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A118G Mu Opioid Receptor polymorphism increases inhibitory effects on $Ca_V2.2$ channels

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HIGHLIGHTS

- We tested the impact of N40D hMOR polymorphism on $Ca_V2.2$ inhibition.
- N40D reduce the agonist concentration needed to inhibit $Ca_V2.2e37a$ by hMOR.
- N40D effect on the agonist requirements is independent on exon 37a/b $Ca_V2.2$ site.
- N40D does not modify the voltage dependency of the inhibitory effect on $Ca_V2.2e37a$.

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ABSTRACT

Single nucleotide polymorphisms (SNPs) in the human *OPRM1* gene result in common variants of Mu Opioid Receptors (hMORs). The A118G SNP occurs at high frequency in certain human populations and produces an aminoacidic substitution: N40D (hMOR-N to hMOR-D) at protein level. N40D is reported to alter pain thresholds and morphine efficacy. hMORs inhibit $Ca_V2.2$ channels (N-type currents) at presynaptic nociceptor terminals in dorsal horn, thus reducing calcium influx, transmitter release, and transmission of noxious signals. Nociceptors express different splice isoforms of $Ca_V2.2$. Isoforms distinguished by the presence of alternatively spliced exon e37a are of interest because channels containing e37a are particularly enriched in nociceptors. Recent studies showed that $Ca_V2.2e37a$ is more sensitive to inhibition by Mu Opioid Receptors than the ubiquitous splice variant $Ca_V2.2e37b$. Here, we evaluate the effect of hMOR-N and hMOR-D on cloned $Ca_V2.2e37a$ channels expressed in mammalian cells. We observe that hMOR-D inhibits $Ca_V2.2e37a$ currents at agonist concentrations 4-fold lower than those needed to inhibit $Ca_V2.2e37a$ currents by the same degree via hMOR-N. We observe little difference in hMOR-D and hMOR-N inhibition of $Ca_V2.2e37b$ currents. Our study demonstrates that this common site of *OPRM1* polymorphism affects the inhibitory actions of MORs on both major $Ca_V2.2$ isoforms expressed in nociceptors.

1. Introduction

Human Mu Opioid Receptors (hMORs) are encoded by the *OPRM1* gene. MORs are G protein-coupled receptors with high affinity for endogenous opioids [28]. MORs inhibit $Ca_V2.2$ (N-type) voltage-gated calcium channels (VGCCs) at presynaptic terminals of nociceptors, which represents one of their therapeutically important sites of action as analgesics. By inhibiting $Ca_V2.2$ channel activity, MORs inhibit calcium influx into presynaptic terminals, inhibit transmitter release, and down-regulate transmission in the pain pathway [10,22]. hMORs are receptors for morphine, a common analgesic used to treat severe pain. Common single nucleotide polymorphisms (SNPs) in the human *OPRM1* gene underlie the expression of hMORs variants in humans. A common SNP located at nucleotide position 118 in exon 1 of the *OPRM1* gene results in an asparagine (hMOR-N) to an aspartate substitution (hMOR-D) in the extracellular domain of the hMOR protein (N40D). The frequency of this allele varies among different human populations ranging from 4 to 48% [20]. Multiple labs have investigated the relevance of the hMOR-D and hMOR-N variants to pain pathways, and have reported conflicting results [6,14]. Fillingim et al. found that the occurrence of the rare hMOR-D variant correlates with a higher pressure-pain threshold in healthy individuals, and they failed to observe this correlation with other painful stimuli [15]. Janicki et al. found that chronic pain patients are less likely to express hMOR-D than non-chronic pain patients. Moreover, these authors found

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that within the subgroup of chronic pain patients with the highest opioid requirements, individuals carrying the hMOR-D allele need lower opioid doses to relieve pain suggesting that hMOR-D has a protective effect against chronic pain [18]. On the other hand, Campa et al. found that patients suffering from cancer pain were less responsive to morphine when they carried at least one copy of hMOR-D allele, as compared to patients carrying hMOR-N allele in homozygous combination [7]. These conflicting results reveal a complex scenario and the molecular mechanisms underlying these behavioral phenotypes are unknown.

Recent studies suggest that hMOR-N and hMOR-D may differentially inhibit native $Ca_V2.2$ calcium currents, although there are conflicting data. One study assessed the ability of cloned hMOR-N and hMOR-D to inhibit endogenous VGCCs in sympathetic neurons. Higher concentrations of DAMGO and morphine were needed to inhibit VGCCs in neurons expressing hMOR-N compared to hMOR-D [25]. In contrast to these findings, higher concentrations of morphine were needed to inhibit VGCCs in trigeminal ganglia neurons from mice engineered to only express the hMOR-D variant. Additionally, the maximum inhibition of trigeminal calcium currents by morphine was lower in hMOR-D mice [24]. We hypothesized that some of these differences could be explained by the fact that different neurons express different isoforms of $Ca_V2.2$ channels and some sites of alternative splicing influence the channel's responsiveness to MORs [1,29]. One site of alternative splicing of particular interest in *Cacna1b*, the gene that encodes $Ca_V2.2$, involves a mutually exclusive pair of exons, e37a and e37b. E37a has restricted expression and is enriched in nociceptors while e37b is expressed throughout the nervous system [3]. MORs inhibit both e37a and e37b containing $Ca_V2.2$ channels but only $Ca_V2.2e37a$ channels are inhibited by a persistent voltage-independent pathway [29]. Additionally, morphine has reduced analgesic actions in mice that lack e37a-$Ca_V2.2$ channels [1].

Here, we used cloned channels and receptors in a mammalian expression system to directly compare the ability of hMOR-D and hMOR-N to inhibit $Ca_V2.2e37a$ and $Ca_V2.2e37b$ channels. Lower concentrations of DAMGO were required to achieve the same degree of inhibition of both $Ca_V2.2$ isoforms in cells expressing hMOR-D compared to hMOR-N. Our data suggest that patients expressing hMOR-D variants have higher sensitivity to the analgesic actions of MOR activation compared to those expressing hMOR-N.

2. Methods

Clones and transient transfection: HEK293 cells were grown in DMEM (EMEVE) and 10% Fetal Bovine Serum (Internegocios) and split when 70% confluent. Voltage-gated calcium channel subunits $Ca_V2.2e37a$ or $Ca_V2.2e37b$, $Ca_V\beta_3$, $Ca_V\alpha_2\delta$, as well as e-GFP and hMOR-N or hMOR-D containing plasmids were transfected into HEK293 cells using Lipofectamine 2000 (Invitrogen). After transfection, cells were kept in culture for 24 h. Calcium channel subunits and e-GFP clones were kindly provided by Dr. D. Lipscombe (Brown University), and hMOR clones by Dr. V. Ruiz Velasco (Penn State University).

Electrophysiology: We performed whole-cell patch-clamp recording. Extracellular solution (mM): 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 140 choline chloride, pH 7.4 with CsOH. Internal solution (mM): 126 CsCl, 10 EGTA, 1 EDTA, 10 HEPES, 4 NaATP, 4 $MgCl_2$, pH 7.2 with CsOH. Series resistances were less than 5 MΩ.

Voltage protocols: Cells were held at −100 mV to remove closed-state inactivation. Test potentials (to 10 mV for 15 ms) were applied every 10 s. We applied prepulses to +80 mV for 15 ms, 12 ms before the test potential. This prepulse protocol is maximally effective in removing all voltage-dependent inhibition of $Ca_V2.2e37b$ (94.66 ± 3.45% of total, $n = 7$ cells, value not different from 100%, $p = 0.1737$) and has no effect on control current values (data not shown). We used the MOR specific agonist, DAMGO ([D-Ala2, N-MePhe4, Gly-ol]-enkephalin) (Sigma), at concentrations ranging from 0.001 to 5000 nM.

Statistics: Data are expressed as mean ± standard error and statistical significance was examined by Student's *t*-test and *p* values <0.05 were considered significantly different (OriginPro 8). The DAMGO concentration–response curves were fitted with the Hill equation and EC50 were compared with Fisher's *F*-test ($p < 0.05$) (GraphPad Prism 5).

3. Results

We generated concentration-inhibition curves for DAMGO inhibition of $Ca_V2.2$ currents in cells expressing hMOR-N or hMOR-D together with $Ca_V2.2e37a$ (Fig. 1). We measured calcium currents using 2 mM Ca^{2+} as the charge carrier using whole-cell

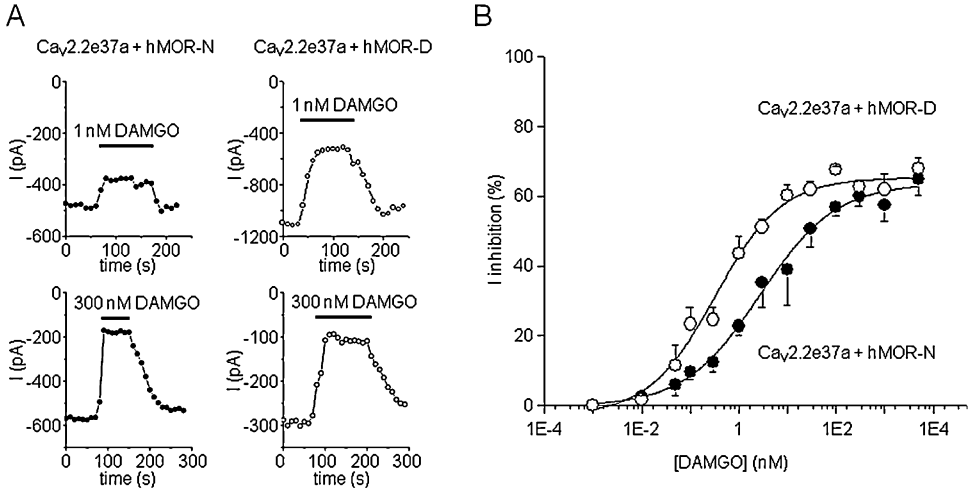

Fig. 1. DAMGO concentration–response curves of hMOR-N and hMOR-D mediated inhibition of $Ca_V2.2e37a$ currents. (A) The figure displays the time courses of peak $Ca_V2.2e37a$ current (evoked at +10 mV from a holding of −100 mV) inhibition by application of 1 (upper panels) or 300 nM (lower panels) of DAMGO in HEK293 cells expressing $Ca_V2.2e37a$ and MOR-N (left panels) or $Ca_V2.2e37a$ and MOR-D (right panels). (B) DAMGO concentration–response curves of $Ca_V2.2e37a$ current inhibition in HEK293 cells co-expressing $Ca_V2.2e37a$ and hMOR-N (closed circles; $n = 77$ cells), or hMOR-D (open circles; $n = 73$ cells). Lines represent fitted Hill equations for $Ca_V2.2e37a$ and hMOR-N ($r^2 = 0.87$) and for $Ca_V2.2e37a$ and hMOR-D ($r^2 = 0.85$).

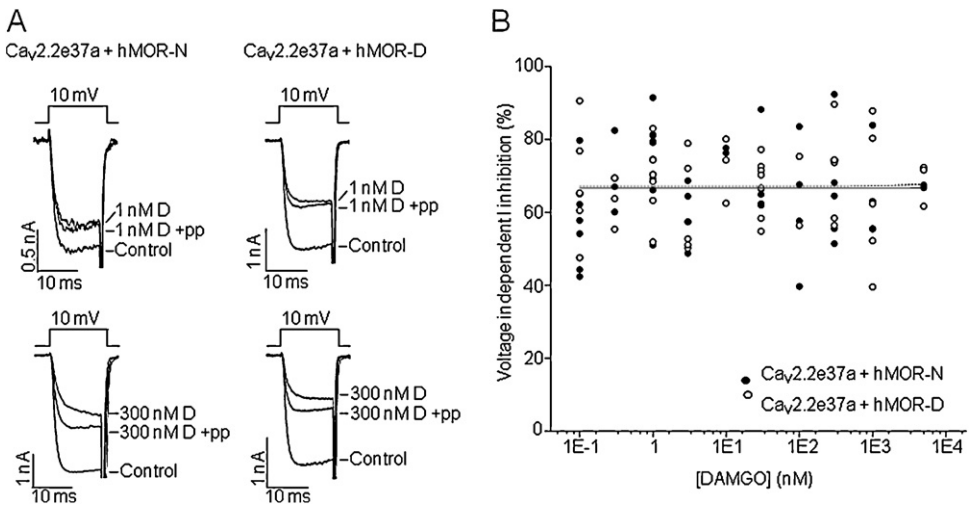

Fig. 2. Voltage dependency of hMOR-N and hMOR-D mediated inhibition of $Ca_V2.2e37a$ currents at different DAMGO concentrations. (A) Representative $Ca_V2.2e37a$ current traces evoked at +10 mV from holding potential of −100 mV for 15 ms in control condition, in presence of DAMGO 1 (upper panels) or 300 nM (lower panels), with (D +pp) or without (D) the application of a +80 mV pre-pulse in HEK293 cells expressing $Ca_V2.2e37a$ and hMOR-N (left panels) or $Ca_V2.2e37a$ and hMOR-D (right panels). (B) Scatter plots of % of $Ca_V2.2e37a$ voltage independent inhibition versus DAMGO concentration acting on hMOR-N (closed circles; $n = 45$), or hMOR-D (open circles; $n = 49$). Both lineal regression lines (solid for $Ca_V2.2e37a$ and hMOR-N and dotted for $Ca_V2.2e37a$ and hMOR-D data set) have slopes not significantly different from 0 (Fisher's tests), and equals *Y*-intercepts (Fisher's test; $Ca_V2.2e37a$ and hMOR-N = 66.61 ± 2.06%; $Ca_V2.2e37a$ and hMOR-D = 67.19 ± 1.76%; $p = 0.8661$).

patch recording. Peak $Ca_V2.2$ current amplitudes were indistinguishable between cells expressing hMOR-N and hMOR-D (−49.57 ± 7.46 versus −54.59 ± 8.51 pA/pF, respectively). DAMGO at concentrations above 10^{-11} M inhibited $Ca_V2.2$ currents rapidly and reversibly in cells expressing hMORs (Fig. 1A). DAMGO had no effect on $Ca_V2.2$ currents in cells that were not expressing hMORs (data not shown). At saturating concentrations of DAMGO, its effects on $Ca_V2.2$ currents were not distinguishable in cells expressing hMOR variants. However, at lower subthreshold concentrations, inhibition by DAMGO was greater in cells expressing hMOR-D compared to hMOR-N (Fig. 1). We fit DAMGO inhibition curves with the Hill equation and estimated EC_{50}s for DAMGO's effects in cells expressing hMOR-N and hMOR-D at 2.72 ± 1.04 and 0.32 ± 0.12 nM (Fisher's test; $p < 0.001$). Maximum inhibition (63.7 ± 4.0% for hMOR-N and 65.3 ± 2.3% for hMOR-D) and Hill coefficients (0.56 ± 0.12 for hMOR-N and 0.63 ± 0.12 for hMOR-D) were not significantly different.

G protein-coupled receptors including hMORs use two functionally and molecularly distinct pathways to inhibit $Ca_V2.2$ channels. We used strong depolarizing prepulses of 15 ms to +80 mV to remove all voltage-dependent inhibition in the presence of DAMGO, isolating the voltage-independent inhibition [17]. We measured the amount of voltage-dependent and voltage-independent inhibition of $Ca_V2.2$ currents mediated by different concentrations of DAMGO in cells expressing hMOR-D or hMOR-N. We found that approximately 65% of DAMGO's inhibitory actions on $Ca_V2.2$ currents use a voltage-independent inhibitory pathway which is resistant to pre-pulse depolarization. The fraction of voltage-independent inhibition by DAMGO was the same at all concentrations tested and regardless of hMOR subtypes (Fig. 2A and B).

Finally, we compared the inhibitory actions of DAMGO on $Ca_V2.2e37a$ and $Ca_V2.2e37b$ currents because we know from previous studies that e37 influences G protein inhibition of $Ca_V2.2$. We found no splice variant-specific difference in the patterns of inhibition: DAMGO was more effective at inhibiting both $Ca_V2.2e37a$ and $Ca_V2.2e37b$ channels in cells expressing hMOR-D compared to those expressing hMOR-N at non-saturating concentrations (Fig. 3).

4. Discussion

We show here that the common human MOR polymorphism N40D influences the ability of hMORs to inhibit $Ca_V2.2$ channels. We also show that the increased sensitivity of the hMOR-D variant to DAMGO is observed in both $Ca_V2.2$ splice isoforms expressed in nociceptors (e37a and e37b). The e37a/e37b site of alternative splicing is conserved among mammalian *Cacna1b* genes including human [16,21] and e37a is expressed in nociceptors of rat and mouse. It is therefore highly likely that both isoforms are expressed in nociceptors of humans. Nociceptors expressing $Ca_V2.2e37a$ channels have larger $Ca_V2.2$ currents compared to those that only express $Ca_V2.2e37b$ channels and they are more sensitive to inhibition by G protein-coupled receptors including MORs [3,8]. Recent studies showed that the spinal level analgesic actions of morphine are reduced in mice lacking e37a [1]. Our data suggest that individuals carrying the G118 (hMOR-D) allele will have enhanced sensitivity to MOR activation which could add to the functional impact of $Ca_V2.2e37a$ channels [1,33].

We speculate that the increased ability of DAMGO to inhibit $Ca_V2.2$ channels through hMOR-D compared to hMOR-N is related to a change in agonist binding and/or receptor activation. It is difficult to pinpoint the precise mechanism explaining the differential effect of DAMGO on hMOR-D and hMOR-N because agonist binding influences receptor activation and receptor activation influences agonist binding [9]. Previous studies have failed to demonstrate a higher DAMGO binding affinity to hMOR-D than hMOR-N [2]. We doubt the possibility of a differential coupling between G protein and $Ca_V2.2$ channel because the voltage dependence of the inhibition does not change and the maximum effect is the same using both hMOR variants. Another plausible explanation for the higher potency of hMOR-D could be a differential expression level that could lead to a spare receptor effect and a left shift in the concentration inhibition curve. This is very unlikely because several studies, in different systems, have demonstrated that hMOR-D expression level is lower than hMOR-N [23,36]. Changes in expression level could be masking a higher difference in DAMGO potency on hMOR-D and hMOR-N inhibition of $Ca_V2.2$. Additional possibilities include a higher DAMGO effect in a subpopulation of receptors with different activation properties, such as homo-dimers [34].

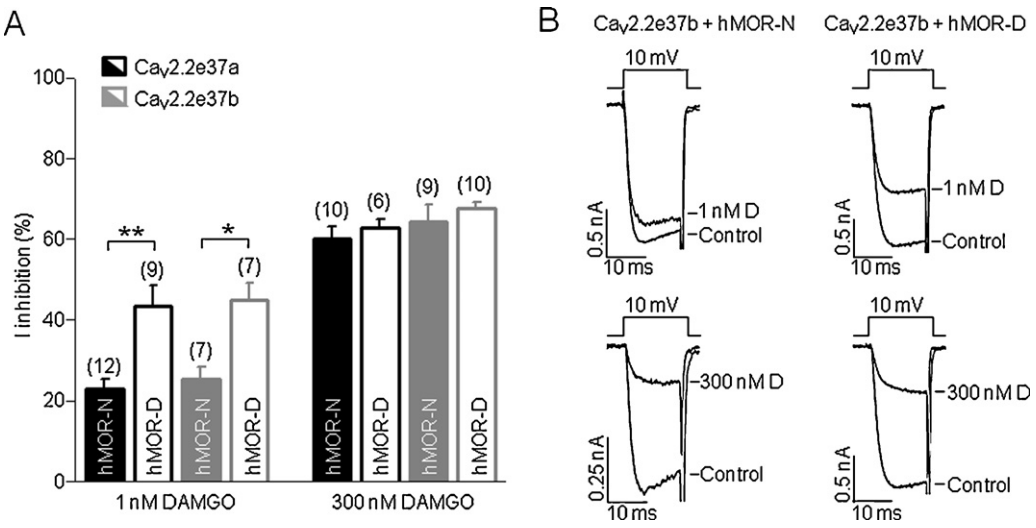

Fig. 3. Comparison of hMOR-N and hMOR-D mediated inhibition of $Ca_V2.2e37a$ and $Ca_V2.2e37b$ currents. (A) Calcium current inhibition at 1 and 300 nM DAMGO in HEK293 cells expressing $Ca_V2.2e37a$ and hMOR-N, $Ca_V2.2e37a$ and hMOR-D, $Ca_V2.2e37b$ and hMOR-N, or $Ca_V2.2e37b$ and hMOR-D. Asterisk symbols mean statistical difference between groups (*t*-test; $^{**}p = 0.0007$, $^{*}p = 0.0032$). (B) Representative calcium current traces (evoked at +10 mV from a holding potential of −100 mV during 15 ms) in control conditions and 1 (upper panels) or 300 nM DAMGO application (D, lower panels) in HEK293 cells expressing $Ca_V2.2e37b$ and hMOR-N, (left panels) and $Ca_V2.2e37b$ and MOR-D (right panels).

There is now ample evidence that the impact of N40D on MOR mediated calcium channels inhibition is both agonist- and system-dependent. In sympathetic neurons injected with hMOR-N or hMOR-D mRNA, hMOR-D is more potent at inhibiting native calcium currents when the agonist is DAMGO or morphine but no differences are observed with morphine-6-glucuronide and endomorphin I [25]. Our findings with DAMGO are most consistent with those studies [25]. Although qualitatively similar, we used DAMGO concentrations that were 100-fold lower to inhibit $Ca_V2.2$ currents compared to those used in the Margas et al. study. Different cellular environments or different auxiliary subunit compositions of channels could underlie the differences in DAMGO doses requirements. On the other hand, in humanized mice expressing hMOR-D or hMOR-N, Mahmoud et al. showed that hMOR-D is less effective than hMOR-N when assaying total calcium currents inhibition by morphine in trigeminal neurons, with no potency differences between the polymorphisms when applying DAMGO or Fentanyl [24,30]. These mixed data could be due to functional selectivity of agonists. This means that different agonists could activate different associated signaling pathways. This phenomenon has been described for several GPCRs, including MOR [35,37]. The discrepancy between the actions of agonists in different cellular models may also be due to the fact that the total calcium current assayed in different neuron types have different proportions of N-type calcium channels and different $Ca_V2.2$ isoforms. It would be interesting to address if agonist type influences the left shift in potency mediated by N40D in our system.

A complex situation is expected at the therapeutic level for MOR agonists since the N40D substitution also could affect hMOR biology at several levels including glycosylation, membrane trafficking [26,27], agonist affinity [4], expression levels [36] or efficacy of signaling to G proteins [13]. Several clinical studies have reported that morphine is less effective at producing analgesia in patients with one or two copies of the G118 (hMOR-D) allele of the *OPRM1* gene compared to those homozygous for A118 (hMOR-N) [5–7,14]. These studies included patients with cancer [7,19] and those suffering post-surgery pain [11,12,31,32]. However, at least one report, in agreement with our study, concluded that in chronic pain, lower doses of morphine were effective in pain management in patients carrying the G118 allele (hMOR-D) [18]. These diverse results from association studies reveal pain as a complex trait. Thus the greater efficacy of hMOR-D to inhibit $Ca_V2.2e37a$ channels that we know are expressed in nociceptors could contribute to the differential agonist requirement in some patients with chronic pain.

5. Conclusion

Our studies demonstrate that N40D has a gain of function effect on hMOR-mediated inhibition of presynaptic calcium channels, a relevant mechanism that controls pain pathway activity.

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