

## Detecting the mu opioid receptor in brain following SDS-PAGE with multiple approaches

Peng Huang, Lee-Yuan Liu-Chen

Department of Pharmacology and Center for Substance Abuse Research, Temple University School of Medicine, Philadelphia, PA19140

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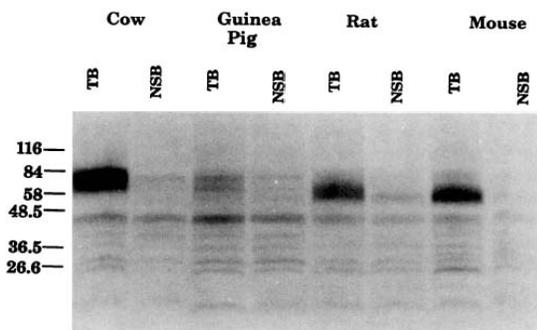
## 1. ABSTRACT

In general, it has been difficult to obtain antibodies which are useful for immunoblotting of endogenous seven-transmembrane receptors (7TMRs) despite the claims made by many companies on commercially available antibodies. In this review, we will use the mu opioid receptor (MOPR) in brain as an example to underscore the importance of using knock-out (K/O) mice and multiple independent approaches (ligand affinity-labeling, receptor phosphorylation and immunoblotting) in identifying 7TMRs following sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE). The rigor and convergence of pharmacological and biochemical data provide confidence on the unequivocal identification of MOPR. The distinct relative molecular masses (Mr's) and band patterns are largely due to variations in the extent of N-glycosylation in different cell lines, brain regions and species.

## 2. INTRODUCTION

In literature, the MOPR bands detected by nonimmunological or immunological methods following SDS-PAGE can be divided into two categories: broad and diffuse ones with higher relative molecular masses (Mr's) vs. sharp ones with lower Mr's. Some researchers reported the MOPR as sharp band(s) with Mr's between 40 and 60 kDa in mouse or rat neuronal tissues by immunoblottings [For examples, see (1) and (2)]. In contrast, as summarized in this review, other researchers including our group have demonstrated the MOPR mainly as a diffuse band by covalent labeling with [<sup>3</sup>H]beta-funaltrexamine ([<sup>3</sup>H]beta-FNA), receptor phosphorylation and immunoblotting with anti-MOPR antibodies. This single broad band has a Mr range between 58 to 97 kDa and the median Mr above 62 kDa, depending on cell lines, brain regions and species. The broad and diffuse nature of the protein bands indicates that MOPRs are glycoproteins. Upon deglycosylation to

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**Figure 1.** Covalent labeling with [ $^3\text{H}$ ]beta-FNA of MOPRs in bovine striatal membranes and guinea pig, rat, and mouse brain membranes. MOPR in membranes were incubated with 5 nM [ $^3\text{H}$ ]beta-FNA in the absence and presence of 10 microM naloxone for total binding (TB) and nonspecific binding (NSB), respectively. For each species, equal amounts of protein were loaded for total binding and nonspecific binding, i.e., 7.3 mg for the cow, 4.2 mg for the guinea pig, 3.9 mg for the rat, and 5.2 mg for the mouse. Radioactivities loaded in each lane were 9900 dpm (cow, TB), 4200 dpm (cow, NSB), 5700 dpm (guinea pig, TB), 3300 dpm (guinea pig, NSB), 5400 dpm (rat, TB), 3000 dpm (rat, NSB), 8400 dpm (mouse, TB), and 3600 dpm (mouse, NSB). Molecular mass standards are in kDa. Note that for each species there was one radiolabeled protein band in which the labeling was greatly reduced by naloxone. [Reprinted with permission from Figure 2 of Liu-Chen *et al.* (4)].

remove N-linked glycans, MOPRs became sharp bands with Mr's close to the theoretical molecular mass (~43 kDa) of the deduced amino acid sequences.

### 3. LIGAND AFFINITY-LABELING: THE [ $^3\text{H}$ ]BETA-FNA-LABELED MOPR IS HETEROGENEOUS IN MR'S DUE TO DIFFERENTIAL N-LINKED GLYCOSYLATION, DEPENDING ON SPECIES AND EXPRESSION SYSTEMS

In 1987, Liu-Chen and Phillips (3) showed that [ $^3\text{H}$ ]beta-FNA (5 nM) bound specifically and covalently to MOPR in bovine striatal membranes, which was blocked by naloxone and selective MOPR ligands, but not by selective delta opioid receptor (DOPR) or kappa opioid receptor (KOPR) ligands. SDS-PAGE under denaturing and reducing conditions followed by fluorography demonstrated that among several protein bands labeled by [ $^3\text{H}$ ]beta-FNA in the total binding preparation, there was one band in which labeling was greatly reduced by 10 microM of naloxone. [ $^3\text{H}$ ]beta-FNA-labeled MOPR migrated as a diffuse and broad band with a molecular weight range of 68 to 97 kDa, indicating that the MOPR is a glycoprotein. Subsequently, Liu-Chen and colleagues (4) showed that, in brain membranes of guinea pigs, rats, and mice, [ $^3\text{H}$ ]beta-FNA similarly covalently labeled MOPRs. The MOPRs migrated as broad and diffuse protein bands of different Mr's in these species (Figure 1), in the order (high to low) of cow, guinea pig, rat, and mouse. The medians of Mr's were 77, 72, 67, and 66 kDa for the cow, guinea pig,

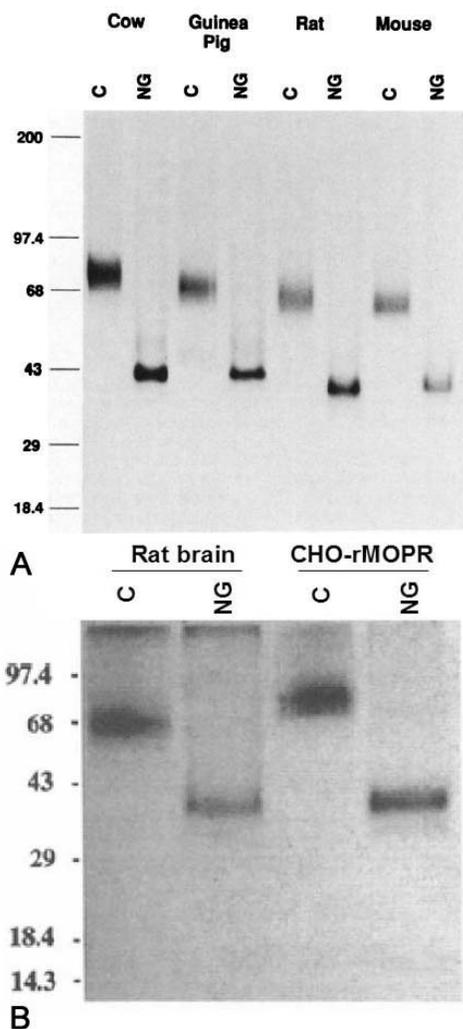
rat, and mouse, respectively (Figure 1) (4).

[ $^3\text{H}$ ]beta-FNA-labeled brain membranes were solubilized and subjected to wheat germ agglutinin (WGA) affinity chromatography. MOPR was purified ~30 fold and in the total labeling preparation there was one broad and highly radioactively labeled protein band (Figure 2A, C lanes) (3,4), whereas in the nonspecific labeling preparation only a very faintly labeled band was observed in the same molecular mass range (data not shown). No other labeled protein band was observed in either preparation. These results indicate that the MOPR is the only [ $^3\text{H}$ ]beta-FNA-labeled protein in the eluate following WGA affinity chromatography. In each species, the labeled MOPR migrated as a broad band with Mr's of 70-88 kDa (median, 77 kDa), 66-80 kDa (median, 72 kDa), 60-75 kDa (median, 67 kDa), and 60-72 kDa (median, 66 kDa) for the cow, guinea pig, rat, and mouse, respectively (Figure 2A, C lanes) (4). To determine whether the variation in the Mr of MOPR among these four species was due to different degrees of glycosylation, WGA eluates were treated with *N*-peptide:N-glycosidase F (PNGase F) to remove N-linked glycans. After treatment, the labeled protein band of higher Mr's disappeared and there was one highly radioactively labeled sharp band for each species. This band had similar Mr's for all four species: 43 kDa for the cow and guinea pig, 39 kDa for the rat, and 40 kDa for the mouse (Figure 2A, NG lanes) (4), indicating that the variation in the Mr's of MOPRs appears to be largely due to different degrees of N-glycosylation. This was confirmed when MOPRs were cloned from these species. Amino acid sequences deduced from MOPR-1 cDNA clones for different species have similar numbers of amino acid (aa) residues: cow, 401 aa; human and guinea pig, 400 aa; rat and mouse, 398 aa. Although there are only 2- or 3-aa difference between cow/guinea pig and rat/mouse, there is 3-4 kDa difference in the Mr's. The reason for this discrepancy is not entirely clear. Amino acid sequence comparison between rat and bovine MOPRs shows that the two, although highly homologous, have some differences in the N-terminal domains. The sequence differences may lead to differential SDS binding and cause anomalous behaviors in SDS-PAGE.

The cloned rat MOPR-1 and endogenous MOPR in the rat brain were also compared (5). As shown in Figure 2B, [ $^3\text{H}$ ]beta-FNA specifically labeled one band with mass of 70-89 kDa (median: 80 kDa) in membranes of Chinese hamster ovary cells stably expressing the rat MOPR (CHO-rMOPR) (lane 3), and one band with mass of 61-78 kDa (median: 67 kDa) in the WGA-purified rat brain preparation (lane 1) (5). After deglycosylation with PNGase F to remove N-linked glycans, both protein bands in the CHO-rMOPR membranes and the rat brain preparation became sharp bands of 40 kDa (Figure 2B, lanes 2 and 4) (5). These results indicate that the difference in the Mr of cloned receptors expressed in CHO cells and native receptors reflects differences in the extent of N-glycosylation.

The studies using [ $^3\text{H}$ ]beta-FNA labeling (4,5) were consistent with the finding of Eppler *et al.* (6) in

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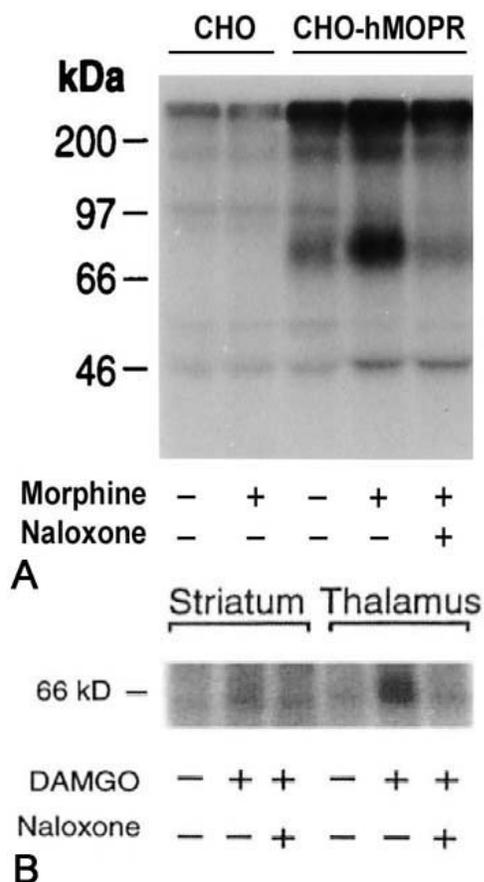
**Figure 2.** (A) Effect of PNGase F on [ $^3$ H]beta-FNA-labeled MOPR in the WGA affinity-purified brain membrane preparations of the cow, guinea pig, rat, and mouse. WGA eluates of the four species (each containing ~10,000 dpm) were treated with PNGase F for 6 h and subjected to SDS-PAGE and fluorography. C, Control; NG, treated with PNGase F. Amounts of radioactivity and protein loaded in each lane were approximately 10,000 dpm and 200 microG, respectively. Molecular mass standards are in kDa. (Reprinted from Figure 6B of Liu-Chen *et al.*, ref. (4), with permission of copyright 1993 The American Society for Pharmacology and Experimental Therapeutics). (B) Deglycosylation by PNGase F of [ $^3$ H]beta-FNA-labeled MOPR in the WGA affinity-purified rat brain preparation and CHO-rMOPR membranes. The WGA affinity-purified rat brain preparation and solubilized CHO-rMOPR membranes were treated with or without PNGase F for 6 h and subjected to SDS-PAGE and fluorography. Lanes 1 and 2, rat brain WGL preparation, control and PNGase F-treated, respectively, 12,400 dpm and 300 microG of protein each. Lanes 3 and 4, CHO-rMOPR, control and PNGase F-treated, respectively, 24,000 dpm and 600 microG of protein each. Exposure time was 5 days. [Reprinted with permission from Figure 3 of Chen *et al.*, ref. (5)].

1993. Using ligand affinity-labeling, these researchers showed that (epsilon-biotinyl-Lys32) beta-endorphin bound to an opioid receptor protein in rat brain membranes, which was blocked by naloxone and (D-Ala2, N-MePhe4,Glyol5)enkephalin (DAMGO), but not by (D-Pen2,D-Pen5)enkephalin (DPDPE). The receptor-ligand complex was solubilized and the receptor was purified with immobilized streptavidin and wheat germ agglutinin. The purified glycoprotein had a broad Mr range of 60-70 kDa, which became ~40-kDa after deglycosylation. Partial amino acid sequence identified this protein to be the MOPR.

### 4. AGONIST-PROMOTED MOPR PHOSPHORYLATION: PHOSPHORYLATED MOPR WAS SHOWN AS BANDS WITH MEDIAN MR'S ABOVE 65 KDA

Agonist-induced MOPR phosphorylation was first demonstrated by Wang and colleagues in 1996 for the cloned human MOPR (hMOPR) (7). Both CHO-hMOPR and nontransfected CHO cells incubated for 2 h with [ $^{32}$ P]orthophosphoric acid to label the ATP pool and stimulated with 1 microM morphine for 20 min. Cells were solubilized, immunoprecipitated with specific polyclonal anti-MOPR antisera generated against the C-terminal last 18 amino acids of human MOPR (383-400) resolved on SDS-PAGE. Autoradiography revealed that in addition to a number of common phosphoprotein bands, CHO-hMOPR displayed a broad 70-85 kDa phosphoprotein not found in control CHO cells (Figure 3A). There was a modest level of basal receptor phosphorylation and morphine treatment increased it 5-fold. Naloxone (10 microM) blocked this morphine-induced phosphorylation (Figure 3A). The phosphorylated MOPR band pattern in Chinese hamster ovary (CHO) cells were confirmed in human embryonic kidney (HEK293) cells and Neuro 2A cells by several groups later following activation of MOPR by agonists such as DAMGO, including Carman *et al.* (8), El Kouhen *et al.* (9), Zhang *et al.* (10), and Schmidt *et al.* (11). It is noteworthy that morphine-induced phosphorylated MOPR band were not observed in HEK293 cells unless GRK2 (G protein-coupled receptor kinase 2) was overexpressed (8,10).

Wang and colleagues subsequently developed a method to detect phosphorylation of the MOPR in the rat brain by labeling striatum and thalamus slices with [ $^{32}$ P]orthophosphoric acid and immunoprecipitating the receptor with anti-MOPR antiserum (12). They identified phosphorylated MOPR as a broad and diffuse band with the median Mr of 66 kDa in thalamus (Figure 3B). In the thalamus, there was basal MOPR phosphorylation, and DAMGO enhanced it by three-fold which was blocked by the antagonist naloxone (Figure 3B). The level of MOPR phosphorylation in the striatum was much lower than that in the thalamus (Figure 3B). No MOPR phosphorylation was found in the cerebellum, even with DAMGO stimulation (data not shown), which is consistent with previous reports that the cerebellum has no MOPR.



**Figure 3.** (A) Agonist-promoted phosphorylation of the MOPR: autoradiogram of phosphoproteins extracted from control CHO cells (lanes 1 and 2) and human MOPR-expressing CHO cells (lanes 3-5) Cells were pretreated for 20 min at 37 °C with 1 microM morphine (lanes 2 and 4) or 1 microM morphine and 1 microM naloxone (lane 5), immunoprecipitated with anti-MOPR antibodies and separated on SDS-PAGE. Dried gels were exposed to X-ray films for 2 days, and size standards were derived from prestained markers electrophoresed in adjacent lanes. [Modified with permission from Figure 3 of Zhang *et al.*, ref. (7)]. (B) Agonist-promoted phosphorylation of MOPR in the rat striatum and thalamus. Tissue slices were pretreated for 20 min at 30 °C with no drug (basal), 1 microM DAMGO with or without 2 microM naloxone as indicated. Tissues were solubilized, immunoprecipitated with anti-MOPR antibodies, and separated on an SDS-PAGE gel. Dried gels were exposed to X-ray films for 3 days. [Reprinted with permission from Figure 2A of Deng *et al.*, ref. (12)].

**5. WESTERN BLOT: MOPR HAS BRAIN REGION-SPECIFIC HETEROGENEITY IN N-GLYCOSYLATION REVEALED BY AN ANTI-MOPR ANTIBODY AND BRAIN TISSUES FROM MOPR-KNOCKOUT MICE**

We have recently generated anti-muC, a rabbit polyclonal anti-MOPR antibody against the sequence

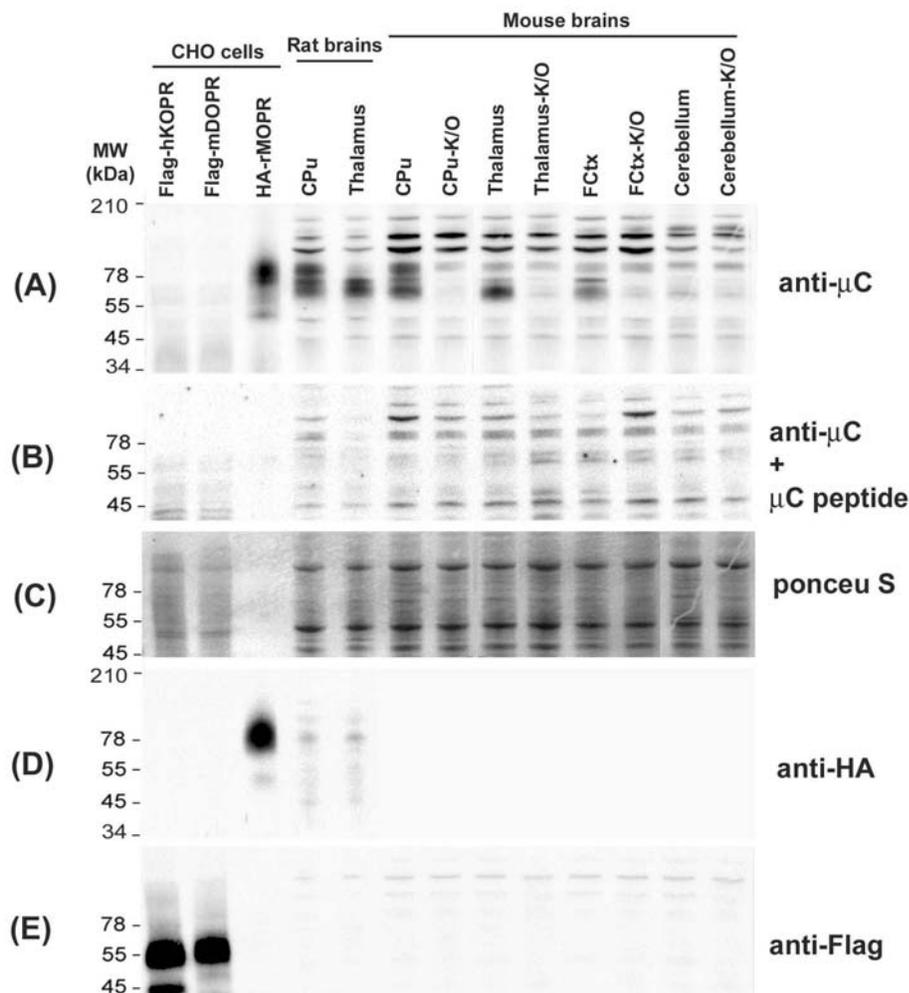
CT<sup>383</sup>NHQLLENLEAETAPLP<sup>398</sup>, which corresponds to the last 16 amino acids of the C-terminal domain predicted from the cloned MOPR-1 (aa383-398 for the rat and mouse MOPR, and aa385-400 for the human MOPR) and which is identical among human, rat and mouse (13). The anti-muC was purified by muC peptide affinity chromatography, and in addition, labeled with biotin (13). By use of this antibody and its biotinylated form, MOPR expressed in CHO cells and endogenous MOPR in brains were detected by immunoblotting unambiguously with high specificity. It also unexpectedly revealed heterogeneity in N-linked glycosylation in some brain regions (13).

Immunoblotting was first carried out in CHO-HA-rMOPR cells with both anti-muC and anti-HA. Anti-muC labeled a major broad band with a median Mr of 78 kDa and a minor lower band of Mr 52 kDa (Figure 4A, lane 3), which were similar to the bands labeled with anti-HA (Figure 4D, lane 3), indicating that anti-muC can be used for immunoblotting. Staining of both bands was blocked by preincubation of the antibody with the muC peptide. Anti-muC and anti-HA detected no specific bands in either CHO-FLAG-hKOPR (human kappa opioid receptor) or CHO-FLAG-mDOPR (mouse delta opioid receptor) cells (Figure 4A and 4D, lanes 1 and 2). Blotting with anti-FLAG antibodies revealed the DOPR and KOPR mainly as broad bands with median Mr's of 58 kDa and 55 kDa, respectively (Figure 4E, lanes 1 and 2). The 78 kDa diffuse band revealed by anti-muC immunoblotting is consistent with an early finding of Surratt *et al.* (14) with untagged MOPR expressed in COS cells and an antibody developed against a similar epitope. This band is also similar to those in publications on immunoblotting of HA-tagged or Flag-tagged MOPR in CHO, Neuro2A or HEK293 cells from different groups (11,15,16,17,18).

Similar immunoblotting was then carried out on brain membranes to identify endogenous MOPR (Figure 4A, Lanes 4-13). Anti-muC detected multiple bands in rat and mouse brain regions including CPu (caudate putamen), thalamus and frontal cortex. The staining of most bands was reduced or abrogated by pre-incubation of anti-muC with the muC peptide, making it difficult to definitively identify which bands are the MOPR. We therefore used MOPR knockout mouse brains as the control. These MOPR knockout mice were generated by Pintar and his colleagues by replacing the exon 1 with the Neo cassette (19).

CPu, thalamus, frontal cortex and cerebellum were dissected from brains of littermates of wild-type and MOPR-knockout (K/O) mice and membranes were prepared. Western blotting revealed that in wild-type mice, anti-muC labeled several bands in the CPu, one of which was absent in the MOPR-K/O mice (Figure 4A, lanes 6 and 7), indicating that this protein band, with a Mr range of 60-84 kDa (median, 74 kDa), represents the MOPR. Similarly, in the thalamus and frontal cortex, one of the bands labeled by anti-muC in the wildtype was not present in the MOPR-K/O mice (Figure 4A, lanes 8, 9 and lanes 10, 11). However, surprisingly, the MOPR bands in the thalamus and frontal cortex were narrower and had lower Mr (58-68 kDa (median, 63 kDa)) (Figure 4A, lanes 8 and 10). The

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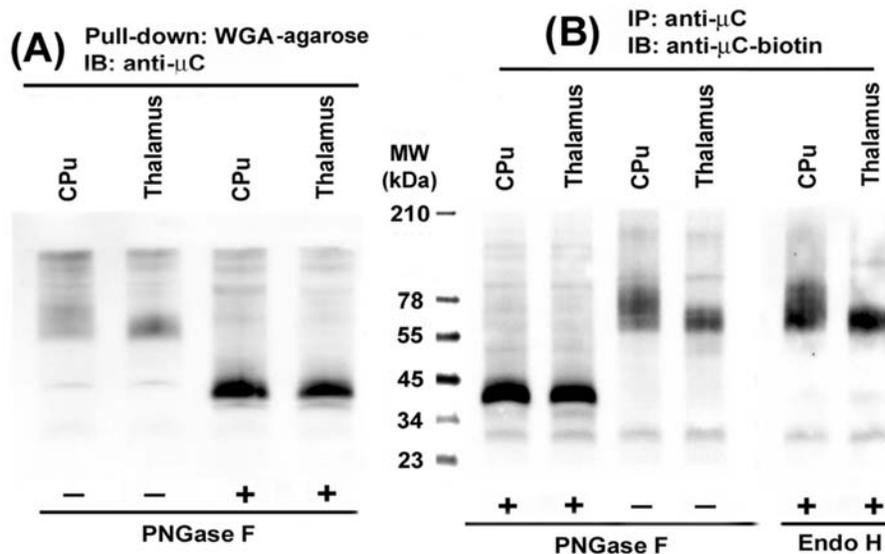


**Figure 4.** Immunoblotting of the rat MOPR stably expressed in CHO cells and the endogenous MOPR in the rat and mouse brains. (A) Immunoblotting was performed with anti- $\mu$ C, a polyclonal anti-MOPR antibody (1:5000, final 0.26 microG/ml) on CHO cells stably expressing FLAG-hKOPR, FLAG-mDOPR and HA-rMOPR (lanes 1-3), membranes prepared from CPu and thalamus of rat brains (lanes 4 and 5) or from CPu, thalamus, frontal cortex (FCtx) and cerebellum of wild-type and MOPR-K/O mice (lanes 6-13). (B) Immunoblotting was performed the same way as in (A), except with anti- $\mu$ C pre-incubated with the  $\mu$ C peptide (0.6 microG/ml). (C) The same membrane in (A) was stained with Ponceu S to show protein loading amounts. (D) The same membrane in (A) was stripped and blotted with an anti-HA monoclonal antibody (HA.11) (1:5000). (E) Immunoblotting was performed with similar protein contents on the membranes in (A) with an anti-Flag polyclonal antibody.

labeling of both kinds of bands was completely blocked by preadsorption of anti- $\mu$ C with the  $\mu$ C peptide (Figure 4B, lanes 6-11). There was no difference in labeling in cerebella of wild-type and MOPR-K/O mice (Figure 4A, lanes 12 and 13). Similar amounts of proteins were loaded for each mouse tissue (30 microG per lane) as demonstrated by Ponceu S staining of the membranes (Figure 4C, lanes 6-13). These results demonstrate that MOPR knockout mouse brains allow us to unequivocally identify MOPR protein bands.

Immunoblotting with anti- $\mu$ C was subsequently performed on membranes of the rat brains (Figure 4A, lanes 4 and 5). Based on the results on the mouse brains, we identified MOPR in the rat CPu as a broad and diffuse

band with a Mr range of 61-84 kDa (median, 75 kDa), and that in the rat thalamus as a narrow and diffuse band (60-72 kDa) with a smaller median Mr of 66 kDa. Both bands were completely blocked by pre-adsorption with the  $\mu$ C peptide (Figure 4B, lanes 4 and 5). Thus, a similar discrepancy in Mr of the MOPR between CPu and thalamus was observed in the rat as that in the mouse (13). The 66-kDa band of MOPR in rat thalamus was in accord with that detected with either antibody or receptor phosphorylation (Figure 3B) by Wang and colleagues (12). In addition, the diffuse MOPR bands were more prominent with much less non-specific signals when immunoblotting was carried out with fractions of lipid rafts preparation (by use of sodium carbonate) for rat CPu and thalamus membranes (data not shown) (13).



**Figure 5.** Deglycosylation of MOPR in rat CPu and thalamus. Membranes of the rat CPu or thalamus were solubilized with 2% Triton X-100. (A) The solubilized preparations were applied to a WGA-agarose column and the bound glycoproteins were eluted with 0.25 M N-acetyl-D-glucosamine. The eluate was left untreated or treated with PNGase F, resolved with 8% SDS-PAGE, and immunoblotted with anti- $\mu$ C (1:5000). (B) The solubilized preparations were immunoprecipitated with anti- $\mu$ C followed by PANSORBIN (Calbiochem), dissolved in 5% SDS/0.4 M DTT and left untreated or treated with PNGase F or Endo H. Samples were analyzed by 8% SDS-PAGE and immunoblotting was performed with anti- $\mu$ C-biotin (1:5000) and anti-biotin-HRP-conjugate (1:5000). [Reprinted with permission from Figure 2 of Huang *et al.* ref. (13)].

PNGase F treatment of the WGA affinity-purified rat brain MOPR, which removes all N-linked glycans, resulted in an increase in the mobility of MOPR in the CPu or thalamus on SDS-PAGE (Figure 5A, lanes 3 and 4), compared with the untreated controls (Figure 5A, lanes 1 and 2). More importantly, the diffuse bands with different widths and median Mr's (Figure 5A, lanes 1 and 2) in the two regions became sharp bands with identical Mr's (~40 kDa) which is in accord with the theoretical molecular mass of MOPR (Figure 5A, lanes 3 and 4). Anti- $\mu$ C-precipitated MOPR of the rat CPu or thalamus was also treated with PNGase F, yielding a similar observation by use of biotinylated anti- $\mu$ C for detection (Figure 5B, lanes 1-4). Thus, the difference in Mr of the MOPR in the thalamus and CPu is due to differential N-linked glycosylation. In addition, Endoglycosidase H (Endo H) treatment, which cleaves N-linked glycans of high-mannose and some hybrid types, caused no mobility changes of the MOPR of CPu and thalamus (Figure 5B, lanes 5 and 6). These results indicate that the MOR in the CPu and thalamus contains complex types N-linked glycans of varying numbers and/or carbohydrate components (13).

Many 7TMRs, including opioid receptors, possess one or more putative N-glycosylation motifs (Asn-X-Ser/Thr) in their amino-terminal extracellular portion. The nucleotide sequences of full-length MOPR-1 cDNAs from rat CPu and thalamus were identical as determined by RT-PCR and DNA sequence determination (data not shown). Thus, rat brain region-specific N-glycosylation was not due to RNA editing of the five potential N-

glycosylation sites in the receptor N-terminal domain.

In CHO cells, in addition to the broad and diffuse 78-kDa band, there was a less diffuse 52-kDa band (Figure 4A and 4D, lane 3); however, in the brain, only the larger band was detected. The 52-kDa band most likely represents glycosylated intermediate form of the MOPR in the intracellular compartments, which was not phosphorylated upon agonist activation (Figure 3A) since phosphorylation occurs to receptors on the cell surface. It should be noted that it was not possible to detect if [ $^3$ H]beta-FNA labeled the 52-kDa band of the MOPR in CHO cells due to the low sensitivity of fluorography for  $^3$ H and only the fully glycosylated MOPR remained following WGA affinity chromatography (5). Therefore, it appears that there are little or no such intermediates in the brain. Previous immunoblotting studies also showed MOPR in rat brain as similar broad and diffuse bands at higher molecular range, by use of the whole brain (20) or one brain region (12).

With different approaches or results from different publications, the detected MOR bands from the same tissue source have slight differences in the estimated Mr's, which may be due to differences in protein markers, buffers (Tris-Glycine vs. Tricine), gel concentration and other gel-running conditions used. In addition, when partially purified MOPR was used, the purification conditions, such as the detergent and buffer which the receptor was in and the affinity chromatography (antibody or ligand) used, may also introduce variations. Nevertheless, three lines of evidence converged well and together supported the identification of MOPR in SDS-PAGE mainly as a single

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broad and diffuse band with varying broadness and different median sizes ranging from 63 kDa to 80 kDa. The Mr's are much larger than the theoretical molecular mass (~43 kDa) of MOPR, largely being ascribed to heterogeneity of N-glycosylation in different cells, brain areas and species. The possible physiological impact of differential N-glycosylation of MOPR may be related to levels of the receptor distributed in cholesterol and glycolipids-enriched membrane subdomains (lipid rafts) in brain, and may affect receptor signaling, phosphorylation and desensitization (13).

### 6. CONCLUSION

Our experience demonstrates that it is difficult to identify endogenous MOPR by immunoblotting, similar to other 7TMRs, due to its low abundance *in vivo* and heavy and heterogeneous glycosylation. Many antibodies against 7TMRs are not suitable for immunoblotting even after purification with antigen affinity chromatography, although they may be useful for immunohistochemistry or immunoprecipitation. It is fortuitous if one can obtain good antibodies for immunoblotting. When attempting to identify protein bands for untagged 7TMRs, one needs to take multiple approaches and determine if there is a convergence of data. Results from knockout animals are particularly useful for identification of the receptor.

### 7. ACKNOWLEDGEMENTS

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**Abbreviations:** 7TMRs, seven transmembrane receptors; aa, amino acid; beta-FNA, beta-funaltrexamine; CHO cells, Chinese hamster ovary cells; CPu, caudate putamen; DAMGO, [D-Ala<sup>2</sup>,N-MePhe<sup>4</sup>,Gly<sup>ol5</sup>]enkephalin; DOPR, delta opioid receptor; DPDPE, [D-Pen<sup>2</sup>,D-Pen<sup>5</sup>]enkephalin; Endo H, endoglycosidase H; HEK293 cells, human embryonic kidney cells; K/O, knockout; KOPR, kappa opioid receptor; GRK2, G protein-coupled receptor kinase 2; MOPR, mu opioid receptor; Mr's, relative molecular masses; PNGase F, Peptide: N-Glycosidase F; SDS-PAGE, sodium dodecyl sulfate - polyacrylamide gel electrophoresis; WGA, wheat germ agglutinin.

**Key Words:** The Mu Opioid Receptor, Brain, Glycosylation, Beta-FNA, Phosphorylation, Immunoblotting, Review

**Send correspondence to:** Lee-Yuan Liu-Chen, Department of Pharmacology, Temple University School of Medicine, 3420 North Broad Street, Philadelphia, PA 19140, USA, Tel: 215-707-4188, Fax: 215-707-7068, E-mail: lliuche@temple.edu

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