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Agonist treatment did not affect association of mu opioid receptors with lipid rafts and cholesterol reduction had opposite effects on the receptor-mediated signaling in rat brain and CHO cells

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Abstract

Lipid rafts are small cholesterol- and glycosphingolipid-enriched membrane subdomains. Here we compared the mu opioid receptor (MOR)–lipid rafts relationship in the rat brain, where neurons have non-caveolae rafts, and in CHO cells stably transfected with HA-rat MOR (CHO-HA-rMOR), which are enriched in caveolae. Membranes of rat caudate putamen (CPu) and thalamus or CHO-HA-rMOR cells were homogenized, sonicated in a detergent-free 0.5 M Na₂CO₃ buffer and fractionated through sucrose density gradients. Western blot and [³H]diprenorphine binding showed that ~70% of MOR in CHO-HA-rMOR was present in low-density (5–20% sucrose) fractions enriched in cholesterol and/or ganglioside M1 (GM1) (lipid rafts) in plasma membranes, whereas about 70% and 45% of MOR in CPu and thalamus, respectively, were associated with lipid rafts. Incubation with a saturating concentration of etorphine or morphine at 37 °C for 30 min failed to change the MOR location in rafts in CHO-HA-rMOR, indicating that the internalized MOR does not move out of rafts, in contrast to the delta opioid receptor. *In vivo*, rafts association of MOR in CPu and thalamus was not affected significantly in rats implanted with two 75-mg morphine pellets for 72 h. In addition, cholesterol reduction by methyl-β-cyclodextrin (MCD) disrupted rafts and shifted MOR to higher density fractions in both CHO-HA-rMOR and CPu membranes. However, MCD treatment had opposite impacts on MOR signaling in the two tissues: it attenuated MOR-mediated [³⁵S]GTPγS binding in CPu but enhanced it in CHO-HA-rMOR.

Keywords

mu Opioid receptor; Lipid rafts; Brain; Cholesterol; Morphine; Methyl-β-cyclodextrin

1. Introduction

The actions of opioid drugs are mediated by at least three types of opioid receptors: mu, delta and kappa (MOR, DOR and KOR, respectively) (Pasternak, 1988). These receptors belong to rhodopsin subfamily of 7-transmembrane receptors (7-TMRs)/G-protein-coupled

receptors (GPCRs) (Bockaert and Pin, 1999). They are coupled via pertussis toxin-sensitive G_i/G_o proteins to many downstream effectors such as adenylate cyclase, potassium channels, calcium channels, and a mitogen-activated protein kinase pathway [for a review, see Law et al., 2000]. Although opioid drugs have long been used as analgesics, their clinical use is limited by the fear of addiction and side effects such as respiratory depression, decreased gastrointestinal motility, sedation, mood changes, and tolerance (Kieffer, 1999). Morphine acts primarily on the MOR (Matthes et al., 1996) and is among the most widely used and abused drugs.

Lipid rafts, including non-caveolae rafts and caveolae, are small cell membrane domains (10–200 nm), which are thought to serve as platforms for many cellular processes (Pike, 2006). They are enriched in cholesterol and glycosphingolipids (e.g., GM1). Recently, lipid rafts were suggested to be renamed as “membrane rafts,” because it has become clear that proteins play a major role in their formation and contribute to their functions (Pike, 2006). While caveolae are relatively stable because the protein caveolins support the structure, non-caveolae rafts are considered to be unstable, heterogeneous and dynamically produced and degraded (Pike, 2006). Electron micrographs show that caveolae are flask-shaped membrane invaginations at plasma membranes in most differentiated cells (Cohen et al., 2004). On the contrary, morphological identification of non-caveolae rafts has been elusive (Shaw, 2006). The existence of non-caveolae rafts is largely based on their biochemical nature of insolubility in nonionic detergents at low temperature and high buoyancy in density gradients (Brown and Rose, 1992), which does not differentiate non-caveolae rafts from caveolae.

Many 7-TMRs/GPCRs have been shown to locate in and regulated by lipid rafts (Pike, 2003; Chini and Parenti, 2004; Ostrom and Insel, 2004), including opioid receptors (Head et al., 2005; Xu et al., 2006; Zhao et al., 2006; Patel et al., 2006; Huang et al., 2007). For example, MOR expressed in HEK293 cells was found by Zhao et al. (2006) to be mainly localized in lipid rafts domains of plasma membranes and this localization was required for MOR-mediated adenylyl cyclase superactivation. Our recent findings demonstrated that the kappa and delta opioid receptor (KOR and DOR, respectively) expressed in CHO cells were mostly associated with and regulated by lipid rafts (Xu et al., 2006; Huang et al., 2007). We have also demonstrated that the acute treatment with a full agonist move some DORs out of lipid rafts in both CHO cells and NG108-15 cells (Huang et al., 2007). However, most of those studies were performed in caveolae-rich non-neuronal cells, while numerous GPCRs, including opioid receptors, are present in neurons in the brain, which were demonstrated to be devoid of caveolae and deficient in its structural protein(s), the caveolins (Cameron et al., 1997).

By the use of an anti-MOR antibody (anti- μ C), we have recently found that the MOR associated with lipid rafts at different levels in rat caudate putamen (CPu) and thalamus, with cholesterol and ganglioside M1 as markers for lipid rafts (Huang et al., in press). In this study, we determined to what extent MOR in CHO-HA-rMOR was localized in lipid rafts. In addition, we investigated rafts association of MOR in cells following acute treatment of CHO-HA-rMOR with agonists and in CPu and thalamus after chronic morphine treatment of rats *in vivo*. Moreover, effects of disrupting rafts by cholesterol reduction on MOR–G protein coupling in the two systems were examined.

2. Results

2.1. MOR in rat brain CPu and rMOR expressed in CHO cells are mainly localized in the lipid rafts from plasma membranes

A modified version of our published procedure was used to prepare lipid rafts (Xu et al., 2006; Huang et al., 2007), which was originally described by Song et al. (1996). Prepared by this method, non-caveolae rafts cannot be distinguished from caveolae.

MOR is present at high levels in several regions in the rat brain, including caudate putamen (CPu), where MORs are located in neurons (Mansour et al., 1988). CPu membranes and CHO-HA-rMOR cells were sonicated on ice in a 500 mM sodium carbonate buffer (pH 11) and fractionated in a 5%/35%/45% discontinuous sucrose density gradient by ultracentrifugation, and twelve 1-ml fractions were collected. A milky band was observed in the low-density region at the junction of 5% and 35% sucrose, i.e., fraction 4 or 5.

For the CPu, the vast majority of opioid receptors, as determined by [³H]diprenorphine binding (Fig. 1A) and cholesterol (data not shown), was found in fraction 5 at the interface of 5%/35% sucrose coincided with the milky band. Flotillin-1 was distributed in all 12 fractions, with the highest level in fraction 5 (Fig. 1B, middle panel). These results are consistent with our previous observations (Huang et al., 2007). In addition, caveolin-1 was observed mainly located in fraction 5 (Fig. 1B, lower panel), but the signal was weak. Moreover, fraction 5 also had most of MOR immunoreactivity by Western blotting (Fig. 1B, upper panel), which we have found to be a diffuse broad band with a median Mr of 75 kDa. For CHO-HA-rMOR cells, in a comparable manner, MOR binding (Fig. 1C) and immunoreactivity (Fig. 1D, upper panel), caveolin-1 (Fig. 1D, middle panel), and flotillin-1 (Fig. 1D, lower panel) peaked in the fraction 5 and distributed predominantly in low-density fractions (1–5). Cholesterol distributed mainly in low-density fractions with a similar pattern, while the majority of proteins were in higher density fractions, particularly fractions 9–12 [data not shown and similar to those for CHO cells in Xu et al., 2006].

In both CPu and CHO-HA-rMOR cells, the low-density fractions (1–5) of the 5%/35%/45% discontinuous sucrose density gradient contained most of MOR binding sites (80–90%) (Figs. 1A and C) and MOR immunoreactivity (Figs. 1B and D, upper panels) and cholesterol (~80%) (Xu et al., 2006; Huang et al., 2007). Thus, a majority of MORs in both tissues are located in buoyant cholesterol-rich membrane domains, namely lipid rafts. As MOR is mainly distributed on plasma membranes in the rat CPu by immunostaining (Wang et al., 2003), and 92% of MOR was located on cell surface in CHO-HA-rMOR cells as determined by receptor binding (Fig. 3B), our results indicate that the MOR-related rafts are derived from plasma membranes.

We have previously observed that fractionation through continuous sucrose density gradient (5–35% on top of sample in 45% sucrose), instead of discontinuous one (5%/35%/45%), facilitated detection of shift of the kappa or delta opioid receptor out of lipid rafts following treatment with an agonist or MCD (Xu et al., 2006; Huang et al., 2007). In our recent publication (Huang et al., 2007), we have shown cholesterol contents in each fraction following continuous gradient centrifugation for both CHO cells and rat brain membranes. Cholesterol contents in CHO cells and in rat caudate putamen membranes are highest in the fraction 1. In CHO cells they gradually reduced to the lowest in fraction 12, whereas in rat caudate putamen membrane cholesterol contents in all other fractions are much lower, each containing <20% of that of the fraction 1. Similar patterns were observed in this study (data not shown). Accordingly, we carried out continuous sucrose gradient to prepare lipid rafts for the following experiments.

2.2. MOR remained in lipid rafts after acute treatment of CHO-HA-rMOR cells with agonists

For control CHO-HA-rMOR cells, the highest level of [^3H] diprenorphine binding was detected in fraction 1 ($34.6 \pm 2.0\%$, $n=3$) (Fig. 2B, control), coincided with the peaks of HA immunoreactivity (Fig. 2A, control in upper panel) and caveolin-1 (Fig. 2A, control in lower panel). The majority (73.0%) of MOR binding sites (Fig. 2B, control) and most of the receptor proteins (Fig. 2A, control in upper panel) were found in fractions 1–4. Fractions 1–4, corresponding to 5 to ~20% sucrose, were defined as the lipid rafts fractions according to Brown and Rose (1992) and Macdonald and Pike (2005).

We then determined whether treatment of CHO-HA-rMOR cells with an agonist affected MOR association with lipid rafts. Incubation of cells for 30 min with etorphine ($1 \mu\text{M}$) or morphine ($10 \mu\text{M}$) did not move MOR out of lipid rafts significantly, as determined by both [^3H]diprenorphine binding (Fig. 2B) and Western blot of the MOR (Fig. 2A, upper panel), neither did DAMGO ($1 \mu\text{M}$) (data not shown). In addition, the agonist treatments did not affect the rafts association of caveolin-1 (Fig. 2A, lower panel).

Under the same conditions, MOR was internalized significantly by etorphine, but not by morphine. The internalized receptors were revealed as punctuated staining in the cytosol by immunostaining with an anti-HA antibody (Fig. 3A, etorphine). The percentages of intracellular MOR quantified by binding were $8.0 \pm 1.4\%$ for control cells, $29.4 \pm 2.9\%$ for etorphine-treated cells, and $8.6 \pm 2.0\%$ for morphine-treated cells (Fig. 3B), indicating that MOR mainly distributed at plasma membranes in control and morphine-treated cells while >20% of MOR underwent internalization after acute etorphine treatment.

2.3. MOR in rat CPu and thalamus remained in lipid rafts after *in vivo* chronic morphine treatment

Chronic administration of morphine or other MOR agonists in rats induces or enhances many stereotypic behaviors including rearing, sniffing and turning, which is thought to involve MOR in the CPu to regulate dopaminergic inputs (Wang et al., 2003). In addition, chronic morphine treatment-induced anti-nociceptive tolerance in rats was reported to involve brain regions such as periaqueductal grey and thalamus (Noble and Cox, 1996).

We have recently found that, in rat brain, there is a higher degree of MOR–lipid rafts association in CPu than that in thalamus. Here, we tested whether *in vivo* chronic morphine treatment differentially affected the rafts association of MOR in rat CPu or thalamus. Sprague–Dawley rats were implanted with placebo or morphine pellets for 72 h, a paradigm commonly used to induce significant physical dependence and tolerance (Bell and Adler, 1988). Rats were killed and CPu or thalamus tissues were collected. Membranes were prepared from CPu and thalamus of vehicle- and morphine-treated rats and subjected to lipid rafts preparation procedures.

For CPu membranes, similar distribution patterns were observed for chronic morphine-treated and control rats. [^3H] diprenorphine binding sites peaked in fraction 1, which contained $26.3 \pm 1.8\%$ and $24.2 \pm 1.2\%$ of total binding sites ($n=3$) for control and treated rats, respectively (Fig. 4B). Fraction 1 also had the highest density of MOR immunoreactivity (Fig. 4A). Fractions 1–4, corresponding to 5–20% sucrose density, contained most of the MOR immunoreactivities (Fig. 4A), 67.8% and 66.4% of [^3H]diprenorphine binding sites for control and treated rats, respectively (Fig. 4B).

For thalamus membranes, neither did we find significant differences between control and morphine-treated rats. There were no prominent peaks found for control and morphine-treated rats in both MOR immunoreactivities (Fig. 4C) and [^3H]diprenorphine binding (Fig. 4D). Fractions 1–4 contained 44.3% and 43.3% of total [^3H]diprenorphine binding (Fig. 4D).

for control and treated rats, respectively. MOR immunoreactivity distributed throughout the 12 fractions without significant preference (Fig. 4C).

For both CPu and thalamus membranes, cholesterol and ganglioside M1 (GM1) were found mainly in low-density fractions and peaked in fraction 1 in a similar manner (Huang et al., *in press*), which was not affected by chronic morphine treatment (data not shown).

Thus, chronic morphine treatment *in vivo* did not significantly change percent of MOR in lipid rafts in rat CPu and thalamus.

2.4. Methyl- β -cyclodextrin (MCD) treatment reduced cholesterol level and shifted the MOR to fractions of higher density

We have previously reported that pretreatment with 2% MCD for 1 h at 37 °C caused $49.6 \pm 3.4\%$ and $76.7 \pm 1.2\%$ reduction in cholesterol content in CHO cells and rat CPu membranes, respectively (Huang et al., 2007). To examine whether cholesterol reduction affected localization of MOR in lipid rafts, we treated CHO-HA-rMOR cells and CPu membranes with 2% MCD, which reduced the cholesterol levels, respectively, similar to those in our previous report (Huang et al., 2007). For CHO-HA-rMOR cells, following MCD treatment, high levels of HA immunoreactivity were shifted from fractions 1–4 to fractions 5–9 (Figs. 5A and B, the upper panels), so was [^3H] diprenorphine binding (data not shown). High contents of caveolin-1 were shifted from fractions 1–6 to fractions 5–10 (Figs. 5A and B, the lower panels). For CPu membranes, MCD treatment shifted [^3H]diprenorphine binding out of fractions 1–3 to fractions 4–10 (data not shown), as we reported recently (Huang et al., 2007). Collectively, in both rat CPu membranes and CHO-HA-rMOR cells, MCD treatment reduced cholesterol, leading to the disruption of lipid rafts and shift of MOR to high-density fractions. Thus, the high cholesterol contents in lipid rafts are necessary to sustain the buoyancy of MOR in the tissues/cells.

2.5. Effects of MCD treatment on MOR-mediated G protein activation

Activation of the MOR enhances [^{35}S]GTP γS binding to pertussis toxin-sensitive G proteins in cell membranes (Traynor and Nahorski, 1995), which has been used as a functional measure for G protein activation. After MCD treatment, the basal binding for CHO cells remained the same, while the basal binding for CPu was only ~50% of that (~2000 dpm) for the untreated sample, indicating that the binding of GTP to G proteins (affinity and/or accessibility) was cholesterol (rafts)-dependent in rat CPu but not in CHO cells. In rat CPu membranes, MCD treatment decreased the E_{max} values (normalized as percent of basal binding) of both DAMGO and morphine without affecting their EC_{50} values significantly (Fig. 6A and Table 1). In contrast, in CHO-HA-rMOR cells, MCD treatment greatly enhanced the E_{max} values of etorphine, DAMGO and morphine without affecting their EC_{50} values significantly (Fig. 6B and Table 1). The EC_{50} values of DAMGO and morphine for the MOR were much higher in CPu membranes than in CHO cells. The EC_{50} values are consistent with our previous finding for CHO cells (Huang et al., 2001a) and that of Maher et al. (2000) for CPu membranes. The discrepancies may be due to different levels of receptors (Whaley et al., 1994) and G proteins and their stoichiometry in different tissues.

3. Discussion

Neurons in the rat brain have non-caveolae rafts (Cameron et al., 1997), while CHO cells have abundant caveolae (Navratil et al., 2003). In this study, we found that (1) ~70% of MOR was located in the rafts of plasma membranes in both rat CPu and CHO-HA-rMOR cells; (2) MOR–rafts association was not changed by incubation of CHO-HA-rMOR cells with agonists; (3) chronic treatment of rats *in vivo* with morphine did not change

distribution of MOR in lipid rafts in CPu and thalamus, which, to the best of our knowledge, represents the first report studying the effects of chronic agonist treatments on rafts association of G-protein-coupled receptors *in vivo*.

In addition, disruption of rafts by MCD treatment attenuated MOR-mediated G protein activation in CPu but enhanced it in CHO-HA-rMOR cells, supporting our hypothesis that lipid rafts sustain $G_{i/o}$ -coupled GPCRs-mediated signaling in caveolin-deficient neuronal cells but inhibit it in caveolin-enriched non-neuronal cells (Huang et al., 2007).

3.1. MOR remained in rafts after agonist treatment in contrast to DOR

In rats treated chronically with morphine with a paradigm that induces tolerance and dependence (Bell and Adler, 1988), rafts association of MOR in the CPu or thalamus remained similar to control rats. In CHO cells, the agonists DAMGO and etorphine did not move the receptor out of lipid rafts (probably caveolae) although they induced significant MOR internalization. Our results were consistent with the recent findings of Zhao et al. (2006) in HEK293 cells.

The finding that agonists do not affect lipid rafts association of MOR is in contrast to our previous findings on DOR which was partially shifted out of rafts by full agonists in CHO cells and NG108-15 cells (Huang et al., 2007). Lipid rafts localization of some GPCRs is not affected by agonist stimulation, for example, endothelin ETA receptor (Chun et al., 1994). Nevertheless, some other GPCRs, such as β_2 -adrenergic receptors (Rybin et al., 2000), which are constitutively localized in lipid rafts, moved out of rafts upon agonist binding and were proposed to leave lipid rafts to be internalized via clathrin-coated pits. The mechanisms for the different behaviors of GPCRs are not clear.

3.2. Possible mechanisms underlying the difference in MCD treatment-induced changes in MOR signaling: CHO-HA-rMOR cells vs. rat brains

MCD treatment shifted MOR out of lipid rafts in both CHO cells and brain membranes, indicating that cholesterol is critical for MOR localization in lipid rafts. However, MCD treatment had opposite effects on receptor-G protein coupling in the two systems. It reduced MOR agonist-promoted [35 S]GTP γ S binding to CPu and thalamus membranes but enhanced it to membranes of CHO cells. These findings are similar to our recent observations that cholesterol depletion had opposite effects on signaling of the DOR in brain membranes and a neuron-like cell line (NG108-15 cells), which lack caveolae, vs. a non-neuronal cell line (CHO cells) enriched in caveolae (Huang et al., 2007). We have demonstrated that in CHO cells caveolin-1 (the structure protein of caveolae) is associated with $G_{\alpha i}$ (Xu et al., 2006; Huang et al., 2007). Caveolins have been shown to inhibit G_{α} activities (Li et al., 1995). MCD treatment attenuates the association between caveolins and $G_{\alpha i}$, leading to disinhibition of $G_{\alpha i}$ and thus a higher degree of receptor-mediated $G_{\alpha i/o}$ protein activation in CHO cells (Xu et al., 2006; Huang et al., 2007). In contrast, since there are little caveolins and caveolae in neurons (Cameron et al., 1997), such a scenario does not occur in CPu membranes. Caveolae are relatively large immobile raft domains with a diameter of 65 nm that can occupy 4–35% of the plasma membrane depending on cell type (Parton, 2003), whereas non-caveolae rafts, at least those occupied by GPI-anchored proteins, are mobile and smaller with a diameter less than 10 nm (Sharma et al., 2004). According to a recent simulation study, segregating proteins in caveolae vs. non-caveolae rafts was predicted to have quite different functional consequences (Nicolau et al., 2006). In both systems, however, cholesterol is an important factor for organizing signaling molecules in lipid rafts, at least opioid receptors and G proteins.

We have recently shown that MCD treatment reduced DAMGO-promoted [^{35}S]GTP γ S binding in CPu to a greater extent than in thalamus (Huang et al., in press), which correlates with the higher degree of MOR association with lipid rafts in CPu than in thalamus, further supporting the notion that lipid rafts sustain MOR–G protein coupling in neuronal tissues.

3.3. The relative molecular mass values (M_r) of MOR vary in different tissues

In SDS-PAGE gel, MOR in rat CPu migrated as a broad and diffuse band with an M_r 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 M_r of 66 kDa, which is consistent with our previous findings. The difference is due to different degrees of N-glycosylation of MOR (Huang et al., in press). For HA-MOR stably expressed in CHO cells, the diffuse band detected ranged from 72 to 84 kDa with a median M_r of 78 kDa.

In summary, the majority of MOR is located in plasma membrane lipid rafts in the rat CPu and CHO cells, while less of MOR in rat thalamus is rafts associated. Lipid rafts sustained MOR-mediated G protein activation in rat brain membranes but appeared to inhibit it in CHO cells. Agonist treatment *in vitro* or *in vivo* does not affect MOR localization in rafts.

4. Experimental procedure

4.1. Materials

[^3H]diprenorphine (58 Ci/mmol) and [^{35}S]guanosine 5-(γ -thio) triphosphate (GTP γ S) (1250 Ci/mmol) were purchased from Perkin-Elmer Co. (Boston, MA). Morphine and etorphine were provided by the Drug Supply System of National Institute on Drug Abuse (NIDA). Naloxone, DAMGO, sodium carbonate, 2-morpholinoethanesulfonic acid (MES), glycerol, ethylenediamine tetraacetic acid (EDTA), ethylene glycol-*bis*(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), dithiothreitol (DTT), PMSF, GDP, GTP γ S, and methyl- β -cyclodextrin (MCD) were purchased from Sigma Co. (St. Louis, MO). For phosphate assay, hydrogen peroxide, Fisk-Subbarrow reducer and phosphate standard were obtained from Sigma (St. Louis, MO) and ammonium molybdate was purchased from Fisher (Newark, DE). Ammonium persulfate was purchased from Bio-Rad Laboratories (Hercules, CA). Anti-GM1 polyclonal antibody was purchased from Calbiochem (San Diego, CA). Anti-caveolin-1 monoclonal antibody (clone 2297) and anti-flotillin-1 monoclonal antibody were obtained from BD Transduction Laboratories (San Jose, CA). Immobilon-P transfer membrane was from Millipore Corporation (Billerica, MA). Goat anti-mouse IgG conjugated with horseradish peroxidase (HRP) was from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Goat anti-rabbit IgG conjugated with HRP, SuperSignal West Pico Chemiluminescent Substrate Solution and RestoreTM Western Blot Stripping Buffer were from Pierce Co. (Rockford, IL). Mini CompleteTM Protease Inhibitor Cocktail was from Roche (Nutley, NJ). HA.11 was a product of Covance (Cumberland, VA). Placebo and 75-mg morphine pellets were from the National Institute of Drug abuse (NIDA).

4.1.1. Antibody—Anti- μC was rabbit polyclonal antibody generated against a synthetic peptide corresponding to the last 16 amino acids (383–398) of the C-terminal domain predicted from the cloned rat MOR-1 (GenBank: NM_013071), which has been proven to detect MOR in rat brain by Western blot successfully (Huang et al., in press).

4.1.2. Cell lines—the clonal CHO cell line stably expressing HA-rMOR was established as described previously (Huang et al., 2001b), with a B_{max} value of 1.8 pmol/mg membrane protein. CHO-HA-rMOR cells were cultured in Dulbecco's modified Eagle's medium F12 HAM supplemented with 10% fetal calf serum, 0.1 mg/ml geneticin, 100 U/ml penicillin

and 100 µg/ml streptomycin in a humidified atmosphere consisting of 5% CO₂ and 95% air at 37 °C.

4.2. Rats and the chronic morphine treatment

Male Sprague–Dawley rats, initially weighing 250–275 g, were housed in groups of three and acclimated for 7 days prior to surgery. Each group of animals were lightly anesthetized with isoflurane and implanted subcutaneously with two placebo or 75-mg morphine pellets in the mid-dorsal tissue (Bell and Adler, 1988). Approximately 72 h after pellet implantation, the animals were decapitated and brains were collected, which were dissected immediately to obtain CPu or frozen for later dissection. All rats were grouped housed (3 rats per cage) in a temperature/humidity-controlled environment that was maintained on a 12-h light/dark cycle. Animals received food and water *ad libitum* throughout each study. All experiments were conducted in accordance with the National Institutes of Health guidelines for the Care and Use of Laboratory Animals and with an approved animal protocol from Temple University School of Medicine Institutional Animal Care and Use Committee. All efforts were made to minimize the number of animals used and any potential suffering.

4.3. Rat brain CPu membrane preparation

The frozen meninges-stripped SD rat brains were purchased from Pel-Freeze Biologicals (Rogers, AR). Rat brains were also collected from control or chronic morphine-treated rats as above. The caudate putamen tissues were dissected and homogenized in ~8 vol 10 mM TEL buffer (10 mM Tris, 1 mM EGTA, 0.32 M sucrose, 10 mM glucose and 10 µM leupeptin, pH 7.4). After centrifugation at ~920×*g* for 10 min, the supernatant was saved and centrifuged again at ~100,000×*g* for 20 min. The pellet was resuspended in 5 mM TEL buffer (5 mM Tris, 5 mM EDTA, 5 mM EGTA and 10 µM leupeptin, pH 7.4) and stayed on ice for 30 min. After homogenization to disrupt synaptosomes, the homogenate was centrifuged at ~100,000×*g* for 30 min. The brown tight pellet at bottom was discarded and the top layer of loose pellet was saved for lipid rafts isolation as below. All procedures were performed at 4 °C or on ice.

4.4. Lipid rafts preparation

Detergent-free preparation of lipid rafts using sodium carbonate was conducted according to Song et al. (1996) with some modifications. One notable modification is the use of 8 ml of 5–35% continuous sucrose gradient on top of 4 ml of 45% sucrose in centrifugation, in addition to 5%/35%/45% discontinuous gradient, to facilitate detection of receptor shift following treatment with an agonist or MCD (Xu et al., 2006; Huang et al., 2007) [for a diagram, see Fig. S1 in Huang et al., 2007]. Cells were grown to confluence in 100-mm tissue culture plates, harvested, and centrifuged at 300×*g* for 5 min. Cell pellets or rat brain CPu membranes were resuspended in 2 ml of 500 mM sodium carbonate buffer (pH 11) and homogenized using a Thomas loose fitting Teflon homogenizer (10 strokes) followed by sonication (eight 20-s bursts at 6 W) on ice using a Fisher Sonic Dismembrator 60. Two ml of 90% sucrose prepared in MES-buffered saline (MBS) (20% glycerol, 150 mM NaCl, 2 mM EDTA, 25 mM MES, pH 6.5) were added to the homogenized samples yielding 45% sucrose in a total volume of 4 ml. An 8-ml continuous gradient of 5–35% sucrose prepared in MBS buffer containing 0.25 M sodium carbonate (for discontinuous gradient, 4 ml of 5% sucrose and 4 ml of 35% sucrose) was layered on the top of the 45% fraction. Isopycnic ultracentrifugation was then carried out at 39,000 rpm (~190,000×*g*) using a Beckman ultracentrifuge and a SW 41 rotor for 16–20 h at 4 °C. Following ultracentrifugation, twelve 1-ml fractions were collected from the bottom of the gradient tube using a peristaltic pump.

4.5. Western blot

Immunoblotting of MOR in rat brain, HA-rMOR, caveolin-1 and flotillin-1 was carried out according to our published method (Li et al., 2001; Xu et al., 2006), using anti- μ C and monoclonal antibodies against the HA-tag (HA.11), caveolin-1 (clone 2297) and flotillin-1, respectively.

4.6. Reduction of membrane cholesterol content by 2% of methyl- β -cyclodextrin (MCD) treatment

For CHO-HA-rMOR cells, cells were incubated with MCD for 1 h at 37 °C in the serum-free medium, while control cells received serum-free medium alone. Following incubation, MCD was removed by washing 3 times in cold PBS. The cells were then harvested and cell membranes prepared following our previous procedures (Huang et al., 2001a). For rat brain CPu, brain membranes (2 mg of protein) were prepared as above and then suspended in 20 ml of 5 mM TEL buffer with or without 2% MCD and incubated at 37 °C for 1 h. The membranes were then centrifuged at $\sim 100,000\times g$ at 4 °C for 30 min. The pelleted membranes were washed twice by suspension in the 20 ml TEL buffer and centrifugation at $\sim 100,000\times g$ at 4 °C for 30 min.

4.7. Ligand binding to opioid receptors

Binding was performed on each fraction of rat brain CPu membranes or CHO-HA-rMOR cell preparations after sucrose gradient centrifugation with [3 H]diprenorphine (1 nM, $\sim 120,000$ – $150,000$ dpm) in 1 ml of 50 mM Tris-HCl buffer/1 mM EGTA (pH 7.4) (TE buffer) at room temperature for 1 h in duplicate according to our published procedure (Xu et al., 2006; Huang et al., 2007). Nonspecific binding was defined as binding in the presence of naloxone (10 μ M).

4.8. [35 S]GTP γ S binding

Determination of [35 S]GTP γ S binding to G proteins was carried out using a modified procedure of Zhu et al. (1997). CHO-HA-rMOR cells were incubated with vehicle or 2% MCD as described and harvested, and membranes were prepared as described previously. Rat brain CPu membranes were treated with MCD as described. Membranes (10 μ g protein) were incubated in a reaction buffer (50 mM HEPES, 100 mM NaCl, 5 mM MgCl₂, 1 mM EDTA) containing [35 S]GTP γ S (100,000–150,000 dpm, 80–100 pM) and GDP (10 μ M for CHO-HA-rMOR cells; 80 μ M for rat brain CPu) with or without DAMGO or morphine (10^{-12} to 10^{-5} M) in a total volume of 0.5 ml in duplicate for 60 min at 30 °C. Nonspecific binding (~ 200 dpm) was determined in the presence of 10 μ M GTP γ S. Bound and free [35 S]GTP γ S were separated by filtration with GF/B filters under reduced pressure. Radioactivity was determined by liquid scintillation counting with a counting efficiency of 95%.

4.9. Fluorescence microscopy

Briefly, CHO-HA-rMOR cells were grown on Lab-Tek II Slide Chambers. Before immunostaining, cells were first treated with or without a test drug (1 mM etorphine or 10 mM morphine), at 37 °C for 30 min, and then fixed in 4% paraformaldehyde for 15 min at room temperature, then quenched with three washes of TBS. Cells were permeabilized with 0.1% Triton X100 in a blocking solution (4% normal goat serum in TBS). After washing, cells were incubated sequentially with monoclonal anti-HA (HA.11 from Covance) and goat anti-mouse IgG (H+L) conjugated Alexa Fluor 488 (1:1000 dilution, from Molecular Probes) for 30 min, respectively. Cells were mounted with Slow Fade mounting medium and coverslips were sealed with nail polish. Fluorescence microscopy was performed using a Nikon Eclipse TE300 microscope fitted with a 60 $\times$ oil objective lens.

4.10. Determination of intracellular receptors

Intracellular receptors were assessed according to a modification of a previously described procedure (Huang et al., 2001b). CHO-HA-rMOR cells were cultured with or without 1 mM etorphine or 10 mM morphine, washed three times with 1× PBS buffer, and harvested. Binding was performed on intact cells in suspension in 1× PBS buffer. Total receptor levels were assessed by binding with 0.4 nM [³H]diprenorphine in the presence or absence of 10 mM naloxone, while surface receptors were measured by binding with 0.4 nM [³H]diprenorphine in the presence or absence of 4 μM CTAP. Binding was performed at room temperature for 60 min, and bound and free radioactivity was separated by filtration with cold 1× PBS.

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Abbreviations

CHO	Chinese hamster ovary cells
CPu	caudate putamen
DAMGO	[D-Ala ² ,N-MePhe ⁴ ,Glyol ⁵]enkephalin
DOR	delta opioid receptor
GM1	ganglioside M1
7-TMRs/GPCRs	seven-transmembrane receptors/G-protein-coupled receptors
HA	influenza hemagglutinin epitope tag (YPYDVPDYA)
KOR	kappa opioid receptor
MCD	methyl-β-cyclodextrin
MOR	mu opioid receptor
Mr	relative molecular mass

References

- Bell J, Adler MW. Comparison of peripheral and central administration of naloxone in precipitating abstinence in morphine-dependent rats. *Drug Alcohol Depend.* 1988; 21:189–194. [PubMed: 3168761]
- Bockaert J, Pin JP. Molecular tinkering of G protein-coupled receptors: an evolutionary success. *EMBO J.* 1999; 18:1723–1729. [PubMed: 10202136]
- Brown DA, Rose JK. Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. *Cell.* 1992; 68:533–544. [PubMed: 1531449]
- Cameron PL, Ruffin JW, Bollag R, Rasmussen H, Cameron RS. Identification of caveolin and caveolin-related proteins in the brain. *J Neurosci.* 1997; 17:9520–9535. [PubMed: 9391007]
- Chini B, Parenti M. G-protein coupled receptors in lipid rafts and caveolae: how, when and why do they go there? *J Mol Endocrinol.* 2004; 32:325–338. [PubMed: 15072542]
- Chun M, Liyanage UK, Lisanti MP, Lodish HF. Signal transduction of a G protein-coupled receptor in caveolae: colocalization of endothelin and its receptor with caveolin. *Proc Natl Acad Sci U S A.* 1994; 91:11728–11732. [PubMed: 7972131]
- Cohen AW, Hnasko R, Schubert W, Lisanti MP. Role of caveolae and caveolins in health and disease. *Physiol Rev.* 2004; 84:1341–1379. [PubMed: 15383654]

- Head BP, Patel HH, Roth DM, Lai NC, Niesman IR, Farquhar MG, Insel PA. G-protein-coupled receptor signaling components localize in both sarcolemmal and intracellular caveolin-3-associated microdomains in adult cardiac myocytes. *J Biol Chem*. 2005; 280:31036–31044. [PubMed: 15961389]
- Huang P, Kehner GB, Cowan A, Liu-Chen LY. Comparison of pharmacological activities of buprenorphine and norbuprenorphine: norbuprenorphine is a potent opioid agonist. *J Pharmacol Exp Ther*. 2001a; 297:688–695. [PubMed: 11303059]
- Huang P, Li J, Chen C, Visiers I, Weinstein H, Liu-Chen LY. Functional role of a conserved motif in TM6 of the rat mu opioid receptor: constitutively active and inactive receptors result from substitutions of Thr6. 34(279) with Lys and Asp. *Biochemistry*. 2001b; 40:13501–13509. [PubMed: 11695897]
- Huang P, Xu W, Yoon SI, Chen C, Chong PL, Liu-Chen LY. Cholesterol reduction by methyl-beta-cyclodextrin attenuates the delta opioid receptor-mediated signaling in neuronal cells but enhances it in non-neuronal cells. *Biochem Pharmacol*. 2007; 73:534–549. [PubMed: 17141202]
- Kieffer BL. Opioids: first lessons from knockout mice [Review]. *Trends Pharmacol Sci*. 1999; 20:19–26. [PubMed: 10101958]
- Law PY, Wong YH, Loh HH. Molecular mechanisms and regulation of opioid receptor signaling. *Annu Rev Pharmacol Toxicol*. 2000; 40:389–430. [PubMed: 10836142]
- Li J, Chen C, Huang P, Liu-Chen LY. Inverse agonist up-regulates the constitutively active D3.49(164)Q mutant of the rat μ opioid receptor by stabilizing the structure and blocking constitutive internalization and down-regulation. *Mol Pharmacol*. 2001; 60:1064–1075. [PubMed: 11641435]
- Li S, Okamoto T, Chun M, Sargiacomo M, Casanova JE, Hansen SH, Nishimoto I, Lisanti MP. Evidence for a regulated interaction between heterotrimeric G proteins and caveolin. *J Biol Chem*. 1995; 270:15693–15701. [PubMed: 7797570]
- Macdonald JL, Pike LJ. A simplified method for the preparation of detergent-free lipid rafts. *J Lipid Res*. 2005; 46:1061–1067. [PubMed: 15722565]
- Maher CE, Selley DE, Childers SR. Relationship of mu opioid receptor binding to activation of G-proteins in specific rat brain regions. *Biochem Pharmacol*. 2000; 59:1395–1401. [PubMed: 10751548]
- Mansour A, Khachaturian H, Lewis ME, Akil H, Watson SJ. Anatomy of CNS opioid receptors. *Trends Neurosci*. 1988; 11:308–314. [PubMed: 2465635]
- Matthes HW, Maldonado R, Simonin F, Valverde O, Slowe S, Kitchen I, Befort K, Dierich A, Le Meur M, Dolle P, Tzavara E, Hanoune J, Roques BP, Kieffer BL. Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the mu-opioid-receptor gene (see comments). *Nature*. 1996; 383:819–823. [PubMed: 8893006]
- Navratil AM, Bliss SP, Berghorn KA, Haughian JM, Farmerie TA, Graham JK, Clay CM, Roberson MS. Constitutive localization of the gonadotropin-releasing hormone (GnRH) receptor to low density membrane microdomains is necessary for GnRH signaling to ERK. *J Biol Chem*. 2003; 278:31593–31602. [PubMed: 12791688]
- Nicolau DV Jr, Burrage K, Parton RG, Hancock JF. Identifying optimal lipid raft characteristics required to promote nanoscale protein–protein interactions on the plasma membrane. *Mol Cell Biol*. 2006; 26:313–323. [PubMed: 16354701]
- Noble F, Cox BM. Differential desensitization of mu- and delta-opioid receptors in selected neural pathways following chronic morphine treatment. *Br J Pharmacol*. 1996; 117:161–169. [PubMed: 8825358]
- Ostrom RS, Insel PA. The evolving role of lipid rafts and caveolae in G protein-coupled receptor signaling: implications for molecular pharmacology. *Br J Pharmacol*. 2004; 143:235–245. [PubMed: 15289291]
- Parton RG. Caveolae-from ultrastructure to molecular mechanisms. *Nat Rev, Mol Cell Biol*. 2003; 4:162–167. [PubMed: 12563293]
- Pasternak, GW. *The Opiate Receptors*. The Humana Press; Clifton, NJ: 1988.

- Patel HH, Head BP, Petersen HN, Niesman IR, Huang D, Gross GJ, Insel PA, Roth DM. Protection of adult rat cardiac myocytes from ischemic cell death: role of caveolar microdomains and delta opioid receptors. *Am J Physiol, Heart Circ Physiol*. 2006; 291:H344–H350. [PubMed: 16501018]
- Pike LJ. Lipid rafts: bringing order to chaos. *J Lipid Res*. 2003; 44:655–667. [PubMed: 12562849]
- Pike LJ. Rafts defined: a report on the Keystone Symposium on Lipid Rafts and Cell Function. *J Lipid Res*. 2006; 47:1597–1598. [PubMed: 16645198]
- Rybin VO, Xu X, Lisanti MP, Steinberg SF. Differential targeting of beta-adrenergic receptor subtypes and adenylyl cyclase to cardiomyocyte caveolae. A mechanism to functionally regulate the cAMP signaling pathway. *J Biol Chem*. 2000; 275:41447–41457. [PubMed: 11006286]
- Sharma P, Varma R, Sarasij RC, Ira, Gousset K, Krishnamoorthy G, Rao M, Mayor S. Nanoscale organization of multiple GPI-anchored proteins in living cell membranes. *Cell*. 2004; 116:577–589. [PubMed: 14980224]
- Shaw AS. Lipid rafts: now you see them, now you don't. *Nat Immunol*. 2006; 7:1139–1142. [PubMed: 17053798]
- Song KS, Li S, Okamoto T, Quilliam LA, Sargiacomo M, Lisanti MP. Co-purification and direct interaction of Ras with caveolin, an integral membrane protein of caveolae microdomains. Detergent-free purification of caveolae microdomains. *J Biol Chem*. 1996; 271:9690–9697. [PubMed: 8621645]
- Traynor JR, Nahorski SR. Modulation by mu-opioid agonists of guanosine-5'-O-(3-[³⁵S]thio)triphosphate binding to membranes from human neuroblastoma SH-SY5Y cells. *Mol Pharmacol*. 1995; 47:848–854. [PubMed: 7723747]
- Wang H, Cuzon VC, Pickel VM. Ultrastructural localization of delta-opioid receptors in the rat caudate-putamen nucleus during postnatal development: relation to synaptogenesis. *J Comp Neurol*. 2003; 467:343–353. [PubMed: 14608598]
- Whaley BS, Yuan N, Birnbaumer L, Clark RB, Barber R. Differential expression of the beta-adrenergic receptor modifies agonist stimulation of adenylyl cyclase: a quantitative evaluation. *Mol Pharmacol*. 1994; 45:481–489. [PubMed: 8145734]
- Xu W, Yoon SI, Huang P, Wang Y, Chen C, Chong PL, Liu-Chen LY. Localization of the kappa opioid receptor in lipid rafts. *J Pharmacol Exp Ther*. 2006; 317:1295–1306. [PubMed: 16505160]
- Zhao H, Loh HH, Law PY. Adenylyl cyclase superactivation induced by long-term treatment with opioid agonist is dependent on receptor localized within lipid rafts and is independent of receptor internalization. *Mol Pharmacol*. 2006; 69:1421–1432. [PubMed: 16415176]
- Zhu J, Luo LY, Chen C, Liu-Chen LY. Activation of the cloned human κ opioid receptor by agonists enhances [³⁵S] GTP γ S binding to membranes: determination of potencies and efficacies of ligands. *J Pharmacol Exp Ther*. 1997; 282:676–684. [PubMed: 9262330]

Discontinuous Sucrose Gradients **Fig. 1.**

Localization in lipid rafts of the mu opioid receptor (MOR) in membranes of the rat caudate putamen (CPu) and CHO-HA-rMOR cells. CPu membranes prepared from 6 frozen meninges-stripped rat brains or three 100-mm dishes of CHO-HA-rMOR cells were sonicated in 0.5 M sodium carbonate buffer (pH 11) and then fractionated through a discontinuous sucrose gradient (5%/35%/45%) by ultracentrifugation as described in Experimental procedures. Twelve 1-ml fractions were collected and each fraction was subjected to (A and C) [³H]diprenorphine (~1 nM) bindings using naloxone (10 μM) to define nonspecific binding. Two 100-μl aliquots from each fraction were used in binding in duplicate as described in Experimental procedures. Data are expressed as percent of total specific [³H]diprenorphine binding. (B and D) Immunoblottings with anti-μC, anti-HA(HA. 11), anti-flotillin-1 and anti-caveolin-1 antibodies, respectively. Data shown in this image are representatives of the two experiments performed with similar results.

Fig. 2.

Lipid rafts distribution of HA-rMOR expressed in CHO cells was not affected by acute agonist treatment. CHO-HA-rMOR cells were incubated at 37 °C in the absence and presence of 1 μ M etorphine or 10 μ M of morphine for 30 min. Cells were collected and 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 Experimental procedures. Twelve fractions from continuous sucrose gradients were collected and each fraction was subjected to (A) immunoblotting of HA-rMOR (the upper panel) with a monoclonal anti-HA antibody (HA.11) and caveolin-1 (the lower panel) with an anti-caveolin-1 monoclonal antibody. Each image represents one of the two experiments performed with similar results. (B) [3 H]diprenorphine (~1 nM) binding and data were expressed as percent of total specific [3 H]diprenorphine binding. Three 100-mm dishes of confluent CHO-HA-rMOR cells were used for fractionation and two 100- μ l aliquots of each 1-ml fraction were used in binding in duplicate. The average [3 H]diprenorphine-specific binding in the fraction 1 was ~6300 dpm/100 μ l. Data are shown as mean $\pm$ SEM from three independent experiments.

Fig. 3. In CHO-HA-rMOR cells, the MOR was internalized by acute treatment of etorphine, but not morphine. (A) Immunofluorescence imaging by use of the anti-HA monoclonal antibody, HA.11, as described in the Experimental procedures. (B) Quantification of intracellular receptors by intact cell binding assay as described in the Experimental procedures.

Fig. 4.

Lipid rafts distribution of the MOR in rat CPU and thalamus membranes was not affected by chronic *in vivo* morphine treatment. Three adult male Sprague–Dawley rats (250–275 g) were implanted subcutaneously with two control (placebo) or 75-mg morphine pellets in the middorsal region under light isoflurane anaesthesia. The animals were decapitated at 72 h after implantation and the CPU and thalamus tissues collected. Brain membranes were prepared from each region and fractionation for lipid rafts was performed as in Fig. 2 legend. Twelve fractions from continuous sucrose gradients were collected and each fraction was subjected to immunoblotting of the MOR in rat CPU (A) and thalamus (C) with anti- μ C antibody against a synthetic peptide corresponding to rMOR (381–398). The result represents one of the two experiments performed with similar results. [3 H] diprenorphine (~1 nM) binding of rat CPU (B) and thalamus (D) using naloxone (10 μ M) to define nonspecific binding. Two 100- μ l aliquots from each fraction were used in binding in duplicate as described in Experimental procedures. Data are expressed as percent of total specific [3 H]diprenorphine binding. Data are shown as mean $\pm$ SEM from three independent experiments.

Fig. 5. Effects of MCD treatment on MOR and caveolin-1 distribution in CHO-HA-rMOR cells. Cells were treated with (A) serum-free medium or (B) 2% MCD for 1 h at 37 °C as described in Experimental procedures, then collected and subjected to lipid rafts preparation through continuous sucrose gradients as described in Fig. 2 legend. Twelve 1-ml fractions were collected and for each fraction, HA-rMOR and caveolin-1 were detected by immunoblottings with the anti-HA antibody, HA.11 and an anti-caveolin-1 monoclonal antibody. Each image represents one of the two experiments performed with similar results.

Fig. 6.

MCD pretreatment has different effects on agonist-induced $[^35\text{S}]\text{GTP}\gamma\text{S}$ binding to membranes of (A) rat brain CPu and (B) CHO-HA-rMOR cells. Cells or rat CPu membranes were incubated with vehicle or 2% MCD, membranes were prepared and $[^35\text{S}]\text{GTP}\gamma\text{S}$ binding experiments were performed as described in Experimental procedures. Data are normalized as percent of basal $[^35\text{S}]\text{GTP}\gamma\text{S}$ binding (in the absence of agonists) and shown as mean $\pm$ SEM of 3–5 experiments performed in duplicate and summarized in Table 1. The basal $[^35\text{S}]\text{GTP}\gamma\text{S}$ binding was ~ 2000 dpm for non-treated membranes from both CHO cells and CPu. After MCD treatment, the basal binding for CHO cells remained the same, however, the basal binding for CPu was only $\sim 50\%$ of that for the non-treated sample.

Table 1

Effects of MCD pretreatment on EC_{50} and E_{max} values of mu agonists in stimulating [35 S]GTP γ S binding to membranes of CHO-HA-rMOR cells and rat CPu

	Control		2% MCD treated	
	EC_{50} (nM)	E_{max} (Percent of basal)	EC_{50} (nM)	E_{max} (Percent of basal)
<i>CHO-HA-rMOR cells</i>				
Etoprophine	0.34±0.12	201±10.0	1.0±0.25	278±10.2 *
DAMGO	8.5±1.3	206±5.3	21.9±6.9	291±10.5 *
Morphine	18±7.0	178±8.5	49±10	255±3.8 *
<i>Rat caudate putamen</i>				
DAMGO	1123±976	272±8.9	1088±670	181±4.0 *
Morphine	1010±490	180±9.8	1535±589	123±4.8 *

Cells or CPu membranes were incubated with vehicle or 2% MCD, and [35 S]GTP γ S binding experiments were performed as described in Experimental procedures. Data are shown as mean±SEM of 3–5 experiments performed in duplicate.

* $P < 0.01$, by Student's two-tailed t tests, compared with each control.