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Modulation of Ca^{2+} Channels by Heterologously Expressed Wild-type and Mutant Human μ-Opioid Receptors (hMOR) Containing the A118G Single Nucleotide Polymorphism

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ABSTRACT

The most common single nucleotide polymorphism (SNP) of the human \(\mu\)-opioid receptor (hMOR) gene occurs at position 118 (A118G) and results in substitution of asparagine to aspartate at the N-terminus. The purpose of the present study was to compare the pharmacological profile of several opioid agonists to heterologously expressed hMOR and N-type \(Ca^{2+}\) channels in sympathetic neurons. cDNA constructs coding for wild-type and mutant hMOR were microinjected in rat superior cervical ganglion neurons and N-type \(Ca^{2+}\) channel modulation was investigated employing the whole-cell variant of the patch clamp technique. Concentration-response relationships were generated with the following selective MOR agonists: DAMGO, morphine, morphine-6-glucuronide (M-6-G) and endomorphin I. The estimated maximal inhibition for the agonists ranged from 52 to 64% for neurons expressing either hMOR subtype. The rank order of potencies for estimated \(EC_{50}\) values (nM) in cells expressing wild-type hMOR was: DAMGO (31) >> morphine (76) \(\approx\) M-6-G (77) \(\approx\) endomorphin I (86). On the other hand, the rank order in mutant-expressing neurons was: DAMGO (14) >> morphine (39) >> endomorphin I (74) \(\approx\) M-6-G (82), with a two-fold leftward shift for both DAMGO and morphine. The DAMGO-mediated \(Ca^{2+}\) current inhibition was abolished by the selective MOR blocker, CTAP, and by pertussis toxin pretreatment of neurons expressing either hMOR subtype. These results suggest that the A118G variant MOR exhibits an altered signal transduction pathway and may help explain the variability of responses to opiates observed with carriers of the mutant allele.
INTRODUCTION

The human mu (μ)-opioid receptor (hMOR) is the primary target of opioid analgesics employed in the treatment of pain and drug addiction. The hMOR is a product of the OPRM1 gene and also a member of the G protein-coupled receptor superfamily. Short-term activation of MOR leads to voltage-gated Ca\(^{2+}\) channel inhibition, G protein-gated inwardly rectifying K\(^{+}\) (GIRK) channel opening and adenylyl cyclase inhibition via pertussis toxin (PTX)-sensitive G\(\alpha\) (G\(\alpha_{i/o}\)) protein subunits (Bailey and Connor 2005). This results in a decrease of both neuronal excitability and neurotransmitter release. However, the long-term use of opioid analgesics can lead to tolerance, drug dependence and addiction.

The human OPRM1 gene has been reported to undergo at least ten single nucleotide polymorphisms (SNP) within the open reading frame and over 100 in non-coding regions (for review see Lötsch and Geisslinger 2005). The most common SNP within the OPRM1 coding region occurs at position 118 (A118G) in Exon I and results in an amino acid change from asparagine (N) to aspartate (D) at position 40 of the receptor. Asparagine is one of five putative glycosylation sites located on the extracellular N-terminal domain of the receptor (Mestek et al. 1995). The A118G polymorphism occurs with an allelic frequency ranging from 10-40% (Szeto et al. 2001; Kim et al. 2004; Lötsch and Geisslinger, 2005, dependent on population studied). Several clinical studies have shown that the presence of A118G polymorphism is associated with opiate effectiveness observed in patients (Shi et al. 2002; Skarke et al. 2003; Romberg et al. 2004; Romberg et al. 2005; Klepstad et al. 2004; Lötsch and Geisslinger, 2005; Janicki et al. 2006) as well as susceptibility to drug addiction (Bond et al. 1998; Szeto et al. 2001). On the other hand, some studies have also reported a lack of a correlation between the presence of the A118G SNP and drug addiction (Gelernter et al. 1999; Franke et al. 2001; Arias et al. 2006).
Few in vitro studies have examined the effect of the A118G polymorphism on receptor function and the findings have been conflicting. One report found that the mutant hMOR expressed in AV-12 cells had a 3-fold higher binding affinity for β-endorphin than the wild-type opioid receptor (Bond et al. 1998). In addition, the β-endorphin-mediated GIRK channel activation was three times more potent in mutant-expressing Xenopus oocytes than those expressing wild-type hMOR. Conversely, two separate studies have shown that the mutant hMOR expressed in either COS cells (Befort et al. 2001) or HEK 293 cells (Beyer et al. 2004) did not demonstrate significant changes in binding affinity, potency or signaling mechanisms when compared to wild-type receptors. More recently, it has been reported that CHO cells transfected with the mutant hMOR exhibited lower mRNA and protein expression levels (Zhang et al. 2005). The authors also reported that mutant 118G allele mRNA levels in human brain tissue were lower than the wild-type allele.

The purpose of the present study was to investigate the role that the A118G polymorphism plays in N-type Ca\textsuperscript{2+} channel modulation by various MOR agonists in rat sympathetic superior cervical ganglion (SCG) neurons. The majority of Ca\textsuperscript{2+} current in SCG neurons is carried by N-type Ca\textsuperscript{2+} channels (Ikeda 1991) and SCG neurons do not natively express µ-opioid receptors. Therefore, this model system offers an appropriate null background within a neuronal cellular context. More importantly, N-type Ca\textsuperscript{2+} channels play a major role in neurotransmitter release and have been shown to be modulated by MOR in central and sensory neurons (for review see Law et al. 2000). In the present report, wild-type and mutant hMOR were heterologously expressed in SCG neurons and the pharmacological profile of various MOR agonists was determined in order to ascertain whether the N40D mutation exhibits a differential modulation of
N-type Ca\(^{2+}\) channels. In this report, the first-described, or “prototype” hMOR and “A118G variant” gene products are referred throughout as wild-type and mutant hMOR, respectively.
MATERIALS AND METHODS

Neuron isolation

Superior cervical ganglion (SCG) neurons from adult rats were prepared using the method described previously (Ruiz-Velasco and Ikeda 2000). The experiments carried out were approved by the Institutional Animal Care and Use Committee (IACUC). Male Wistar rats (175-225 g) were anaesthetized with CO₂ and then decapitated using a laboratory guillotine. The neurons were enzymatically dissociated as described (Ruiz-Velasco and Ikeda 2000). The isolated neurons were resuspended in Minimal Essential Medium (MEM), supplemented with 10% fetal calf serum, 1% glutamine and 1% penicillin-streptomycin solution (all from Invitrogen, Carlsbad, CA). The dissociated neurons were plated onto 35 mm polystyrene tissue culture plates coated with poly-L-lysine and stored in a humidified incubator (95% air and 5% CO₂) at 37°C.

cDNA microinjection

Microinjection of cDNA plasmids was performed with an Eppendorf 5246 microinjector and 5171 micromanipulator (Brinkmann Instruments, Inc., Westbury, NY) 3-5 h after plating the neurons as described previously (Ikeda 2004). Plasmids coding for wild-type (Guthrie cDNA Resource Center, Sayre, PA) and mutant hMOR were subcloned in pcDNA3.1 (Invitrogen) and injected at concentrations of 5, 20 and 200 ng/µl. The mutant hMOR construct was prepared by site-directed mutagenesis (TOP Gene Tech., Montreal, Canada). Wild-type and mutant plasmid sequences were confirmed by automated oligonucleotide sequencing. The “enhanced” green fluorescent protein (pEGFP-N1; BD Biosciences, Clontech, Palo Alto, CA) cDNA was co-injected at 5 ng/µl to allow for identification of successfully injected neurons.

Electrophysiology and data analysis
Ca^{2+} currents were recorded at room temperature (21-24°C) employing the whole-cell patch-clamp technique within 24 hr following nuclear microinjection of vectors. The recording pipettes were pulled from borosilicate glass capillaries (Corning 7052; Garner Glass, Claremont, CA) on a Flaming-Brown (P-97) micropipette puller (Sutter Instrument Co., Novato, CA), coated with Sylgard (Dow Corning, Midland, MI) and fire polished with a microforge. SCG whole-cell Ca^{2+} currents were acquired with a patch-clamp amplifier (Axopatch 200B, Axon Instruments, Foster City, CA), analog filtered at 5-10 kHz (-3 dB; 4-pole lowpass Bessel filter) and digitized employing custom designed software (S5) on a PowerMacG4 computer (Apple Computer, Cupertino, CA) equipped with an 18-bit analog-to-digital converter board (ITC18, Instrutech Corp., Port Washington, NY). The cell’s series resistance (80-85%) and membrane capacitance were electronically compensated. Data and statistical analyses were performed with the Igor Pro (Lake Oswego, OR) and drc package from the R statistical programming environment (R Development Core Team) software packages, respectively with P < 0.05 considered statistically significant. Summary graphs and current traces were produced with the Igor Pro and Canvas 8.0 (Deneba Software, Miami, FL) software packages.

The pipette solution contained (mM): 120 N-methyl-D-glucamine, 20 tetraethylammonium hydroxide (TEA-OH), 11 EGTA, 10 HEPES, 1 CaCl_2, 4 Mg-ATP, 0.3 Na_2GTP, and 14 tris creatine phosphate. The pH was adjusted to 7.2 with methanesulfonic acid and the osmolality was 293–302 mosmol/kg. The external solution consisted of (mM): 145 TEA-OH, 140 methanesulfonic acid, 10 HEPES, 15 glucose, 10 CaCl_2, and 0.0003 tetrodotoxin. The pH was adjusted to 7.4 with TEA-OH and the osmolality was 320–325 mosmol/kg.

The concentration-response curves were determined by the sequential application of increasing concentrations of the receptor agonist. No more than three different concentrations
were employed with each cell to avoid desensitization. The results were pooled and each point represents the mean ± SEM. The concentration-response curves were fit to the Hill equation: 

\[ I = \frac{I_{\text{MAX}}}{1 + \left(\frac{\text{IC}_{50}}{[\text{ligand}]^n}\right)^{n_H}} \]

where \( I \) is the percentage inhibition, \( I_{\text{MAX}} \) is maximum inhibition, \( \text{IC}_{50} \) is half-inhibition concentration, \([\text{ligand}]\) is agonist concentration and \( n_H \) is the Hill coefficient.

**Solution and drugs**

Stock solutions of norepinephrine (NE)-bitartrate, [D-Ala\(^2\),NMe-Phe\(^4\),gly-ol\(^5\)]-enkephalin (DAMGO), morphine-6-glucuronide (M-6-G), morphine, endomorphin I and D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH\(_2\) (CTAP) (all from Sigma Chemical Co., St. Louis, MO) were prepared in H\(_2\)O and diluted in the external solution to their final concentrations prior to use. *Bordetella pertussis* toxin (PTX, List Biological Laboratories, Inc., Campbell, CA) was added to the culture medium (12-20 h) at a final concentration of 500 ng/ml.
RESULTS

Expression of wild-type and mutant hMOR in rat SCG neurons

In the present study, we examined the functional effects of the hMOR mutation (N40D) by comparing the modulation of N-type Ca\textsuperscript{2+} channels by the wild-type and mutant hMOR heterologously expressed in acutely dissociated rat SCG neurons. The initial experiments were carried out to determine the effective concentration of the hMOR cDNA constructs required to obtain coupling with Ca\textsuperscript{2+} channels without altering other native G protein-coupled receptor signaling pathways (i.e. \(\alpha_2\)-adrenergic receptors). Ca\textsuperscript{2+} currents were evoked every 5 s with a double-pulse voltage protocol (shown in Figure 1A) consisting of two identical test pulses (to +10 mV from a holding potential of -80 mV) separated by a large depolarizing conditioning pulse to +80 mV (Elmslie et al. 1990; Ikeda 1991). The Ca\textsuperscript{2+} current inhibition was measured isochronally 10 ms after initiation of the prepulse in the absence and presence of the agonist. Figure 1A shows the time course of both pre- and postpulse Ca\textsuperscript{2+} current amplitude before and after external application of the high affinity MOR agonist, DAMGO and NE. Also shown is the Ca\textsuperscript{2+} current facilitation, which is the ratio of the postpulse to prepulse current. Exposure of the cell to DAMGO (10 \(\mu\)M) did not result in Ca\textsuperscript{2+} channel inhibition (current traces 1 and 3). On the other hand, stimulation of the \(\alpha_2\)-adrenergic receptor by NE (10 \(\mu\)M) resulted in inhibition of Ca\textsuperscript{2+} channel currents by 50% (current traces 5 and 7). The NE-induced inhibition was greater during the prepulse (trace 7) than the postpulse (trace 8), indicating a voltage-dependent inhibition of the currents--characterized by kinetic slowing of the prepulse current and enhancement of the postpulse current. Thus, the post/pre ratio increased from 1.27 to 2.21 in the presence of NE (Figure 1A).
Figure 1B shows time course of Ca\(^{2+}\) current inhibition of a neuron microinjected with the wild-type hMOR cDNA (20 ng/µl). Application of DAMGO resulted in robust inhibition of the Ca\(^{2+}\) currents (traces 1 and 3) that was both voltage-dependent (post/pre ratio of 2.85) and comparable to the modulation observed with NE (traces 5 and 7). When SCG neurons were microinjected with 200 ng/µl wild-type hMOR cDNA (Figure 1C), the coupling between Ca\(^{2+}\) channels and either hMOR subtype was comparable to that observed with 20 ng/µl cDNA. Nevertheless, the NE-induced Ca\(^{2+}\) current inhibition was lower (traces 5 and 7) than that typically observed in the other groups. This is presumably a result of an alteration of native G protein signaling pathways and has been previously observed with ORL1 opioid (Beedle et al. 2004) and cannabinoid receptors (Vasquez and Lewis 1999). The NE-mediated Ca\(^{2+}\) current inhibition in neurons microinjected with 5, 20 and 200 ng/µl mutant hMOR cDNA was similar to that observed with wild-type hMOR-expressing cells (data not shown). Figure 1D is a summary graph showing the DAMGO- and NE-mediated Ca\(^{2+}\) current inhibition of uninjected and neurons microinjected with 5, 20 and 200 ng/µl wild type or mutant hMOR cDNA. Although microinjection of 5 ng/µl did not result in an alteration of coupling components between NE-activated \(\alpha_2\)-adrenergic receptors and Ca\(^{2+}\) channels, the DAMGO-mediated Ca\(^{2+}\) current inhibition was variable. Also, nuclear microinjection of 200 ng/µl of either hMOR cDNA resulted in a significantly lower (\(P < 0.05\)) NE-induced Ca\(^{2+}\) channel inhibition. Thus, for all subsequent experiments described, 20 ng/µl hMOR cDNA was chosen as the concentration that would maintain a consistent receptor-channel stoichiometry without altering native signaling pathways.

Next, we wanted to determine whether the signaling proteins that couple N-type Ca\(^{2+}\) channels and the N40D mutant receptor were different from the wild-type hMOR. As mentioned
above, MOR are coupled with members of the G\alpha_i/G\alpha_o subfamily that are pertussis toxin (PTX)-sensitive. Figure 2Ai and 2Bi shows current traces of neurons expressing wild-type and mutant hMOR, respectively. Bath application of DAMGO (10 \mu M) resulted in inhibition of Ca^{2+} currents by 75 and 78\%, respectively. On the other hand, Figure 2Aii and Bii shows that overnight PTX pretreatment of the neurons decreased the DAMGO-mediated Ca^{2+} current inhibition. The mean Ca^{2+} current inhibition (\pm SEM) in PTX-treated cells was significantly (P < 0.01) reduced in both wild-type (56 \pm 6\% vs. 9 \pm 3\%) and mutant (61 \pm 9\% vs. 9 \pm 2\%) expressing neurons (Figure 1C). These results suggest that both receptor subtypes modulate N-type Ca^{2+} channels via Go_{i/o} G protein subunits.

Pharmacological profile of opioid ligand-mediated Ca^{2+} current inhibition in wild-type and mutant hMOR-expressing SCG neurons

In the next set of experiments, the concentration-dependent Ca^{2+} current inhibition by DAMGO was determined in neurons heterologously expressing wild-type or mutant hMOR. Ca^{2+} currents were evoked employing the voltage protocol described in Figure 1A. Figure 3A shows the time course of Ca^{2+} current inhibition by 0.003, 0.03 and 3 \mu M DAMGO in neurons expressing the wild-type hMOR. The time course shown in figure 3B is that of a neuron expressing mutant hMOR receptors exposed to 0.03, 0.3 and 3 \mu M DAMGO. Again, both plots show that the DAMGO-mediated Ca^{2+} current inhibition is voltage-dependent. The DAMGO concentration-response curves for the wild-type (closed circles) and mutant (open circles) hMOR are plotted in Figure 3C. The data were fit to the Hill equation. The EC_{50}, maximum inhibition and Hill coefficient (\pm SEM) obtained were 30.8 \pm 8.5 and 14.2 \pm 3.7 nM, 64.4 \pm 3.4 and 61.6 \pm 2.5\%, and 0.8 and 1.2 for wild-type (n = 4-11) and mutant (n = 4-16) hMOR-expressing cells, respectively. Thus, the data plotted in Figure 3C show that DAMGO displayed a significantly
higher potency (P = 0.002) for mutant hMOR-expressing neurons but similar efficacy for both receptor subtypes.

To confirm that the DAMGO-mediated Ca\(^{2+}\) current inhibition was mediated by MOR, the ability of the receptor antagonist D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH\(_2\) (CTAP) to block the coupling response was examined. Figure 4A illustrates the time course of Ca\(^{2+}\) current inhibition by DAMGO in an SCG neuron expressing wild-type hMOR. Bath application of DAMGO (1 \(\mu\)M) caused a 73% inhibition of the Ca\(^{2+}\) current (traces 1 and 3). Following a recovery period, the cell was pretreated with CTAP (10 \(\mu\)M) for 120 s. When both CTAP and DAMGO were coapplied, the DAMGO-mediated Ca\(^{2+}\) current inhibition was completely abolished (traces 5 and 7). In seven neurons tested, the mean DAMGO-mediated inhibition was 62 \(\pm\) 5% prior to CTAP pretreatment. Figure 4B shows similar blocking effects by CTAP of the DAMGO-induced Ca\(^{2+}\) current inhibition of an SCG neuron expressing the mutant hMOR receptor (cf. traces 1 and 3 with traces 5 and 7). Before CTAP pretreatment, the mean inhibition of Ca\(^{2+}\) currents (\(\pm\) SEM) following exposure to DAMGO was 66 \(\pm\) 4% (n = 5). The results summarized in Figure 4C suggest that both heterologously expressed MOR modulate N-type Ca\(^{2+}\) channel currents in SCG neurons.

The alkaloid morphine is one of the most widely used pain relievers, and a recent study has reported that carriers of the A118G allele required higher doses of morphine to achieve pain relief (Klepstad et al. 2004). Therefore, in the next set of experiments, the concentration-dependent Ca\(^{2+}\) current inhibition by morphine was determined in SCG neurons expressing each receptor subtype. The time course of morphine-mediated Ca\(^{2+}\) current of wild-type and mutant hMOR-expressing neurons is shown in Figure 5A and 5B, respectively. Ca\(^{2+}\) currents were evoked as described above and increasing concentrations of morphine were applied to the
external bath solution. Figure 5C illustrates the morphine concentration-response relationship for both hMOR-expressing group of cells. The Hill equation was again employed for data analysis. The EC$_{50}$, maximum inhibition (± SEM) and Hill coefficient values for wild-type hMOR-expressing neurons were 75.5 ± 26.0 nM, 50.2 ± 3.4% and 1.3, respectively (n = 3-11). On the other hand, the EC$_{50}$, maximum inhibition (± SEM) and Hill coefficient values of neurons microinjected with mutant hMOR cDNA were 39.5 ± 18.9 nM, 61.1 ± 5.2% and 0.8, respectively (n = 3-8). Overall, these results demonstrate that morphine exhibits a higher potency (P = 0.13) with mutant hMOR-expressing cells than those expressing wild-type hMOR, as well as a slightly higher efficacy (Figure 5C).

Morphine-6-glucuronide (M-6-G) is a metabolic morphine byproduct that is also capable of activating MOR. In this set of experiments, the M-6-G concentration-response relationship was examined in neurons expressing either hMOR receptor subtype. Figure 6A shows the Ca$^{2+}$ current inhibition as a function of increasing M-6-G amounts. Data analysis showed that the EC$_{50}$, maximum inhibition (± SEM) and Hill coefficients were similar in magnitude for wild-type and mutant hMOR-expressing neurons: 76.9 ± 26.0 and 82.1 ± 33.7 nM (P = 91), 58.8 ± 4.6 and 56.0 ± 5.1% and 0.8 and 0.8, respectively. Next, the endogenous MOR receptor highly selective partial agonist, endomorphin I was tested. Figure 6B shows the concentration-response relationship for those curves generated by wild type and mutant hMOR-expressing neurons. The values (± SEM) were: 86.3 ± 19.7 and 73.6 ± 21.0 nM (P = 0.64), 61.0 ± 3.3 and 59.9 ± 3.2%, and 1.4 and 0.9, respectively. Overall these results suggest that M-6-G and endomorphin I do not show significant differences in modulating N-type Ca$^{2+}$ channel currents following activation of either wild-type or mutant hMOR.
DISCUSSION

Mu (μ) opioid receptors are the main target of opiates used in the treatment of acute and chronic pain. However, the dose of opioid analgesics required to relieve pain are highly variable (Mayer and Hollt 2006; Janicki et al. 2006). Although pain perception and bioavailability contribute to the variability observed clinically, genetics is now considered an important factor that can affect patient response to analgesics. For example, the gene coding for the hMOR has been found to undergo several SNP. The most frequently occurring SNP occurs at nucleotide 118 (A→G) and leads to a change of the amino acid asparagine to aspartate (N40D) and elimination of a putative glycosylation site at the N-terminus (Bond et al. 1998; Lötsch and Geisslinger 2005). It is believed that the A118G SNP may play a significant role in the variability of the clinical effectiveness of opiates, susceptibility for drug addiction and sensitivity of pain patients to develop long-term pain symptoms (Lötsch and Geisslinger 2005; Janicki et al. 2006).

The purpose of the present study was to examine the role that the mutant (N40D) hMOR plays in N-type Ca\textsuperscript{2+} channel modulation in sympathetic neurons. Since opioid alkaloids, such as morphine, and opioid peptides mediate pain inhibition throughout the nervous system partly by activating GIRK channels and inhibiting high voltage-gated Ca\textsuperscript{2+} channels, we took advantage of our expression system (i.e. SCG neurons) that would allow us to study the coupling mechanisms within a neuronal context. The pharmacological profile of the high affinity agonist DAMGO showed that mutant hMOR-expressing cells exhibited a 2-fold higher potency in Ca\textsuperscript{2+} channel inhibition than neurons expressing the wild-type receptor, while both group of neurons displayed similar efficacies. In addition, the DAMGO-mediated Ca\textsuperscript{2+} current inhibition was blocked by the MOR blocker CTAP and by pretreatment with PTX. Our results are also consistent with those observed with coupling of mutant hMOR and another G protein effector, GIRK channels (Bond
et al. 1998). In that study, evidence was also provided to show that the binding of the endogenous opioid, β-endorphin, had a three-fold higher binding affinity for the mutant hMOR than for the wild-type hMOR.

Three in vitro studies have previously reported that DAMGO binding parameters in cell membrane preparations were not different between wild-type and mutant hMOR in AV-12 (Bond et al. 1998), COS (Befort et al. 2001) and HEK293 cells (Beyer et al. 2004; in this study mutant hMOR expression levels were lower, discussed below). Thus the N40D mutation does not appear to affect the binding of agonists to the mutant receptor, but rather alters the signal transduction events or receptor dimerization. For instance, the study by Befort et al. (2001) also reported that the DAMGO binding characteristics to another hMOR SNP, (T802C), were not different from wild-type hMOR-expressing COS cells. However, they found that [35S]GTP-γS binding (a measure of G protein signaling) was reduced in the mutant-hMOR-expressing cells. Our results are consistent with an apparent change in signaling mechanism(s) that couple N40D hMOR and Ca2+ channels. It should be noted that under our experimental conditions we are not able to determine protein levels, and thus, a decrease in surface expression (i.e. less receptor reserve) of mutant hMOR relative to wild-type receptors cannot be ruled out.

Alternatively, it may be that substitution of the putative glycosylation site at the N-terminus alters the ability of mutant MOR to form dimers. Homo- and heterodimerization is a phenomenon that has been found to occur with several GPCR, including MOR (Rios et al. 2001). For instance, β1-adrenergic receptors contain one glycosylation site on the N-terminus (N15), and it has been shown that the N15A mutant receptor exhibited a decreased ability to form homodimers when compared to wild-type receptors as well as a reduction in cell surface expression (He et al. 2002). In a subsequent study, it was demonstrated that dimer formation
between the N15A mutant $\beta_1$ adrenergic receptor and $\alpha_2$ adrenergic receptor (containing a double mutation to block glycosylation) was significantly enhanced when compared to dimerization of both wild-type receptors (Xu et al. 2003). Whether dimerization of mutant hMOR is altered or inhibited by the loss of this sugar moiety requires further investigation.

Since morphine is the most commonly employed opiate analgesic, the coupling of Ca$^{2+}$ channels to morphine-activated wild-type and mutant hMOR was also examined in this study. Neurons expressing mutant receptors exhibited a greater than two-fold increase in potency when compared to wild-type hMOR-expressing cells while the efficacy was similar in both groups. A similar observation was reported to occur with oocytes heterologously expressing GIRK channels and mutant hMOR, though $\beta$-endorphin was the agonist employed (Bond et al. 1998). The EC$_{50}$ value for $\beta$-endorphin-mediated GIRK channel activation was three times lower in mutant hMOR-expressing oocytes. Reports from clinical studies are conflicting with regard to the presence of the 118G allele and morphine’s analgesic effect (for review see Lötsch and Geisslinger 2005). In a subgroup of chronic pain patients homozygous for the wild-type allele, we have observed that the morphine requirement for pain relief was significantly higher than patients carrying the mutant allele (Janicki et al. 2006). On the other hand, in a group of healthy volunteers, it has been shown that the amount of morphine required to achieve pupil-constricting effects was not different between carriers of either allele (Lötsch et al. 2002a). In another report, however, a 2.1 and 3.6 rightward shift of morphine potency was observed in heterozygous and homozygous carriers of the mutant allele, respectively (Skarke et al. 2003). A study of cancer pain patients reported that a higher dose of morphine was necessary for pain relief of those homozygous for the 118G allele (Klepstad et al. 2004). The mechanism for these differences remains unclear.
The modulation of Ca^{2+} currents by the active morphine metabolite M-6-G or the opioid peptide endomorphin I was not significantly different in neurons expressing either hMOR subtype. These results are consistent with the observations reported to occur in HEK293 (Beyer et al. 2004) and AV-12 cells (Bond et al. 1998). Nevertheless, a study employing volunteers found that carriers of the mutant allele showed a decreased potency with regard to M-6-G-induced pupil constriction when compared to homozygous wild-type carriers (Lötsch et al. 2002a). Yet, another study found that 118G-carrying healthy volunteers reported less nausea and vomited less frequently when M-6-G was used as the opioid agonist (Skarke et al. 2003). And in a study of 2 patients with renal failure, it was observed that the patient carrying the G118 allele was able to better tolerate increased plasma levels of M-6-G than the homozygous wild-type patient (Lötsch et al. 2002b). These studies suggest that some of the side-effects associated with M-6-G may offer some protection to 118G carriers. The mechanism is presently unknown.

A recent *in vitro* study reported that Chinese hamster ovary (CHO) cells transfected with the mutant hMOR had significantly lower MOR mRNA levels and protein expression than wild-type-transfected cells (Zhang et al. 2005). The authors suggested that their findings were indicative of a loss of function by the N40D hMOR. Lower mutant hMOR expression levels were also observed to occur in HEK293 cells (Beyer et al. 2004). The results of the present study and those previously reported (Bond et al. 1998; Befort et al. 2001) are not consistent with a loss of MOR function. The discrepancies in the studies indicate that characterization of the mutant hMOR is dependent on the cell system employed. In fact, Befort and coworkers (2001) found that the expression levels of wild-type and mutant hMOR in COS cell membranes were not different, nor was there a significant change in the DAMGO-induced down-regulation of both receptor subtypes. These differences, however, highlight the advantage of employing SCG
neurons as an expression system to study the functional coupling of the N40D hMOR to natively expressed ion channels and G protein subunits. The temporal resolution (i.e. seconds) in our study allows for the measurement of the Gβγ-mediated, membrane-delimited and voltage-dependent modulation of Ca\(^{2+}\) channels. On the other hand, biochemical assays are normally determined over longer periods. Finally, the dissimilarities may be due to the fact that some cell lines do not express proteins that are involved in mRNA and/or protein processing, folding or trafficking that normally occur in neurons (for review see Bailey and Connor 2005). For instance, in rat and bovine tissue it has been reported that tissue expression of the atrial natriuretic peptide receptor, guanylyl cyclase-A, is influenced by the degree of N-glycosylation and is not uniform across tissues, such as brain, kidney and lung (Müller et al. 2002).

In summary, our results indicate that wild-type and mutant hMOR can be successfully expressed in rat SCG neurons, an expression system that can be employed to further examine the signal transduction elements that couple N40D hMOR and N-type Ca\(^{2+}\) channels. The potencies of the DAMGO- and morphine-mediated Ca\(^{2+}\) channel inhibition were shifted leftward in mutant hMOR-expressing neurons when compared to cells expressing the wild-type receptor. Coupling of both receptor subtypes to Ca\(^{2+}\) channels was PTX-sensitive and blocked by pretreatment of CTAP. Finally, no significant differences were observed in Ca\(^{2+}\) channel modulation by M-6-G and endomorphin I in neurons expressing either hMOR subtype.
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FIGURE LEGENDS

**Figure 1:** Concentration-response for wild-type (WT) hMOR cDNA microinjected and DAMGO- or NE-mediated Ca\(^{2+}\) current inhibition in rat SCG neurons. A, B and C, time courses of Ca\(^{2+}\) current amplitude inhibition acquired from the sequential application of DAMGO (10 µM) and NE (10 µM) in uninjected neurons and neurons injected with 20 and 200 ng/µl wild-type hMOR cDNA, respectively. Superimposed Ca\(^{2+}\) current traces (shown to the right) evoked with the ‘double-pulse’ voltage protocol (shown in Figure 1A, top) in the absence (lower trace) or presence (upper trace) of DAMGO and NE for uninjected (A), WT hMOR-injected (20 ng/µl cDNA, B) and WT hMOR-injected (200 ng/µl cDNA, C) neurons. Currents were evoked every 5 or 10 s. D, summary graph of mean (± SEM) Ca\(^{2+}\) current inhibition produced by application of NE and DAMGO in uninjected cells and neurons microinjected with 5, 20 and 200 ng/µl WT or MUT hMOR cDNA. Inhibition was determined from the Ca\(^{2+}\) current amplitude measured isochronally at 10 ms into the prepulse (+10 mV) in the absence or presence of DAMGO or NE. Numbers in parenthesis indicate the number of experiments. * P < 0.01 compared to neurons injected with 5 or 20 ng/µl cDNA.

**Figure 2:** DAMGO-mediated Ca\(^{2+}\) current inhibition in hMOR-expressing neurons is pertussis toxin (PTX)-sensitive. Superimposed Ca\(^{2+}\) current traces in the absence and presence of 10 µM DAMGO in control (Ai and Bi) and PTX-treated (500 ng/ml) neurons (Aii and Bii). Currents were evoked every 10 s with the ‘double-pulse’ voltage protocol (shown in Figure 1A, top). C, summary of mean (± SEM) Ca\(^{2+}\) current inhibition produced by 10 µM DAMGO. Inhibition was determined from the Ca\(^{2+}\) current amplitude measured isochronally at 10 ms into the prepulse (+10 mV) in the absence or presence of DAMGO. * P < 0.01 vs. non-PTX treated neurons, Student’s t-test. Numbers in parentheses indicate the number of experiments.
**Figure 3:** DAMGO concentration-response relationship in SCG neurons expressing wild-type (WT) and mutant (MUT) hMOR. A and B are time courses of Ca\(^{2+}\) current amplitude inhibition for pre- (●) and postpulse (○) acquired from the sequential application of DAMGO (0.003-3 µM). Currents were evoked every 5 s with the ‘double-pulse’ voltage protocol (shown in Figure 1A, top). Inhibition was determined from the Ca\(^{2+}\) current amplitude measured isochronally at 10 ms into the prepulse (+10 mV) in the absence or presence of DAMGO. Filled bars indicate the application of DAMGO. C. Concentration-response curves in neurons expressing WT (closed circles, n = 4-11) and MUT (open circles, n = 4-16) hMOR. Each point represents the mean (± SEM) Ca\(^{2+}\) current inhibition. The smooth curves were obtained by fitting the data to the Hill equation.

**Figure 4:** The MOR antagonist, CTAP, blocks the DAMGO-mediated Ca\(^{2+}\) current inhibition in SCG neurons expressing wild-type (WT) and mutant (MUT) hMOR. A and B are time courses of Ca\(^{2+}\) current amplitude inhibition for pre- (●) and postpulse (○) acquired from the sequential application of DAMGO (1 µM), CTAP (10 µM), and DAMGO (1 µM) + DAMGO (10 µM). Currents were evoked every 5 s with the ‘double-pulse’ voltage protocol (shown in Figure 1A, top). Inhibition was determined from the Ca\(^{2+}\) current amplitude measured isochronally at 10 ms into the prepulse (+10 mV) in the absence or presence of DAMGO, and absence or presence of DAMGO + CTAP. The numbered Ca\(^{2+}\) current traces in each time course are shown to the right. C, summary graph of mean (± SEM) Ca\(^{2+}\) current inhibition produced by application of DAMGO and DAMGO + CTAP. Numbers in parenthesis indicate the number of experiments.

**Figure 5:** Morphine concentration-response relationship and Ca\(^{2+}\) current inhibition in wild-type (WT) and mutant (MUT) hMOR-expressing neurons. A and B are time course of Ca\(^{2+}\) current as a function of increasing concentration/application of morphine (0.03, 0.3 and 3 µM). Filled
circles are pre- and open circles are postpulse currents. Currents were evoked every 10 s employing the voltage protocol described in Figure 1A, top. C, summary dose-response for morphine in wild-type (●, solid line) and mutant (○, dashed line) hMOR-expressing neurons. Each point on the curve represents the mean ± SEM from 3-8 neurons. The smooth curves were obtained by fitting the data to the Hill equation.

**Figure 6:** Concentration-response for morphine-6-glucuronide- (M-6-G) and endomorphin I-mediated Ca²⁺ current inhibition in WT and MUT hMOR-expressssing neurons. Plots of concetration response curves for M-6-G (A) and endomorphin I (B) in wild-type (●, solid line) and mutant (○, dashed line) hMOR-expressing neurons. The smooth curves were obtained by fitting the data to the Hill equation. Each point on the curve represents the mean ± SEM from 3–11 neurons.
Figure 2

A WT hMOR

i. Control

ii. PTX-treated

B MUT hMOR

i. Control

ii. PTX-treated

C Summary

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PTX-treated</th>
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<tbody>
<tr>
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<td>(4)</td>
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<tr>
<td>MUT hMOR</td>
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</tbody>
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* Significant difference
Figure 3

A WT hMOR

B MUT hMOR

C Summary
A WT hMOR

B MUT hMOR

C Summary
Figure 5

A WT hMOR

B MUT hMOR

C Summary
Figure 6

A Morphine-6-glucuronide

B Endomorphin I