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Biochemical and Biophysical Research Communications 365 (2008) 82–88
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Brain region-specific N-glycosylation and lipid rafts association of the rat mu opioid receptor

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Received 27 September 2007
Available online 31 October 2007

Abstract

The mu opioid receptor (MOR) in the rat and mouse caudate putamen (CPu) and thalamus was demonstrated as diffuse and broad bands by Western blot with a polyclonal antibody against a C-terminal peptide of MOR, which were absent in the cerebellum and brains of MOR-knockout mice. The electrophoretic mobility of MOR differed in the two brain regions with median relative molecular masses (Mr's) of 75 kDa (CPu) vs. 66 kDa (thalamus) for the rat, and 74 kDa (CPu) vs. 63 kDa (thalamus) for the mouse, which was due to its differential N-glycosylation. Rat MOR in CPu was found mainly associated with low-density cholesterol- and ganglioside M1 (GM1)-enriched membrane subdomains (lipid rafts), while the MOR in the thalamus was present in rafts and non-rafts without preference. Cholesterol reduction by methyl-β-cyclodextrin decreased DAGMO-induced [^{35}S]GTPγS binding in rat CPu membranes to a greater extent than in the thalamus membranes.

Keywords: Opioid receptors; Glycosylation; N-Glycans; Lipid rafts; Brain

Opioid drugs act on at least three types of opioid receptors: mu, delta, and kappa (MOR, DOR, and KOR), respectively [1]. They are coupled via pertussis toxin-sensitive G_i/G_o proteins to a variety of effectors that include adenylate cyclase, potassium channels, calcium channels, and mitogen-activated protein kinase pathways (for a review, see [2]). Morphine acts primarily on the MOR [3] and is among the most widely abused drugs. MOR is widely distributed in neurons throughout the brain and spinal cord [4,5].

Many G protein-coupled receptors (GPCRs), including opioid receptors, possess one or more putative N-glycosylation motifs (Asn-X-Ser/Thr) in their amino-terminal extracellular portion. N-glycosylation has been found to regulate some GPCRs with regard to their export trafficking [6,39], ligand-induced internalization [7,39] and degradation [8,39]. Recently, specific sugar binding receptors (lectins) have been shown to be important for the surface expression of N-glycosylated membrane proteins including GPCRs [9–11].

Lipid rafts are small membrane domains (10–200 nm) enriched in cholesterol and glycosphingolipids (e.g., GM1) [12]. They are thought to serve as platforms for various cellular processes and considered to be unstable, heterogeneous, and dynamically produced and degraded [12]. Law

Abbreviations: aa, amino acid; CHO, Chinese hamster ovary cells; CPu, caudate putamen; DAMGO, [D-Ala^2,*N*-MePhe^4,Glyol^5]enkephalin; DOR, delta opioid receptor; Endo H, endoglycosidase H; GM1, ganglioside M1; GPCRs, G protein-coupled receptors; KOR, kappa opioid receptor; K/O, knockout; MCD, methyl-β-cyclodextrin; MOR, mu opioid receptor; Mr, relative molecular mass; WGA, wheat germ agglutinin.

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doi:10.1016/j.bbrc.2007.10.128

and colleagues have found that MOR expressed in HEK293 cells were mainly localized in lipid rafts subdomains of plasma membranes and this localization was required for MOR-mediated adenylyl cyclase superactivation [13], a cellular adaptation observed upon chronic opioid treatment.

In this study, we generated and characterized an anti-MOR antiserum for immunoblotting of MOR and found the MOR in the brain was heterogeneous with regard to its N-glycans and rafts association, unexpectedly.

Materials and methods

Materials. [^{3}H]Diprenorphine (58 Ci/mmole) and [^{35}S]guanosine 5-(γ-thio)triphosphate (GTPγS) (1250 Ci/mmole) were purchased from Perkin-Elmer Co. (Boston, MA). Lectin from *Triticum vulgaris* (wheat germ agglutinin/WGA)-Agarose and methyl-β-cyclodextrin (MCD) were purchased from Sigma Co. (St Louis, MO). Anti-GM1 polyclonal antibody and PANSORBIN was purchased from Calbiochem (San Diego, CA). HA.11 was a product of Covance (Cumberland, VA). *Anti-μC* is a rabbit polyclonal anti-MOR antibody against the sequence CT^{383}NHQLEN-LEAETAPLP^{398}, which corresponds to the last 16 amino acids (383–398) of the C-terminal domain predicted from the cloned rat MOR-1 (GenBank: NM_013071) and which is identical among human, rat, and mouse. The antibody was developed and purified by use of μC peptide affinity chromatography, as described in our previous report [14]. *Biotinylated anti-μC:* biotinylation of the anti-μC by sulfo-NHS-LC-Biotin was carried out according to the instructions (Pierce). The clonal CHO cell line stably expressing HA-rMOR (*CHO-HA-rMOR*) was established and cultured as described previously [15], with a B_{max} value of 1.8 pmol/mg membrane protein. *MOR-knockout (K/O) mice* were originally developed in the lab of Dr. John Pintar by disruption of exon-1 of the MOR-1 gene through homologous recombination [16]. *Brains:* the frozen meninges-stripped brains of mix-gender Sprague–Dawley rats were purchased from Pel-Freeze Biologicals (Rogers, AR). Brains were also collected from male Sprague–Dawley rats and littermates of female wild-type and MOR-K/O mice.

Most of following methods are our published ones as cited below. There are brief descriptions for some of them in figure legends and results. Brain region dissection, brain membrane preparation and solubilization [17], WGA affinity chromatography [18] and immunoprecipitation of MOR by anti-μC [14] were carried out at 4 °C, as cited, respectively. Treatments of MOR with PNGase F and Endo H followed the manufacturer's protocols (New England Biolabs).

Detergent-free preparation of lipid rafts using sodium carbonate was conducted according to our published method [17] following Song et al. [19] with some modifications. Determination of cholesterol and ganglioside M1 (GM1) contents, reduction of cell membrane cholesterol content by 2% of methyl-β-cyclodextrin (MCD) treatment, [^{3}H]diprenorphine binding, and [^{35}S]GTPγS binding were carried out following our published methods, respectively [17].

Results

Western blotting of the MOR in CHO-HA-rMOR cells and in brains

For CHO-HA-rMOR cells, anti-μC-labeled proteins migrated as a major broad band with a median Mr of 78 kDa and a minor lower band of Mr 52 kDa (Fig. 1A, left panel), which are similar to the bands labeled by HA.11 (Fig. 1A, right panel). Both antibodies detected no specific bands in either CHO-FLAG-hKOR or CHO-FLAG-mDOR cells (Fig. 1A).

To detect endogenous MOR, CPu, thalamus, and cerebellum were dissected from mouse brains of wild-type and MOR-knockout (K/O) littermates, and membranes were prepared. Western blotting revealed that in wild-type mice, anti-μC labeled several bands in the CPu, one of which was absent in the MOR-K/O mice (Fig. 1B, upper panel, lanes 1 and 2), indicating that this protein band, with an Mr of 60–84 kDa (median, 74 kDa), represents the MOR. Similarly, in the thalamus, one of the bands labeled by anti-μC in the wild-type was not present in the MOR-K/O mice (Fig. 1B, upper panel, lanes 3 and 4). However, surprisingly, the MOR band in the thalamus was narrower and had lower Mr [58–68 kDa (median, 63 kDa)] (Fig. 1B, upper panel, lane 3). The labeling of both bands was completely blocked by preadsorption of anti-μC with the μC peptide (Fig. 1B, middle panel). There was no difference in labeling in cerebella of wild-type and MOR-K/O mice (Fig. 1B, upper panel, lanes 5 and 6). The amount of protein loaded (30 μg per lane) for each sample was similar as demonstrated by Ponceu S staining of the membranes (Fig. 1B, lower panel).

Immunoblotting with anti-μC was also performed on membranes of the rat CPu and thalamus. MOR in the rat CPu migrated as a broad and diffuse band with an Mr range of 61–84 kDa (median, 75 kDa), while MOR in the rat thalamus was resolved as a narrow and diffuse band (60–72 kDa) with a smaller median Mr of 66 kDa (Fig. 1C, left panel). Both bands were completely blocked by preadsorption with the μC peptide (Fig. 1C, right panel). Thus, a similar discrepancy in Mr of the MOR between CPu and thalamus was observed in the rat.

Solubilization of the rat CPu or thalamus membranes and purification of the MOR by WGA affinity chromatography or immunoprecipitation

Membranes of the rat CPu or thalamus were solubilized and partially purified with WGA affinity chromatography, which enriched the MOR approximately 30-fold [18]. In addition, the MOR was partially purified by immunoprecipitation with anti-μC followed by PANSORBIN.

Western blotting of WGA affinity-purified materials with anti-μC showed that the MOR in the CPu migrated as a broad and diffuse band with a median Mr of 75 kDa (Fig. 2A, lane 1). Blotting of immunoprecipitated complex with biotinylated anti-μC yielded similar results (Fig. 2B, lane 3). In contrast, the MOR in the thalamus migrated as a narrow and diffuse band with a median Mr of 66 kDa (Fig. 2A, lane 2 and 2B, lane 4).

Treatment of the MOR from rat CPu or thalamus with glycosidases

PNGase F treatment of the WGA affinity-purified materials, which removes all N-linked glycans, resulted in an increase in the mobility of MOR in the CPu or thalamus on SDS–PAGE (Fig. 2A, lanes 3 and 4), compared with

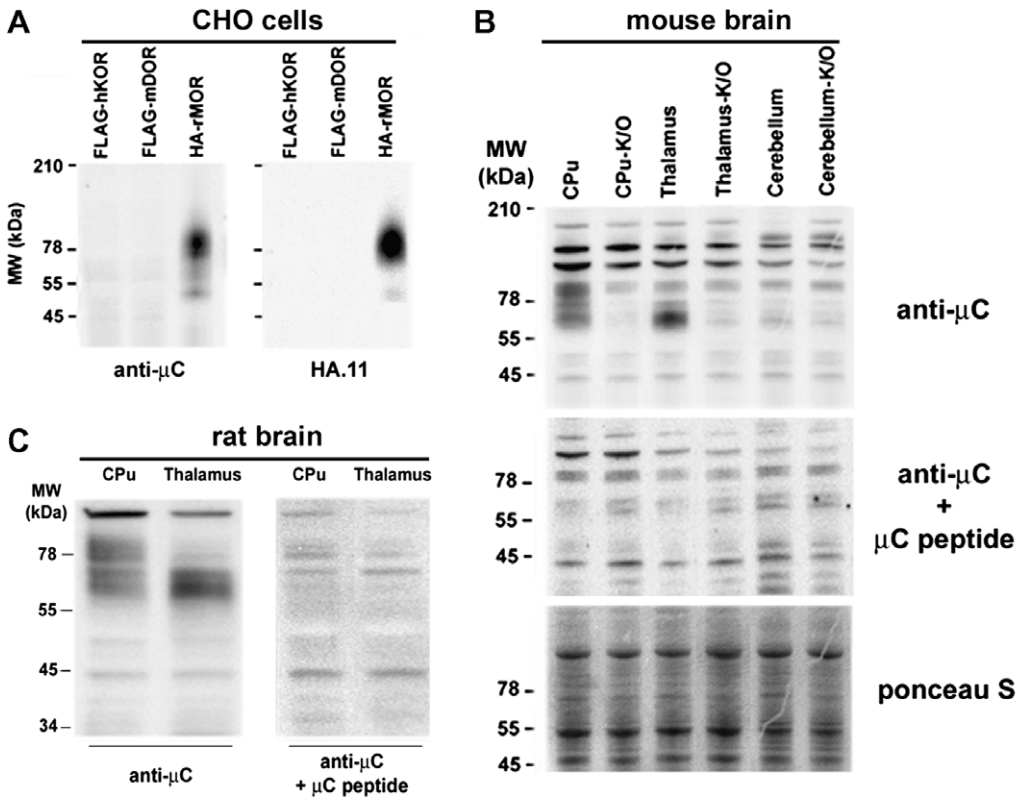

Fig. 1. Immunoblotting of the HA-rMOR stably expressed in CHO cells (A) and the endogenous MOR in CPu and thalamus of the mouse (B) or rat (C) brain. HA-rMOR, FLAG-hKOR, and FLAG-mDOR stably expressed in CHO cells were blotted by anti-μC, a polyclonal anti-MOR antibody (1.3 mg/ml) (1:5000) (A, left panel). The same membrane was stripped and blotted with an anti-HA monoclonal antibody (HA.11) (1:5000) as described in Materials and methods (A, right panel). Membranes prepared from CPu, thalamus, and cerebellum of wild-type and MOR-K/O mice were blotted with anti-μC (1:5000) (B, upper panel) or the same antibody (1:5000) preincubated with the μC peptide (0.6 μg/ml) (B, middle panel) as described in Materials and methods. The same blot used in the upper panel of (B) was stained by Ponceu S to show protein loading amounts (B, lower panel). In addition, CPu and thalamus were dissected from frozen rat brains and membranes were prepared. Western blot was performed with anti-μC (1:5000) (C, left panel) or anti-μC (1:5000) preincubated with the μC peptide (0.6 μg/ml) as described in Materials and methods (C, right panel). Each of these figures represents one of the three experiments performed with separate batches of tissues.

the untreated controls (Fig. 2A, lanes 1 and 2). More importantly, the diffuse bands with different widths and median Mr's (Fig. 2A, lanes 1 and 2) in the two regions became sharp bands with identical Mr's (43 kDa) (Fig. 2A, lanes 3 and 4). Anti-μC-precipitated MOR of the CPu or thalamus was also treated with PNGase F, yielding a similar observation (Fig. 2B, lanes 1–4). Thus, the difference in Mr of the MOR in the thalamus and CPu is due to differential N-linked glycosylation.

In addition, Endo H treatment, which cleaves N-linked glycans of high-mannose and some hybrid types, caused no mobility changes of the MOR of CPu and thalamus (Fig. 2B, lanes 5 and 6). These results indicate that the MOR in the CPu and thalamus contains different complex type N-linked glycans and is likely to be located in trans-Golgi and/or plasma membranes.

The association of MOR and lipid rafts in rat brain: CPu vs. thalamus

We carried out fractionation using continuous sucrose gradient (5–35% on top of sample in 45% sucrose) to prepare lipid rafts of CPu or thalamus membranes as described previously [17,20].

For rat CPu membranes, cholesterol level (Fig. 3E, CPu) and GM1 immunoreactivity (Fig. 3F, CPu) peaked in fraction 1, which had the highest buoyancy. In addition, fraction 1 contains the highest levels of [^{3}H]diprenorphine binding sites ($28.3 \pm 1.4\%$ of total binding sites, $n = 3$) (Fig. 3B) and MOR immunoreactivity (Fig. 3A). Fractions 1–4, corresponding to 5–20% sucrose, were defined as the lipid rafts fractions [21,22]. Fractions 1–4 contained $62.4 \pm 3.9\%$ of total cholesterol (Fig. 3E, CPu), the overwhelming majority of GM1 (Fig. 3F, CPu), $69.4 \pm 4.1\%$ of [^{3}H]diprenorphine binding sites (Fig. 3B), and most of the MOR immunoreactivity (Fig. 3A).

For rat thalamus membranes, cholesterol level (Fig. 3E, thalamus) and GM1 immunoreactivity (Fig. 3F, thalamus) peaked in fraction 1. Fractions 1–4 contained $68.4 \pm 6.8\%$ of total cholesterol (Fig. 3E, thalamus) and the majority of GM1 (Fig. 3F, thalamus), similar to CPu membranes. However, there was no prominent peak observed for [^{3}H]diprenorphine binding (Fig. 3D) and MOR immunore-

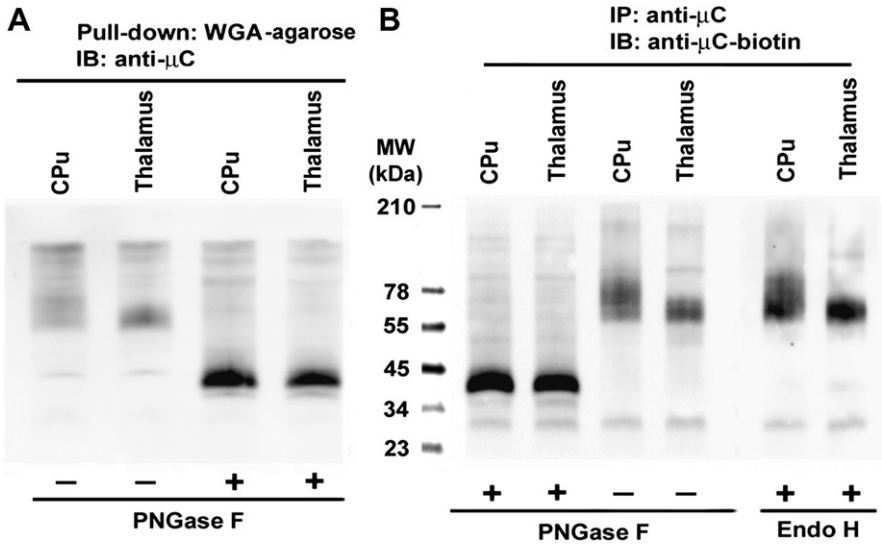

Fig. 2. Deglycosylation of MOR from rat CPu and thalamus. Membranes of the rat CPu or thalamus were solubilized with 2% Triton X-100. (A) The solubilized preparations were applied to a WGA-agarose column and the bound glycoproteins were eluted with 0.25 M *N*-acetyl-D-glucosamine. The eluate was left untreated or treated with PNGase F, resolved with 8% SDS–PAGE, and immunoblotted with anti-μC (1:5000). (B) The solubilized preparations were immunoprecipitated with anti-μC followed by PANSORBIN (Calbiochem), dissolved in Denatured buffer (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-μC-biotin (1:5000) and anti-biotin-HRP-conjugate (1:5000). Each figure shown represents one of two independent experiments.

activity (Fig. 3C). Fractions 1–4 contained 44.3 ± 3.2% of [^{3}H]diprenorphine binding (Fig. 3D) and MOR immunoreactivity distributed throughout the 12 fractions without significant preference (Fig. 3C).

Thus, a majority (~70%) of the MOR in rat CPu membranes is localized in lipid rafts, while less (~44%) in rat thalamus membranes are rafts associated.

Effects of MCD treatment on MOR-mediated G protein activation in rat CPu or thalamus

We have shown that MCD treatment reduces cholesterol in lipid rafts preferentially, leading to disruption of lipid rafts in different tissues and cell lines including rat brain membranes [17,20], and pretreatment with MCD followed by MCD-conjugated cholesterol was shown to restore cell cholesterol contents and reverse all the MCD effects [17,20]. In rat CPu membranes, MCD treatment significantly decreased the E_{max} value (% above the basal level) of DAMGO-promoted [^{35}S]GTPγS binding without affecting its EC_{50} value (~1 μM). The E_{max} after treatment (81 ± 4.0%) was ~42% of control E_{max} (172 ± 8.9%) (Fig. 4). In rat thalamus membranes, MCD treatment decreased the E_{max} value of DAMGO, but to a less extent, without affecting its EC_{50} value (~1 μM). The E_{max} after treatment (146 ± 5.8%) was ~74% of control E_{max} (197 ± 10.8%) (Fig. 4). It is noteworthy that, after MCD treatment, the basal [^{35}S]GTPγS binding was only ~50% of that (~2000 dpm) of the untreated samples for both rat CPu and thalamus membranes, indicating that the binding of GTP to G proteins (affinity and/or accessibility) was cholesterol (rafts)-dependent.

Discussion

To the best of our knowledge, this is the first report to show that the post-translational modification of a GPCR in the brain by N-glycosylation is region-specific. This is also the first demonstration that a membrane protein is associated with lipid rafts to varying degrees in different brain areas.

Affinity-purified rabbit polyclonal anti-MOR antibody, anti-μC, recognizes MOR in Western blot with high specificity

The Mr's of the MORs in the mouse and rat CPu detected with anti-μC are similar to those detected by [^{3}H]β-funaltrexamine labeling [18] or an antiserum against a 15-aa peptide corresponding to amino acid 384–398 predicted from cloned rat MOR-1 [5], for which the whole brains were used. In addition, the Mr of the MOR in the rat thalamus in this study is consistent with that detected by receptor phosphorylation [23]. In contrast, there are reports showing by immunoblotting the MOR as sharp band(s) with Mr between 40 and 60 kDa in mouse or rat neuronal tissues (For examples, see [24] and [25]). We believe that, because of our use of MOR-K/O brain tissues and wild-type cerebellum as negative controls, MOR-1 is defined by immunoblotting as a single diffuse band with apparent Mr ranging from 58 to 84 kDa depending on brain regions in the mouse or rat.

There are multiple splice variants of the MOR [26]. Most of the splice variants have different C-terminal domains. Since anti-μC was raised against the extreme C-terminal 16-aa (383–398) peptide of rat MOR-1, which

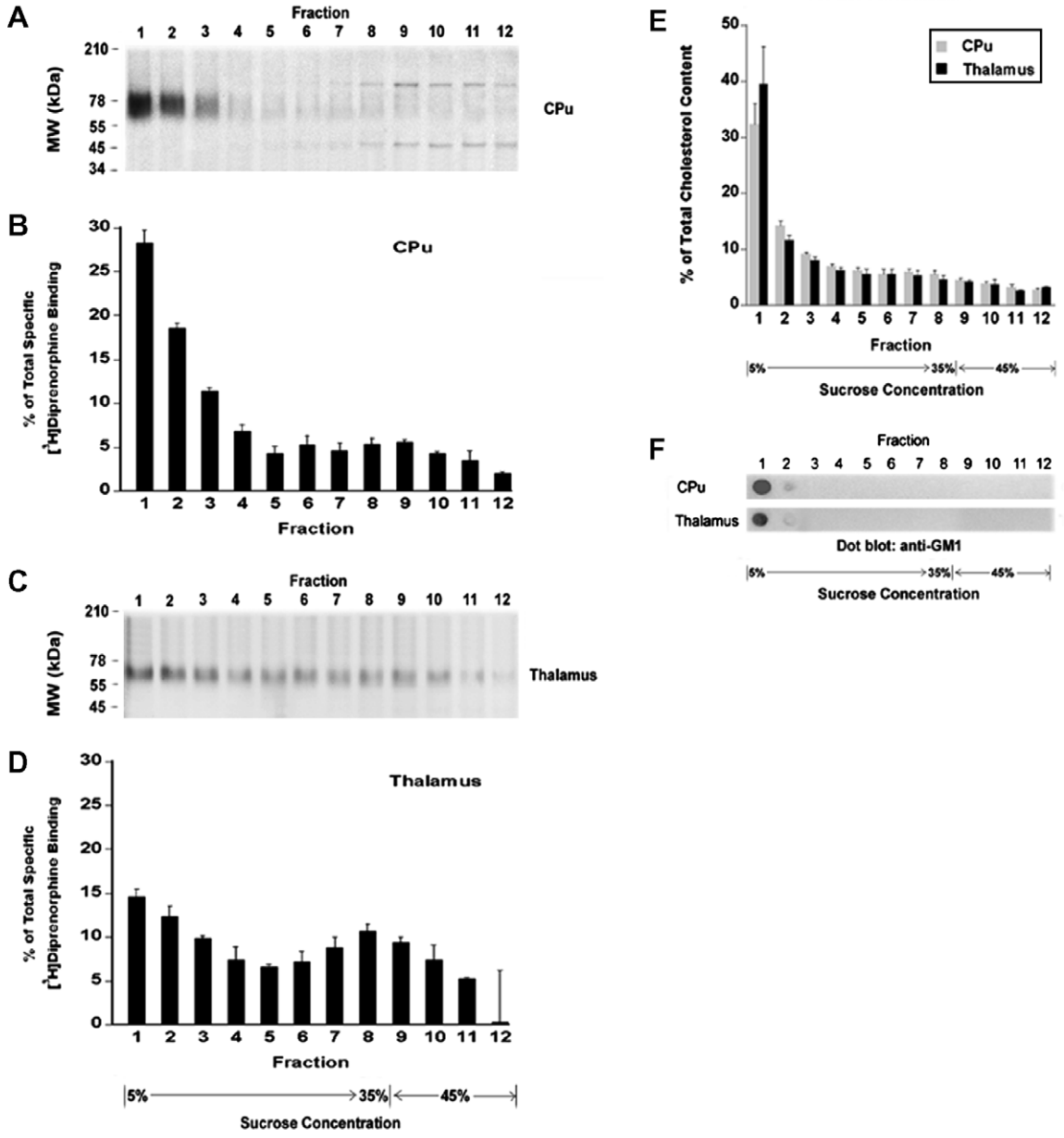

Fig. 3. MOR in the rat CPu and thalamus is associated with lipid rafts differently. Rat CPu or thalamus membranes were sonicated in 0.5 M sodium carbonate buffer (pH 11) and then fractionated through a continuous sucrose gradient (Fractions 1–8: 5–35%; fractions 9–12: 45%) by ultracentrifugation as described in Materials and methods. Twelve 1-ml fractions were collected and each fraction was subjected to: (A, C) Immunoblotting of the MOR with anti-μC in (A) CPu and (C) thalamus. (B, D) [^{3}H]Diprenorphine (~1 nM) binding using naloxone (10 μM) to define nonspecific binding in (B) CPu and (D) thalamus. Two 100-μl aliquots from each fraction were used in binding in duplicate as described in Materials and methods. Data are expressed as % of the sum of specific [^{3}H]diprenorphine binding. (E) Determination of cholesterol contents. Data are expressed as the ratios of [cholesterol in each fraction]/[total cholesterol content]. (F) Determination of GM1 levels by a dot-blot assay with an anti-GM1 antibody. In this set of experiments, membranes of CPu or thalamus prepared and combined from three fresh rat brains were used for fractionation. Data in (B), (D), and (E) are shown as means ± sem of and those in (A), (C), and (F) represent one of the three experiments performed with separate batches of fresh brain tissues.

shares four residues (TNHQ) with the C-terminal variants, the antibody is not likely to react with those 3′ variants. Thus, anti-μC is largely specific to MOR-1, the predominant form of MOR.

Brain region heterogeneity in N-glycans of the MOR is reminiscent of our previous observation that there are species variations in Mr of β-[^{3}H]funaltrexamine-labeled MOR and the disparities are attributed to different degrees of N-linked glycosylation [18].

No RNA editing of MOR-1 occurs in either rat CPu or thalamus

The full-length cDNA sequences of MOR-1 from rat CPu and thalamus were same as shown by RT-PCR (data not shown). Thus, brain region-specific N-glycosylation was not due to RNA editing of the five potential N-glycosylation sites in the receptor N-terminal domain.

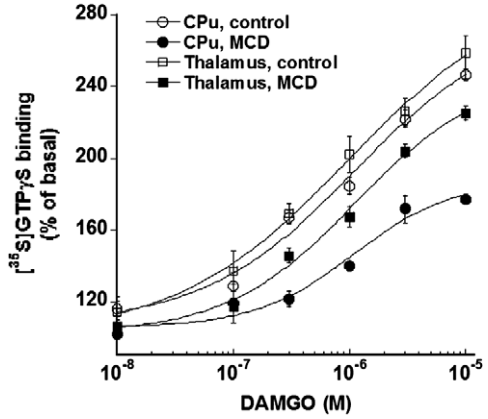

Fig. 4. Effects of cholesterol reduction on DAMGO-induced [^{35}S]GTPγS binding to membranes of rat CPu and thalamus. Rat CPu or thalamus membranes were incubated with vehicle or 2% MCD and [^{35}S]GTPγS binding experiments were performed as cited in Materials and methods. Data are normalized as % of basal [^{35}S]GTPγS binding (in the absence of DAMGO) and shown as means ± sem of 3–5 experiments performed in duplicate using separate batches of tissues. The basal [^{35}S]GTPγS binding was ~2000 dpm for untreated membranes of both regions. After MCD treatment, the basal binding for membranes of both regions was only ~50% of that for the untreated ones.

Thalamus: an exception regarding opioid receptors–lipid rafts relationship

By use of the same non-detergent rafts preparation method employed in this study, we and others have found that mu, delta, or kappa opioid receptors are mainly associated with lipid rafts in several tissues/cells, including rat brain CPu, human placenta, rat cardiac myocytes, NG108-15 cells, HEK293 cells, and CHO cells [13,17,20,27,28]. In this regard, the MOR in the rat thalamus is an exception. Moreover, MOR-mediated G protein activation in the rat thalamus was attenuated to a much less extent by cholesterol reduction, in comparison with MOR in the rat CPu. These results indicate that coupling of rafts-associated MOR to G proteins is more cholesterol-sensitive, in comparison with that of MOR in non-rafts. Thus, MOR is able to interact with Gi/o proteins in as well as out of lipid rafts, via cholesterol-dependent and -independent or less dependent manners, respectively.

Immuno-electron microscopy revealed that MOR in the rat CPu was predominantly distributed along non-synaptic portions of the dendritic plasma membranes of medium spiny neurons [29], which, based on our results, would be lipid rafts-enriched portions. Indeed, Sheng and colleagues showed that lipid rafts exist abundantly in dendrites of cultured hippocampal neurons [30]. Whether MOR in the rat thalamus adopts a similar or less restrictive localization has not been studied; nevertheless, our findings may imply a wider distribution of MOR in different membrane subdomains in this brain region, which is under investigation.

Although size, constitution, and dynamics of lipid rafts in neurons may not be fully elucidated for some time to come, the observations of brain region-specific rafts association of MOR, a GPCR, and its varying degrees of cholesterol-dependent signaling appear to support the concept of lipid rafts as functional membrane subdomains, which have been proposed to participate in the neuronal signaling, cell adhesion, axon guidance, and synaptic transmission [31].

Is N-glycosylation and/or lipid rafts involved in differential regulation of MOR in the brain after opioid treatment?

Studies have shown differences in agonist-induced MOR regulation in various brain regions including CPu and thalamus. Wang and colleagues observed that DAMGO-induced MOR phosphorylation in the CPu was much lower than that in the thalamus [23]. Childers and colleagues reported that, after chronic heroin administration in rats, MOR desensitization was detected in some brain regions including thalamus and periaqueductal gray, but not in others including CPu and nucleus accumbens, as determined by DAMGO-stimulated [^{35}S]GTPγS binding [32]. Moreover, Noble and Cox found that MOR desensitization, defined as a reduced ability of agonists to inhibit adenylyl cyclase activity following chronic morphine treatment, occurred in thalamus and periaqueductal gray, but not in CPu and nucleus accumbens [33]. Thus, a question arises as to whether the heterogeneity of MOR in N-glycosylation and/or rafts-distribution plays a role in such differential MOR phosphorylation and desensitization in different brain regions. In in vitro cellular systems, expression of G protein-coupled receptor kinase 2 (GRK2) enhanced phosphorylation and regulation of MOR [34,35], and most of GRK2 was shown to be not associated with rafts [36]. Since GRK2 is ubiquitously expressed in the brain, this regulation was expected to occur in vivo [37]. Thus, a simple hypothesis is that thalamus has more MOR in non-rafts membranes, where the bulk of GRK2 resides, and thereby gets phosphorylated and desensitized more readily than MOR in CPu, which is mostly located in rafts and less accessible to the kinase. As some glycoproteins have been postulated to be stabilized in lipid rafts by speculative rafts-associated lectins (carbohydrate-binding proteins) [38], it is conjectural that the lower degree of rafts localization of the MOR in the thalamus may result from a lower level of glycosylation.

Law and colleagues suggested that lipid rafts were required as the platform to organize a protein complex for MOR-mediated adenylyl cyclase superactivation [13]. It would be intriguing to study whether MOR induced adenylyl cyclase superactivation in CPu to a higher extent than in thalamus because of more MOR in lipid rafts.

In conclusion, MOR is differentially modified by N-glycosylation and associated with lipid rafts in the rat CPu vs. thalamus. Whether and how such variations relate to each other and affect MOR function and regulation in a brain region-specific manner remains to be investigated.

Acknowledgment

This work was supported by National Institutes of Health Grants: R01 DA17302 and P30 DA13429.

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