

Impact of A118G Polymorphism on the Mu Opioid Receptor Function in Pain

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Abstract

Mu Opioid Receptor (MOR) activation by exogenous or endogenous agonists causes reduction of pain threshold after a noxious stimulus, relieving pain sensation. MOR is encoded by *OPRM1* gene and its messenger RNA suffers extensible modifications by alternative splicing and single nucleotide polymorphisms (SNPs). A118G (N40D) is the most frequent encoding MOR SNP in humans. In this review we discuss the impact of this polymorphism at molecular, cellular and clinical levels. Since some SNPs are unequally distributed among human populations, we also discuss the utility of A118G as an ethnicity marker among worldwide human populations. As an example, we evaluate A118G frequency in an Argentinean human population and compare it with worldwide frequencies extracted from HapMap database.

Keywords: Mu opioid receptors; A118G polymorphism; N40D polymorphism; Voltage gated calcium channels; Population studies

Introduction

Morphine was isolated from opium more than 200 years ago, and it currently remains as the most used analgesic and reference point to compare new analgesic drugs [1]. Morphine belongs to a large family of endogenous and exogenous compounds known as opioids that bind to 4 different seven-transmembrane G-protein coupled receptors: delta, kappa, orphanine and mu opioid receptor. Morphine's highest affinity corresponds to the Mu Opioid Receptor (MOR) encoded by *OPRM1* gene which also is the target of endogenous opioids: enkephalins and β -endorphins. MOR is expressed mainly at the nervous system and its activation causes reduction of pain threshold after a noxious stimulus without anesthesia and pain relieving sensation.

There are several isoforms of MOR at the protein level generated by *OPRM1* messenger RNA (mRNA) alternative splicing and/or by single nucleotide polymorphisms (SNPs). In humans, the most frequent encoding MOR SNP is the replacement of adenine at position 118 for a guanine (A118G) [2]. A118G causes the substitution of an asparagine by an aspartate at position 40 (MOR-N to MOR-D; N40D), which is placed at exon 1 in the extracellular region of the receptor before its first transmembrane loop [3].

Although there are many reports investigating N40D effect on MOR activity in pain, the impact of this SNP on the receptor function and its patho-physiological meaning remain elusive. At molecular level, we and others have reported differences in the agonist affinity and potency, intracellular cascades and effectors involved in MOR effects [4-11]. On the other hand, clinical studies have demonstrated changes in pain sensation and morphine requirements under different pain conditions [12-16]. Moreover, population studies showed a clear correspondence between the A118G frequency and pain parameters reported for human populations. Here we summarize the most relevant studies and discuss the cases where the results are controversial. In the population studies section, we also discuss the possibility that 118G allele may be useful to identify differences in the genetic lineage among human populations given its unequal worldwide distribution. In this regard, we present an illustrative analysis of novel data collected from Argentinean human population.

A118G modifies MOR activity

The amino acid substitution N40D is located at the extracellular N-terminal domain before the first transmembrane loop [3]. There

are several reports describing how A118G SNP impacts on MOR function including changes in expression level, agonist's affinity and intracellular pathways involved.

Due to the extracellular location of N40D substitution, differences in agonist binding and efficacy are predicted. Indeed, MOR ligand β -endorphin has a 3 times higher affinity for MOR-D than for MOR-N, while there are no changes for other opioids (enkephalins, dynorphins and DAMGO) between the two MOR variants [4]. In more recent reports, Befort et al. [5] and Beyer et al. [6] found a smaller difference: less than 2-fold increase in affinity. The discrepancy among these studies could be due to differences in the cell types used. Bond and colleagues found the larger difference measuring the β -endorphin binding by displacement of [³H] DAMGO in AV-12 cells stably transfected with human MOR-N and MOR-D. On the other hand, Beyer et al. determined the β -endorphin binding with the same method but in HEK293 cells stably transfected with each receptor variant, while Befort and colleagues measured the β -endorphin binding by displacement of [³H] diprenorphine in COS cells transiently transfected with each receptor variant.

Another predictable difference among the isoforms is a change in protein level and localization since the correct folding and trafficking to the membrane of G protein coupled receptors (GPCRs) depend on its extracellular domains. Zhang et al. [12] found a 200 % decrease of MOR-D mRNA levels as compared to MOR-N mRNA levels in postmortem brains samples of heterozygous humans. In the same study, *in vitro* experiments showed a similar difference in mRNA levels (Table 1) for CHO cells transiently transfected with human MOR-N and MOR-D (1.5 times lower for MOR-D40). Interestingly, a more prominent difference for membrane protein levels was observed: 10-fold lower for MOR-D than for MOR-N [17] (Table 1). These data

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MOR properties	MOR-N vs. MOR-D	Experimental conditions	Ref.
β -endorphin affinity	MOR-N < MOR-D	[³ H]DAMGO displacement in stably transfected AV-12 cells	[4]
morphine affinity	MOR-N = MOR-D	[³ H]DAMGO displacement in stably transfected AV-12 cells [³ H]diprenorphine displacement in transiently transfected COS cells [³ H]DAMGO displacement in stably transfected HEK293 cells	[4] [5] [6]
DAMGO affinity	MOR-N = MOR-D	[³ H]DAMGO displacement in stably transfected AV-12 cells [³ H]diprenorphine displacement in transiently transfected COS cells [³ H]DAMGO displacement in stably transfected HEK293 cells	[4] [5] [6]
GIRK activation	MOR-N < MOR-D	Potassium current measured by voltage-clamp in <i>Xenopus</i> oocytes injected with mRNA coding for each receptor variants and GIRK.	[4]
CaV2.2 inhibition	MOR-N < MOR-D	Whole-cell calcium currents in transiently transfected HEK293 cells Whole-cell calcium currents in SCG neurons microinjected with each receptor variants	[8] [7]
PKA activity	MOR-N = MOR-D	By application of morphine in stably transfected Neuro2A cells	[10]
ERK1/2 phosphorylation	MOR-N = MOR-D	Western blot after application of morphine in stably transfected Neuro2A cells	[10]
Gi/o activity	MOR-N < MOR-D	[³⁵ S]GTP γ S binding evoked by increasing concentrations of DAMGO in transiently transfected COS cells	[5]
MOR mRNA level	MOR-N > MOR-D	Real-time PCR in postmortem brains samples of heterozygous humans and in transiently transfected CHO cells	[17]
MOR protein level	MOR-N >> MOR-D	Western blot in transiently transfected CHO cells	[17]

Table 1: Summary of the N40D impact on several MOR functional properties.

suggest that the decrease of MOR protein level caused by the N40D polymorphism involves posttranslational mechanisms such changes in protein folding and/or trafficking. In this regard, the asparagine at the 40th position has been proposed as a potential N-glycosylation site [18]. GPCR N-glycosylation is necessary for correct activity of chaperone proteins that control the folding and trafficking of GPCRs. A recent report showed differences in N-glycosylation levels between the human MOR-D and the human MOR-N variants [19] and a reduction in the half-life of the receptor protein by pull chase studies [19]. Thus, changes in N-glycosylation could explain the difference observed in expression levels, including the discrepancy between mRNA and protein level changes observed. On the other hand, other authors failed to see differences in the protein levels in transiently transfected COS cells [5].

Several reports also show that the N40D polymorphism modifies the intracellular pathways involved downstream MOR activation. MOR couples to Gi/o proteins and its acute activation reduces protein kinase A (PKA) activity and increases the Extracellular Receptor Kinases 1 and 2 (ERK1/2) phosphorylation [20-22]. Befort et al. [5] have quantified the [³⁵S] GTP γ S binding evoked by increasing concentrations of DAMGO in COS cells transiently transfected with human MOR-N and MOR-D. The apparent EC50 was similar for both receptor variants but the maximal response (efficacy) was higher for MOR-D than for MOR-N indicating that the amino acid replacement lead to an increase in G protein activation by the receptor (Table 1) [5]. In another study, Deb et al. [10] found that application of morphine in Neuro2A cells stably transfected with MOR-N or MOR-D caused a similar decrease on PKA activity and similar levels of pERK1/2 (Table 1). Chronic exposure to MOR agonists causes receptor adaptation including cAMP and PKA up regulation [9,23]. In response to chronic treatment with morphine, Neuro2A cells expressing MOR-N showed high PKA activity and decreased ERK1/2 phosphorylation levels. In contrast, Neuro2A cells expressing MOR-D have no changes in those parameters, as compared to basal conditions, when exposed to chronic treatment with morphine indicating absence of compensatory effects when MOR-D is expressed [10].

The inhibition of pain sensation by MOR is, in part, due to hyperpolarization and decreased neurotransmission mediated by increased potassium and reduced calcium conductances in pain pathways neurons [24,25]. N40D substitution modifies MOR effect on neuronal ion channels. MOR activates G protein activated inward rectifier

K channels (GirK) by increasing its conductance [26]. Bond et al. [4] found that the activation of GirK currents was 3-fold larger for MOR-D than for MOR-N [4] (Table 1). Regarding calcium channels, MOR activation inhibits presynaptic calcium channels on nociceptive neurons. Nociceptors terminals are located at the dorsal horn of the spinal cord, where they synapse onto ascending spinal cord neurons. In this first synapse, stimulation of presynaptic MOR impairs excitatory neurotransmitter release and so, pain sensation. Some of the main targets of the MOR cascade are the voltage gated calcium channels (Ca_v2.2 and Ca_v2.1) that control synaptic vesicles release. Nociceptors mainly express Ca_v2.2, and, in particular, two mutually exclusive alternative splicing variant of this channel: Ca_v2.2e37a and Ca_v2.2e37b [27]. These two isoforms differ in 14 amino acids of the intracellular C terminus of the channel, and the CaV2.2e37b is ubiquitously expressed in the nervous system while the Ca_v2.2e37a is almost exclusively express in a subset of nociceptors [27]. Moreover, the exon 37a containing isoform has a higher sensitivity to G protein mediated inhibition due to phosphorylation at tyrosine 1747 which is absent in exon 37b. We demonstrated that MOR-D inhibits either Ca_ve37a or Ca_v2.2e37b isoforms with a 4-fold smaller apparent EC50 for the agonist DAMGO than MOR-N [8] (Table 1). Thus, the difference in potency for DAMGO of MOR variants is independent of the CaV2.2 isoform under study. Margas et al. [7] showed that native Ca_v currents were also more sensitive to inhibition by MOR-D than MOR-N. They studied the effect of MOR-N and MOR-D activation on Ca_v currents from superior cervical ganglia (SCG) neurons which are enriched in Ca_v2.2 channels [7]. They microinjected cDNA coding for MOR-N and MOR-D into SCG isolated neurons and found a higher potency of DAMGO on cells expressing MOR-D than MOR-N.

The studies discussed above show a higher potency for MOR-D than MOR-N to inhibit Ca_v channels. However, one study has reported the opposite result in humanized mice expressing human MOR-N or MOR-D. This study found that Ca_v currents from trigeminal ganglion neurons are inhibited at lower morphine doses when the neurons are injected with human MOR-D mRNA as compared with the inhibition observed in neurons injected with MOR-N mRNA [11]. The discrepancy between this report and the previously mentioned studies could be due to the fact that trigeminal ganglia have several Ca_v types contributing to the voltage gated calcium currents [28,29] while Lopez Soto et al. [8] analyzed Ca_v2.2 transfected in HEK293 cells and Margas et al. [7] studied SGC neurons where the Ca_v2.2 are the predominant voltage gated channels.

Correlation between pain sensation and analgesic requirements and the MOR N40D polymorphism

Studies performed *in vitro* have been very important to understand the molecular basis of the changes on MOR functionality due to N40D substitution. Additionally, genetically modified mouse models have been essential to investigate the physiological implications of the A118G SNP in a highly controlled experimental setting. Then, pain studies with human populations have well stressed the impact of A118G SNP on pain sensation and analgesic requirements. In this section we discuss the available mouse models and the more relevant clinical and population paper describing the impact of N40D in human pain treatment.

Mouse genome has an A118G equivalent polymorphism at position 112 (A112G). This substitution exchanges an asparagine residue to an aspartic acid residue at position 38 (N38D), homologous to position 40 of human MOR [30]. Mague et al. [30] taking advantage of genetic engineering techniques has produced knock-in mouse lines that are homozygous A/A or G/G at position 112 of *opmr1*. The authors found that MOR mRNA and protein levels were lower for G/G mice than for A/A mice in brain regions related to pain without changes in binding affinity for several agonists [30]. Moreover, the molecular mass of brain MOR was smaller in A/A mice as compared to G/G mice, presumably due to a decreased N-linked glycosylation in MOR-D variant. Using the hot plate test as a nociception assay, the authors tested the impact of A112G substitution in pain sensation and morphine requirements to relieve pain and found no differences in pain threshold between A/A and G/G mice. Interestingly, morphine potency was lower for G/G mice than A/A mice when anti-nociception was tested. Increasing the noxious thermal stimulus unmasks a greater baseline jumping behavior and lower EC50 for morphine-mediated anti-nociception in G/G mice compared with A/A mice [30]. As we previously mentioned, two humanized mouse lines also have been generated, where exon 1 of mouse *oprm1* gene was replaced by exon 1 of human OPRM1 gene, containing A allele or G allele exclusively [31]. Here the behavioral observations were also opposite to what was expected since: authors found a higher morphine requirement for G/G humanized mice and no differences when fentanyl was used as a MOR agonist. One explanation for these discrepancies is that human-mouse chimera receptor could have molecular consequences that carry artificial differences or similarities for polymorphism effect on MOR function. Nevertheless, the differences among the strategies demonstrated the complexity of the pain neuronal circuit's integration.

Tolerance to pain and analgesic requirements are variable parameters among human individuals. This has been confirmed by many experimental and clinical pain trials but the mechanisms involved are still not fully understood. Since inter-individual pain sensitivity differences have a heritable component [32], *OPRM1* gen polymorphisms emerged as a molecular candidate.

Experimental pain studies with A118G SNP have been documented. This kind of studies has the advantage of producing pain in a measurable and controlled environment with the caveat that the pain does not involve a patho-physiological process. In healthy volunteers, one study has shown that G allele is associated with higher pressure pain thresholds when different pain modalities, including thermal, mechanical, and ischemic stimulus, were evaluated [33]. A sex-genotype interaction was found in terms of heat pain perception: G allele is associated to lower pain ratings in men and higher pain ratings in women. Oertel et al. [34] assessed electrically and chemically induced pain under serial doses of alfentanil. Tolerance to electrical

stimuli and alfentanil doses requirements revealed a decreased opioid analgesia in G allele carriers as compared to A/A homozygous [34]. Similarly, opioids have a reduced analgesic effect in G/G homozygous under chemically induced pain. Romberg et al. [35] also tested experimental electrically induced pain under morphine-6-glucuronide (M6G)-induced analgesia in healthy volunteers [35]. They found a poor response to M6G analgesia in G allele carriers in comparison with A/A homozygous. Despite the caveat of the small sample number, these studies performed in human subjects are important to understand the impact of A118G in human pain.

There are numerous clinical studies assessing A118G impact in human pain tolerance and relief by opioids. As we presented in this section, available data is inconsistent maybe as a product of the diverse nature of the original pathological condition producing each painful state.

Gynecological and child birth pain are common models used to evaluate pain tolerance and analgesia in humans. Zhang et al. [12] found that G allele presence correlates with a lower threshold of pain tolerance to electrical stimulation in women Chinese gynecological patients. Moreover, G/G homozygous women needed more fentanyl for analgesia than heterozygous and A/A homozygous [12]. A118G impact on pain in women during child birth has been often reported. One study found a 1.5 fold lower responsiveness to opioid in A/A women compared to G carriers during labor analgesia [13]. Also, Sia et al. [14] had examined the impact of A118G in a study evaluating pain management in women that underwent a caesarian section surgery. Their results showed higher pain score and morphine requirements for G/G patients than A/G and A/A patients [14]. In contrast, the opposite result has been reported in similar postoperative pain conditions [15]. Thus, differences in pain threshold and opioids requirement among gynecological pain patients carrying G and A alleles exist but there is not a clear pattern among studies performed.

There are several studies testing the impact of A118G SNP in cancer pain. To interpret these studies is necessary to consider the source of pain diagnosis since pain can vary dramatically among cases. Cancer pain patients with an ongoing chronic morphine treatment need more morphine to relieve pain when they carry the G allele. In this report, an intriguing piece of information is that heterozygous patients feel more pain than A/A and G/G groups [16]. A possible explanation to this is that no differences in G/G individuals were detected because the sample size of this group was very small in this study (n = 4). Campa et al. have found that A allele carrying patients with cancer pain responded better to an opioid-based therapy than G/G or G/A patients [36].

On the other hand, Janicky et al. have inquired about A118G polymorphism in acute and chronic pain conditions [37]. In contrast to other results, they have found no significant association between A118G genotype and opioid dose required during or after laparoscopic abdominal surgery. Also they failed to detect differences in pain scores during the late postoperative recovery period. Moreover they analyzed morphine requirement in no cancer chronic pain patients. They found that in a subgroup with the highest opioid usage, the A/A homozygous required higher opioid doses than G allele carriers. Additionally, when they compared acute pain patient group and chronic pain patient group, they found that A118G SNP frequency was ~50% statistically lower in chronic pain group. So, the authors suggest that A118G SNP can be associated to a protective effect against chronic pain [37]. Carrying the G allele has been previously correlated with a protecting function against respiratory depressive effects [34], opioid dependence [4] and alcoholism [38,39]. In opposition, several reports failed to find

the same correlations [35,40-42]. On the other hand the G allele has been related to lower sedative effect by the synergic interaction between two opioid derived drugs, propofol and remifentanyl, used as anesthetics in surgeries [43].

Thus, it is clear that A118G impacts in pain sensation and opioids requirement since many reports showed differences among the genotypes. Due to the heterogeneity in the experimental conditions, it is difficult to arrive to a conclusive state about how this polymorphism modifies pain physiology in humans.

The A118G distribution across human populations

Despite the inter-individual differences in pain sensation, a correlation between pain and human ethnicity exists (inter-populations differences) [44]. Moreover, clinical studies revealed ethnic background as a crucial determinant in analgesic requirements in several pain states such as post-operative [15], chronic [45,46] and cancer pain [47,48]. On the other hand, the duration of opioids analgesia is different among patients with different ethnic backgrounds [49]. This cumulative information allows postulating ethnic identity as a predictor factor of pain sensitivity and analgesic requirements in humans [15,44].

Data supporting correlation between MOR polymorphisms and ethnicity in humans has been reported for several SNP [4,50,51]. In particular, imbalance in A118G distribution among human populations with different ethnic background has been reported in several studies [4,15,40,49,51]. Moreover, Bond et al. [4] found that the negative correlation between addiction to opiates and G allele frequency is observed only in one of the ethnic groups (Hispanic) analyzed [4]. This implies that the impact of A118G in human physiology could depend on the ethnic background.

Thus, there is a link between ethnicity and A118G distribution in human populations. As an illustration of this fact, we compared A118G SNP frequencies in human populations from the HapMap project database. In our analysis, we included a novel set of admixed European-Amerindian population samples that we collected from Corrientes, a north province of Argentina (CTES).

Methods

90 samples (CTES) of unrelated volunteer donors were collected from Corrientes City (Corrientes province, Argentina). Sampling consisted of blood or buccal swabs, and DNA extraction was performed following phenol-chloroform procedure of Sambrook et al. or Chelex 100 [52] (BIO-RAD Laboratories, CA, USA). Each individual signed written consent statement form. This study is approved by the Ethics Committee for Biomedical Research from the Multidisciplinary Institute of Cell Biology (IMBICE).

A118G SNP (dbSNP Accession No. rs1799971) genotyping was made by PCR-RFLP as previously described by Gelernter et al. [40]. As control 10 samples were sequenced by MacroGen Inc. (Seoul, Korea).

Statistical analyses including deviation from Hardy-Weinberg equilibrium (HWE; exact test, $p < 0.05$) and Analysis of Molecular Variance (AMOVA; 1023 permutation, $p < 0.05$) were carried out with the software Arlequin v3.5. To test genetic structure we included 10 International Hap Map Project population samples (www.hapmap.org): Utah residents with Northern and Western European ancestry from the CEPH collection (CEU), Han Chinese in Beijing, China (CHB), Chinese in Metropolitan Denver, Colorado (CHD), Gujarati Indians in Houston, Texas (GIH), Japanese in Tokyo, Japan (JPT), Mexican ancestry in Los Angeles, California (MEX), Maasai in Kinyawa, Kenya (MKG), Tuscan in Italy (TSI), African ancestry in Southwest USA (ASW) and Yoruba in Ibadan, Nigeria. Luhya in Webuye, Kenya (LWK) population sample were excluded because A118G SNP was not genotyped.

Results

We evaluated if A118G SNP frequencies were distributed randomly within the population of samples analyzed. We first analyzed the CTES population samples we collected. Sample size was 90 including 42 men (46.67%) and 48 women (53.33%). The allelic A118G frequency in CTES population sample was 18.89 %, and genotypic frequencies were A/A = 65.56%, A/G = 31.11% and G/G = 3.33%. Gender genotype frequencies were A/A = 59.52%, A/G = 40.48% and G/G = 0% for men, and A/A = 70.83%, A/G = 22.92% and G/G = 6.25% for women. CTES frequencies were in Hardy Weinberg equilibrium (HWE; Observed heterozygosity = 0.31111 and expected heterozygosity = 0.30813; Exact test, $p = 1.000$). Moreover, in the ten HapMap population samples analyzed, all SNP frequencies were also in HWE [53].

We then performed an AMOVA test to evaluate if A118G SNP distribution could reveal a genetic structure among worldwide populations. We tested the eleven population samples all together and found a population structure, with 15.68 % of variability among populations (Table 2, $p < 0.001$). To identify the most probable population structure we grouped them by continental genetic background. We defined three clusters: African (ASW, YRI and MKG), Asian (CHB, CHD, JPT and GIH), and European-American (CTES, CEU, TSI and MEX). Since American populations have an extensive genetic admixture pattern produced by Amerindians-Europeans intermarriage [54,55], we decided to pool American and European populations. Within each cluster A118G SNP failed to detect population substructures (all p values > 0.09). This result validates our criteria to group populations with a naturally genetic background, at least for A118G distribution. Next, we showed that A118G SNP is useful to identify population structure when clustering by continental ethnic background. In order to distinguish the variation among the three defined groups, we performed an AMOVA test and found a 20.19% of variation among groups (Table 2, $p < 0.001$). Accordingly, we also detected 79.62% of variation within populations (Table 2, $p < 0.001$) without significant variation among populations within groups. A limitation of our study is the limited sample size. On the other hand,

Structure tested	% of genetic variability		
	Within populations	Among populations (within groups)	Among groups
All	84.31*	15.68*	-
European – American	100.25852	-0.25852	-
Asiatic	99.55105	0.44895	-
African	99.36067	0.63933	-
Three groups	79.62*	0.19	20.19*

Table 2: Analysis of Molecular Variance (AMOVA) among worldwide populations. Values indicate % of variance component in hierarchical levels in the genetic structures tested. *denotes statistically significant values (significance test: 1023 permutations, $p < 0.05$).

we could not exclude that other MOR SNPs in linkage disequilibrium with A118G could be counting for the genetic structure detected.

In conclusion, we were able to detect a worldwide population genetic structure by analyzing A118G SNP genetic variance. Moreover A118G frequency distributions within populations correlate with ethnic genetic background. This finding may be related to the shared genetic history by populations on the same continent and the large inter-group differences among the human population groups analyzed.

Conclusion

A118G is a relevant human polymorphism that changes MOR physiology and impact on pain sensation and opioids requirements. Here, we analyzed the distribution of alleles A and G in different human populations including a novel Argentinean population supporting the notion that A118G could be useful to determine the ethnic background in a human population. Futures studies about this and others opioids system polymorphisms could contribute to develop individual and population targeted therapies to manage pain conditions.

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