Characterization of an engineered water-soluble variant of the full-length human mu opioid receptor

Jin Xi[a,*], Jie Xiao[a,*], Jose Manuel Perez-Aguilar[b,d], Jinglei Ping[c], A.T. Charlie Johnson, Jr.[c], Jeffery G. Saven[b], Renyu Liu[a,#]

[a] Department of Anesthesiology and Critical Care, University of Pennsylvania, Philadelphia PA 19104, USA
[b] Department of Chemistry, University of Pennsylvania, Philadelphia PA 19104, USA
[c] Department of Physics and Astronomy, University of Pennsylvania, Philadelphia PA 19104, USA
[d] School of Chemical Sciences, Meritorious Autonomous University of Puebla (BUAP), Puebla, Puebla 72570, Mexico

***Contributed equally as co-first authors**

[#] Corresponding Authors:
Renyu Liu, MD; PhD. Associate Professor, Department of Anesthesiology and Critical Care, Perelman School of Medicine at the University of Pennsylvania
336 John Morgan building, 3620 Hamilton Walk, Philadelphia, PA 19104
Phone: 2157461485; FAX: 2153495078
Email: RenYu.Liu@pennmedicine.upenn.edu

Jeffery G. Saven, PhD. Professor, Department of Chemistry, University of Pennsylvania, 231 S. 34th Street, Philadelphia, PA 19104. Phone: 215-573-6062. Email: saven@sas.upenn.edu

Funding Statement: R.L. acknowledges NIH Grant R01 (1R01GM111421). J.G.S. acknowledges additional support from the Penn Laboratory for Research on the Structure of Matter (NSF DMR-1720530) and NSF CHE 1709518. Peptide modelling and design employed the Extreme Science and Engineering Discovery Environment (XSEDE), which is supported by NSF grant no. ACI-1053575, under grant no. J.P. and A.T.C.J. acknowledge support from the National Science Foundation through grant EFRI 2-DARE 1542879.

Running title: A water soluble variant of human full-length mu opioid receptor

Key words
GPCR |human mu opioid receptor | Water-soluble variant | Thermostability

Abstract

Background: A water-soluble variant of the transmembrane domain of the human mu opioid receptor (wsMOR-TM) was previously characterized. This study explored whether the full-length version of the engineered water-soluble receptor, (wsMOR-FL), could be overexpressed in *E. coli* and if it would retain water solubility, binding capability and thermostability.

Methods: wsMOR was over-expressed and purified in *E. coli* BL21(DE3) cells (EMD/Novagen) as we reported previously for the wsMOR-TM. Both native N and C termini were added back to the highly engineered wsMOR-TM. Six His-tag was added in the N terminus for purification purposes. The wsMOR-FL was characterized using atomic force microscope for its monomeric state, circular dichroism for its secondary structure and thermostability. Its binding with naltrexone is also determined.

Results: Compared to the native human MOR, wsMOR-FL displays similar helical secondary structure content and comparable affinity (nM) for the antagonist naltrexone. The secondary structure of the receptor remains stable within a wide range of pH (6-9). In contrast to the transmembrane portion, the secondary structure of full-length receptor tolerated a wide range of temperature (10-90 ºC). The receptor remains predominantly as a monomer in solution, as directly imaged using atomic force microscopy.

Conclusion: This study demonstrated that functional full-length water-soluble variant of human mu receptor can be over-expressed and purified using an *E. coli* over-expression system. This provides a novel tool for the investigation of structural and functional properties of the human MOR. It appears that the N- and C-termini of the protein may play an important role in strengthening the thermostability of the protein in this specific variant.

Introduction

Although the transmembrane domains (TM) of the opioid receptors having been crystallized and high-resolution structures are available,[1,2,3,4,5] structures of the full-length receptors remain elusive. For these structural studies, receptors were engineered with a stabilizer domain attached to the receptor for the purpose of crystallization. The over-expression of the human mu opioid receptor (MOR) in *E. coli* has been very challenging. In an attempt to massively reproduce functional human MOR, we successfully engineered a water-soluble variant of the TM of MOR (wsMOR-TM).[6] This was accomplished by mutating lipid-contacting surface residues on the TM, so as to resemble those expected in a globular protein of similar size; this variant sequence was subsequently expressed in large quantities in *E. coli*. This water-soluble receptor variant has comparable molecular features to the human native MOR.[6] Moreover, we were also able to characterize opioid and wsMOR-TM interactions in a lipid and label-free system.[7,8]

While the orthosteric binding site of the opioid receptors for opioid ligands is located in the core of the TM helical bundle, the role of both N- and C-termini in the receptor's function cannot be overlooked. These termini play critical roles in the molecular and pharmacological function of the receptor as well. For instance, variations in the N-terminus might affect the analgesic effects of heroin.[9] Furthermore, both the extracellular N-terminus and the intracellular C-terminus play critical roles in ligand interaction, G protein binding and activation, phosphorylation, and subsequent signal transduction.[10,11] Research has dominantly focused on the TM domain of the protein due to the relatively simple topology of the system without considering the effects of the flexible N- and C-termini. Yet, it is clear that in order to have a complete characterization, not only the function of the extracellular N-terminus and intracellular C-terminus need to be fully uncovered but also how their interaction with the TM domain modulates the receptor´s function. Hence, even though the resolution of the crystal structure of the TM segment of the mouse MOR has provides important insights into the function of the TM domain,[2,5,12] the role of the N- and C-termini needs to be investigated vigorously to have an integral mechanistic understanding of MOR. Moreover, even though the mouse MOR TM structure has provided significant information regarding the receptor, no human MOR structure is available yet.[2,5,12]

Thus, as a first step toward performing structure-function relationship studies in human MOR, it is critical to develop a protocol for the robust over-expression of the full-length receptor. In this study, we are proposing an alternative system that may be used as a surrogate for the native human MOR, which fulfills some of the requirements needed to reach this goal. We obtained a water-soluble variant of the full-length human mu receptor (wsMOR-FL) from an *E. coli* over-expression system. We compared the thermostability of the wsMOR-FL with its TM-only version, and thus demonstrated the role of N and C termini on receptor thermostability.

Material and Methods

The human MOR sequence was retrieved from the UniProtKB server (P07550).[13] All required reagents, ligand, and native MORs expressed in HEK293 cells for affinity determination were purchased from Cisbio Bioassays (Bedford, MA). All other chemicals were reagent grade or higher and obtained from Sigma-Aldrich (St. Louis, MO, USA).

Expression and purification of wsMOR-FL. The receptor was over-expressed and purified in *E. coli* BL21(DE3) cells (EMD/Novagen) as we reported previously for the TM water-soluble version, wsMOR-TM. [6] However, both native N and C termini were added back to the highly engineered receptor for the comparison of the sequences. The alignment of the sequence is presented as Table 1.

Table 1. Sequences of the full length native (MOR) and water-soluble mu opioid receptor (wsMOR)

MOR	MGHHHHHHSSGLVPRGSHMDSSAAPTNASNCTDALAYSSCSPAPSPGSWVNLSHLDGNLS
wsMOR	MGHHHHHHSSGLVPRGSHMDSSAAPTNASNCTDALAYSSCSPAPSPGSWVNLSHLDGNLS
MOR	DPCGPNRTDLGGRDSLCPPTGSPSMITAITIMALYSIVCVVGLFGNFLVMYVIVRYTKMK
wsMOR	DPCGPNRTDLGGRDSLCPPTGSPSMITAIKIHEEYKKVCEEGKKGNKLVMEVIVRYTKMK
MOR	TATNIYIFNLALADALATSTLPFQSVNYLMGTWPFGTILCKIVISIDYYNMFTSIFTLCT
wsMOR	TATNIYIFNLAKADALAESTLPFQSVNKLMGTWPFGTILCKKVISIDYYNMFTSIFTLCT
MOR	MSVDRYIAVCHPVKALDFRTPRNAKIINVCNWILSSAIGLPVMFMATTKYRQGSIDCTLT
wsMOR	MSVDRYIAVCHPVKALDFRTPRNAKEENEKNWKLSSEIGKPVEKKATTKYRQGSIDCTLT
MOR	FSHPTWYWENLLKICVFIFAFIMPVLIITVCYGLMILRLKSVRMLSGSKEKDRNLRRITR
wsMOR	FSHPTWYWEDKLKDEVFKKAFEEPVKKIKECYGLMILRLKSVRMLSGSKEKDRNLRRITR
MOR	MVLVVVAVFIVCWTPIHIYVIIKALVTIPETTFQTVSWHFCIALGYTNSCLNPVLYAFLD
wsMOR	MVLVVVEVFIKCWTEIHKYVKEGKLVTIPETTFQTVSWHECIAKGYKNSCENPKLYEELD
MOR	ENFKRCFREFCIPTSSNIEQQNSTRIRQNTRDHPSTANTVDRTNHQLENLEAETAPLP
wsMOR	ENFKRCFREFCIPTSSNIEQQNSTRIRQNTRDHPSTANTVDRTNHQLENLEAETAPLP

N- and C-termini are highlighted in yellow. Letters in red highlight mutated residues in the wsMOR.
Green highlights the restriction sites.

No significant modifications of the method were performed except for adding and N- and C-termini to the sequence (highlighted in yellow in Table 1). A six His-tag was added in the N terminus for purification purposes. The modification of the residues and the structure comparison is presented in Figure 1.

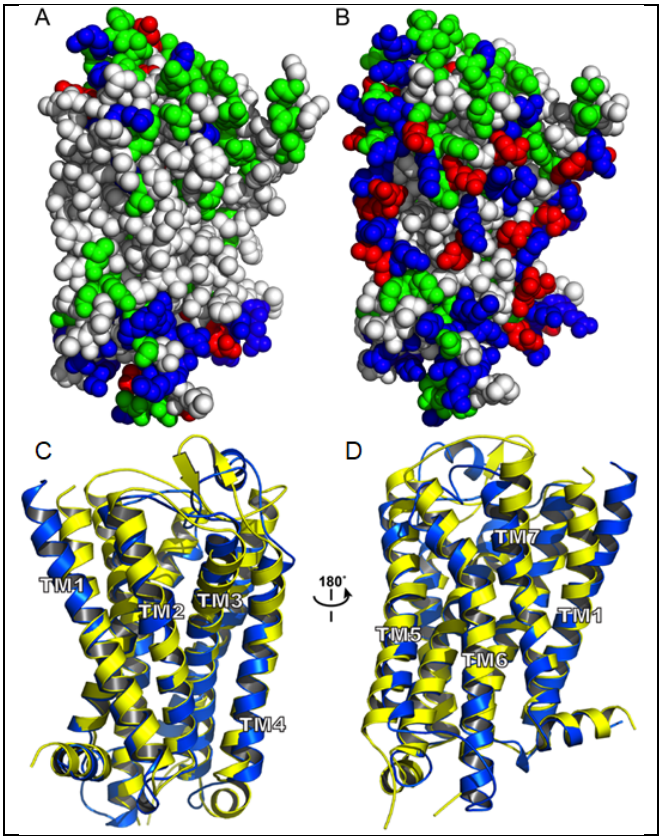

Figure 1. Structure comparison of the water-soluble mu opioid receptor (wsMOR). A) The structure model of the native opioid receptor (modeled from 4DKL); B) The engineered wsMOR structure. Residues are colored by amino acid types: hydrophilic in green (GNQSTY); hydrophobic in white (ACFILMPVW); basic in blue (HKR); and acidic in red (DE). C) and D) Superposition of the mouse mu opioid receptor (yellow from 4KDL) and the wsMOR-TM model (blue). Residues targeted for mutation are those having solvent exposure more than 40% based on calculated estimation. The probabilities of the amino acids at each of the targeted positions are determined by maximizing an effective entropy function described previously from our group and others [14]. The details of the wsMOR design and modeling process have appeared previously [6].

Monomeric states from atomic force microscopy (AFM). We used tapping mode AFM to determine the proportion of monomer and polymers of the wsMOR-FL particles in solution conditions (MFP-3D, Asylum Research, CA) as we described previously. [6,15]AFM is considered a reliable method to investigate protein polymers.[16,17] Solutions containing wsMOR-FL in 20 mM sodium phosphate with 130 mM sodium chloride and 5 mM 2-mercaptoethanol at a pH of 7.0 were incubated for 1 hour on the surface. The surface was washed for 3 min twice with deionized water to remove loosely bound or free protein molecules.

Secondary structure and stability. The secondary structure content was determined using Circular dichroism (CD) (Chirascan, AppliedPhotophysics Limited, Leatherhead, UK). The scan speed was set as 1 nm/s (1 mm path length). Corresponding buffer blanks without receptor were used to subtract the signal from raw data to ensure proper calibration for each assay. At least three sets of experiments were performed to ensure reproducibility and reduce the signal to noise ratio. The helical content of the wsMOR-FL was determined using a spectra deconvolution software.[18]. CD spectroscopy at various temperatures from 10 °C to 90 °C in increments of 2 °C per min were recorded using 6 μM of wsMOR-FL in buffer containing 5 mM sodium phosphate with a pH of 7.0. The temperature-dependent thermostability was analyzed using GraphPad 8.1.2 (GraphPad Software, Inc. La Jolla).

Affinity determination. The affinity of the wsMOR-FL with naltrexone, an antagonist of wild-type MOR, was determined using the same method as we have reported previously.[6] Native MOR on HEK293 cells was used to compete with wsMOR-FL for fluorescent naltrexone (naltrexone that contains the d2 dye as a fluorescent reporter), to establish the competition efficacy as a means to estimate the affinity. The fluorescence emission was detected after excitation at 337 nm at both 620 and 665 nm emission. Affinity for the wsMOR-FL was estimated by obtaining a dose–response curve using the GraphPad 8.1.2. Three separate repeats were obtained to ensure reproducibility.

Statistical Analysis: Data are presented as mean ± SD. Non-paired t-test was used to compare the potential difference between affinities. P less than 0.05 is considered statistically significant.

Results

Overexpression and purification of wsMOR-FL.

Consistent with what has been reported, we were unable to express the native full-length human MOR in *E. coli* despite using same codons used for wsMOR-FL. wsMOR-FL was, however, successfully expressed and purified with high yield. It can be isolated with high purity by affinity chromatography (Fig 1B).[6] To purify wsMOR-FL, we use ~0.1% sodium dodecyl sulfate (SDS), to initiate the purification process as we did in the case of the TM-only version of the wsMOR. The purified wsMOR-FL was clear in solution at ~6 mg/mL in 130 mM NaCl, 20 mM $NaHPO_4$, pH=7.0, after multiple dialyses to remove as much SDS as possible. This is comparable to what we observe with wsMOR-TM, which we have reported previously. AFM results indicate that the wsMOR-FL remains predominantly as a monomeric entity in this solution as shown in Figure 2.

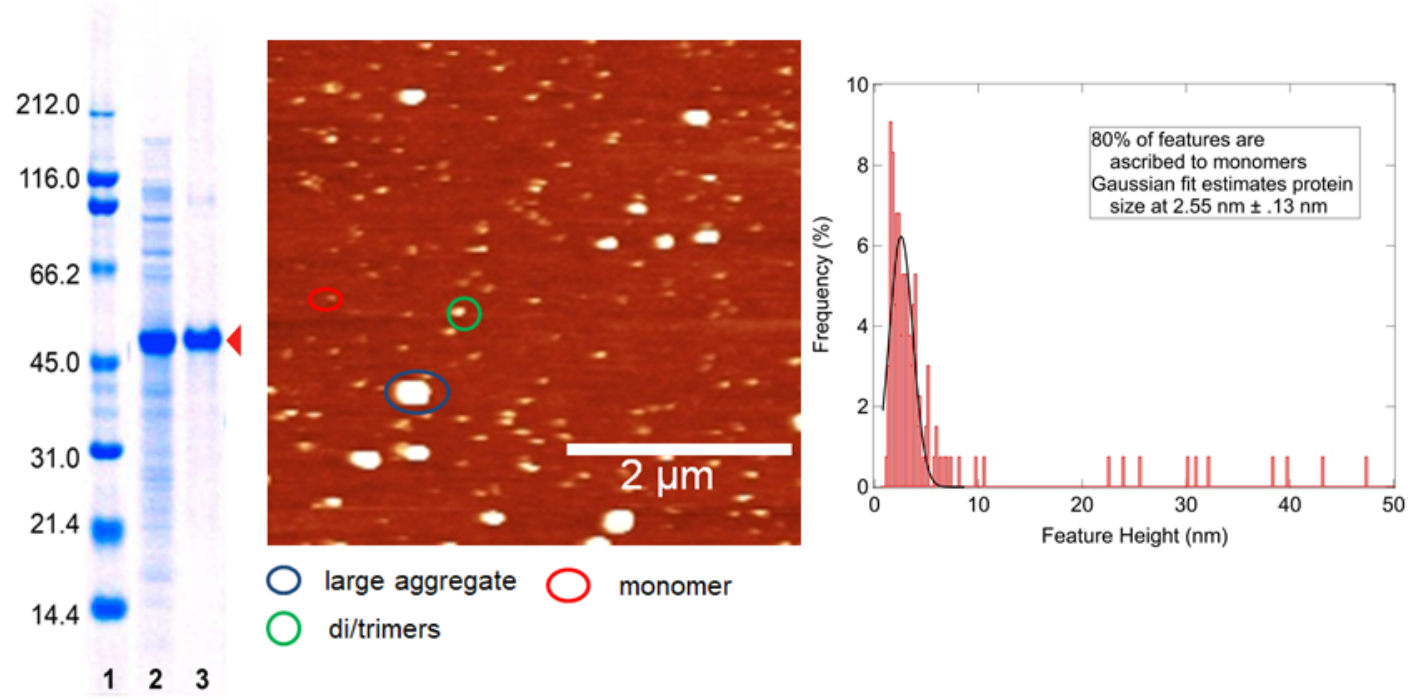

Figure 2. Purification and characterization of receptor. A) A SDS-PAGE gel for wsMOR-FL is shown where lane 1 correspond to the standard, lane 2 to expressed wsMOR-FL in the crude material, and lane 3 to purified wsMOR-FL. The band corresponding to the wsMOR-FL is indicated by a red arrow at appropriate molecular weight. **B)** Tapping mode Atomic Force Microscopy (AFM) image of mu receptor protein on Si/SiO2 substrate. An example of a monomer, dimer and protein aggregate are indicated. Monomer-sized features represent approximately 80% of surface features. Z scale is 8 nm. **C)** Histogram of height of surface features. Monomers are most prevalent, and the Gaussian fit estimates the size of an individual protein monomer as 2.55 nm ± 0.13 nm.

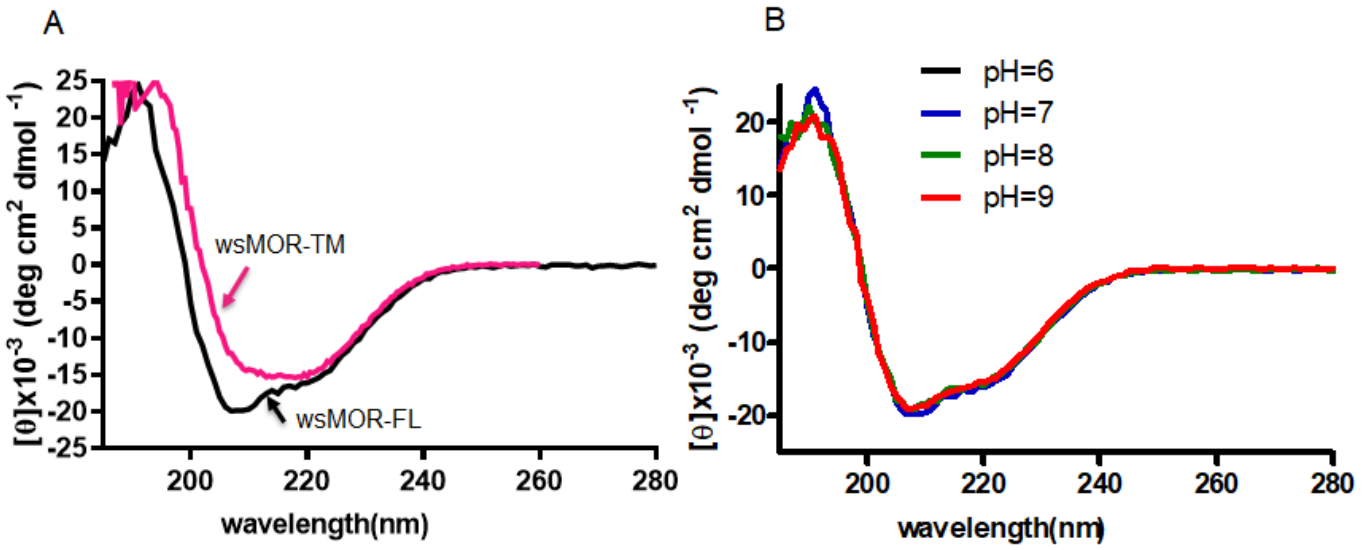

Figure 3. Circular dichroism (CD) spectra of the full-length water-soluble human mu opioid receptor (wsMOR-FL). A: CD spectra comparison between wsMOR-FL and its transmembrane domain only (wsMOR-TM). B: No significant CD spectra changes within the pH range from 6~9, indicating stable secondary structure within the pH tested.

Structure and stability of wsMOR-FL. The CD spectra indicated that the receptor contains a helical content of 37.6% (estimations over the range 205 to 260 nm), similar to that of the native full-length MOR (40.6%). A clear difference in the CD spectra between that for wsMOR-TM and wsMOR-FL is noted (Fig 3A), but each is consistent with helical structure. No significant helical content change was observed with a wide pH range from 6 to 9, indicating stable secondary structure within the pH tested (Fig 3B). While the wsMOR gradually lost ellipticity with the increase of the temperature, wsMOR-FL remains relatively stable within the temperatures evaluated, indicating a significant improvement in thermostability (Fig 4). High concentration (8M) of urea failed to totally unfold the protein. Adding 2-mercaptoethanol in the presence of a high concentration (X M) of urea resulted in loss of ellipticity at 222 nm, consistent with unfolding of the receptor and indicating that disulfide bonds contributed to the stability of the receptor.

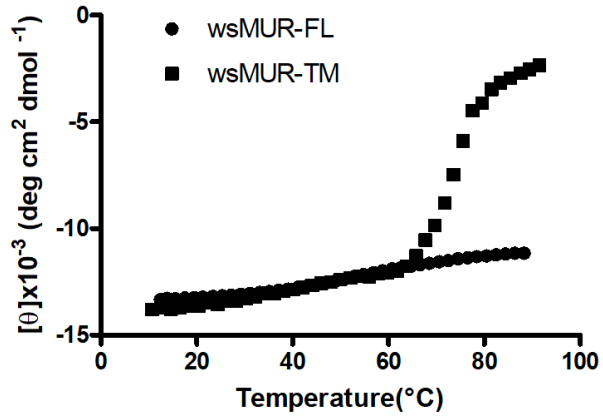

Figure 4. Mean residue ellipticity at 222 nm of wsMOR-FL in buffer solution (5 mM sodium phosphate, pH=7.0) as a function of temperature, from 10 to 90 °C. The spectrum of wsMOR-TM showed significant change near 62 °C and an almost complete loss in molar ellipticity at 90 °C as we reported previously.

wsMOR-FL binding assay. As shown in Figure 5, the ratio of fluorescence emission decreased with incremental concentrations of wsMOR-FL. The K_i of wsMOR-FL were 64 ± 9 nM for naltrexone similar to that for its TM version, wsMOR-TM (67 ± 3 nM, p=0.16 from a non-paired *t* test, Fig 5). No significant fluorescence emission changes were observed with the addition of human serum albumin, a soluble protein with a similar helical structure served as a negative control.

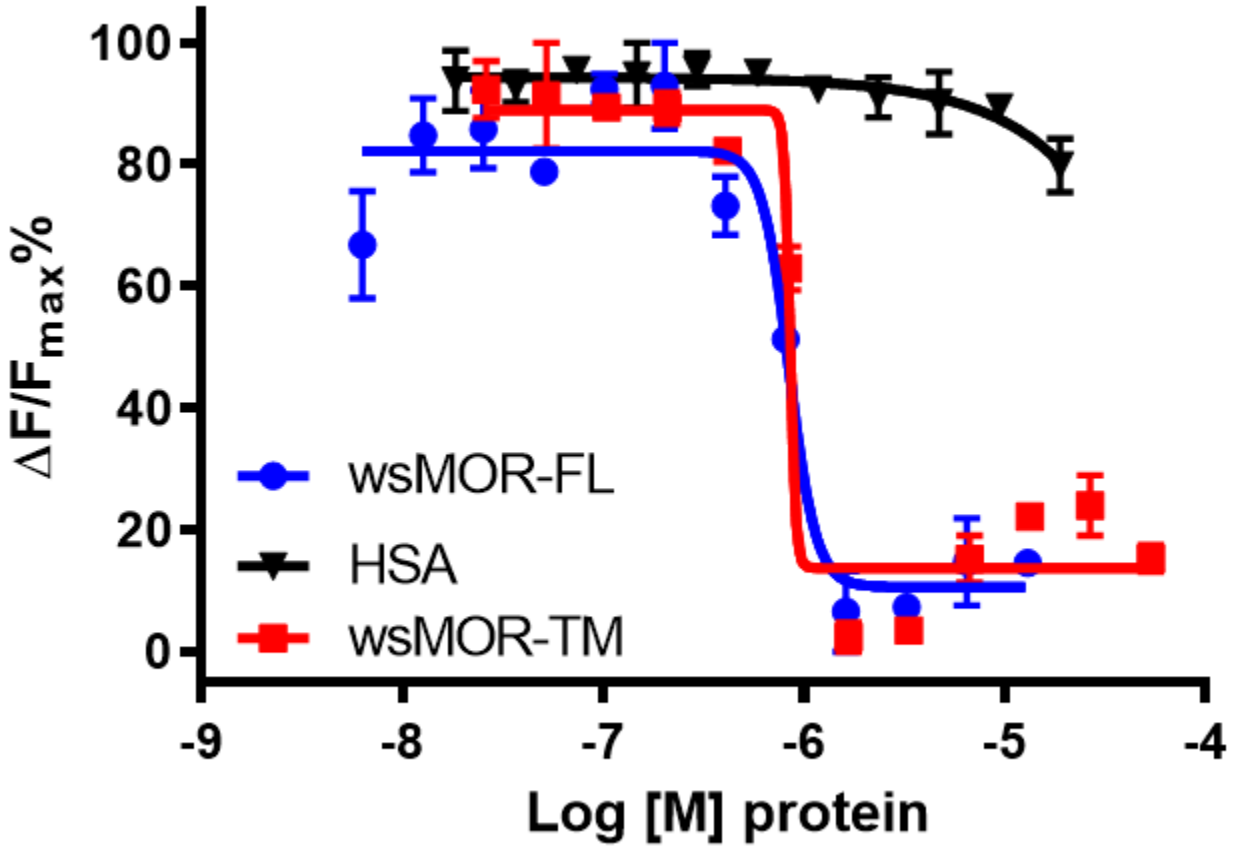

Figure 5. Binding competition assay between the human μ opioid receptor (MOR) expressed in HEK293 cells and the MOR water-soluble variants (wsMOR-FL and wsMOR-TM). Inhibition of the native MOR constitutive signal in the presence of increasing concentrations of wsMOR-FL (blue dots) in sodium phosphate buffer. The Transmembrane portion of the receptor (wsMOR-TM) was used as a positive control (red square). HSA (human serum albumin, inverted black triangles), a water-soluble helical protein, was used as a negative control. Data is used to calculate fluorescence emission ratios, and represent the mean ± standard error of mean of quadruplicates. At least three separate experiments are performed to calculate the affinities.

Discussion

In this study, we have successfully demonstrated that a water-soluble version of the full-length MOR (wsMOR-FL) can be obtained from the *E. coli* heterologous expression system in large (mg) quantities. Also, the inclusion of the extracellular N-terminus and the intracellular C-terminus not only increases the thermodynamic stability of the system, relative to the TM-only water-soluble variant (wsMOR-TM), but also maintains the integrity of the structure over a wide range of pH.[6]

Production of the wsMOR-FL. To express the human mu opioid receptor in *E. coli* in an inclusion body has been reported.[19] Production of large amounts of native full-length human MOR without a fusion partner in *E. coli* has been difficult, the potential toxicity of the native MOR to the bacteria expression system has been discussed.[6,19,20] Some hydrophobic residues (especially those on the exterior surface) of the receptor in the TM domain might be key factors preventing *E. coli* from expressing the receptor. We observed that attempting to express the native receptor stops the growth of the bacteria and consequently, prevents the production of the protein. This is unlikely a codon issue since the same codon was used for the native and wsMOR, but other expression strategies could be employed. Reducing the number of hydrophobic residues on the exterior surface of the receptor successfully allowed the over-expression of the receptor in this hererologous expression system for large production purposes. Our previous efforts to generate a water-soluble variant of MOR was focused on the TM domain as part of efforts to increase the solubility as well as to evaluate its ligand affinity capabilities. From this study, it is clear that adding the entire N- and C-termini did not prevent protein expression, indicating that both termini are not a limiting factor to over-express human MOR in *E. coli*. In other words, we suggest that the native sequence of the N- and C-termini are not the limiting factors affecting the capability to produce large quantities of human native MOR in the bacteria over-expression system. The ability to express and purify large amounts of full-length human MOR from *E. coli* should offer an alternative tool to study structure-function relationships for the receptor in a lipid-free environment. The presence of the extracellular N-terminus and intracellular C-terminus may play critical roles in the molecular and pharmacological function of the protein, especially for the activation of G protein-dependent and G protein-independent signaling pathways. The structure of both termini is unknown, and their function in the G protein-coupled receptors is still unclear, especially for the case of the N terminus.[10] This study provides a novel tool to investigate the structure of the receptor in aqueous solution and to explore in detail the contributions of N- and C-termini to structural stability, protein folding, and function of the receptor.

The structure and thermostability of wsMOR-FL. The CD spectra of the wsMOR-FL demonstrated a predominantly helical structure (37.6%), which is comparable with that for the native full-length MOR expressed in yeast (40.6%).[21,22] Clear differences for the CD spectra between both water-soluble variants, wsMOR-FL and wsMOR-TM, is observed; this is caused solely by the addition of the N- and C-termini.[6] However, it is unclear from this experiment whether the incorporation of the N- and C-termini affected the overall tertiary structure of the receptor. Yet, the N- and C-termini play a critical role in the thermostability of the receptor, since thermostability of wsMOR-FL is much greater than that of wsMOR-TM. Since both termini were included, it is unclear at this point which terminus plays a more dominant role, or if both N- and C-termini are needed to improve the receptor's thermostability. Future studies could be performed by expressing variants containing only the extracellular N-terminus or only the intracellular C-terminus to compare stabilities, structure and binding affinities. Intrinsic disulfide bonds in the human MOR play an important role in the stability of the protein and ligand binding capability. Two cysteine residues forming a conserved disulfide bond between TM3 and the extracellular loop 2, are highly conserved among G protein-coupled receptors, including human MOR. This might affect the conformation of the membrane-spanning domains and ligand binding selectivity.[23] This study confirms that the disulfide bond contributes significantly to the stability of the receptor, since a complete protein unfolding was only achieved with a reducing agent in the presence of a high concentration of urea. While sucrose could be used to stabilize the protein as we demonstrated previously, the intrinsic stability of the protein is critical for its overall structure and function in solution conditions. [15]

Ligand binding for wsMOR-FL. The results clearly indicate that wsMOR-FL has ligand binding affinities in the nanomolar range, which are comparable to those in the TM-only version (wsMOR-TM). This indicates that the N- and C-termini do not contribute significantly to the ligand affinity in this highly isotropic system lacking the lipid environment. Future efforts should focus on whether such highly engineered receptors maintain G protein activation properties in the absence of lipid bilayers.

In conclusion, wsMOR-FL was successfully over-expressed and purified from an *E. coli* protein over-expression system. The inclusion of the native N- and C-termini to the wsMOR did not affect the capability to express the protein nor the binding properties of the water-soluble receptor since they remained similar to that of wsMOR-TM. Yet, by addition the two termini, the thermostability of the receptor was significantly improved. This indicates that the N- and C-termini play a critical role in enhancing the thermostability of the receptor. This wsMOR-FL provides a unique tool to investigate the opioid interactions with the full-length receptor without the need of the lipid environment. Further studies are clearly warranted.

Acknowledgement.
Authors appreciate the paper editing from John R Grothusen, PhD at the Department of Anesthesiology and Critical Care, Perelman School of Medicine at the University of Pennsylvania. The authors also appreciate the outstanding technical support from Qingchen Meng, Ph.D.; at the Department of Anesthesiology and Critical Care at the University of Pennsylvania. TG-CHE110041. J.M.P-A. acknowledges the computing time granted by LANCAD and CONACYT on the supercomputer xiuhcoatl at CGSTIC CINVESTAV.

Conflict of Interest statement: No conflict of interest needs to be reported.

References

1. Granier S, Manglik A, Kruse AC, Kobilka TS, Thian FS, et al. (2012) Structure of the delta-opioid receptor bound to naltrindole. Nature 485: 400-404.
2. Manglik A, Kruse AC, Kobilka TS, Thian FS, Mathiesen JM, et al. (2012) Crystal structure of the micro-opioid receptor bound to a morphinan antagonist. Nature 485: 321-326.
3. Thompson AA, Liu W, Chun E, Katritch V, Wu H, et al. (2012) Structure of the nociceptin/orphanin FQ receptor in complex with a peptide mimetic. Nature 485: 395-399.
4. Wu H, Wacker D, Mileni M, Katritch V, Han GW, et al. (2012) Structure of the human kappa-opioid receptor in complex with JDTic. Nature 485: 327-332.
5. Huang W, Manglik A, Venkatakrishnan AJ, Laeremans T, Feinberg EN, et al. (2015) Structural insights into micro-opioid receptor activation. Nature 524: 315-321.
6. Perez-Aguilar JM, Xi J, Matsunaga F, Cui X, Selling B, et al. (2013) A computationally designed water-soluble variant of a G-protein-coupled receptor: the human mu opioid receptor. PloS one 8: e66009.
7. Ping J, Vishnubhotla R, Xi J, Ducos P, Saven JG, et al. (2018) All-Electronic Quantification of Neuropeptide-Receptor Interaction Using a Bias-Free Functionalized Graphene Microelectrode. ACS Nano 12: 4218-4223.
8. Naylor CH, Kybert NJ, Schneier C, Xi J, Romero G, et al. (2016) Scalable Production of Molybdenum Disulfide Based Biosensors. ACS Nano 10: 6173-6179.
9. Xu J, Xu M, Hurd YL, Pasternak GW, Pan YX (2009) Isolation and characterization of new exon 11-associated N-terminal splice variants of the human mu opioid receptor gene. Journal of neurochemistry 108: 962-972.
10. Coleman JL, Ngo T, Smith NJ (2017) The G protein-coupled receptor N-terminus and receptor signalling: N-tering a new era. Cell Signal 33: 1-9.
11. Deng HB, Yu Y, Pak Y, O'Dowd BF, George SR, et al. (2000) Role for the C-terminus in agonist-induced mu opioid receptor phosphorylation and desensitization. Biochemistry 39: 5492-5499.
12. Koehl A, Hu H, Maeda S, Zhang Y, Qu Q, et al. (2018) Structure of the micro-opioid receptor-Gi protein complex. Nature 558: 547-552.
13. Jain E, Bairoch A, Duvaud S, Phan I, Redaschi N, et al. (2009) Infrastructure for the life sciences: design and implementation of the UniProt website. Bmc Bioinformatics 10: 19.
14. Slovic AM, Kono H, Lear JD, Saven JG, DeGrado WF (2004) Computational design of water-soluble analogues of the potassium channel KcsA. Proc Natl Acad Sci U S A 101: 1828-1833.
15. Zhao X, Perez-Aguilar JM, Matsunaga F, Lerner M, Xi J, et al. (2014) Characterization of a computationally designed water-soluble human mu-opioid receptor variant using available structural information. Anesthesiology 121: 866-875.
16. Gaczynska M, Karpowicz P, Stuart CE, Norton MG, Teckman JH, et al. (2016) AFM Imaging Reveals Topographic Diversity of Wild Type and Z Variant Polymers of Human alpha1-Proteinase Inhibitor. PloS one 11: e0151902.
17. Zhang Y, Hashemi M, Lv Z, Williams B, Popov KI, et al. (2018) High-speed atomic force microscopy reveals structural dynamics of alpha-synuclein monomers and dimers. The Journal of chemical physics 148: 123322.
18. Poschner BC, Reed J, Langosch D, Hofmann MW (2007) An automated application for deconvolution of circular dichroism spectra of small peptides. Anal Biochem 363: 306-308.
19. Ma Y, Kubicek J, Labahn J (2013) Expression and purification of functional human mu opioid receptor from E.coli. PLoS One 8: e56500.
20. Stanasila L, Massotte D, Kieffer BL, Pattus F (1999) Expression of delta, kappa and mu human opioid receptors in Escherichia coli and reconstitution of the high-affinity state for agonist with heterotrimeric G proteins. European journal of biochemistry 260: 430-438.
21. Muller I, Sarramegna V, Milon A, Talmont FJ (2010) The N-terminal end truncated mu-opioid receptor: from expression to circular dichroism analysis. Appl Biochem Biotechnol 160: 2175-2186.

22. Muller I, Sarramegna V, Renault M, Lafaquiere V, Sebai S, et al. (2008) The full-length mu-opioid receptor: a conformational study by circular dichroism in trifluoroethanol and membrane-mimetic environments. J Membr Biol 223: 49-57.
23. Zhang P, Johnson PS, Zollner C, Wang W, Wang Z, et al. (1999) Mutation of human mu opioid receptor extracellular "disulfide cysteine" residues alters ligand binding but does not prevent receptor targeting to the cell plasma membrane. Brain Res Mol Brain Res 72: 195-204.