Allelic Expression Imbalance of Human mu Opioid Receptor (OPRM1) Caused by Variant A118G*

Revised for publication, May 4, 2005, and in revised form, July 20, 2005. Published, JBC Papers In Press, July 26, 2005, DOI 10.1074/jbc.M504942200

Ying Zhang, Danxin Wang, Andrew D. Johnson, Audrey C. Papp, and Wolfgang Sadée1

From the Program in Pharmacogenomics, Department of Pharmacology, College of Medicine and Public Health, The Ohio State University, Columbus, Ohio 43210

As a primary target for opioid drugs and peptides, the mu opioid receptor (OPRM1) plays a key role in pain perception and addiction. Genetic variants of OPRM1 have been implicated in predisposition to drug addiction, in particular the single nucleotide polymorphism A118G, leading to an N40D substitution, with an allele frequency of 10–32%, and uncertain functions. We have measured allele-specific mRNA expression of OPRM1 in human autopsy brain tissues, using A118G as a marker. In 8 heterozygous samples measured, the A118 mRNA allele was 1.5–2.5-fold more abundant than the G118 allele. Transfection into Chinese hamster ovary cells of a cDNA representing only the coding region of OPRM1, carrying adenosine, guanosine, cytidine, and thymidine in position 118, resulted in 1.5-fold lower mRNA levels only for OPRM1-G118, and more than 10-fold lower OPRM1 protein levels, measured by Western blotting and receptor binding assay. After transfection and inhibition of transcription with actinomycin D, analysis of mRNA turnover failed to reveal differences in mRNA stability between A118 and G118 alleles, indicating a defect in transcription or mRNA maturation. These results indicate that OPRM1-G118 is a functional variant with deleterious effects on both mRNA and protein yield. Clarifying the functional relevance of polymorphisms associated with susceptibility to a complex disorder such as drug addiction provides a foundation for clinical association studies.

Drug addiction is a complex disorder with a strong genetic component (1, 2). Serving as a primary target for opioid drugs and peptides, the mu opioid receptor (OPRM1) mediates the effects of morphine and heroin (3, 4). By impinging on dopaminergic pathways, OPRM1 also plays a role in addiction to other drugs of abuse, such as cocaine, nicotine, and alcohol (5, 6).

Because of its central role in drug addiction, numerous studies have addressed potential contributions of polymorphisms in the gene encoding OPRM1 to addiction susceptibility (7, 8). Among multiple single nucleotide polymorphisms (SNPs)1 in OPRM1, C77T (A6V), and A118G (N40D) are well studied nonsynonymous SNPs located at the N terminus of the receptor. In particular, A118G, with an allele frequency of 10–32% in different ethnic groups (9), has been associated with susceptibility to heroin, nicotine, and alcohol addiction (10–12). However, other studies have failed to corroborate these associations (13, 14), possibly as a result of population admixture.

The A118G polymorphism has also been linked to differences in pharmacological properties of OPRM1. In a Swedish population, the G118 variant was shown to correlate with poor response to naltrexone in the treatment of alcoholism (15). Moreover, OPRM1-antagonist naloxone elicited an increased cortisol response in individuals with a G118 allele (16, 17). In transfected cells, OPRM1-Asp40 was reported to have 3-fold higher affinity for β-endorphin than OPRM1-Asn40 (18), suggesting a gain of function, but subsequent studies have failed to corroborate these results (19, 20). In vitro transfection studies, however, indicated that the G118 allele might be associated with lower OPRM1 protein expression than the A118 allele (20, 41). Therefore, the functional significance of the A118G variant of OPRM1 remained unresolved.

cis-Acting functional polymorphisms that affect transcription, mRNA processing, mRNA stability, and protein translation, rather than primary protein structure, may represent a main cause of human phenotypic variability (21–24). Measuring allelic imbalance of mRNA expression has proven to be a powerful tool in detecting such cis-acting polymorphisms (25–27). In this approach, one measures the expression of each of two alleles simultaneously in a target tissue of heterozygous individuals, thereby, eliminating the influence of trans-acting factors. In this study, we have measured allelic OPRM1 mRNA expression, using A118G as the indicator SNP, in human brain tissues (autopsies). We have also determined mRNA and protein expression in CHO cells, after transfection of plasmids carrying the OPRM1 coding region, with A, G, C, and T substituted at position 118. The results demonstrate that the A118G substitution in OPRM1 causes significantly reduced yields of mRNA and receptor protein, indicating a loss of function.

EXPERIMENTAL PROCEDURES

Human Brain Tissues—87 human post-mortem brain tissue samples were obtained from different sources as follows: 16 from Cooperative Human Tissue Network, Philadelphia, PA, and 12 from the Department of Pathology, Ohio State University (mainly cortical tissues samples); 10 human pons tissues from the Brain and Tissue Bank, University of Maryland, Baltimore, MD, and 49 pons section slides from Stanley Research Medical Foundation (Bethesda, MD).

Genomic DNA and RNA Preparation—Genomic DNA and RNA were prepared from frozen brain samples or pons section slides. Tissue samples were treated with sucrose-Triton and digested with proteinate K, followed by sodium chloride precipitation of protein and ethanol purification of DNA. Total RNA was extracted with TRIzol reagent (Invitrogen) and purified using Qiagen RNeasy columns according to the manufacturer’s instructions. Before reverse transcription, total RNA was treated with DNase I (Ambion) at 37 °C for 30 min. Complementary DNA (cDNA) was generated from 1 μg of total RNA with Superscript II reverse transcriptase (Invitrogen), using oligo(dT) and OPRM1-specific primers targeting a region 3′ of A118G (primer for cDNA synthesis: 5′-CAGGTCCGTTGGTTCC).

1 The abbreviations used are: SNP, single nucleotide polymorphism; CHO, Chinese hamster ovary; ActD, actinomycin D.

2 The number of individuals carrying the variant allele in the genotyping and in the human autopsy brain tissues, using A118G as the indicator SNP, in human brain tissues (autopsies). We have also determined mRNA and protein expression in CHO cells, after transfection of plasmids carrying the OPRM1 coding region, with A, G, C, and T substituted at position 118. The results demonstrate that the A118G substitution in OPRM1 causes significantly reduced yields of mRNA and receptor protein, indicating a loss of function.

* This work was supported by National Institutes of Health Grant DA018744. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed: 5072 Graves Hall, 333 W. 10th Ave., Dept. of Pharmacology, The Ohio State University, Columbus, OH 43210. Tel: 614-292-5593; Fax: 614-292-7232; E-mail: address: sadee-1@medctr.osu.edu.

2 The abbreviations used are: SNP, single nucleotide polymorphism; CHO, Chinese hamster ovary; ActD, actinomycin D.

3 The abbreviations used are: SNP, single nucleotide polymorphism; CHO, Chinese hamster ovary; ActD, actinomycin D.

4 The number of individuals carrying the variant allele in the genotyping and in the human autopsy brain tissues, using A118G as the indicator SNP, in human brain tissues (autopsies). We have also determined mRNA and protein expression in CHO cells, after transfection of plasmids carrying the OPRM1 coding region, with A, G, C, and T substituted at position 118. The results demonstrate that the A118G substitution in OPRM1 causes significantly reduced yields of mRNA and receptor protein, indicating a loss of function.

5 The number of individuals carrying the variant allele in the genotyping and in the human autopsy brain tissues, using A118G as the indicator SNP, in human brain tissues (autopsies). We have also determined mRNA and protein expression in CHO cells, after transfection of plasmids carrying the OPRM1 coding region, with A, G, C, and T substituted at position 118. The results demonstrate that the A118G substitution in OPRM1 causes significantly reduced yields of mRNA and receptor protein, indicating a loss of function.

6 The number of individuals carrying the variant allele in the genotyping and in the human autopsy brain tissues, using A118G as the indicator SNP, in human brain tissues (autopsies). We have also determined mRNA and protein expression in CHO cells, after transfection of plasmids carrying the OPRM1 coding region, with A, G, C, and T substituted at position 118. The results demonstrate that the A118G substitution in OPRM1 causes significantly reduced yields of mRNA and receptor protein, indicating a loss of function.

7 The number of individuals carrying the variant allele in the genotyping and in the human autopsy brain tissues, using A118G as the indicator SNP, in human brain tissues (autopsies). We have also determined mRNA and protein expression in CHO cells, after transfection of plasmids carrying the OPRM1 coding region, with A, G, C, and T substituted at position 118. The results demonstrate that the A118G substitution in OPRM1 causes significantly reduced yields of mRNA and receptor protein, indicating a loss of function.

8 The number of individuals carrying the variant allele in the genotyping and in the human autopsy brain tissues, using A118G as the indicator SNP, in human brain tissues (autopsies). We have also determined mRNA and protein expression in CHO cells, after transfection of plasmids carrying the OPRM1 coding region, with A, G, C, and T substituted at position 118. The results demonstrate that the A118G substitution in OPRM1 causes significantly reduced yields of mRNA and receptor protein, indicating a loss of function.
Genotyping of OPRM1—A118G and C17T were scored in genomic DNA prepared from 87 brain tissue samples using SNAPSHOT (Applied Biosystems, CA) as described below.

SNAPSHOT and Quantitative Analysis of Allelic Ratios in mRNA and Genomic DNA—SNAPSHOT was performed as described previously (28). In brief, a section of DNA or cDNA surrounding the SNP (~100 bp) was amplified by PCR using amplification primers (forward: 5'-GTTTGCCTGGGTCACTTGTGC, reverse: 5'-GGGGTACCCGATGGACAGCAGCG-3') under the following PCR conditions: 95 °C for 2 min, followed by 30 cycles of 95 °C for 15 s, 60 °C for 1 min, and 72 °C 1 min. For the analysis of C17T, the following PCR primers were used, under the same conditions (forward: 5'-CCCTGACTCAATCCATTGAGC-3', reverse: 5'-GAGTGACCGCCAAGGCACTAGGT-3'). After PCR, residual primers and unincorporated dNTPs were inactivated by incubating at 37 °C for 3 h with 5 units of Antarctic Phosphatase and 2 units of Exol (New England Biolabs). The purified PCR products were then analyzed using a primer extension method (SNAPSHOT). The extension primers were designed to anneal to the amplified template DNA immediately adjacent to the A118G site. Subsequent extension with DNA polymerase added a single fluorescent dideoxyribonucleoside triphosphate (ddNTP) complementary to the nucleotide at the polymorphic site. The extended primers, labeled with different fluorescent dyes were run on an ABI 3730 capillary electrophoresis instrument, and analyzed with Gene Mapper 3.0 software (Applied Biosystems, Foster City, CA). Peak area ratios were calculated to measure the relative amount of DNA (cDNA) of the two alleles. Control experiments with known mixtures of plasmids carrying different nucleotides in the A118 position demonstrated that peak areas and ratios were linearly related to added amounts of DNA (cDNA). Because of differences in fluorescent yield and terminator dye incorporation, measured peak area ratios differed from unity when a 1:1 ratio of allelic DNA was added, depending on the incorporated fluorescent dye. For each brain tissue, peak area ratios were measured for both genomic DNA and mRNA (cDNA). Assuming that the two alleles were present in equal amounts in genomic DNA, measured DNA and cDNA ratios were normalized to the average of genomic DNA ratios using the equation: normalized ratio = measured DNA (or cDNA) ratio/mean of genomic DNA ratio. For genomic DNA, none of the samples significantly deviated from the expected unity, indicating that the alleles were present in equal quantities in the tissues analyzed (absence of gene dosage effects on allelic expression levels). As an added control, extension primers for A118G were used in both orientations in separate analyses, and DNA and mRNA (cDNA) ratios were calculated in the same fashion to test for any artifacts that may have been introduced by fluorescent dye bias (forward primer: 5'-ACTGATCGACCTTGCCACCTTAGATGGC-3', reverse primer: 5'-ACTGATCGACTGACCTAGATGGC-3').

For C17T, the extension primer was 5'-ATTGCGTTGGTGGCGG-3'. Allelic ratios of A118G/G118 and C17/T17 were measured in all homozygous brain tissues (8 samples for A118G and 1 sample for C17T) by determining peak area ratios. For cDNA preparations, each mRNA sample was converted to cDNA in three separate experiments, and each cDNA sample was analyzed at least twice using SNAPSHOT.

mRNA Analysis Using Real-time PCR—To quantify mRNA expression of the genotyped samples, we first set up a standard curve using various amount (50 fg to 100 pg) of plasmid DNA (OPRM1), performed with real-time PCR with the same PCR amplification primers used for SNAPSHOT, with 12.5 μl of 2× PCR SYBR master mixture (3 mM MgCl2, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 200 μM dUTP, 0.625 units of AmpliTaq Gold, and 0.25 units of AmpErase UNG). The mixtures were run on an ABI 7000 with the same PCR conditions, and threshold cycles were measured. OPRM1 cDNA levels were calculated from the obtained standard curve. As an internal control, ß-actin was amplified for the same samples, and expression levels were determined by a standard curve using different dilutions of a mixture composed of cDNAs from all analyzed samples. The cDNA expression level of each sample was expressed as the ratio of OPRM1 over ß-actin. To detect any possible DNA contamination, real-time PCR was also performed in RNA control samples in the absence of reverse transcriptase during cDNA synthesis.

mRNA Secondary Structure Prediction—The mRNA secondary structure of OPRM1 (reference sequence AY521028) was predicted using Mfold (Unix and Web versions 3.12) (29). Custom-designed Perl scripts created new sequences representing every possible transition SNP in the coding region (n = 1203), including A118G. Sequences were folded in an automated manner with Mfold (Unix Version). The maximum number of folding parameter was set on the optimum of the wild-type sequence (n = 19). For a given RNA sequence, the resulting single-stranded counts measured the number of times each nucleotide was unpaired across all predicted secondary structures (30). We calculated the change in single-stranded counts at each nucleotide in each set of SNP structures relative to wild-type structures (1203 differences). Differences in single-stranded counts for each SNP sequence were analyzed regionally (in nucleotide windows surrounding the SNP) and globally (across the full sequence) as a measure of the relative amount of structural variation predicted to occur in response to a given SNP. Full-length RNA structures for A118, G118, C118, and T118 were predicted using the Mfold web server. All structures (optimal and suboptimal) were visualized, and heuristics were considered to determine motif changes in the A118G region.

OPRM1 Expression Constructs and Mutagenesis—A 1.2-kb fragment containing the human OPRM1 coding region cDNA was subcloned into pcDNA3 vector (Invitrogen, CA) using KpnI and XhoI sites. PCR was used to introduce the mutations at position 118 using the following mutagenesis primers (the mutated sites are in bold): A118G (T,C), 5'-CACCCTAGATGGGGATCC-3', with a BamHI site. The generated 473-bp sequence of OPRM1 was amplified with PCR using the mutagenesis primers and a common reverse primer (5'-CGGGATCCATGGAGAATTC-3', with a BamHI site). The generated 473-bp fragments, together with a common forward primer (5'-GGGGATCCCGATGACGACGACG-3'), were used to amplify a 581-bp fragment of OPRM1. Digestion with KpnI and BamHI and ligation into a 3' fragment from wild type OPRM1 in pcDNA3 vector yielded full-length coding regions. For each construct, 2 clones were isolated. All constructs were confirmed by DNA sequencing.

Cell Culture and Transient Transfection—CHO-K1 cells were cultured in F-12 nutrient medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C with 5% CO2. Twenty-four hours before transfection, cells were seeded into 6- or 12-well dishes. Transfections were performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol.

mRNA Stability Assay—Twenty-four hours after transfection, actinomycin D (ActD) (10 μg/ml) was added to inhibit transcription, and the cells were collected 1, 2, 6, and 12 h after treatment. Control cells were cultured for the same time period without ActD treatment. For plasmid DNA preparation, cells were trypsinized and collected. Plasmid DNA was prepared using a Qiagen DNA miniprep kit. For RNA preparation, cells were lysed with TRIzol reagent and prepared as described above. OPRM1 mRNA was quantified by real-time PCR using PCR amplification primers as described above for SNAPSHOT.

OPRM1 Binding Assay—CHO cells in 12-well plates (polylysine coated) were transfected with 2 μg of wild type or variant OPRM1
plasmids. Twenty-four hours after transfection, cells were washed twice with phosphate-buffered saline, incubated with 2 \( \mu \text{M} \) [3H]diprenorphine (Amersham Biosciences, CA, 50 Ci/mmol) in Tris-HCl buffer (50 mM, pH 7.5) for 1 h at 37 °C. Then, cells were washed three times with cold phosphate-buffered saline and dissolved with 1 N NaOH. The cell lysates were transferred to scintillation vials and radioactivity was determined with a LS-6500 Multipurpose scintillation counter (Beckman Coulter). Nonspecific binding was determined by adding naloxone (20 \( \mu \text{g} \) ) before adding [3H]diprenorphine, which was subtracted from total binding.

**Western Blotting Analysis**—Twenty-four hours after the transfection of wild type and variant OPRM1 into CHO cells plated in 6-well plates, cells were dissolved in 250 \( \mu \text{l} \) of 2X SDS loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue, 5% 2-mercaptoethanol (Bio-Rad), and incubated at 95 °C for 10 min. Samples (40 \( \mu \text{l} \) ) were run on an 8% SDS-polyacrylamide gel. Western blotting was performed using rabbit antibodies directed against the OPRM1 C-terminal (Incstar, Stillwater, MN), followed by detection with ECL Hyperfilm kit (Amersham Biosciences). The membrane was then stripped with stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl, pH 6.7), followed by incubation with mouse anti-\( \alpha \)-tubulin antibodies (Oncogene Research Products, San Diego, CA) and detection with ECL Hyperfilm kit.

**Statistical Analyses**—Data are expressed as mean ± S.D. Statistical analysis was performed using the program GraphPad Prism version 3.00 for Windows, GraphPad Software (San Diego, CA).

**RESULTS**

**Genotyping Results**—We have genotyped 87 samples for A118G and C177T using SNAPSHOT results. For SNP A118G, 76 were AA (87.4%), 8 were heterozygous AG (9.2%), and 3 were GG (3.4%). For SNP C177T, only one heterozygote was detected, all others were CC. The C177T heterozygote was homozygous for A118. Allelic frequencies were at the lower end of previously observed frequencies in various ethnic populations (8, 18).

**Allelic Expression of OPRM1 in Human Brain, Using A118G as the Indicator SNP**—We identified 8 heterozygous samples for OPRM1 A118G, suitable for allelic expression analysis. Genomic DNA and total RNA were isolated from brain tissues, and mRNA was reverse-transcribed to cDNA using a gene-specific primer and oligo(dT). cDNA synthesis using only oligo(dT) failed in brain tissue extracts because the coding region is far removed from the poly(A) tail of OPRM1, and because of partial mRNA degradation. After PCR amplification of the cDNA generated from the gene-specific primer (located in proximity of A118 and oligo(dT), we used SNAPSHOT to measure the peak area ratios of A118/G118 for genomic DNA and cDNA. Control experiments with mixtures of plasmids carrying A118 and G118-OPRM1 demonstrated that the measured ratios were proportional to amounts of each plasmid DNA added (Pearson analysis \( r = 0.992 \), \( p < 0.001 \), 0.997, \( p < 0.001 \), and 0.998, \( p < 0.0001 \), respectively. Data are mean ± S.D., \( n = 3 \).

**Allelic Expression Imbalance of OPRM1**

**FIGURE 1. Correlation between fluorescence peak area measured by SNAPSHOT and the ratios of added plasmid DNAs.** Different amounts of A118-OPRM1 plasmid were mixed with G118-OPRM1 (panel a), C118-OPRM1 (panel b), or T118-OPRM1 (panel c) to obtain ratios of 2.8, 4.6, 5.5, 6.4, and 8.2. The plasmid mixtures were amplified and detected with SNAPSHOT using different nucleotides at position 118 as indicators. The measured relative amounts of each OPRM1 variant present in the mixture were expressed as the peak area ratio of A/G, A/C, or A/T. The peak area ratios for each combination were plotted against the plasmid DNA ratio. Pearson \( r \) analysis for A/G, A/C, and A/T were 0.992, \( p < 0.001 \), 0.997, \( p < 0.001 \), and 0.998, \( p < 0.0001 \), respectively. Data are mean ± S.D., \( n = 3 \).
TABLE ONE
Peak area ratios for mRNA and DNA for 8 A118G heterozygous brain samples analyzed by SNaPshot

| Sample ID | Forward primer extension primer | Reverse primer extension primer |
|-----------|---------------------------------|---------------------------------|
|           | A/G peak area ratios (A118G)    | DNA (n = 3) mRNA (cDNA) (n = 6) | DNA (n = 3) mRNA (cDNA) (n = 3) |
|           | Observed ratios                 | Normalized ratios               | Observed ratios                 | Normalized ratios               |
| DNA       | mRNA (cDNA)                     | DNA                            | mRNA (cDNA)                     | DNA                            |
| 1         | 1.03 ± 0.25                     | 2.13 ± 0.41                    | 0.96 ± 0.27                     | 2.02 ± 0.38                    | 3.59 ± 0.40                     | 5.44 ± 0.30                     | 1.03 ± 0.11                     | 1.56 ± 0.30                     |
| 2         | 1.22 ± 0.33                     | 2.33 ± 0.27                    | 1.13 ± 0.36                     | 2.18 ± 0.25                    | 3.59 ± 0.16                     | 9.22 ± 0.60                     | 1.03 ± 0.05                     | 2.78 ± 0.17                     |
| 3         | 1.26 ± 0.26                     | 2.72 ± 0.23                    | 1.17 ± 0.17                     | 2.53 ± 0.21                    | 3.27 ± 0.09                     | 8.96 ± 0.04                     | 0.93 ± 0.03                     | 2.56 ± 0.01                     |
| 4         | 1.00 ± 0.11                     | 2.14 ± 0.02                    | 0.92 ± 0.12                     | 1.98 ± 0.01                    | 3.40 ± 0.35                     | 7.17 ± 0.28                     | 0.97 ± 0.10                     | 2.05 ± 0.08                     |
| 5         | 0.87 ± 0.21                     | 1.56 ± 0.48                    | 0.81 ± 0.22                     | 1.51 ± 0.44                    | 3.34 ± 0.16                     | 6.52 ± 0.18                     | 0.95 ± 0.05                     | 1.86 ± 0.05                     |
| 6         | 1.21 ± 0.09                     | 1.65 ± 0.40                    | 1.12 ± 0.08                     | 1.57 ± 0.37                    | 3.54 ± 0.60                     | 6.42 ± 1.01                     | 1.01 ± 0.17                     | 1.83 ± 0.29                     |
| 7         | 1.04 ± 0.13                     | 1.86 ± 0.01                    | 0.96 ± 0.14                     | 1.72 ± 0.00                    | 4.45 ± 0.13                     | 5.40 ± 0.35                     | 1.27 ± 0.04                     | 1.54 ± 0.10                     |
| 8         | 1.22 ± 0.07                     | 1.93 ± 0.21                    | 1.13 ± 0.08                     | 1.80 ± 0.19                    | 3.10 ± 0.18                     | 5.43 ± 0.51                     | 0.89 ± 0.05                     | 1.55 ± 0.15                     |
| Mean ± S.D. | 1.11 ± 0.13                    | 1.00 ± 0.13                    |                                  |                                | 3.50 ± 0.18                     | 1.00 ± 0.12                     |

TABLE TWO
Peak area ratios for mRNA and DNA for one C17T heterozygous brain sample analyzed by SNaPshot

| Sample ID | Observed ratios | Normalized ratios |
|-----------|-----------------|------------------|
| DNA (n = 3) | mRNA (cDNA) (n = 3) | DNA | mRNA (cDNA) |
| 9         | 1.03 ± 0.03     | 1.13 ± 0.04      | 1.00 ± 0.03 | 1.10 ± 0.04 |

higher than the DNA ratio at 24 h after transfection (p < 0.01) (Fig. 3a), again indicating that OPRM1-A118 yielded higher mRNA expression than OPRM1-G118, even though the plasmids contained only the coding region of OPRM1.

To determine whether the functional change is specific to the A118G transition, we constructed OPRM1 expression vectors with A118C and A118T substitutions. CHO cells were co-transfected with equal amounts of OPRM1-A118 and either OPRM1-C118 or OPRM1-T118. Twenty-four hours after transfection, DNA and total RNA were isolated, and the ratios of A118/C118 and A118/T118 were measured in DNA and RNA samples. Control experiments with mixtures of two plasmids (A118+C118 or A118+T118 with 5 different ratios for A/C or A/T, 2:8, 4:6, 5:5, 6:4, and 8:2) showed the peak area ratios of A118/C118 and A118/T118 measured by SNaPshot were proportional to the amount of plasmid DNA added (A/C: Pearson r = 0.997, p < 0.001; A/T: Pearson r = 0.998, p < 0.0001) (Fig. 1, b and c). Measured DNA peak area ratios for A/C and A/T differed from unity (A/C: 3.82 ± 0.2, n = 4; A/T: 0.35 ± 0.05, n = 4) (TABLE THREE), because of different incorporation efficiency and fluorescence yield of different dyes (the ABI3730 uses only one excitation wavelength). After normalization to the DNA ratios (adjusted to unity), the mRNA (cDNA) ratios of A118/C118 and A118/T118 did not differ significantly from that in DNA samples, in contrast to the A118/G118 ratio (Fig. 3a). This demonstrates that the effect at position 118 on OPRM1 mRNA expression is specific to G118.

mRNA Turnover of OPRM1-A118 and OPRM1-G118—To test the mRNA turnover rate of OPRM1, cells transfected with wild type OPRM1 were treated with 10 μg/ml ActD for different time periods, and the amount of OPRM1 mRNA was determined with real-time PCR. OPRM1 mRNA levels declined after ActD treatment with a half-life of ~1.2 h (Fig. 3b), determined by non-linear regression analysis. To test
Allelic Expression Imbalance of OPRM1

FIGURE 3. Panel a, differential mRNA expression of OPRM1 variants in transiently transfected CHO cells. Equal amounts of OPRM1 plasmids containing A118 were co-transfected into CHO cells with plasmids containing G118, C118, or T118. Cells were collected at 24 h after transfection and plasmid DNA and total RNA isolated. The ratios of A118/G118, A118/C118, or A118/T118 in cDNA samples were measured with SNaPshot and normalized with the respective plasmid DNA ratios (TABLE THREE). Data are from three independent experiments with duplicate cDNA preparation and SNaPshot assays. Data are mean ± S.D. compared with the DNA ratio, **, p < 0.01 (one-way analysis of variance with Bonferroni post-test). Panel b, time course of OPRM1 mRNA levels after actinomycin D (10 μg/ml) treatment. Amount of mRNA remaining after ActD is shown as % control, yielding a half-life of 1.2 h (one-phase exponential decay); data are mean ± S.E., n = 4. Panel c, mRNA ratios of A118-OPRM1/G118-OPRM1 at different times after ActD treatment. 24 h after transfection equal amounts of A118-OPRM1 and G118-OPRM1 into CHO cells, ActD (10 μg/ml) was added, and RNA/cDNA and plasmid DNA were isolated at different time points (1, 2, 6, and 12 h). The mRNA ratios were detected by SNaPshot, normalized to DNA ratios measured at each time point. Data are mean ± S.D., n = 4.

TABLE THREE

| Observed ratios | Normalized ratios |
|-----------------|------------------|
| DNA (n = 4) | mRNA (cDNA) (n = 6) | DNA (n = 4) | mRNA (cDNA) (n = 6) |
| A/G | 1.06 ± 0.02 | 1.61 ± 0.04 | 1.00 ± 0.02 | 1.52 ± 0.03 |
| A/C | 3.82 ± 0.21 | 3.94 ± 0.38 | 1.00 ± 0.06 | 1.03 ± 0.10 |
| A/T | 0.35 ± 0.05 | 0.29 ± 0.04 | 1.00 ± 0.14 | 0.81 ± 0.12 |

whether OPRM1-A118 and -G118 mRNA decay differently, we treated cells with ActD 24 h after co-transfection with the same amounts of OPRM1-A118 and -G118 plasmids, then collected the cells and isolated plasmid DNA and total RNA at different times after treatment (0, 1, 2, 6, and 12 h). cDNA ratios were determined with SNaPshot. If the differential expression between OPRM1-A118 and -G118 were associated with altered mRNA stability and turnover, one would expect to see increasing A118/G118 ratios after ActD treatment. However, compared with untreated RNA samples, ActD treatment did not increase the ratio of A118/G118 in mRNA (compared with untreated control, p > 0.05, Student’s t tests) (Fig 3c). Therefore, altered mRNA turnover does not appear to account for the differential expression of OPRM1-A118 and -G118.

OPRM1 Protein Expression Measured with Receptor Binding and Western Blotting—Twenty-four hours after transfection with OPRM1 plasmids carrying the four nucleotide substitutions at position 118, receptor binding sites were measured with [3H]diprenorphine (2 nM). Shown in Fig 4a, [3H]diprenorphine binding was significantly reduced only for the G118 variant (p < 0.01) (G118, 13.5 ± 2.4 fmol/mg; A118, 174 ± 33 fmol/mg; C118, 182 ± 40 fmol/mg; T118, 161 ± 49 fmol/mg). The more than 10-fold difference in protein levels between OPRM1 Asp40 (G118) and three other OPRM1 variants with different nucleotides at position 118 (A, C, and T) is considerably greater than the observed mRNA ratio (1.5-fold). This result is consistent with results on protein expression obtained with Western blotting. With similar total protein loading (represented by α-tubulin, Fig 4b), OPRM1-Asp40 yielded barely detectable expression, in contrast to the other three variants (Fig 4b). To determine whether OPRM1-G118 has dominant effects on the expression or degradation of the expressed protein, we measured [3H]diprenorphine binding in CHO cells co-transfected with OPRM1-A118 and -G118. Co-transfected cells displayed approximately half the tracer binding (137 ± 27 fmol/mg) compared with cells transfected with wild-type OPRM1-A118 only (251 ± 51 fmol/mg) (Fig 4c), whereas OPRM1-G118-transfected cells again showed less than 10% of binding.

mRNA Secondary Structure Predictions—Using mRNA secondary structure predictions with Mfold (29), we modeled predicted changes in structure predictions with Mfold (29), we modeled predicted changes in base pairing behavior for all possible transitions in each nucleotide position of OPRM1, by calculating single-stranded count differences from wild type both locally (varying nucleotide windows around each SNP) and globally (across all nucleotides). The results showed G118 trending toward a greater predicted structural difference than the average transition (3 nt local difference = 29, 3 nt local difference mean = 15.1 ± 11.7, skew = 0.73; global value = 1593, global value mean = 1218 ± 628, skew = 0.22). Although this approach can reveal SNP positions predicted to be more likely to alter structure, it has limited descriptiveness because it employs linear windows to describe secondary changes. Thus, examination of other heuristics as well as visualization of optimal and suboptimal structures is necessary at positions of interest (30). Doing such analysis for variations at the OPRM1-G118 position indicated a well predicted helix (nt 102–107; 114–120) in the G118 mRNA.
that did not exist in predictions for A118, C118, and T118 structures (Fig. 5). Moreover, optimal and suboptimal structures for the A118, C118, and T118 mRNA commonly contained a loop motif in this region that did not appear in any predicted G118 structures (Fig. 5). These results indicate that the G118 transition has the potential to affect local folding, and hence mRNA functions.

**DISCUSSION**

The present study documents a significant functional change caused by the substitution of A118 with G118 in OPRM1, a common SNP in human populations. This substitution appears to affect at least two distinct mechanisms, involving mRNA expression in human autopsy brain tissues and in transfected cells, and translation into functional protein, observed *in vitro*. The collection of brain tissue samples analyzed was not designed to permit comparative analysis of OPRM1 protein content, because of the quality of the tissues, the different anatomical locations, and the rather low number of homozygous G118 samples. Allelic mRNA ratio analysis was successful because we utilized a gene-specific primer close to the allelic site for cDNA generation. For OPRM1 protein analysis as a function of A118G genotype, a larger prospective trial with anatomically well-defined brain sections will be required.

Allele-specific expression analysis revealed a 2-fold difference in expression of the wild-type A118 variant over the G118 variant in autopsy brain samples of heterozygous individuals. Because environmental factors are canceled out by measuring allelic mRNA expression ratios, with one allele serving as the control for the other in individual target tissues, this result provides compelling evidence for the existence of a *cis*-acting factor determining mRNA levels. We also developed a SNaPshot assay for OPRM1-C17T as an indicator SNP. Even though only one brain tissue was heterozygous for C17T, this single sample was informative because it was homozygous for A118. Lack of allelic expression imbalance in this sample indicates that C17T does not contribute, and moreover, is consistent with the notion that A118G is causative.

We also attempted to quantify the absolute levels of OPRM1 mRNA, known to vary between different brain areas (32), presumably via trans-acting regulatory factors. Real-time PCR results yielded highly variable levels for samples taken from different cortical lobes. To minimize the effects of tissue-specific expression, we also used samples from a well defined region (pons) and observed less variation within genotypes. This analysis suggested a trend in mRNA levels in the following order AA > AG > GG, but the sample size was insufficient for statistical analysis, in consideration of the quality of the available tissue samples, and additional variability introduced by potential trans-acting factors. These results demonstrate that the analysis of allelic expression imbalance can be performed successfully in small numbers of target tissues because one allele serves as the control for the other. Therefore, the analytical SNaPShot procedure must be repeated independently so that statistical analysis can be performed for each individual sample.

To permit interpretation of clinical association studies, it is critically important to identify the functional polymorphism underlying the observed allelic expression imbalance of OPRM1. Because each of the 8 heterozygous samples measured showed a similarly elevated A118/G118 ratio in human brain tissues, the functional polymorphism must be in linkage disequilibrium with A118G, or A118G itself is responsible for this functional difference. Previous genotype and haplotype studies had failed to detect any known candidate SNP of sufficient frequency in linkage disequilibrium with A118G that could account for this result (7, 8). Therefore, we tested the role of A118G in transcription and translation, regardless of its proposed effect on Β-endorphin ligand binding and G protein coupling (18). To study the mechanism for the allelic expression imbalance, we constructed expression vectors carrying only the coding region of OPRM1, excluding effects of untranslated regions and promoter regions, with A, G, T, and C in position 118. Measurements of allelic mRNA ratios after co-transfection of OPRM1-A118 with the G118, T118, and C118 variants revealed that only the A118/G118 ratio differed significantly from unity. This ratio is somewhat lower than what had been observed in brain tissue, probably reflecting different tissues, and confirming that the A to G transition affects mRNA levels. Inserting C118 or T118 into OPRM1 failed to affect mRNA expression compared with the wild-type A118. These results demonstrate that substitution with G118 alone, in the absence of any other regulatory regions, causes a significant change in mRNA expression.

Altered mRNA stability and turnover could have accounted for these results, as demonstrated for a synonymous SNP, C957T, in the D2 dopamine receptor (33). However, analysis of mRNA decay after treatment with ActD failed to reveal any significant difference in turnover between the A118 and G118 variants. Therefore, mRNA stability does not appear to cause differential mRNA expression. Alternatively, the A118G substitution could have affected transcription or processing of heterogeneous nuclear RNA into mature mRNA (34). Polymorphisms could induce mRNA decay at several steps during heterogeneous nuclear RNA maturation and pioneer protein synthesis in the nucleus (35–37). Further studies are required to clarify the underlying mechanisms of the observed allelic mRNA expression imbalance.

To determine whether A118G affects translation and receptor protein yield, we measured opioid receptor binding sites for the A118 and G118 OPRM1 variants expressed in CHO cells. The wild-type OPRM1-Asn40 (A118) yielded 10-fold more binding sites than OPRM1-Asp40 (G118). Results from Western blotting also demonstrated a much lower protein yield for OPRM1-Asp40, indicating that the low number of bind-
ing sites was not caused by changes in tracer affinity ([3H]diprenorphine was added at saturating concentrations). Because this allelic difference is substantially greater than the allelic mRNA ratio, the G118 substitution, substituting Asp with Asp, appears also to affect translation, or post-translational processing and turnover of OPRM1 protein. Similarly, Beyer et al. (20) had reported considerably greater protein expression after stable transfection of OPRM1-A118 compared with -G118 in HEK293 cell clones (7-fold) but attributed this difference to variations in selecting single clones. The two other possible substitutions at position 118 (A → C or A → T) failed to affect protein expression and binding, similar to what was observed with mRNA expression. These results support the notion that the non synonymous SNP A118G, changing the codon from AAC to GAC, has a substantial effect on both mRNA and protein levels of OPRM1. Whether translation is affected by A118G, or the amino acid substitution Asn to Asp affects protein turnover, remains to be clarified. Because opioid receptors are known to dimerize (38), we further tested whether cotransfection of wild-type with the G118 variant would affect protein levels by conveying instability to a potential heterodimer formed between them. As the functional protein levels (measured with tracer binding) of cotransfected cells were exactly between those of A118- and G118-transfected cells, we were unable to detect any negative dominant effects of G118 in this experiment, indicating that the defect either affects translation, or a potential heterodimer has normal stability.

We were unable to ascertain whether in human brain tissues, allelic differences in OPRM1 protein levels are more pronounced than have been expected solely from the 2-fold difference in allelic mRNA levels. This requires analysis of a sufficient number of homozygous tissues of the same brain region for OPRM1-A118 and -G118. In vivo image analysis of OPRM1 expression (for example, positron emission tomography) would be a useful tool to test these results in human brain.

Previous studies have suggested possible mechanisms affecting translation. Specific sequences in exons can control translation through formation of hairpin structures as shown for the angiotensin type 1a receptor (39). Predicted differences in mRNA structure as a result of SNPs have been associated with altered RNA and protein levels (33). For OPRM1, the A118G transition could similarly change the secondary structure of the mRNA and affect its maturation or translation. Our analysis of global and local mRNA structures of wild-type and variant OPRM1 indicate that the A118G transition likely causes secondary structure alteration, which could have resulted in the observed difference in transcription, mRNA maturation, and translation.

Whereas this study documents strong effects of A118G on OPRM1 receptor functions, we have confirmed an allelic imbalance only at the level of mRNA in human brain tissues. Nevertheless, the magnitude of the functional changes observed in vitro and in vivo suggests that the A118G SNP is relevant to OPRM1 function in human subjects. Because we were able to assess allelic expression in only a limited number of subjects in the present study, we cannot exclude the possibility that additional regulatory polymorphisms may exist that could contribute to genetic differences in OPRM1 expression.

Taken alone, clinical association studies using single polymorphisms as indicators are often confounded by the contributions of multiple additional factors in different subject populations. Whereas the evidence has been growing that A118G is associated with substance abuse and response to naltrexone therapy (10, 12, 15, 40), our finding of a clear functional effect strengthens the conclusion that A118G does play a role in susceptibility to substance abuse. However, the interpretation of physiological implications of this result needs to be revisited, from a previously suggested gain of function (18) to a loss of OPRM1 function as shown here.