SHORT REPORT

Melanocortin-1 receptor gene variants affect pain and μ-opioid analgesia in mice and humans

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Background: A recent genetic study in mice and humans revealed the modulatory effect of *MC1R* (melanocortin-1 receptor) gene variants on κ-opioid receptor mediated analgesia. It is unclear whether this gene affects basal pain sensitivity or the efficacy of analgesics acting at the more clinically relevant μ-opioid receptor.

Objective: To characterise sensitivity to pain and μ-opioid analgesia in mice and humans with non-functional melanocortin-1 receptors.

Methods: Comparisons of spontaneous mutant C57BL/6-*Mc1r*[e/e] mice to C57BL/6 wildtype mice, followed by a gene dosage study of pain and morphine-6-glucuronide (M6G) analgesia in humans with *MC1R* variants.

Results: C57BL/6-*Mc1r*[e/e] mutant mice and human redheads—both with non-functional MC1Rs—display reduced sensitivity to noxious stimuli and increased analgesic responsiveness to the μ-opioid selective morphine metabolite, M6G. In both species the differential analgesia is likely due to pharmacodynamic factors, as plasma levels of M6G are similar across genotype.

Conclusions: Genotype at *MC1R* similarly affects pain sensitivity and M6G analgesia in mice and humans. These findings confirm the utility of cross species translational strategies in pharmacogenetics.

The melanocortin-1 receptor (MC1R) is a rather unlikely candidate to influence acute pain and opioid analgesia, being best known for its role in skin/hair pigmentation[1] and immunomodulation.[2] However, quantitative trait locus (QTL) mapping studies in mice led us to the consideration of MC1R involvement in κ-opioid analgesia in female but not male mice[3] based on the chromosomal location of the mouse *Mc1r* gene and the demonstration of MC1R mRNA and protein in the periaqueductal grey,[4] a midbrain locus of crucial relevance to endogenous analgesic circuitry.[5] We found that the κ-opioid analgesia phenotype of the female *Mc1r*-null C57BL/6-*Mc1r*[e/e] ("recessive yellow", *e/e*) mutant mouse was similar to that of redheaded women with multiple, protein inactivating *MC1R* variants.[3]

The clinical utility of this finding is clearly limited by the uncommon application of κ-opioid acting drugs in pain therapy. On the other hand, drugs acting at the μ-opioid receptor represent the mainstay of clinical treatment of moderate to severe pain. We reasoned that a fuller characterisation of these mutants might reveal other, more clinically relevant phenotypes. To these ends, we tested mutant mice and then humans of various hair colours for their sensitivity to pain and μ-opioid analgesia.

METHODS

Subjects

Naïve, male and female adult (6–10 week old) mice of the C57BL/6J and C57BL/6J-*Mc1r*[e/e] strains were used. Mice were housed with same sex littermates in groups of two to four. Animal experiments were approved by the local animal care and use committee at McGill University.

A total of 44 Dutch and three Scottish volunteers (23 men, 24 women, median age 22, range 18–35 years) participated in this study. All subjects were healthy, and none had participated in pain research previously. All women were on oral contraceptives. Human studies were approved by the local institutional review board at Leiden University Medical Center.

Nociceptive assays

Mice were tested for their basal sensitivity on six different assays of acute and tonic nociception. Detailed protocols have been published previously.[6] In the tail withdrawal test, mice were lightly restrained and the latency to vigorous removal of the distal half of the tail from 49°C or 47°C water was measured. In the hot plate test, mice were placed on a 53°C or 50°C metal surface within a Plexiglas cylinder, and the latency to first hindpaw lick or shake measured. In the paw withdrawal test, mice were placed atop a glass surface in small Plexiglas enclosures, and a high intensity heat lamp was focused on the right or left mid-plantar hindpaw. The latency to withdraw from the heat stimulus was measured. In the tail clip test, mice were lightly restrained and a binder clip (500 *g* force) was applied to their tail near the base. The mouse was immediately removed from the holder onto a table top and the latency to lick, bite, or grab the clip was measured. In the abdominal constriction test, mice received an i.p. injection of 0.9% acetic acid, were placed in Plexiglas observation cylinders, and the number of reflexive constrictions of the abdominal musculature counted for 30 min. Data in each assay were obtained by only one experimenter.

Human subjects were tested by same sex experimenters. After arrival of human subjects in the laboratory, an arterial line was placed in the left or right radial artery under local anaesthesia (for blood sampling) and an intravenous line was inserted in the contralateral arm (for drug infusion). Acute pain was induced by an electrical current through two surface electrodes (Red Dot, 3M, London, ON, Canada) placed on the skin over the tibial bone (shin bone) of the left leg. The electrodes were attached to a computer interfaced current stimulator. The noxious stimulus was a 10 Hz tetanic pulse with a duration of 0.1 ms. The intensity of the noxious stimulation was increased from 0 mA in steps of 0.5 mA per

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Abbreviations: AD, antinociceptive dose; α-MSH, α-melanocyte stimulating hormone; AUEC, area under the time-effect curve; M6G, morphine-6-glucuronide; MC1R, melanocortin-1 receptor

1 s (cut off: 128 mA). The subjects were instructed to press a button on a control box when no further increase in stimulus intensity was acceptable to them and that current was defined as pain tolerance. Upon pressing the button, the stimulus train ended. After a 1 h training session, data acquisition began.

Drugs

Morphine sulfate and morphine-6-glucuronide (M6G) were generously provided by Sabex 2000 (Boucherville, QC, Canada) and CeNeS (Cambridge, UK), respectively. In mice, both drugs were dissolved in saline and administered subcutaneously in an injection volume of 10 ml/kg. The dose given to all human subjects was 0.3 mg/kg (two thirds given as a bolus over a 90 s period, the remainder as a continuous infusion over 58.5 min).

Analgesia

Naïve mice were tested at each of four doses (5, 7.5, 10, or 15 mg/kg) for morphine, and each of three doses (5, 10, or 15 mg/kg) for M6G, to construct dose-response curves. Sample sizes in these experiments were n = 7–24/genotype/sex/dose. Mice were tested for baseline sensitivity on the 49°C tail withdrawal test, immediately injected with morphine or M6G, and then retested at 15, 30, and 60 min post-injection for morphine, and additionally at 120, 180, 240, and 300 min post-injection for the longer acting M6G.

Prior to drug infusion in humans, three baseline tests were performed. After the drug infusion was initiated at time t = 0, the pain tests were performed at the following times: t = 5, 10, 20, 30, 40, 50, 60, 65, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 220, 240, 260, 280, 300, 320, 340, and 360 min.

Pharmacokinetic analysis

Mice of both genotypes and both sexes were injected with morphine (10 mg/kg) or M6G (10 mg/kg), and left undisturbed in their home cages until sacrificed by decapitation at 15, 60, or 180 min post-injection. Trunk blood was collected on ice and immediately centrifuged for 10 min at 3500/min to isolate plasma, which was then stored at $-20°C$ until analysis. Plasma morphine and M6G concentrations were determined with liquid chromatography tandem mass spectrometry. The lower and upper limits of quantification were set at 1.25 and 5000 ng/ml, respectively. Each drug/genotype/sex/time condition was run in triplicate.

At fixed times (t = 2, 5, 10, 20, 30, 40, 50, 60, 62, 65, 70, 80, 90, 105, 120, 150, 180, 240, 300, and 360 min after start of drug infusion), 5 ml of arterial blood in human subjects was drawn for determination of plasma concentrations of M6G. At time points where blood sampling coincided with pain assessment, the pain testing preceded the sampling. Finally, in each subject, an extra sample of blood was obtained for genotyping. Plasma M6G concentrations were determined as
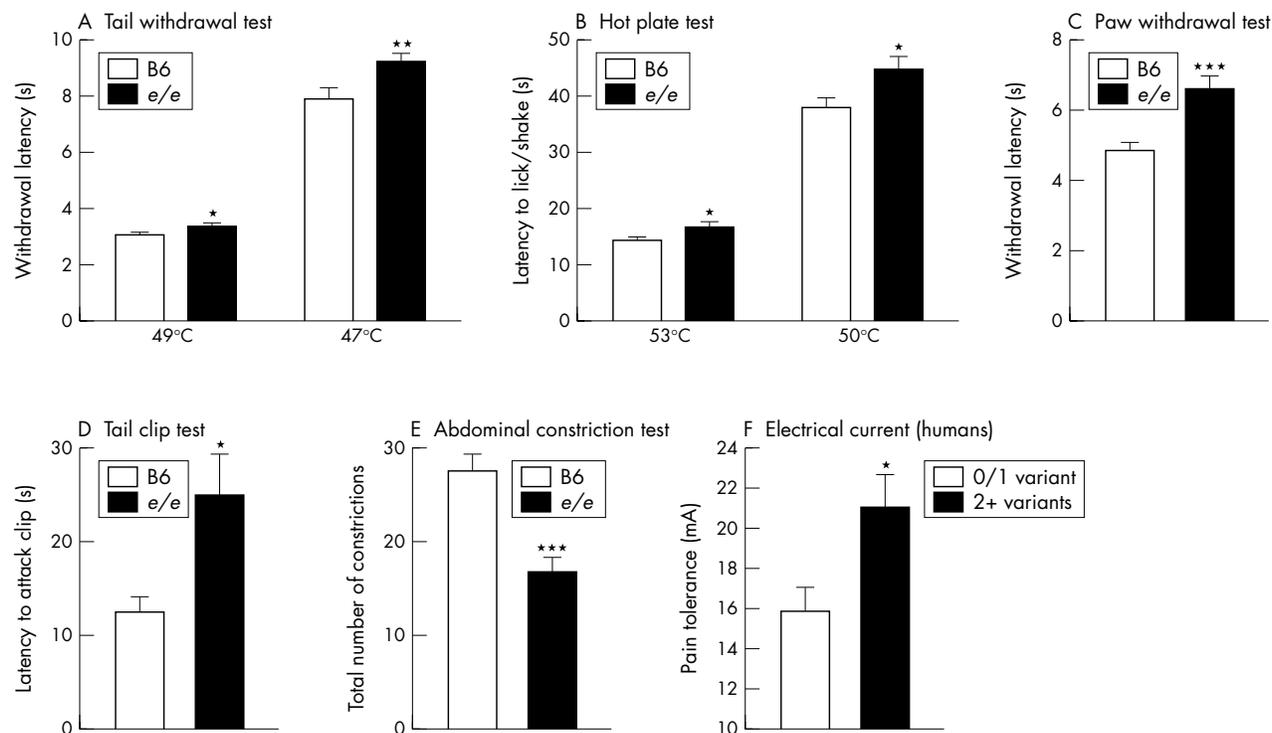

Figure 1 Effect of MC1R functionality on basal nociceptive and pain sensitivity in mice and humans. Graphs A–E compare recessive yellow (*e/e*) mutant mice of both sexes with their wildtype counterparts, C57BL/6J (B6). Graph F compares human subjects of both sexes with functional MC1Rs (0/1 variant) to red-haired subjects with non-functional MC1Rs (2+ variants). Bars represent mean±SEM in all graphs. Sample sizes in these experiments were n = 9–31/genotype/sex, except for the 49°C tail withdrawal test, which represents the compilation of baseline latencies from 2 years of separate experiments using this assay (n = 72–185/genotype/sex). (A) Latencies to withdraw the distal half of the tail from 49°C (left) or 47°C (right) water. Values are the mean of eight latency determinations at four time points (0, 30, 60, 90 min). (B) Latency to lick or shake/flutter a hind paw after being placed on a hot plate set at 53°C (left) or 50°C (right). (C) Latency to withdraw from a radiant heat source aimed from below at the plantar surface of the hind paw. Values are the mean of 32 separate determinations, each separated by at least 5 min. (D) Latency to attack (that is, attempt to remove) a 500 g binder clip attached to the tail near the base. (E) Total number of abdominal constrictions (writhes) observed in a 30 min period after injection of 0.9% acetic acid. (F) Baseline tolerance of electrical current (10 Hz pulse; 0.1 ms duration, 0.5 mA steps) applied to the skin of the left leg. *p<0.05; **p<0.01; ***p<0.001. Data from both sexes combined are shown; significant effects of genotype were seen in both sexes in all assays except the 49°C and 47°C tail withdrawal test, where only male *e/e* mice differed from male B6.

described above, with the coefficient of variation varying from 4 to 8% over the calibration range of 2–10 000 ng/ml.

Genotyping

The *MC1R* polymorphism detection strategy involved sequencing the 5′ two thirds of the open reading frame, where all of the known variants occur except for D294H, which was specifically screened for using a *Taq1* loss of digestion strategy. See Mogil *et al*[3] for details.

Data analysis

Nociceptive assay data in mice were analysed by two way ANOVA (genotype, sex). Where significant interactions were obtained, genotype or sex differences were evaluated by two tailed Student's t test. Half maximal antinociceptive doses (AD_{50}s) and associated 95% confidence intervals were calculated from % analgesia (area under the curve) data using the method of Tallarida and Murray.[7]

For each human subject, the area under the time-effect curves (AUEC) was calculated using the trapezoidal rule and standardised by the length of the study (360 min). The data were separated by genotype with one group consisting of subjects with at least two variant alleles of the *MC1R* gene previously associated with MC1R loss of function (and, thus, red hair) (ins29, R151C, R160W, D294H) and the other group consisting of subjects with the consensus sequence, one

variant allele only, or two variant alleles at amino acids not associated with MC1R loss of function (V60L, V92M, and R163Q)[8] (see table 2 in Appendix 1 for details).

A two way ANOVA (genotype, sex) was performed on baseline pain, AUEC, and plasma M6G concentration data. Where significant interactions were obtained, genotype or sex differences were evaluated by two tailed Student's t test.

RESULTS

As shown in fig 1, *e/e* mutants displayed reduced nociceptive sensitivity. On most assays, a significant effect of genotype was observed in both sexes; the exception was the tail withdrawal test, in which only male *e/e* mice displayed significantly higher latencies. The reduced sensitivity of *e/e* mutants did not appear to be opioid mediated, since the differences persisted after pretreatment with naloxone (10 mg/kg, s.c.) (data not shown).

Next, we constructed analgesic dose-response curves for *e/e* and C57BL/6 mice of both sexes given systemic morphine (5–20 mg/kg) and its bioactive metabolite, M6G (5–15 mg/kg). Although there has been some confusion in the literature, it is now quite clear that M6G acts largely at the μ-opioid receptor,[9] albeit likely at one coded for by an alternatively spliced transcript of the *Oprm* gene.[10] As shown in fig 2A and B for M6G (and table 1 in Appendix 1), *e/e* mutants of both sexes displayed significantly greater inhibition of thermal
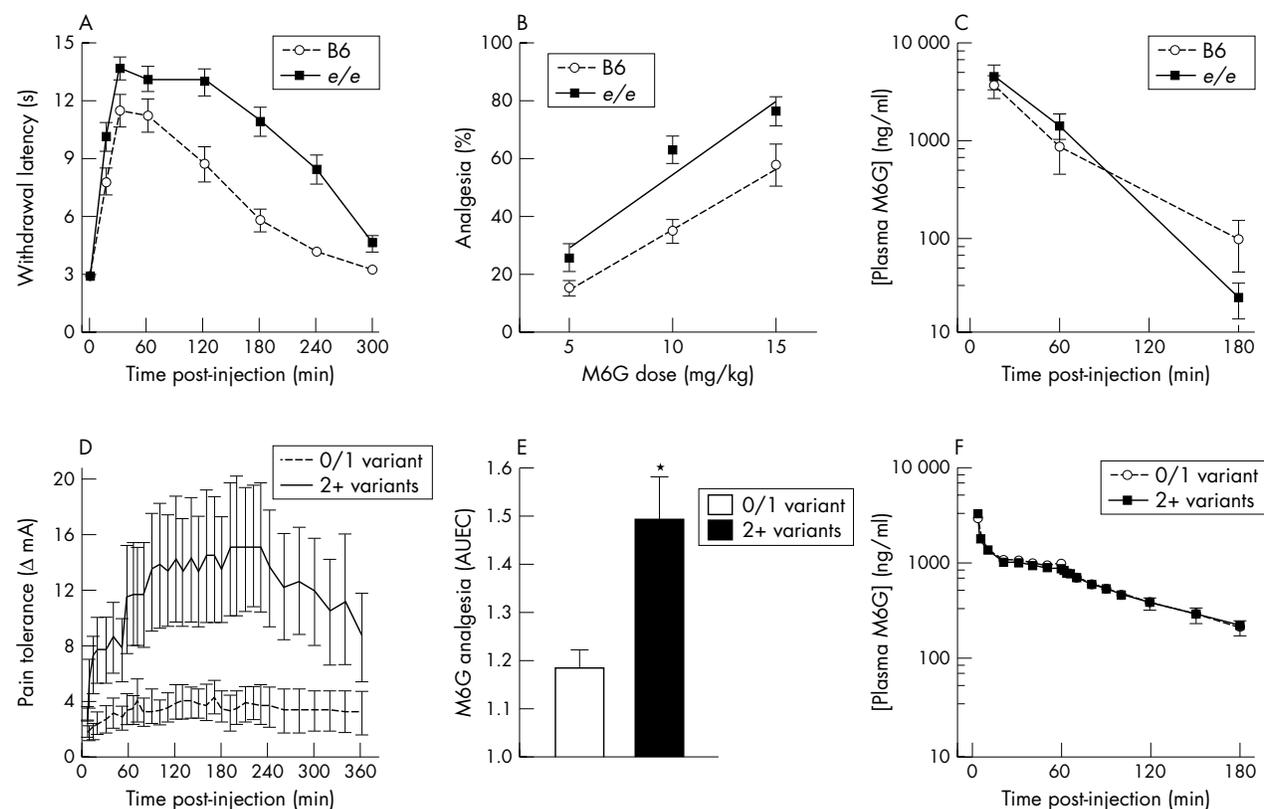

Figure 2 Effect of MC1R functionality on the efficacy of M6G at inhibiting thermal nociception in mice and electrical current pain in humans. Graphs A–C compare recessive yellow (*e/e*) mutant mice of both sexes with their wildtype counterparts, C57BL/6J (B6). Graphs D–F compare human subjects of both sexes with functional MC1Rs (0/1 variant) to red-haired subjects with non-functional MC1Rs (2+ variants). Symbols and bars represent mean ± SEM in all graphs. (A) Latency to withdraw the distal half of the tail from 49°C water before and at various time points after injection of 10 mg/kg (s.c.) M6G. (B) Dose-response relationships for M6G (5, 10, or 15 mg/kg) analgesia. Symbols represent % analgesia, calculated with respect to the area under the time latency curve shown in A, using the trapezoidal rule and with reference to a hypothetical mouse with the same baseline latency but displaying cut off (15 s) latencies at all post-injection time points. (C) Plasma M6G concentrations at 15, 60, and 180 min after injection of 10 mg/kg M6G in naïve B6 and *e/e* mice. We also observed no genotype differences in plasma morphine or M6G concentrations after injection of 10 mg/kg morphine (data not shown). (D) Change in pain tolerance of human subjects at various time points after the administration of 0.3 mg/kg M6G. (E) M6G analgesia expressed as area under the time-effect curve (AUEC relative to baseline currents) shown in D. *p<0.05. (F) Plasma M6G concentrations in human subjects at various time points after injection of 0.3 mg/kg M6G.

nociception from these drugs than did their C57BL/6 counterparts at all doses tested. The M6G data were particularly robust, with differences in both the peak analgesic effect and the duration of analgesia.

Given the strength of the M6G finding in mice, we attempted a translational genetic association study in humans with this clinically useful analgesic. A total of 22 subjects (11 men, 11 women) had two or more variant alleles of the *MC1R* gene in amino acids known to abolish MC1R functionality (R151C, R160W, and D294H); all of these subjects had red hair and fair (type I or II) skin. The remaining 25 subjects (12 men, 13 women) had zero or only one variant alleles at these loss of function loci, and thus possessed functional MC1Rs (see table 2 in Appendix 1), even though seven of these subjects also had red hair (presumably due to genes other than *MC1R*). Baseline pain tolerance differed significantly between genotypes, with greater currents tolerated by *MC1R* variant subjects, 20.9 (SEM 1.7) mA, compared to control subjects, 15.8 (1.2) mA (p = 0.018; fig 1F). There was no significant main effect of sex or genotype×sex interaction.

Analgesic responses after M6G were greater in *MC1R* variant subjects compared to control subjects. The areas under the time-effect (pain tolerance relative to baseline) curves were 1.18 (0.04) mA for control subjects versus 1.49 (0.09) mA for *MC1R* variant subjects (p = 0.003; fig 2D). These values indicate that M6G produced an average increase in tolerable current of 18% (or 4 mA) and 49% (or 10 mA) above baseline in control and *MC1R* variant subjects, respectively (fig 2E). There was no significant main effect of sex or genotype×sex interaction.

The genotypic differences observed were related to the pharmacodynamics of M6G acting at μ-opioid receptors, since plasma M6G concentrations did not differ between genotypes in either mice (fig 2C) or humans (fig 2F) at multiple time points post-M6G injection.

DISCUSSION

Adequate pain control in acute pain patients is of vital importance. Poor pain relief may result in complications and prolonged hospitalisation, and the patient may ultimately develop chronic pain or complex pain syndromes.[11] Pain control is often difficult due to large between-patient variability in pain responses and analgesic efficacy.[12] In this study, we demonstrate a significant link between *MC1R* gene sequence, pain tolerance, and efficacy of the μ-opioid, M6G. In striking agreement with the mouse mutant studies, we observed greater M6G induced analgesic responses in those red-haired humans who are also loss of function ''mutants'' at the *MC1R* gene.

The observation that in humans and mice, the M6G/MC1R interaction is sex independent contrasts with our earlier finding that *MC1R* status affected pentazocine (κ-opioid) analgesia in females only.[3] Whether these are general differences or are restricted to the specific μ- and κ-opioids already tested requires further investigation. The decreased pain sensitivity in MC1R non-functioning humans and *e/e* mice compared to controls suggests that endogenous activation of the MC1R may counteract an endogenous pain inhibition system; that is, produce anti-analgesia. Our failure to observe this difference in basal pain sensitivity previously[3] is likely due to a variable genotypic effect size across different pain modalities (thermal and ischemic pain[3] *v* electrical current pain).

Our choice to study the role of *MC1R* in M6G analgesia in our human population was obvious based on the results of the mouse studies. We believe that these data nicely illustrate the power of direct mouse to human translation in genetic studies of pain. Demonstration of the possible role of *MC1R* in human clinical pain awaits large scale genetic association studies. We suggest that such association studies are more profitably attempted after the successful demonstration of a pharmacogenetic effect against experimental laboratory pain, which can be more carefully controlled.

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APPENDIX 1

Table 1 Half-maximal analgesic doses (AD_{50}s) and potency ratios for morphine (5, 7.5, 10, and 15)* and M6G (5, 10, and 15 mg/kg)† inhibition of 49°C tail withdrawal nociception in *e/e* mutant mice and their wildtype counterparts, C57BL/6

Drug	Strain	Sex	AD_{50}, mg/kg‡	Potency ratio, sex§	Potency ratio, genotype¶
Morphine	C57BL/6	M+F	11.3 (10.2 to 12.6)		
	M only		10.7 (8.9 to 12.5)		
	F only		11.9 (10.4 to 13.6)	0.9 (0.7 to 1.1)	
	e/e	M+F	8.5 (7.8 to 9.3)		1.3 (1.2 to 1.5)**
	M only		8.3 (7.2 to 9.6)		1.3 (1.0 to 1.6)**
	F only		8.7 (7.7 to 9.8)	1.0 (0.8 to 1.2)	1.4 (1.2 to 1.6)**
M6G	C57BL/6	M+F	13.3 (11.4 to 15.5)		
	M only		17.8 (13.1 to 24.3)		
	F only		11.9 (9.7 to 14.4)	1.5 (1.0 to 2.2)*	
	e/e	M+F	8.0 (7.1 to 9.0)		1.7 (1.4 to 2.0)**
	M only		9.0 (7.6 to 10.6)		1.7 (1.3 to 2.4)**
	F only		6.9 (5.7 to 8.3)	1.3 (1.0 to 1.7)*	1.6 (1.3 to 2.2)**

*Based on % analgesia scores over a 60 min testing period; three way ANOVA revealed significant main effects of genotype ($F_{1,195} = 13.3$, $p<0.001$) and dose ($F_{4,195} = 37.1$, $p<0.001$) only.

†Based on % analgesia scores over a 300 min testing period; three way ANOVA revealed significant main effects of genotype ($F_{1,101} = 20.9$, $p<0.001$) and dose ($F_{2,195} = 37.9$, $p<0.001$) only. AD_{50}s calculated on data from the first 60 min (not shown) revealed M6G to be approximately twice as potent as morphine in all groups in this assay.

‡AD_{50}s calculated using the method of Tallarida and Murray[7]; values in parentheses are 95% confidence intervals.

§The ratio of male AD_{50}:female AD_{50}; values in parentheses are 95% confidence intervals.

¶The ratio of C57BL6 AD_{50}:*e/e* AD_{50}; values in parentheses are 95% confidence intervals.

**Significantly different from 1.0, $p<0.05$.

F, female; M, male.

Table 2 Human subject genotypes and phenotypes

Genotype	No. of variants	Genotype*	n (%)†	Phenotype‡
+/+ (consensus sequence)	0	0/1	10 (21%)	All non-redhead
V60L/+	1	0/1	2 (4%)	Both non-redhead
V92M/+	1	0/1	1 (2%)	Non-redhead
R151C/+	1	0/1	3 (6%)	1 Non-redhead; 2 redheads
R160W/+	1	0/1	3 (6%)	2 Non-redheads; 1 redhead
V60L/V60L	2	0/1	1 (2%)	Non-redhead
V60L/V92M	2	0/1	1 (2%)	Non-redhead
V60L/D294H	2	0/1	1 (2%)	Redhead
V92M/D294H	2	0/1	1 (2%)	Redhead
R151C/R163Q	2	0/1	1 (2%)	Redhead
R160W/R163Q	2	0/1	1 (2%)	Redhead
ins29/ins29	2	2+	1 (2%)	Redhead
R151C/R151C	2	2+	3 (6%)	All redheads
R151C/R160W	2	2+	12 (26%)	All redheads
R151C/D294H	2	2+	1 (2%)	Redhead
R160W/R160W	2	2+	4 (9%)	All redheads
R151C/R151C *and* R160W/+	3	2+	1 (2%)	Redhead
Totals		0/1: 25	47	Non-redheads: 18
		2+: 22		Redheads: 29

*Experiments in transfected cell lines have determined that the following *MC1R* variants are unable to induce cyclic AMP production when stimulated by α-melanocyte stimulating hormone (α-MSH) or a long lasting analogue, NDP(Nle,[4] D-Phe[7])-MSH (see Schaffer and Bolognia[8]): R142H (Arg142His), R151C (Arg151Cys), R160W (Arg160Trp), D294H (Asp294His), and the insertion mutations ins29 and ins179. The R142H and insertion mutations are very rare (<1% allele frequency), and were not seen in this study except for one subject homozygous for ins29. Subjects with two (or in one case, three) total variants at ins29, R151C, R160W, and/or D294H (all redheads) were thus classified in the non-functional MC1R genotype group (''2+''). The V60L (Val60Leu) and V92M (Val92Met) are somewhat common mutations, but do not lead to MC1R loss of function, and thus subjects with these *MC1R* variants were classed with the functional MC1R genotype group (''0/1''). R163Q (Arg163Gln) has not yet been tested for cAMP stimulation; we conservatively classified this variant as not affecting MC1R function. It should be noted that the genotypic effect on both baseline sensitivity and M6G analgesia is significant at the $p<0.05$ level regardless of the classification of R163Q containing subjects. We also assayed for the K65N (Lys65Asn) and D84E (Asp84Glu) variants, but found none.

†Percentages do not add up to 100% due to rounding. Allele frequencies in these subjects were: +/+ (wildtype): 31%; ins29: 2%; V60L: 6%; V92M: 3%; R151C: 27%; R160W: 26%; R163Q: 2%; D294H: 3%. These values agree well with those previously reported in the literature.

‡Subjects classified as ''redheads'' had red hair ranging from orange to auburn, fair (type I/II) skin, and blue or green eyes. Twenty two of the 29 redheads (76%) possessed two or more MC1R inactivating variants, also in excellent agreement with the existing literature.