagonists, such as MK-801, reverse morphine hyperalgesia.[11,12] Because NMDA antagonists also potentiate opioid analgesia, they might attenuate hyperalgesia indirectly, by increasing the latent opioid analgesia obfuscated by the concurrent increased nociception. However, this possibility is not supported by the demonstration that MK-801 reverses morphine hyperalgesia in naltrexone-pelleted mice.[11,12]

In humans, morphine undergoes hepatic glucuronidation to more water-soluble compounds, facilitating their renal elimination.[13] One of these metabolites, morphine-6β-glucuronide (M6G), displays affinity at μ-opioid receptors equal to that of morphine and is a potent opioid analgesic in humans and mice.[13-16] However, data from some studies suggest that acute M6G doses can cause hyperalgesia. In the first two studies, a single acute M6G injection reduced tail-withdrawal latencies by up to 40% in mice lacking exons 1 and/or 2 of the μ-opioid receptor.[16,17] In a third study, low M6G doses (10 and 20 mg/70 kg) progressively increased the time to rescue analgesic medication in patients after orthopedic surgery, whereas a higher dose (30 mg/70 kg) caused a subsequent decrease in the time to rescue medication,

> ◇ This article is featured in "This Month in Anesthesiology." Please see this issue of ANESTHESIOLOGY, page 9A.

> ◆ This article is accompanied by an Editorial View. Please see: Lötsch J: Pleiotropic effects of morphine-6β-glucuronide. ANESTHESIOLOGY 2009; 110:1209–10.

which may be considered a manifestation of hyperalgesia.[18] Finally, we recently demonstrated in an open-label study that a single injection of M6G increased pain ratings in healthy volunteers subject to a cutaneous heat pain assay.[19] Because M6G hyperalgesia was not the specific aim of these studies, several questions remain. Specifically, it is not known whether M6G causes hyperalgesia independently of opioid receptor activity, or whether NMDA receptors contribute to this effect. Furthermore, because only acute doses of M6G were injected these studies, it is not known what effect longer M6G delivery paradigms might have on nociception. These questions cannot be addressed by simply extrapolating from studies with morphine because morphine metabolism in mice does not yield M6G. Furthermore, morphine conjugation in rodents and humans also yields morphine-3-glucoronide (M3G), a pronociceptive metabolite thought to underlie morphine hyperalgesia.[10–12,20,21] If both morphine metabolites are indeed pronociceptive, it would not be possible to distinguish between their hyperalgesic effects in human subjects treated with morphine.

Here, we addressed these issues by assaying nociceptive sensitivity in mice and human volunteers injected with an acute M6G dose. The contribution of opioid receptors to M6G hyperalgesia was assessed by treating subjects concurrently with an opioid receptor antagonist. Additional evidence was obtained by testing TrKO mice devoid of $\mu$-, $\kappa$-, and $\delta$-opioid receptor types under identical conditions. The long-term consequences of M6G infusion on nociception was also assessed by assaying nociception daily in mice subject to 6 days of continuous M6G infusion. For both acute and chronic M6G treatment conditions, the ability of the noncompetitive NMDA receptor antagonist MK-801 to reverse hyperalgesia in mice was tested. Because MK-801 can potentiate latent M6G analgesia concurrent with hyperalgesia, mice in this treatment condition were also simultaneously treated with naltrexone.

## Materials and Methods

### Animal Studies

**Subjects and Nociceptive Assay.** All procedures were approved by the College of Staten Island/City University of New York Institutional Animal Care and Use Committee (New York, New York) and conform to guidelines of the International Association for the Study of Pain. Adult male CD-1 mice were purchased (Charles Rivers, Kingston, NY), whereas TrKO mice (gift of John Pintar, Ph.D., Professor, Robert Wood Johnson Medical School, Piscataway, New Jersey) were derived by cross-breeding mice singly deficient in the genes coding for $\mu$, $\delta$, and $\kappa$ receptors using standard homologous recombination techniques.[22,23] Accordingly, B6129F$_1$ mice were bred and served as TrKO controls. The combinatorial mice are devoid of brain or spinal cord [$^3$H]naloxone

receptor labeling, indicating the complete absence of any $\mu$-, $\delta$-, or $\kappa$-opioid receptor subtype, and lack gross behavioral or anatomical alterations.[22,23] Mice were maintained on a 12:12 h light–dark cycle in a climate-controlled room with free access to food and tap water. Each subject was used once, and for all studies a minimum of six mice were used in every experimental group. The tail-withdrawal test of D'Amour and Smith was chosen for its stability in the context of repeated testing.[10–12,24] Tails of the mice were immersed in water maintained at $47.3° \pm 0.2°C$, which elicits preopioid baseline latencies between 9 and 11 s, minimizing possible floor effects during hyperalgesia. Latency withdrawal was recorded twice at 30-s intervals and averaged. A cutoff latency of 30 s was used to prevent tissue damage. Nociception was tested near midphotophase to reduce circadian effects on the test results.[25]

**Drug Delivery.** Morphine-6$\beta$-glucuronide (National Institute on Drug Abuse Drug Supply Program, Bethesda, MD) and MK-801 (Sigma-Aldrich, St. Louis, MO) were dissolved in saline and injected subcutaneously. Acute doses were injected in a volume of 10 ml/kg, whereas continuous infusion was achieved using osmotic pumps (Alzet model 2001; Alza, Mountain View, CA).[10–12] The pumps were implanted during oxygen–isoflurane anesthesia through a small dorsal midline incision. Osmotic pumps afford continuous opioid infusions and control for hyperalgesia associated with withdrawal in opioid-dependent subjects that potentially confounds experiments in which chronic opioid treatment is accomplished *via* repeated acute injections.[26] Pellets containing 30 mg of the general opioid receptor antagonist naltrexone or a placebo formulation (National Institute on Drug Abuse Drug Supply Program) were wrapped in nylon mesh and subcutaneously implanted in the nape of the neck 24 h before M6G delivery by acute injection or continuous infusion. In rats, 30-mg naltrexone pellets substantially elevate naltrexone plasma levels 1 h after implantation, and sustain pharmacologically active levels of naltrexone such that there is a greater than 50-fold rightward shift of the morphine analgesia dose–response curve 8 days later.[27] In mice, naltrexone pellets completely abolished the analgesic effect of an acute 10-mg/kg morphine injection starting 24 h after implantation (coinciding here with start of M6G infusion) and for a minimum of 7 additional days.[11]

**Study Design.** Nociception was assayed before (*i.e.*, baseline) and at 30-min intervals for 120 min after an acute M6G (10 mg/kg) or saline injection in CD-1 mice implanted with naltrexone or placebo pellets. TrKO mice and their B6129F$_1$ controls were subject to the identical acute injection protocol with the exception that they were not implanted with pellets of any kind. The effect of continuous M6G (1.6 mg/kg/24 h) or saline infusion on nociception was tested for 6 consecutive infusion days in separate groups of CD-1 mice implanted

with naltrexone or placebo pellets. In these groups, withdrawal latencies were measured before the start of infusion (baseline) and on each subsequent day. Finally, the ability of an acute MK-801 (0.05 mg/kg) dose to reverse M6G hyperalgesia was tested in separate groups of CD-1 mice implanted with naltrexone pellets. The MK-801 dose chosen for study does not increase tail-withdrawal latencies in naive or saline-infused mice.[10,12,28] For the acute M6G condition, mice were first injected with MK-801 and then an acute M6G dose (10 mg/kg) 30 min later. Nociception was assayed immediately before the M6G injection (baseline) and at 30-min intervals for the next 2 h. Mice subject to continuous M6G infusion (1.6 mg/kg per 24 h) were assayed for nociception before infusion (baseline) and on day 4 (t = 0), at which time all mice were hyperalgesic in agreement with the continuous infusion study above. Immediately after assaying nociception on day 4, MK-801 was injected and nociception was reassessed at 30-min intervals for 2 h. Control mice in both acute and chronic M6G conditions were injected with saline vehicle instead of MK-801.

**Data Analysis.** Withdrawal latencies were analyzed (GB Stat; Dynamic Microsystems, Silver Spring, MD) using a three-way analysis of variance (pretreatment [placebo–naltrexone pellets] × drug [saline–M6G] × post-drug interval) except for latencies obtained after MK-801 injection, which were subject to two-way analysis of variance (treatment [MK-801–saline] × posttreatment interval). The Fisher least significant difference method (protected *t* tests) was used for *post hoc* comparisons. *P* values less than 0.05 were considered significant. All animal values are reported as group mean ± SEM withdrawal latencies.

*Human Studies*

**Subjects.** Forty human volunteers (aged 18–39 yr; 20 women and 20 men; body mass index <30 kg/m²) were recruited to participate in the studies after approval of the protocols was obtained from the Leiden University Medical Center Human Ethics Committee (Commissie Medische Ethiek, Leiden, The Netherlands) and after giving written informed consent. All candidates underwent a physical examination, and only healthy subjects without a history of illicit drug use or psychiatric illness were allowed in the study. All subjects were advised not to eat or drink for at least 8 h before the start of the study.

The subjects were randomly allocated to one of four treatment groups. Twenty subjects were injected with 0.4 mg/kg intravenous M6G; 10 of these (5 men) had an intravenous background infusion of naloxone (0.04-mg/kg bolus, followed by 0.04 mg · kg⁻¹ · h⁻¹), and the 10 others (5 men) had an intravenous background of

normal saline. Twenty subjects were injected with intravenous placebo (0.9% NaCl); 10 of these subjects (5 men) had an intravenous background infusion of naloxone (0.04-mg/kg bolus, followed by 0.04 mg · kg⁻¹ · h⁻¹), and the 10 others (5 men) had a intravenous background infusion of normal saline. The naloxone–saline infusion started 30 min before the M6G–placebo infusion and lasted for 2.5 h (end of study). Thermal pain measurements were performed just before the naloxone bolus infusion (t = −30 min), just before the M6G bolus infusion (t = 0 min), and next at 10-min intervals (first hour of the study) and 20-min intervals (second hour of the study).

**Drugs.** Morphine-6β-glucuronide was donated by CeNeS Ltd. (Cambridge, United Kingdom; now Paion AG, Aachen, Germany), and naloxone was purchased from Orpha-Devel GmbH (Pukersdorf, Austria). Placebo–saline (0.9% NaCl) was manufactured by the local pharmacy. Randomization†† and preparation of the syringes was performed by a physician not involved in the study. M6G bolus was infused over 90 s, and naloxone bolus was infused over 120 s.

**Pain Measurements.** Heat pain was induced using a TSA-II device running the WinTSA 5.32 software package (Medoc Ltd., Ramat Yishai, Israel).[29] Using a 3-cm² Peltier element or thermode, the skin of the volar side of the left forearm was stimulated with a gradually increasing stimulus (0.5°C/s). Baseline temperature was set at 32°C. Subjects were asked to rate their pain verbally on a scale from 0 (no pain) to 10 (worst pain imaginable), *i.e.*, a numerical rating scale (NRS). After the subjects were familiarized with the device and NRS scoring, the NRS to three heat stimuli was assessed with the following peak temperatures: 47°, 48°, and 49°C. The lowest stimulus causing an NRS ≥5 and ≤7 was used in the remainder of the study. The test data were discarded. Next, baseline values were obtained in triplicate (the averaged value was used in the data analysis). To prevent frequent stimulation of just one part of the skin, we divided the volar side of the test arm into six zones and moved the thermode from zone to zone (1 to 6 and back) between subsequent stimuli.[29]

**Data Analysis.** In a previous study on the effect of low-dose alfentanil on thermal antinociception using the TSA II device, we observed significant analgesic effects (mean reduction in visual analog score from 8 to 2 cm) in 10 subjects.[29] Based on these data, we initially performed a pilot study (open design) showing that 0.4 mg/kg intravenous M6G was analgesic using an electrical pain assay but hyperalgesic using heat pain,[19] and set the sample size for the current study at n = 10 per treatment level. To assess the effect of the intravenous drug infusion over time, an analysis of variance using a repeated-measures design was performed (factor = NRS). To assess the effect of naloxone *versus* saline treatment, we calculated time-adjusted area-under-the-Δeffect curves

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†† We used randomization lists from wwww.randomization.com. Accessed September 22, 2006.

**Fig. 1. Two-hour time course of tail-withdrawal latencies after a single subcutaneous injection of 10 mg/kg morphine-6$\beta$-glucuronide (M6G; given at t = 0) in mice. (A) CD-1 mice implanted with placebo (squares, n = 6) or naltrexone (NTX) pellets (circles, n = 11) 24 h before M6G injection. (B) Opioid receptor triple knockout mice (TrKO; circles, n = 7) and the 129XB6F1 control animals (squares, n = 7). Significant treatment, time, and time × treatment effects were observed (all $P < 0.001$). Post hoc comparisons: * $P < 0.01$ and ** $P < 0.05$ versus pre-M6G baseline (BL). Data are mean ± SEM latencies obtained before M6G infusion (0) and at 30-min intervals during M6G infusion.**
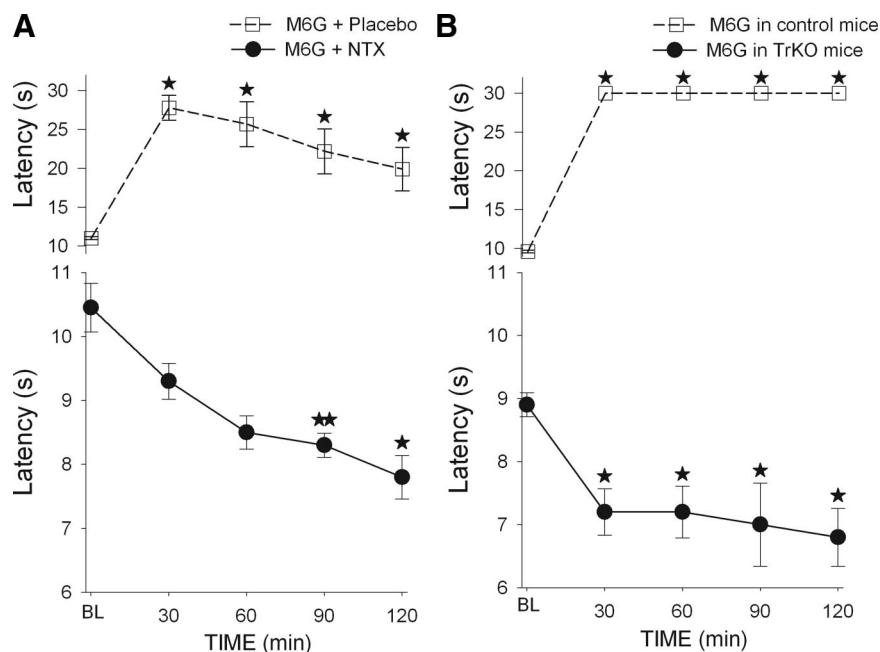
(where $\Delta$effect is the effect above the pre–M6G-placebo value) using the trapezoidal rule and tested the significance of differences by $t$ test. A separate analysis was performed in M6G-treated and placebo-treated subjects. $P$ values less than 0.05 were considered significant. Values reported are mean ± SEM.

## Results

### Animal Studies

**Nociception after Acute M6G Injection.** As illustrated in figure 1A, an acute 10-mg/kg M6G dose increased withdrawal latencies for at least 120 min in CD-1 mice implanted with placebo pellets ($P < 0.01$). In contrast, this potent analgesia was not evident in naltrexone-pelleted controls. Instead, M6G increased nociception, thereby significantly reducing tail-withdrawal latencies from baseline (10.5 ± 0.4 s) at t = 90 min (8.3 ± 0.2 s; $P < 0.05$) and t = 120 min (7.8 ± 0.3 s; $P < 0.01$). Similar results were obtained when assaying nociception after acute M6G (10 mg/kg) injection in TrKO mice and their B6129F$_1$ controls (fig. 1B). M6G caused maximal analgesia for a minimum of 120 min in control mice, whereas it caused only significant hyperalgesia during the same time period in TrKO mice lacking $\mu$-, $\kappa$-, and $\delta$-opioid receptors. For all strains in both acute M6G conditions, saline injection in either placebo- or naltrexone-pelleted mice did not alter withdrawal latencies from baseline values (data not shown for clarity). This finding is consistent with previous reports.[10–12,28,30]

**Nociception during Continuous M6G Infusion.** Continuous subcutaneous M6G infusion (1.6 mg/kg per 24 h) produced no detectable analgesia in either placebo- or naltrexone-pelleted mice. Instead, increased nocicep-

tion was evident starting on infusion day 1 and continued until the end of study on day 6 (figs. 2A and B). The magnitude of this hyperalgesia was at a maximum on infusion day 4, where baseline latencies were reduced
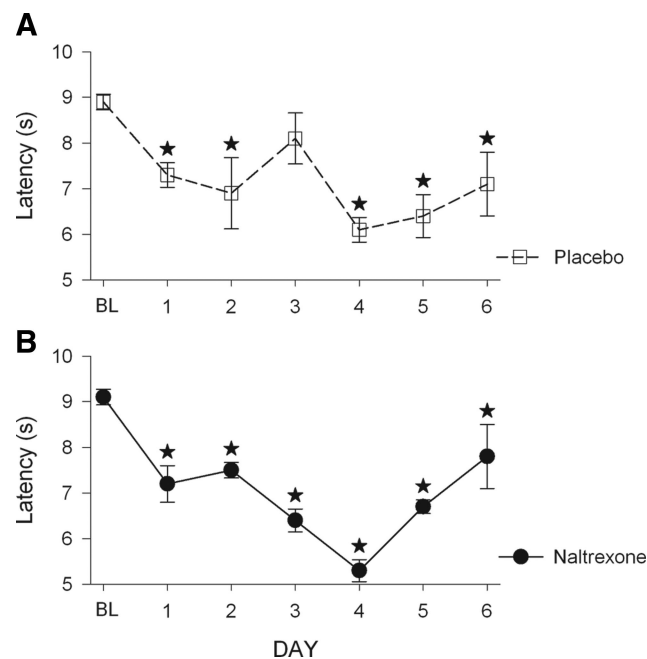


**Fig. 2. Six-day time course of tail-withdrawal latencies in CD1 mice during the continuous subcutaneous infusion of morphine-6$\beta$-glucuronide (M6G; infusion rate = 1.6 mg/kg per 24 h). (A) M6G effect in mice implanted with placebo pellets (squares, n = 6). (B) M6G effect in mice implanted with naltrexone pellets (circles, n = 6). Pellets were implanted 24 h before starting the M6G infusion. Data are mean ± SEM latencies obtained before M6G infusion (i.e., baseline [BL]) and at daily intervals during M6G infusion. Significant main effects were observed for time ($P < 0.0001$) and time × treatment ($P < 0.01$), but not for treatment ($P > 0.05$). Post hoc comparisons: * $P < 0.01$ versus BL.**

## A  Acute



## B  Chronic



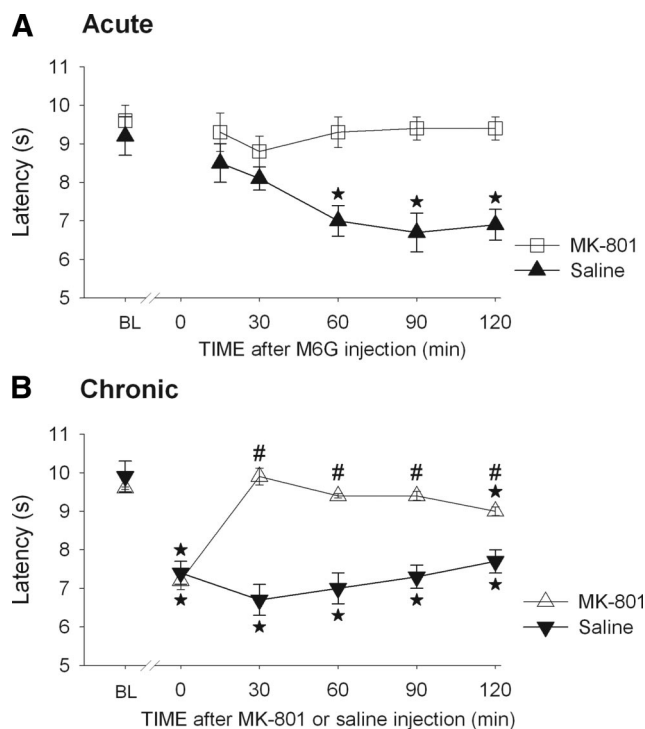**Fig. 3.** Effect of an acute injection of MK-801 on tail withdrawal latencies in CD-1 mice given morphine-6β-glucuronide (M6G) by acute injection (10 mg/kg) (*A*) or CD-1 mice on the fourth day of a continuous infusion of M6G (infusion rate: 1.6 mg/kg per 24 h) (*B*). All mice were implanted with pellets containing the opioid receptor antagonist naltrexone. (*A*) Acute: Thirty minutes after 0.05 mg/kg subcutaneous MK-801 or saline injection, 10 mg/kg M6G was injected at t = 0. Subsequently, tail-withdrawal latencies were followed for 2 h. *Squares* = MK-801 (n = 6); *triangles* = saline (n = 6). (*B*) Chronic: During the continuous subcutaneous infusion of M6G, 0.05 mg/kg subcutaneous MK-801 (*up triangles*, n = 6) or saline (*down triangles*, n = 6) was injected just after the latency measurement on day 4 (pre–MK-801 latencies here shown at *t* = 0). Significant treatment, time, and time × treatment effects were observed (all *P* < 0.01). Data are mean ± SEM. *Post hoc* comparisons: * *P* < 0.01 *versus* pre-M6G baseline (BL). # *P* < 0.01 *versus* t = 0.

from 8.9 ± 0.2 s to 6.1 ± 0.3 s in placebo-pelleted mice (*P* < 0.01; fig. 2A) and from 9.1 ± 0.2 s to 5.3 ± 0.2 s in mice implanted with naltrexone pellets (*P* < 0.01; fig. 2B). The magnitudes of the latency reductions in placebo- and naltrexone-pelleted mice were highly similar throughout the 6 test days and significantly differed from each other on day 3 only. As in previous studies,[10–12] withdrawal latencies did not differ from baseline values during saline infusion in either placebo- or naltrexone-pelleted mice (data not shown for clarity).

**Effect of NMDA Receptor Blockade on M6G Hyperalgesia.** Mice injected with saline 30 min before an acute M6G (10 mg/kg) dose displayed significant reductions in withdrawal latencies relative to baseline at t = 60 min (fig. 3A). In contrast, no hyperalgesia was manifest at any time after the identical M6G dose in subjects injected with MK-801 (0.05 mg/kg) instead of saline. Figure 3B illustrates pain responses in mice subject to continuous M6G infusion. Latencies were significantly

increased relative to preinfusion baseline values (9.6 ± 0.5 s) at t = 0 on day 4 (7.2 ± 0.2 s; *P* < 0.01), whereas a subsequent MK-801 (0.05 mg/kg) injection reversed this hyperalgesia, increasing withdrawal latencies to the baseline values obtained before the start of the M6G infusion within 30 min (9.9 ± 0.1 s; *P* < 0.01 *vs.* t = 0 values) and remaining elevated for at least 120 min. In contrast, injecting saline instead of MK-801 did not alter latencies in a separate group of M6G-infused control mice displaying significant hyperalgesia of approximately equal magnitude on day 4.

### *Human Studies*

The naloxone infusion scheme was designed to achieve a steady state concentration greater than 10 ng/ml, which is assumed to cause full reversal of μ-, κ-, and δ-opioid receptors, even when dealing with high-affinity opioids.[31,32] The estimated Cp naloxone (using the following pharmacokinetic parameter values[32]: $V_1$ = 14.0 l, $V_2$ = 109 l, $CL_1$ = 3.60 l/min, $CL_2$ = 5.05 l/min) is 14 ng/ml throughout the measurement period of the study (t = 0–120 min).

Subjects receiving M6G (0.4 mg/kg intravenous) showed increased pain responses irrespective of the naloxone or saline background infusion (figs. 4A and B), significantly different from baseline (t = 0) from t = 30 to t = 120 min. NRS increased from 6.2 ± 0.2 (t = 0 min) to a maximum of 7.2 ± 0.2 at t = 60 min in the naloxone group (*P* < 0.05), and from 6.0 ± 0.2 (t = 0 min) to a maximum of 7.1 ± 0.3 at t = 100 min in the placebo group (*P* < 0.05). Area-under-the-Δeffect curves did not differ between groups: 0.76 ± 0.27 mA (saline) *versus* 0.66 ± 0.24 mA (naloxone). Subjects receiving placebo–saline (fig. 4C) and placebo–naloxone (fig. 4D) showed no systematic changes in NRS. Area-under-the-Δeffect curves did not differ between the two placebo groups: 0.10 ± 0.15 mA (saline) *versus* −0.16 ± 0.13 mA (naloxone).

## Discussion

The main findings in mice are as follows: (1) Acute M6G injection increases pain sensitivity in mice subject to opioid receptor blockade by naltrexone and in TrKO mice lacking μ-, κ-, and δ-opioid receptors; (2) continuous M6G infusion causes long-lasting (6-day minimum) increases in pain sensitivity that start within 24 h, irrespective of the presence or absence of opioid receptor blockade with naltrexone; and (3) NMDA receptor blockade with MK-801 respectively blocks or reverses the increased pain sensitivity induced by the acute injection or continuous infusion of M6G in naltrexone-pelleted mice. In humans, we observed that a single intravenous injection of M6G increased pain sensitivity for at least 6 h (fig. 4). Furthermore, consistent with our

Fig. 4. Influence of 0.4 mg/kg morphine-6β-glucuronide (M6G) and placebo on experimental heat pain responses in human volunteers during background exposure to saline and background exposure to naloxone. During a saline (*A*) and naloxone (*B*) background infusion, M6G causes an immediate and persistent hyperalgesic response. In contrast, placebo produces no consistency in response independent of the background infusion (*C*, saline, and *D*, naloxone). Naloxone and saline were given as an intravenous bolus of 0.043 mg/kg (*down arrows*), 30 min before M6G or placebo injection (*up arrows*), followed by a continuous infusion of 0.043 mg/kg per hour. Values are mean ± SEM; different *symbols* indicate different groups of 10 subjects. Significant main effects: (*A*) M6G–saline, $P < 0.01$; (*B*) M6G–naloxone, $P < 0.001$. *Post hoc* comparisons: * $P < 0.05$ *versus* t = 0. NRS = numerical rating pain scale.
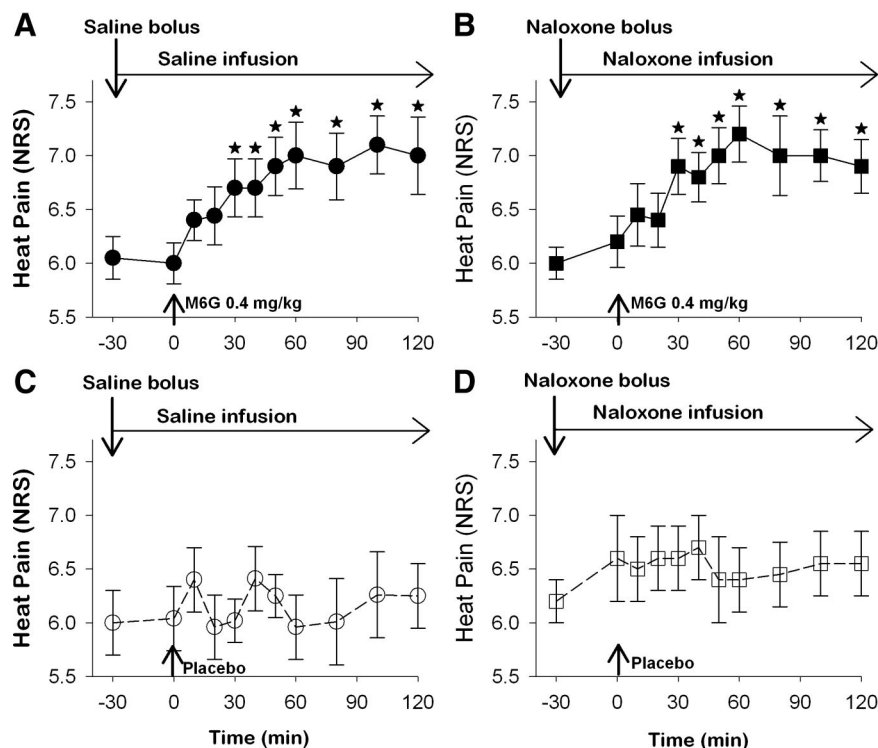


findings in mice, the increased pain sensitivity observed after M6G injection in humans exposed to a noxious thermal stimulus persisted during the simultaneous continuous infusion of naloxone to block opioid receptors.

An array of mechanisms is proposed to underlie opioid-induced hyperalgesia. For example, opioids can 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.[7] Others provide evidence consistent with the hypothesis that hyperalgesia is an adaptive response. In such a scenario, increased nociception is a consequence of an opioid receptor–mediated opponent process acting as a foil to opioid analgesia.[7] A series of studies also describe a system-wide mechanism integrating spinopetal projections from the rostroventral medulla with spinal alterations that modulate primary afferent activity.[6] Despite their diversity, these accounts unanimously characterize hyperalgesia as a consequence of opioid receptor activity or analgesia. However, here we report that M6G hyperalgesia is manifest in mice and humans treated concurrently with high enough doses of opioid antagonist so that opioid receptors (and analgesia) are completely blocked. Furthermore, we observed M6G hyperalgesia in TrKO mice where μ-, κ-, and δ-opioid receptors are altogether absent. Importantly, there were no changes in nociception in naltrexone- or placebo-pelleted mice injected with saline, indicating that hyperalgesia was a consequence of M6G exposure. Therefore, the current data indicate that M6G causes hyperalgesia in

mice and humans that, like morphine and oxymorphone hyperalgesia in mice,[10–12] is independent of concurrent opioid receptor activity or analgesia.

We have previously demonstrated that morphine and oxymorphone can cause hyperalgesia *via* mechanisms unrelated to their opioid activity.[10–12] To this list of clinically relevant opioids we now include M6G, which is currently undergoing phase 3 clinical trials.[18,33] Therefore, despite the fact that all three opioids preferentially act *via* the μ-opioid receptor, their hyperalgesic liability is unrelated to their common opioid receptor pharmacodynamics. We and others have previously speculated that opioid-induced hyperalgesia might result from the conjugation of the parent opioid compound at the 3′-position into pronociceptive glucuronide metabolites.[10–12,20,21] M3G, for example, is the most abundant morphine metabolite[34] and has no detectable affinity at any opioid receptor subtype or analgesic effect,[30,35–38] and systemic M3G doses can decrease tail-withdrawal responses in mice and evoke agitation to even innocuous touch in rats that is not diminished by naloxone.[4,30] M3G accumulation has also long been thought to underlie morphine hyperalgesia in humans. Oxymorphone metabolism as well yields oxymorphone-3-glucuronide, a metabolite similar to M3G.[39] With regard to M6G, itself a morphine metabolite, we are not aware of any reports showing that M6G metabolism directly yields any neuroexcitatory or pronociceptive fragments. However, M6G injection increases M3G levels within 60 min in mice, an effect attributable to the metabolism of morphine that is generated from the enterohepatic circula-

tion of M6G.[40] Here, the onset of hyperalgesia after an acute M6G injection in naltrexone-pelleted mice was generally similar (t = 90 min). Further implicating the contribution of M3G is our finding that the NMDA receptor antagonist MK-801 blocked or reversed hyperalgesia elicited by an acute injection or the continuous infusion of M6G, respectively. Although the M3G binding site and mechanism of action are not known, the neuroexcitatory effects of M3G are thought to involve NMDA receptor activity, and NMDA receptor antagonists dose-dependently reduce M3G symptoms, including enhanced nociception.[35,41,42] However, based on data from a previous study,[40] it is unlikely that the acute M6G dose injected here would result in physiologically relevant concentrations of M3G to cross the blood–brain barrier, although such an accumulation may be possible during continuous M6G infusion. In humans, M3G levels remain undetected after an acute M6G injection, and acute M3G injection in humans was without effect on nociception.[37,38,42] Therefore, at this time, we can only speculate as to whether M3G might contribute to M6G hyperalgesia. Accordingly, any such contribution may be dependent on the duration of M6G exposure (i.e., acute injection or continuous infusion) and the species studied. These issues will comprise the specific aims of future studies.

Regardless of the mechanism underlying morphine, oxymorphone, and M6G hyperalgesia, or whether all three opioids cause hyperalgesia via common mechanisms, the current data suggest that hyperalgesia after M6G has a more rapid onset and is more robust. For example, we previously showed that an acute subcutaneous morphine or oxymorphone injection in TrKO mice at doses identical to M6G doses administered here (10 mg/kg) did not reduce tail-withdrawal latencies even after 120 min.[10] In contrast, here we report that opioid receptor blockade significantly reduced withdrawal latencies within 90 min in CD-1 mice. Furthermore, whereas hyperalgesia caused by continuous morphine infusion in both placebo- and naltrexone-pelleted CD-1 mice is delayed until day 4,[11,12] significant hyperalgesia is already manifest 24 h after the start of continuous M6G infusion, regardless of the concurrent pellet treatment. These data suggest that M6G activates pronociceptive mechanisms more rapidly or efficaciously than either morphine or oxymorphone. This might explain why relatively high doses of M6G are required to elicit an adequate analgesic response in experimental and clinical studies with humans.[13,33,43] That is, the ability of M6G to rapidly evoke significant hyperalgesia in a variety of delivery circumstances may offset any concurrent analgesic effect. This assumption that M6G produces hyperalgesia more rapidly than morphine can be directly tested by assaying thermal pain responses in humans subject to morphine. We are embarking on just such a study, and our preliminary data indeed show that a single intravenous injection of morphine does not cause pain ratings on our thermal assay to increase in a manner similar to that observed here with M6G (E.v.D. and A.D., unpublished experimental data on the effect of intravenous morphine on thermal pain in healthy volunteers, March 4, 2008), suggesting that M6G hyperalgesia to heat pain is more readily manifest than hyperalgesia wrought by morphine. Considering these arguments, the absence of an analgesic response in the heat pain test may be a dose effect. Possibly at higher doses, which overcome any hyperalgesic effect, M6G is analgesic in this nociceptive assay. Using an electrical pain test, M6G antinociception in humans is observed in doses ranging from 0.2 to 0.6 mg/kg.[19,43,44] This then suggests that the thermal nociceptive assays is more sensitive to M6G hyperalgesia than the electrical pain assay. This may be related to differences in nociceptive pathways activated by heat and electrical pain.[45]

Multiple studies show the ability of NMDA receptor antagonists to reverse opioid-induced hyperalgesia,[6,11,12,27] and here the noncompetitive NMDA receptor antagonist MK-801 was effective in blocking or reversing hyperalgesia after acute injection and continuous infusion of M6G, respectively. The current data thus demonstrate that M6G hyperalgesia in mice is dependent on NMDA receptor activity. There is currently no definitive explanation of how NMDA receptor antagonists reverse opioid hyperalgesia. A possible direct interaction of NMDA antagonists with opioid receptors (see Sarton et al.[46]) would be a moot point because we show here in naltrexone-pelleted mice that M6G hyperalgesia is unrelated to opioid receptor activity. For identical reasons, it also cannot be the case that MK-801 reversed M6G hyperalgesia only indirectly, by potentiating latent M6G opioid analgesia concurrent with hyperalgesia. Kilpatrick and Smith[14] reported that although M6G was inactive at two binding sites within the NMDA receptor, suggesting the absence of a direct blockade of M6G activity at these sites, it is not yet possible to rule out M6G activity at other sites within the receptor complex. It has also been suggested that NMDA antagonists block or reverse opioid hyperalgesia by antagonizing NMDA receptors localized to central primary afferent terminals that cause spinal sensitization and increased nociceptive input.[6] To this we add the possible contribution of NMDA receptors at loci upstream or downstream from the site where pronociceptive mechanisms are activated in response to M6G administration. Further studies are needed to address these possibilities.

Although just 5–10% of morphine is metabolized to M6G, M6G plasma concentrations increase rapidly after acute morphine administration and reach relatively high values after chronic treatment, particularly when renal function is compromised. Therefore, M6G may make an important contribution not only to morphine analgesia but, as we demonstrate here, to hyperalgesia as well.

This potential role for M6G as a causative factor of morphine hyperalgesia requires further study. M6G causes analgesia in mice *via* its activity at $\mu$-opioid receptors,[14–16] and M6G is also currently in phase 3 clinical trials as an opioid analgesic. It is thought to possess a pharmacologic profile that imbues it with certain advantages relative to other opioids in the management of pain. The addition of another clinically effective opioid is certainly a welcomed addition to the opioid pharmacopeia. However, despite whatever advantages M6G may afford for the treatment of pain, the current results suggest that the absence of hyperalgesia is not one of them.

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