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# The μ-Opioid Receptor Variant N190K Is Unresponsive to Peptide Agonists yet Can be Rescued by Small-Molecule Drugs[S]

Jean-Philippe Fortin, Lei Ci, Jonathan Schroeder, Carmit Goldstein, Maria Claudia Montefusco, Inga Peter, Steven E. Reis, Gordon S. Huggins, Martin Beinborn and Alan S. Kopin

*Molecular Pharmacology Research Center, Molecular Cardiology Research Institute, Tufts Medical Center, Tufts University School of Medicine, Boston, Massachusetts (J.P.F., L.C., J.S., C.G., M.C.M., G.S.H., M.B., A.S.K.); Department of Genetics and Genomic Sciences, Mount Sinai School of Medicine, New York, New York (I.P.); and University of Pittsburgh, Pittsburgh, Pennsylvania (S.E.R.)*

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## ABSTRACT

The μ-opioid receptor (MOR) plays an important role in modulating analgesia, feeding behavior, and a range of autonomic functions. In the current study, we investigated the degree to which 13 naturally occurring missense mutations affect the pharmacological properties of the human MOR. After expression of each receptor in human embryonic kidney 293 cells, signaling ($G\alpha_{i/o}$-mediated) induced by peptide agonists was assessed using luciferase reporter gene assays. Multiple mutants (S66F, S147C, R260H, R265C, R265H, and S268P) show a significant reduction in agonist potency. At the N190K variant, agonist-mediated signaling was essentially absent. Enzyme-linked immunosorbent assay, microscopic analysis, and radioligand binding assays revealed that this mutant shows markedly reduced cell-surface expression, whereas all other receptor variants were expressed at normal levels. Surface expression of the N190K variant could be increased by incubation with the alkaloid agonist buprenorphine or with either naltrexone or naloxone, structurally related MOR antagonists. We were surprised to find that both putative antagonists, despite being inactive at the wild-type MOR, triggered a concentration-dependent increase in N190K receptor-mediated signaling. In contrast, peptidic ligands failed to promote expression or rescue function of the N190K mutant. Subsequent analysis of the N190K variant in an ethnically diverse cohort identified this isoform in a subgroup of African Americans. Taken together, our studies reveal that the N190K mutation leads to severe functional alterations and, in parallel, changes the response to established MOR ligands. The extent to which this mutation results in physiological abnormalities or affects drug sensitivity in selected populations (e.g., those with chronic pain or addiction) remains to be investigated.

## Introduction

The human μ-opioid receptor (MOR) plays a central role in the modulation of pain perception. This G protein-coupled receptor (GPCR) mediates many of the analgesic and addictive properties of opiate drugs, including morphine (Le Merrer et al., 2009). Activation of the MOR has also been shown to modulate a range of autonomic functions, including body temperature control, intestinal motility, and respiratory drive (Przewłocki and Przewłocka, 2001; Le Merrer et al., 2009). In addition to nociception and autonomic function, long-standing evidence suggests that MOR modulates feeding behavior (Bodnar, 2004). Administration of MOR agonists tends to enhance food intake, whereas antagonists inhibit feeding.

Targeted disruption of the MOR gene in mice abolishes morphine-induced analgesia, as well as the accompanying respiratory depression, constipation, and physical depen-

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**ABBREVIATIONS:** MOR, μ-opioid receptor; GPCR, G protein-coupled receptor; DAMGO, [D-Ala$^2$,*N*-MePhe$^4$,Gly-ol]-enkephalin; β-CNA, β-chlornaltrexamine; CTAP, H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-$NH_2$; HA, hemagglutinin; HEK, human embryonic kidney; WT, wild type; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; NTX, naltrexone; HDL, high-density lipoprotein.

dence (Sora et al., 1997; Matthes et al., 1998; Roy et al., 1998). In addition, work using MOR knockout mice suggests a role for this receptor in modulating body weight. Depending on diet composition, absence of the MOR in rodents confers protection against obesity (Tabarin et al., 2005; Zuberi et al., 2008).

The MOR is activated by a series of endogenous peptides including endomorphin 1 and 2, β-endorphin, leucine-enkephalin, and methionine-enkephalin. In addition, synthetic MOR agonists, including peptide (e.g., DAMGO) and nonpeptide (e.g., morphine, buprenorphine, methadone) compounds, have been identified. At the cellular level, activation of the MOR results primarily in $G\alpha_{i/o}$-mediated inhibition of adenylate cyclase, leading to a reduction in intracellular cAMP, inhibition of calcium channels, and activation of inwardly rectifying potassium channels (North et al., 1987; Moises et al., 1994). In addition, MOR agonist-induced signaling triggers β-arrestin-dependent activation of mitogen-activated protein kinases (Zheng et al., 2008).

It is well established that missense polymorphisms in GPCRs can result in a variety of pharmacologic abnormalities (e.g., alteration in receptor-mediated signaling, affinity, expression) that may in turn predispose to physiologic changes and/or disease (Seifert and Wenzel-Seifert, 2002; Conn et al., 2007). It has been proposed that the occurrence of MOR missense mutations in the human population may underlie variability in the pharmacologic response to endogenous as well as synthetic MOR ligands and at the same time affect susceptibility to the development of drug addiction (Lotsch and Geisslinger, 2005).

In the current study, 13 nonsynonymous single amino acid changes in the MOR were selected for pharmacological analysis from the NaVa (Natural Variants) database, which catalogs known human GPCR polymorphisms (frequency >1%) as well as rarer mutations (Kazius et al., 2008). Our investigations suggest that selected MOR variants show reduced or absent agonist function. Among the abnormal MORs, we demonstrate that the previously uncharacterized N190K isoform has markedly impaired membrane trafficking and as a result does not signal in response to endogenous peptide agonists. Furthermore, our studies reveal that the MOR antagonists naltrexone and naloxone can not only restore cell-surface expression but also induce receptor-mediated signaling of this otherwise "dead" receptor.

## Materials and Methods

**Materials.** DAMGO ([D-Ala$^2$,*N*-MePhe$^4$,Gly-ol]-enkephalin) was purchased from Bachem (Bubendorf, Switzerland). β-Chlornaltrexamine (β-CNA), H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-$NH_2$ (CTAP), naltrexone, naloxone, endomorphin 1, and leucine-enkephalin were from Sigma (Saint Louis, MO). Buprenorphine was obtained from Reckitt Benckiser Pharmaceuticals (Richmond, VA). Cell culture media, fetal bovine serum, and Lipofectamine reagent were obtained from Invitrogen (Carlsbad, CA). Peroxidase-conjugated, anti-hemagglutinin (HA) monoclonal antibody (3F10), and BM-blue, a peroxidase substrate, were purchased from Roche Applied Science (Indianapolis, IN). The plasmids encoding a serum response element ($SRE_{5x}$) or a cAMP response element ($CRE_{6x}$) ligated upstream of a luciferase reporter gene have been described previously (Hearn et al., 2002; Fortin et al., 2010).

**Construction of Human μ-Opioid Receptor Plasmids.** After subcloning of the MOR in pcDNA1.1, missense mutations were introduced into the receptor cDNA using oligonucleotide-directed site-specific mutagenesis as described previously (Bläker et al., 1998). To enable assessment of receptor expression, an HA epitope (YPYDYPDYA) was introduced after the initiator methionine of each receptor isoform. The nucleotide sequences of all receptor coding regions were confirmed by automated DNA sequencing.

**Cell Culture.** Human embryonic kidney (HEK) 293 cells were grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, and 100 μg/ml streptomycin. The cells were maintained at 37°C in a humidified environment containing 5% $CO_2$.

**Luciferase Reporter Gene Assays.** Receptor-mediated signaling via inhibitory $G\alpha_{i/o}$ proteins was assessed using a luciferase reporter gene assay as described previously (Al-Fulaij et al., 2008). In brief, HEK293 cells were plated at a density of 1 to 2 × $10^3$ cells per well onto clear-bottomed, white 96-well plates and grown for 2 days to ~80% confluence. Cells were then transiently transfected using Lipofectamine reagent (Invitrogen) with cDNAs encoding 1) a wild-type or mutant MOR (or an empty expression vector), 2) a cAMP-responsive element-luciferase reporter gene ($CRE_{6X}$-luc), and 3) β-galactosidase, to enable correction for interwell variability. Twenty-four hours after transfection, cells were incubated for 6 h (unless specified otherwise) with or without the appropriate concentration of ligand diluted in serum-free medium supplemented with 0.5 μM forskolin. As an alternative strategy to measure $G\alpha_{i/o}$-mediated signaling, cells were transfected as described above but with the $CRE_{6x}$-luc replaced by a combination of a serum-responsive element-luciferase reporter gene ($SRE_{5x}$-luc) and a chimeric Gα protein ($G\alpha_{q5i}$). Introduction of the five C-terminal residues of $G\alpha_{i/o}$ in the $G\alpha_q$ protein ($G\alpha_{q5i}$) allows detection of $G\alpha_{i/o}$-coupled receptor-mediated signaling using the SRE-luc reporter gene (Conklin et al., 1993). Twenty-four hours after transfection, cells were stimulated for 18 h with or without naltrexone, naloxone, buprenorphine, CTAP, or DAMGO in serum-free medium. In both protocols (using either $CRE_{6X}$-luc or SRE/$G\alpha_{q5i}$ as a reporter system), the medium was gently aspirated after ligand treatment, and luciferase activity was measured using steadylite reagent (PerkinElmer Life and Analytical Sciences, Waltham, MA). A β-galactosidase assay was then performed after adding the enzyme substrate 2-nitrophenyl β-D-galactopyranoside. After incubation at 37°C for 30 to 60 min, substrate cleavage was quantified by measurement of optical density at 420 nm using a SpectraMax microplate reader (Molecular Devices, Sunnyvale, CA). Corresponding values were used to normalize the luciferase data.

**Assessment of Receptor Expression Using ELISA.** Receptor expression levels were determined using a procedure described by Al-Fulaij et al. (2008). In brief, HEK293 cells grown in 96-well plates were transiently transfected with either pcDNA1.1 or a plasmid encoding an HA-tagged WT or mutant MOR receptor. In selected experiments, a dominant-negative mutant of dynamin (DynK44N) construct was cotransfected with the control or receptor encoding plasmids. Forty-eight hours after transfection, the cells were washed once with phosphate-buffered saline (PBS), pH 7.4, and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. Cells were permeabilized with 0.1% Triton X-100 in PBS for 2 min to enable detection of total receptor expression levels; cell-surface expression was measured without the permeabilization step. After washing with PBS/100 mM glycine, the cells were incubated for 30 min in blocking solution (PBS/20% bovine serum). A horseradish peroxidase-conjugated monoclonal antibody (clone 3F10; Roche Applied Science, Indianapolis, IN) directed against the HA-epitope was then added to the cells (1:500 dilution in blocking solution). After 1 h, the cells were washed five times with PBS, and BM-blue (3.3′-5, 5′-tetramethylbenzidine; Roche Applied Science) solution (50 μl/well) was added. After incubation for 30 min at room temperature, conversion of this substrate by antibody-linked horseradish peroxidase was terminated by adding 2.0 M sulfuric acid (50 μl per well). Converted substrate (which correlates with the amount of receptor)

was assessed by measuring light absorbance at 450 nm using a SpectraMax microplate reader (Molecular Devices, Sunnyvale, CA).

**Confocal Microscopy.** HEK293 cells were plated at a density of $5 \times 10^4$ cells/well onto poly-L-lysine–coated 35-mm glass-bottomed dishes (MatTek Corporation, Ashland, MA) and grown for 2 days to ~80% confluence. Cells were then transfected with cDNA encoding the N-terminally HA-tagged MOR (either WT or N190K mutant). Forty-eight hours after transfection, the cells were washed once with PBS and fixed with 4% paraformaldehyde in PBS for 10 min. After an additional two washes with PBS, cells were incubated for 30 min at room temperature in PBS with 10% FBS. Cells were then further incubated for 1h in PBS/10% FBS in the presence of an Alexa Fluor 488-conjugated monoclonal antibody directed against the HA-epitope (Invitrogen) at a final concentration of 1 $\mu$g/ml. The cells were then washed five times with PBS and subsequently kept in the same solution. Images were obtained using confocal miscroscopy (TCS SP2; Leica, Wetzlar, Germany).

**Radioligand Binding Assays.** HEK293 cells were plated at a density of $3 \times 10^4$ cells/well onto 24-well plates and grown for 18 to 24 h to ~80% confluence. Cells were then transiently transfected with either pcDNA1.1 or a plasmid encoding the WT MOR or N190K variant and grown for an additional 48 h. Whole-cell binding studies were initiated by washing cells once with 500 $\mu$l of low-sodium binding buffer (1.25 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5.37 mM KCl, 0.4 mM $KH_2PO_4$, 0.06 M choline chloride, 0.34 mM $Na_2HPO_4$, 5.55 mM *d*-Glucose, 25 mM HEPES, 0.2% bovine serum albumin, 0.15 mM phenylmethylsulfonyl fluoride, and 0.02% $NaN_3$) at room temperature, followed by addition of 500 $\mu$l of the same media. Saturation curves were constructed by adding increasing concentrations of [$^3$H]DAMGO (0–10 nM) to the appropriate wells. After a 3-h incubation at room temperature, the cells were washed three times with ice-cold PBS, solubilized in 0.1 N NaOH, and neutralized with an equal volume of 0.1 N HCl. Radioactivity in the resulting suspension was then measured by scintillation counting. Specific [$^3$H]DAMGO binding to cells expressing the WT or N190K MOR was determined by subtracting nonspecific radioligand binding as determined using cells transfected with the empty expression vector pcDNA1.1. Presence of bound radioligand at the cell surface was confirmed by subjecting cells to an acid wash procedure (Laporte et al., 2002). In brief, cells were washed three times using ice-cold PBS supplemented with 0.2 M acetic acid and 0.150 mM NaCl after the 3-h incubation period at room temperature. This approach revealed that 68 ± 6% ($n$ = 3) of the specifically bound [$^3$H]DAMGO could be washed off, supporting the contention that the majority of radioligand binding occurs at the cell surface (data not shown).

**Cohort.** Heart Strategies Concentrating on Risk Evaluation (Heart SCORE) is a single-site prospective community-based cohort study investigating the mechanisms underlying population disparities in cardiovascular disease (Aiyer et al., 2007; Kelley-Hedgepeth et al., 2008). Our sample includes 1191 subjects (425 African Americans and 766 white persons) who provided consent and a DNA sample. Demographics, physical examination data, and laboratory measurements were obtained as reported elsewhere (Aiyer et al., 2007). This study was approved by the Tufts Medical Center Institutional Review Board.

**Genotyping.** The frequency of the rs34074916 single-nucleotide polymorphism (encoding the N190K variant) in the Heart SCORE cohort was assessed by TaqMan analysis. TaqMan genotyping kits purchased from Applied Biosystems (Foster City, CA) were used according to the manufacturer's instructions. A 7900 real-time PCR system was used for amplification. The reaction volume was 5 $\mu$l and included 10 ng of DNA, 2.5 $\mu$l of Universal PCR master mix (2×), and 0.1 $\mu$l of 40× probes. The reaction conditions were as follows: 95° for 10 min followed by 40 cycles of 15 s at 92°C and 1 min at 60°C. Real-time PCR results were analyzed with the use of the SDS 2.3 program by Applied Biosystems. Presence of the rs34074916 single-nucleotide polymorphism was confirmed by Sanger sequencing.

**Data Analysis.** Prism software (ver. 5.0; GraphPad Software, San Diego, CA) was used for nonlinear curve fitting of receptor signaling and radioligand binding data, and for calculation of half-maximal effective concentrations ($EC_{50}$ values), dissociation constant ($K_d$), and density of binding sites ($B_{max}$). The $pEC_{50}$ and total/surface expression values for each of the mutants were compared with the corresponding control values at the WT receptor using one-way analysis of variance followed by Dunnett's post test (GraphPad INSTAT software). Multivariable linear regression analysis was used to determine the association between cardiovascular risk factors and rs34074916 allele carriage while controlling for age, sex, body mass index (BMI), smoking, alcohol consumption, and medication history. The nominal threshold for statistical significance was set at 0.05. Association analyses were performed using SAS/STAT (SAS Institute, Inc., Cary, NC).

## Results

**Human MOR Variants.** Thirteen nonsynonymous single amino acid substitutions in the human MOR (A6V, N40D, D51N, G63V, S66F, S147C, N190K, R260H, R265C, R265H, S268P, D274N, and V293I) were selected for analysis based on a review of the NaVa database (Kazius et al., 2008) and of the literature (Befort et al., 2001; Wang et al., 2001; Lotsch and Geisslinger, 2005). The position of each amino acid substitution is illustrated in a diagram of the MOR (Fig. 1). To enable pharmacological assessment of each human MOR missense variant, site-directed mutagenesis was used to introduce relevant amino acid substitutions into the corresponding wild-type receptor. Each of the mutant receptor constructs was expressed in HEK293 cells and pharmacologically characterized.

**MOR Variants Show Alterations in DAMGO-Induced Signaling.** MOR-mediated activation of the $G\alpha_{i/o}$ signaling pathway was assessed using a $CRE_{6X}$-luciferase reporter gene assay. Forskolin stimulates adenylate cyclase, thereby triggering an increase in intracellular cAMP levels, which in turn results in CRE-dependent luciferase activity. Simultaneous addition of a saturating concentration of the prototypic MOR agonist DAMGO results in a $G\alpha_{i/o}$-mediated inhibition of forskolin-induced luciferase activity.

As an initial screen, basal and DAMGO-induced signaling were examined at each MOR isoform using a saturating concentration of agonist ($10^{-6}$ M) (Fig. 2A). The effect of

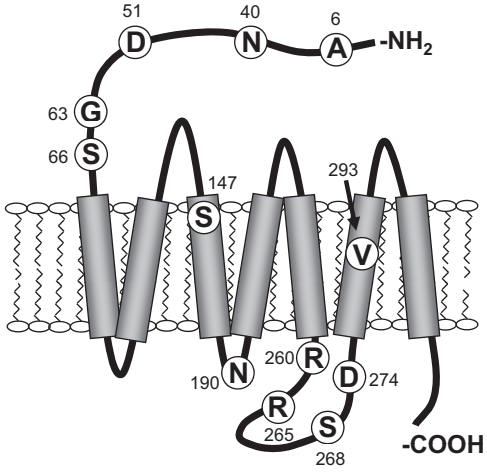


**Fig. 1.** A diagram of the MOR illustrating the position of missense mutations within the receptor protein. Respective residues in the wild-type protein are indicated by the single letter code.

forskolin alone, measured as an index of basal signaling, was similar in cells expressing WT or variant MORs (Fig. 2A, gray bars). At the wild-type receptor, DAMGO blocked 88% of forskolin-induced activity. The signaling efficacy of DAMGO was significantly reduced at R265C, R265H, and S268P relative to wild type and was essentially absent when assessed at the N190K variant (Fig. 2A, black bars). At each of the other variants, DAMGO efficacy was similar to that of wild type.

**Selected Variants Exhibit Reduced Potency of Both Synthetic and Endogenous MOR Agonists.** To further explore the observed alterations in DAMGO-induced signaling, and to investigate whether these changes also apply to endogenous ligand function, concentration-response curves for DAMGO, endomorphin 1, and leucine-enkephalin were generated for each variant and compared with wild type (Table 1; Fig. 2B illustrating selected variants). Five MOR variants showed a significant decrease in potency for

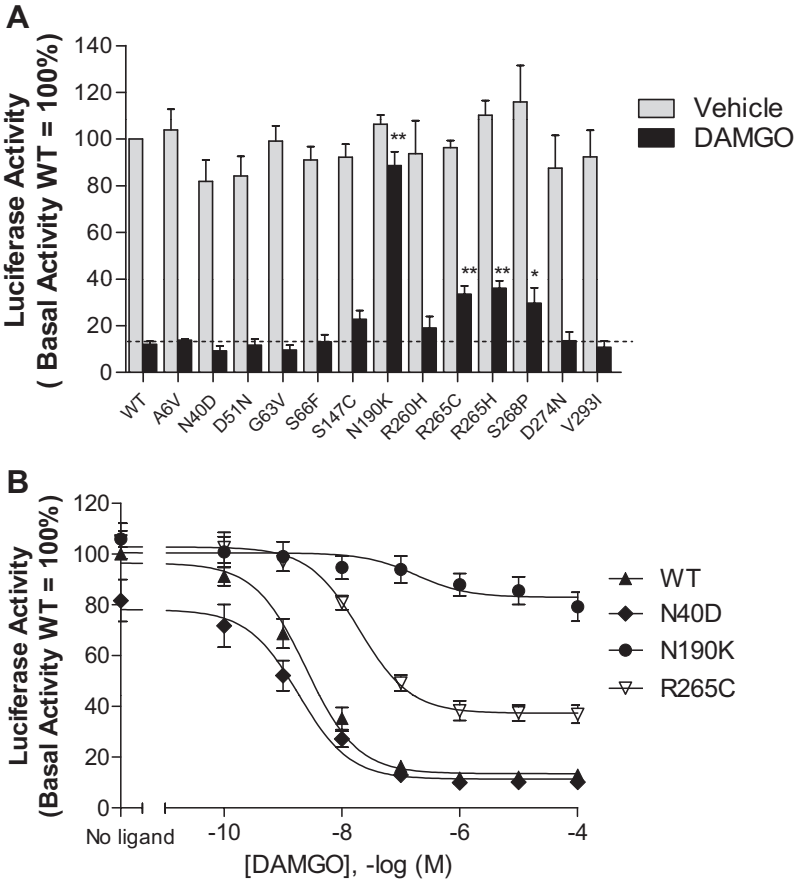


**Fig. 2.** Selected MOR missense mutations alter DAMGO-induced signaling. HEK293 cells were transiently transfected with either the WT or a MOR isoform and a $CRE_{6x}$-Luc reporter gene construct. Twenty-four hours after transfection, cells were incubated for 6 h with or without a saturating concentration of DAMGO (1 $\mu$M) (A) or increasing concentrations of DAMGO (B) diluted in serum-free medium supplemented with 0.5 $\mu$M forskolin. After stimulation, luciferase activity was quantified as described under *Materials and Methods*. All activity values were normalized relative to the forskolin-stimulated maximum at the WT MOR (100% activity). Data represent the mean ± S.E.M. from at least four independent experiments, each performed in triplicate. Significance, efficacy of mutant versus wild-type MOR, analysis of variance followed by Dunnett's post test; *, $p < 0.05$; **, $p < 0.01$.

TABLE 1

Agonist potency at wild-type vs. mutant MORs

All values represent the mean ± S.E.M. from at least five independent experiments.

| Receptor | DAMGO | | Endomorphin 1 | | Leu-Enkephalin | |
|---|---|---|---|---|---|---|
| | $EC_{50}$ | $pEC_{50}$ | $EC_{50}$ | $pEC_{50}$ | $EC_{50}$ | $pEC_{50}$ |
| | *nM* | | *nM* | | *nM* | |
| hMOR | 1.8 | 8.82 ± 0.11 | 1.7 | 8.77 ± 0.02 | 4.2 | 8.44 ± 0.16 |
| A6V | 3.1 | 8.62 ± 0.16 | 2.1 | 8.69 ± 0.07 | 7.1 | 8.23 ± 0.23 |
| N40D | 1.7 | 8.87 ± 0.15 | 1.3 | 8.91 ± 0.07 | 4.0 | 8.47 ± 0.19 |
| D51N | 3.0 | 8.60 ± 0.13 | 1.7 | 8.79 ± 0.08 | 4.9 | 8.40 ± 0.20 |
| G63V | 1.4 | 8.95 ± 0.13 | 1.4 | 8.92 ± 0.11 | 5.8 | 8.46 ± 0.35 |
| S66F | 8.4 | 8.18 ± 0.14* | 5.7 | 8.34 ± 0.13* | 36.8 | 7.66 ± 0.31 |
| S147C | 6.2 | 8.29 ± 0.12 | 4.1 | 8.41 ± 0.08* | 15.2 | 7.88 ± 0.17 |
| N190K | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
| R260H | 25.6 | 7.77 ± 0.18** | 6.3 | 8.26 ± 0.11** | 22.7 | 7.70 ± 0.25** |
| R265C | 18.1 | 7.79 ± 0.11** | 5.4 | 8.30 ± 0.07** | 36.4 | 7.52 ± 0.21* |
| R265H | 12.7 | 7.95 ± 0.11** | 8.4 | 8.14 ± 0.12** | 28.2 | 7.61 ± 0.18* |
| S268P | 6.9 | 8.21 ± 0.11* | 4.2 | 8.41 ± 0.08* | 14.2 | 7.86 ± 0.08 |
| D274N | 0.9 | 9.12 ± 0.13 | 0.6 | 9.31 ± 0.13** | 2.7 | 8.63 ± 0.19 |
| V293I | 2.2 | 8.82 ± 0.18 | 1.7 | 8.84 ± 0.12 | 5.3 | 8.38 ± 0.24 |

N.D., not determined.
* $P < 0.05$, significance versus wild-type MOR value.
** $P < 0.01$, significance versus wild-type MOR value.

DAMGO relative to wild type (S66F, R260H, R265C, R265H, and S268P) (Table 1). A parallel reduction in endomorphin 1 and/or leucine-enkephalin $EC_{50}$ was observed at each of these receptors (Table 1; efficacies shown in Supplemental Fig. 1). Because of the lack of efficacy, corresponding ligand potencies at the N190K variant could not be determined. It is noteworthy that agonist potency tended to be increased at the D274N variant; however, this change reached statistical significance only with endomorphin 1. In contrast to these functional alterations, the $EC_{50}$ values for DAMGO, endomorphin 1, and leu-enkephalin at the A6V, N40D, D51N, G63V, and V293I mutants were comparable with those at the wild-type receptor.

**The N190K Variant Shows Decreased Receptor Expression.** The expression level of HA-tagged wild-type and variant MORs were determined by ELISA (Fig. 3). For each receptor, transfection of increasing amounts of cDNA (0–16 ng) led to an elevation in cell-surface expression. Relative to wild type, the N190K variant showed markedly lower cell-surface expression (~20% of the wild-type level). Total expression, measured in permeabilized cells, was also decreased for the N190K mutant (~50% of the wild-type level; data not shown). In contrast to the N190K variant, the surface expression level of other functionally abnormal receptors was comparable with that of wild type (Fig. 3).

**Absence of Specific DAMGO Binding on Cells Expressing the N190K Variant.** The observed loss of function of the N190K variant was further investigated by radioligand binding experiments using the tracer [$^3$H]DAMGO (Fig. 4). High-affinity saturable radioligand binding was measured using cells expressing the WT MOR ($K_d$, 8.6 nM; $B_{max}$, 8 fmol/well). In contrast, no specific DAMGO binding was detected in parallel studies with the N190K mutant.

**Nonpeptidic Ligands Increase Cell-Surface Expression of the N190K Variant.** Accumulating evidence supports the idea that selected small-molecule ligands may promote enhanced cell-surface expression of wild-type and mutant GPCRs (Conn and Ulloa-Aguirre, 2010). We assessed the effects of the nonpeptidic MOR antagonist NTX on cell-surface expression of the wild-type receptor and the N190K variant using an ELISA (Fig. 5). Treatment with 10 $\mu$M NTX for 18 h led to a marked increase in the cell-surface expression of the N190K mutant. In contrast, surface expression of the wild-type receptor was minimally up-regulated after NTX administration (Fig. 5 and 7).

NTX-induced up-regulation of the N190K variant was further explored using confocal microscopy (Fig. 6). Consistent with our ELISA and radioligand binding data, microscopy provided further evidence that cell-surface expression of the N190K variant was dramatically reduced compared with the wild-type MOR. These experiments also confirmed that prolonged treatment (i.e., 18 h) with NTX markedly increases surface expression of the abnormal N190K mutant.

To assess whether the effect of NTX on expression of the N190K MOR variant is restricted to this opioid ligand, a series of antagonists (naloxone, $\beta$-CNA, CTAP) and agonists (buprenorphine, DAMGO, leucine-enkephalin, endomorphin 1) were tested for their ability to modulate surface expression of the WT MOR and N190K variant (Fig. 7). In parallel experiments, to explore whether decreased expression of the N190K variant results from constitutive endocytosis, we as-

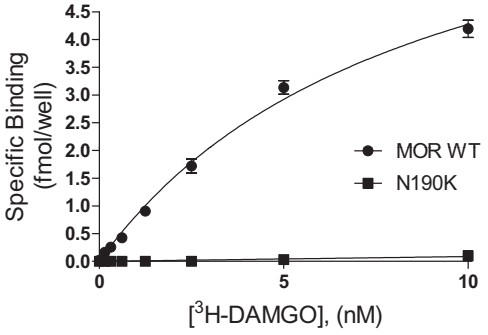


**Fig. 4.** Cells expressing the N190K variant display no specific [$^3$H]DAMGO surface binding sites. HEK293 cells plated onto 24-well plates were transiently transfected with either pcDNA1.1 or a plasmid encoding the WT MOR or N190K variant. Forty-eight hours after transfection, cells were washed and incubated for 3 h at room temperature with the indicated concentrations of [$^3$H]DAMGO. Incubations were performed and terminated as described under *Materials and Methods*. Nonspecific binding, established using HEK293 cells transfected with the pcDNA1.1 plasmid, was subtracted from the total binding values measured at receptor expressing cells. Data represent the mean ± S.E.M. from three independent experiments, each performed in triplicate.

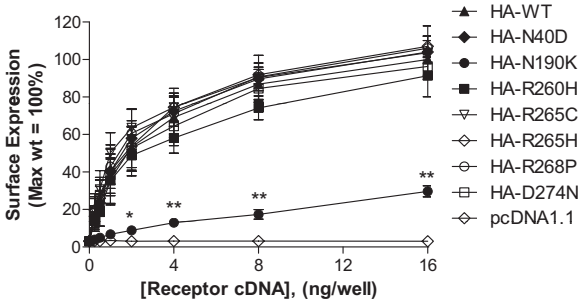


**Fig. 3.** The N190K MOR missense mutation alters cell-surface expression. Cell-surface expression of HA-tagged MORs increases as a function of cDNA concentration. HEK293 cells were transfected with increasing amounts of plasmid encoding either the wild-type or a mutant HA-tagged MOR. After 48 h, surface expression was measured by ELISA as described under *Materials and Methods.* Expression data are shown as a percentage of the maximal value observed at the wild-type MOR (transfection of 16 ng of cDNA/well). Each data point represents the mean ± S.E.M. from at least four independent experiments, each performed in triplicate. Significance, surface expression of WT versus mutant MOR; one-way analysis of variance with Dunnett's post test. **, $p < 0.01$.

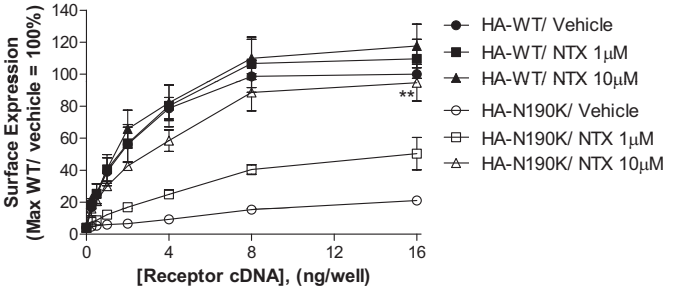


**Fig. 5.** Cell surface expression of the N190K mutant is enhanced by the small-molecule antagonist naltrexone. HEK293 cells were transiently transfected with plasmid encoding an HA-tagged version of the WT or N190K MOR. Twenty-four hours later, cells were treated for 18 h with media containing vehicle or increasing concentrations (1 or 10 $\mu$M) of naltrexone. The levels of surface expression of each HA-tagged receptor were assessed using ELISA as described under *Materials and Methods*. Data represent the mean ± S.E.M. from at least four independent experiments, each performed in triplicate. Significance, surface expression of vehicle-treated WT or mutant MOR versus antagonist treated cells; one-way analysis of variance with Dunnett's post test. **, $p < 0.01$.

sessed the effect of a dominant-negative dynamin mutant, DynK44N. Expression of this construct has been shown to block both spontaneous and agonist-dependent internalization of WT and mutant GPCRs by preventing the formation of endocytic vesicles (Rochdi et al., 2010) (Fig. 7).

Surface expression of the wild-type MOR was not significantly altered after a prolonged incubation with naloxone, β-CNA, buprenorphine, or CTAP (Fig. 7A). In contrast, treatment with each of the peptide agonists (10 μM, 18 h) led to a marked reduction in the number of wild-type MORs displayed at the cell surface. Consistent with its ability to interfere with endocytosis, overexpression of DynK44N efficiently inhibited agonist-induced internalization of the wild-type MOR (Fig. 7A).

As observed with NTX, treatment with 10 μM naloxone, β-CNA, or buprenorphine also led to increased expression of the N190K variant (Fig. 7B). In each case, the compound-induced increase in expression was highly significant but less pronounced compared with the effect of NTX. In contrast, the peptidic antagonist (CTAP) or agonists (DAMGO, leucine-enkephalin, endomorphin 1) did not modify the expression level of this mutant receptor. It is noteworthy that DynK44N potentiated buprenorphine-induced stabilization of the N190K mutant at the cell surface (Fig. 7B), whereas it did not influence expression of this mutant receptor under basal conditions or in response to either NTX, naloxone, or β-CNA. These observations may suggest that modulation of receptor trafficking by the alkaloid agonist buprenorphine is more complex than that induced by the structurally related antagonist ligands. It is possible that buprenorphine induces some degree of receptor internalization in addition to rescuing surface expression of the N190K variant and that the former effect is blocked by DynK44N expression.

**Naltrexone Displays Agonist-like Properties at the N190K Variant.** At the wild-type MOR, the established agonists DAMGO and buprenorphine induced receptor activation reflected as a concentration-dependent inhibition of forskolin-induced luciferase activity (Fig. 8A). In contrast, NTX, naloxone, and CTAP had minimal if any effect, consistent with previous classification of these ligands as antagonists. Assessment of these compounds at the N190K variant revealed that this variant was essentially unresponsive to DAMGO but could still be activated by buprenorphine (Fig. 8B). Surprisingly, two of the three established MOR antagonists (NTX and naloxone) acted as agonists at the N190K variant, showing efficacy similar to that of buprenorphine.

To further support the involvement of inhibitory G proteins in the activity of naltrexone at the N190K variant, we used a complementary assay providing a positive measure of receptor-mediated signaling. This assay relies on coexpression of a chimeric $G\alpha_{q5i}$ protein, which links MOR stimulation to activation of an $SRE_{5x}$-luciferase reporter gene construct (see *Materials and Methods*). As observed with the forskolin inhibition assay (Fig. 8), DAMGO-induced signaling was absent at the N190K variant, whereas at the wild-type receptor, this ligand resulted in concentration-dependent MOR activation (Supplemental Fig. 2). Conversely, NTX did not activate the wild-type MOR but induced a pronounced increase in receptor-mediated activity at the N190K variant (Supplemental Fig. 2). NTX-induced signaling

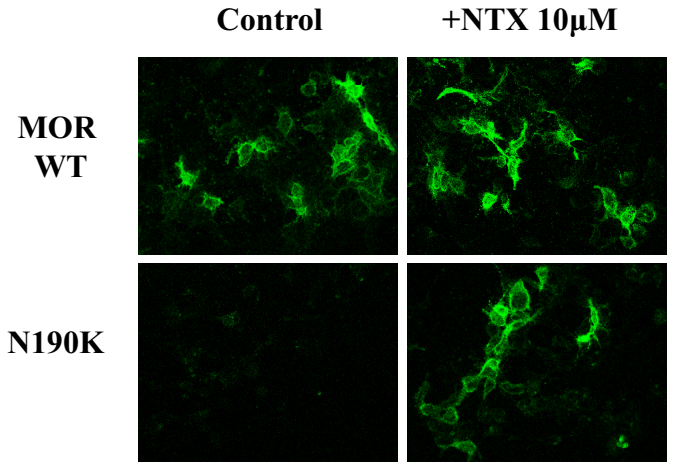


**Fig. 6.** Cell surface expression of the N190K mutant can be rescued by the small molecule naltrexone. HEK293 cells were transiently transfected with a plasmid encoding either an HA-tagged WT or N190K MOR. Twenty-four hours later, cells were treated for 18 h with media containing vehicle or 10 μM naltrexone. The levels of surface expression of each HA-tagged receptor were visualized using confocal microscopy as described under *Materials and Methods*.

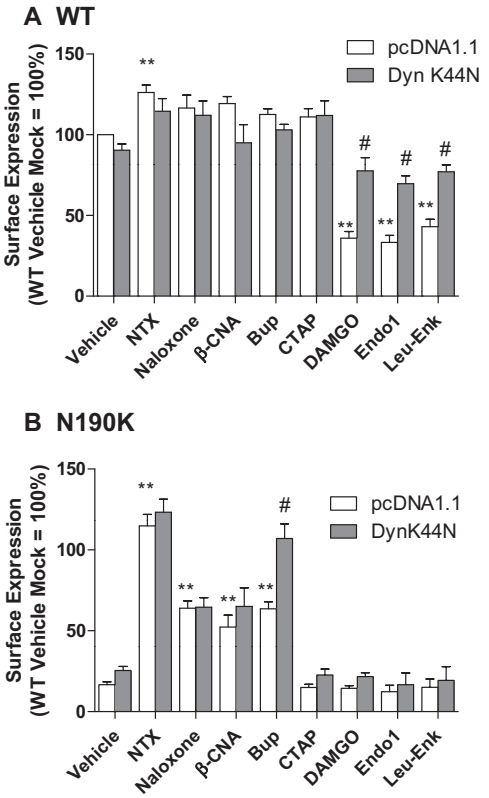


**Fig. 7.** Expression of a dominant dynamin mutant (DynK44N) fails to rescue surface expression of the N190K mutant. HEK293 cells were transiently transfected with either plasmid encoding the HA-tagged WT MOR or N190K variant in combination with pcDNA1.1 or the DynK44N-encoding plasmid. DynK44N-rescued agonist-induced internalization of the WT MOR (A), however had no effect on the surface expression of the N190K variant in either the presence or the absence of ligands (B). After 24 h, cells were treated with media containing vehicle or 10 μM ligand. Surface expression levels of the receptors were assessed by ELISA as described under *Materials and Methods*. Data represent the mean ± S.E.M. from at least four independent experiments, each performed in triplicate. Significance, one-way analysis of variance with Dunnett's post test, surface expression of vehicle versus ligand treated cells, **, $p < 0.01$; surface expression in the absence versus presence of DynK44N, #, $p < 0.01$. Bup, buprenorphine; Endo1, endomorphin 1.

reached a level approximating 75% of the maximal activity triggered by DAMGO at the wild-type MOR.

**The N190K MOR Variant Is Associated with Elevated HDL Cholesterol Levels.** To assess the frequency of the N190K variant in Americans of either African or European descent, we genotyped 1191 subjects within the Heart SCORE cohort. The N190K variant occurred in three African American women (overall frequency of 0.25%), but was not found in people of European ancestry. The Heart SCORE is a community-based observational study of individuals at risk for coronary artery disease. Analysis of metabolic factors recorded on all Heart SCORE participants revealed that N190K carriers had significantly elevated high-density lipoprotein (HDL) levels compared with noncarriers (75.60 ± 7.72 versus 54.79 ± 1.30, $p = 0.0065$). Differences between carriers and noncarriers exceeding 10% were also observed in a subset of other clinical parameters, including body mass index, waist circumference, blood triglyceride levels, and systolic blood pressure. However, none of these changes reached statistical significance (Supplemental Table 1).

## Discussion

The MOR modulates analgesia, feeding behavior, as well as selected autonomic functions including respiratory rate and intestinal motility. It has been proposed that mutations in the MOR gene may underlie observed interindividual variability in the response to opioids (Lotsch and Geisslinger, 2005). In this study, we investigated the effect of naturally occurring polymorphisms on MOR function by comparing the corresponding pharmacological properties with those of the wild-type receptor.

Sequential assessment of 13 missense mutations in the human MOR sequence revealed that selected variants showed significant alterations in ligand-induced signaling. The S66F, S147C, R260H, R265C, R265H, and S268P mutations each resulted in altered DAMGO, endomorphin 1 and/or leucine-enkephalin potency or efficacy compared with wild-type values. Of particular note, receptor mediated signaling in response to these peptides was absent at the N190K isoform. At least four mechanisms may contribute to the observed mutation-induced pharmacological changes: 1) alteration of G protein coupling, 2) modification of the ligand-receptor interaction, 3) abnormal transitioning from the inactive to the active receptor state (a conformational change that triggers second-messenger signaling), and/or 4) defective receptor trafficking (e.g., intracellular trapping) (Beinborn et al., 2004; Conn and Ulloa-Aguirre, 2010).

Previous studies using different signaling readouts (including guanosine 5′-*O*-(3-thio)triphosphate binding assays) showed that the R260H, R265H, and S268P MOR mutations result in a loss-of-function phenotype despite normal surface expression (Koch et al., 2000; Befort et al., 2001; Wang et al., 2001). Our data obtained using a different approach confirm and extend these findings by demonstrating that such abnormalities also apply to the endogenous agonists endomorphin 1 and leucine-enkephalin. Furthermore, we showed that the previously uncharacterized R265C mutation reduces agonist potency to an extent similar to that of the three mutations discussed above. Consistent with the location of corresponding amino acid substitutions in the third intracellular loop, these observations collectively support that these sequence alterations may interfere with receptor/G protein interaction (Befort et al., 2001; Wang et al., 2001). A parallel mechanism (enhanced receptor/G protein interaction) may be responsible for the observed increase in potency observed at the D274N variant. In contrast, altered G protein coupling is unlikely to explain defective signaling of the S66F variant that results from an amino acid substitution within the receptor's N terminus. Given this location, the observed decrease in agonist potency could be due to an alteration in receptor-ligand interaction.

No major pharmacological abnormality was observed at the A6V, N40D, D51N, G63V, and V293I mutants. It must be noted that inconsistent/divergent results regarding ligand affinity and surface expression of the N40D variant have been reported (Kroslak et al., 2007; Oertel et al., 2009). Such differences between studies aimed at characterizing subtle alterations in GPCR function may be explained, at least to some degree, by methodological variation and/or the cellular model system used for receptor analysis (Oertel et al., 2009).

Pharmacological studies of the N190K mutation have not been reported previously. Our results for this second intracellular loop substitution mutant reveal that receptor-mediated signaling in response to endomorphin 1, leucine-enkephalin, or DAMGO was absent. Taken together, ELISA, microscopic, and radioligand binding studies of the N190K

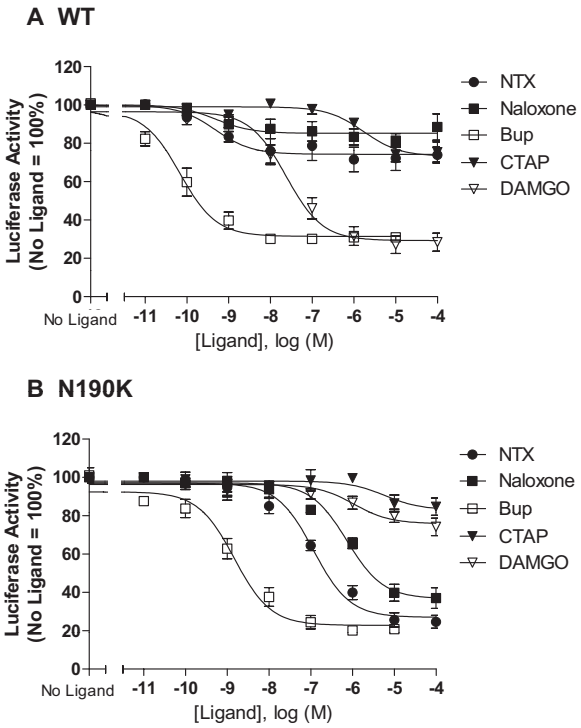


**Fig. 8.** Naltrexone, naloxone, and buprenorphine display agonist activity at the N190K variant. HEK293 cells were transiently transfected with either the WT (A) or the N190K (B) MOR isoform and a $CRE_{6x}$-Luc reporter gene construct. Twenty-four hours after transfection, cells were incubated for 18 h with increasing concentrations of DAMGO, naltrexone, naloxone buprenorphine, or CTAP together with 0.5 μM forskolin. After stimulation, luciferase activity was quantified as described under *Materials and Methods*. DAMGO activates the WT MOR but has minimal if any effect at the N190K variant. Conversely, NTX and naloxone are strong agonists at the N190K variant but show minimal if any activity at the WT MOR. All activity values were normalized relative to the forskolin-stimulated maximum at the WT MOR (= 100%). Data represent the mean ± S.E.M. from at least four independent experiments, each performed in triplicate.

mutant suggest reduced surface expression as a plausible explanation for the lack of signaling. The decrease in N190K expression may be due to mutation-induced structural instability and/or misfolding of the receptor protein. Conformationally defective proteins may either accumulate intracellularly or be targeted to the ubiquitination/ proteosome pathway, which ultimately leads to degradation (Conn et al., 2007). The marked reduction in total expression of the N190K variant (in addition to cell-surface expression) suggests the latter scenario to be the case. It is of note that prolonged treatment with micromolar concentrations of a small molecule MOR antagonist (naltrexone, naloxone, or β-CNA) or agonist (buprenorphine) led to a significant increase in cell-surface expression of the N190K mutant. In contrast, peptidic ligands (antagonist as well as agonist) failed to modulate expression of this variant. Consistent with previous studies on other GPCRs (Conn et al., 2007), our data support the idea that selected small-molecule ligands may cross cell membranes and act intracellularly as pharmacological chaperones, facilitating correct folding and plasma membrane targeting of the MOR protein. Alkaloid ligands, including naltrexone, have previously been shown to promote maturation and membrane insertion of both WT and mutant δ-opioid receptor isoforms that are retained in the endoplasmic reticulum (Leskela et al., 2007).

In addition to increasing surface levels of the N190K MOR variant, we showed that application of selected small molecule antagonists lead to unexpected ligand induced signaling at this mutant receptor. It is possible that the same mutation that impairs surface expression at this receptor variant concomitantly converts naltrexone/naloxone from an antagonist to an agonist. Such mutation-induced alterations in ligand function (albeit without affecting receptor expression) have been reported previously with other GPCRs, including the $\beta_2$-adrenergic and cholecystokinin 2 receptors (Strader et al., 1989; Bläker et al., 1998). Corresponding changes in ligand function may reflect either an alteration in ligand-receptor interaction or an increase in basal receptor activity. The latter may result in a systematic amplification in the activity of receptor selective ligands (resulting in apparent antagonists or weak partial agonists acquiring higher levels of activity) (Samama et al., 1993; Kopin et al., 2003).

If the N190K mutation induces constitutive receptor activity as considered above, it may in parallel trigger down-regulation or impaired expression (e.g., as a consequence of structural instability as reported with other constitutively active GPCRs (Gether et al., 1997; Li et al., 2001; Fortin et al., 2010). In this scenario, the naltrexone-induced rescue of expression could unmask basal receptor signaling thus contributing to the agonist function of this ligand. Consistent with this possibility, Li et al. (2001) have reported that a genetically engineered artificial mutation in the rat MOR (D164Q) confers not only constitutive activation but also increased basal endocytosis. Furthermore, that report demonstrated that surface expression of this constitutively active mutant could be enhanced by naltrexone, possibly by stabilizing the protein structure and by preventing spontaneous internalization (Li et al., 2001).

To explore the possibility that decreased expression of the N190K variant results from constitutive endocytosis, we assessed the effect of DynK44N, a dominant-negative dynamin mutant that is known to block basal and agonist-mediated internalization of many GPCRs. Such a construct was recently shown to prevent constitutive endocytosis, thus uncovering the elevated basal activity of naturally occurring vasopressin V2 receptor variants (Rochdi et al., 2010). Our observation that DynK44N efficiently blocks agonist-induced endocytosis at the wild-type MOR but fails to increase surface expression of the N190K variant under basal conditions suggests that ligand-independent internalization does not explain the diminished expression of this mutant receptor. Taken together, our studies leave open the possibility that multiple mechanisms may underlie NTX function at the N190K mutant (e.g., rescued receptor expression, mutation-induced conversion of this ligand to an agonist, amplification of trace intrinsic activity, unmasking of constitutive activity). The relative contribution of these factors remains to be defined.

The finding that a naturally occurring MOR mutation enables a putative antagonist to trigger receptor mediated signaling is novel. It is noteworthy that an *artificial* substitution of a conserved serine in either the μ- or the δ-opioid receptor has been shown to confer agonist properties to classic antagonists including naltrexone (Claude et al., 1996). This conserved residue (Ser196 in the MOR) is located at the junction of the second intracellular loop and transmembrane domain 4, in the same vicinity as Asn190. In mice genetically engineered to express a S196A MOR construct in the spinal cord, the putative "antagonist" naloxone induced antinociceptive responses without signs of tolerance or dependence (Chen et al., 2007). On the basis of this finding and our observations, it is possible that naltrexone will paradoxically induce antinociceptive actions in individuals harboring the N190K polymorphism.

Genotype analysis of an ethnically diverse cohort revealed occurrence of the N190K mutation in African Americans. It is of note that these persons have significantly elevated HDL cholesterol levels. We regard this association to be hypothesis-generating. A relationship between reduced MOR signaling and HDL, a cardioprotective cholesterol carrier, is plausible in light of recent clinical trials showing that a combination drug including naltrexone and bupropion led to increased HDL levels (Greenway et al., 2009). Genetic studies in additional cohorts as well as more detailed clinical evaluation of persons with the N190K mutation (e.g., assessment of nociception, susceptibility to addiction, and lipid profile) will be needed to define the frequency and phenotypic/pharmacogenomic impact of this functionally abnormal MOR variant in the human population.

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**Address correspondence to:** Dr. Alan S. Kopin, 800 Washington Street, P.O. Box 7703, Boston, MA 02111. E-mail: akopin@tuftsmedicalcenter.org