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Posttranslation Modification of G Protein-Coupled Receptor in Relationship to Biased Agonism

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Abstract

Biased signaling has been reported with a series of G protein-coupled receptors (GPCRs), including α_2 -adrenergic receptor and μ -opioid receptor (OPRM1). The concept of biased signaling suggests that the agonists of one particular receptor may activate the downstream signaling pathways with different efficacies. Thus in an extreme case, agonists might activate different sets of signaling pathways, which provide a new route to develop drugs with increased efficacies and decreased side effects. Among the many factors, posttranslation modifications of receptor proteins have major roles in influencing the biased signaling. Take OPRM1, for example, the phosphorylation and palmitoylation of receptor can regulate the biased signaling induced by agonists. Thus, by modulating these posttranslation modifications, the biased signaling of GPCRs can be regulated. In addition, although it is not considered as posttranslation modification normally, the distribution of GPCRs on cell membrane, especially the distribution between lipid-raft and non-raft microdomains, also contributes to the biased signaling. Thus in this chapter, we described the methods used in our laboratory to study receptor phosphorylation, receptor palmitoylation, and membrane distribution of receptor by using OPRM1 as a model. A functional model was also provided on these posttranslational modifications at the last section of this chapter.

1. INTRODUCTION

The concept on G protein-coupled receptor (GPCR) signaling has changed dramatically over the past several decades (Kenakin, 1995a, 1995b; Urban et al., 2007; Violin & Lefkowitz, 2007). One of the previous concepts classifies the agonists of one particular receptor into: full agonists, partial agonists, neutral agonists, and inverse agonists, depending on their efficacies to activate the receptor (Kenakin, 1995a, 1995b). The fundamental tenet of this receptor theory is that one agonist should activate all the downstream pathways of the receptor with similar efficacies.

Although this concept was widely accepted for many years, it was unable to explain several recent observations which indicate that one agonist may activate the different signaling pathways to different extents (Ahn, Shenoy, Wei, & Lefkowitz, 2004; Gesty-Palmer et al., 2006). Thus, the insufficiency of this concept has been pointed out in recent years (Urban et al., 2007). A new theory of biased signaling (or agonist-selective, agonist-dependent, functional selectivity of agonist) has been proposed so as to accommodate all the observations in GPCR activation. The new theory suggests that one agonist can activate the

downstream signaling pathways with different efficacies. The efficacies of one agonist to activate downstream signaling pathways do not necessarily correlate with those of another agonist. That is, one agonist may prefer to activate one set of signaling pathways, while another agonist may prefer to activate a different set of signaling pathways (Urban et al., 2007; Zheng, Loh, & Law, 2010).

The new theory also has great impact on the new drug development. The biased signaling suggests a possible pathway selectivity of agonist under various conditions. Therefore, if the new agonist only activate the signaling pathways that lead to the desired therapeutic effects, but not the signaling pathways that result in the side effects, this new agonist should be a “better” drug (Zheng, Loh, et al., 2010). Thus, great efforts have been put into the investigation on biased signaling. However, with the large numbers of agonists and numerous downstream signaling pathways activated by GPCRs, it is difficult and time consuming to determine the efficacy in each individual agonist-signaling pathway pair. Therefore, it will be valuable to identify simple methods to predict the biased signaling induced by agonist. Based on our previous reports (Zheng, Chu, Qiu, Loh, & Law, 2008; Zheng, Chu, Zhang, Loh, & Law, 2011; Zheng, Zeng, Zhang, et al., 2010), receptor phosphorylation and receptor membrane distribution can be used to predict the biased signaling.

Using μ -opioid receptor (OPRM1) as an example, agonist-induced OPRM1 phosphorylation correlates well with agonist-induced biased signaling. Agonists that induce low level of receptor phosphorylation prefer to activate PKC-pathway, while agonists that induce high level of receptor phosphorylation prefer to activate β -arrestin-pathway (Zheng, Chu, et al., 2008; Zheng et al., 2011). Thus the ability of agonist to induce OPRM1 phosphorylation will be useful to predict whether it is PKC- or β -arrestin-pathway that is used by the agonist for signal transduction. As discussed previously, the competition between G protein and β -arrestin determines whether agonist selects for the PKC-pathway or the β -arrestin-pathway. Receptor phosphorylation increases the affinity of receptor complex for β -arrestin (Zhang et al., 1998) and favors the selection of β -arrestin-pathway (Zheng, Loh, et al., 2010).

As receptor palmitoylation has been suggested to contribute to the homodimerization and G protein coupling of GPCR (Cherezov et al., 2007), the palmitoylation of receptor also participates in the biased signaling. G protein is normally highly palmitoylated and myristoylation (Elortza et al., 2003; Foster, De Hoog, & Mann, 2003; Linder et al., 1993; Nebl et al., 2002; Oldham & Hamm, 2006). In our reported studies, OPRM1 has been suggested to be anchored to the lipid raft microdomains through the interaction with G protein in the absence of agonist (Zheng, Chu, et al., 2008). In addition, the differential influence of agonists on the membrane distribution of OPRM1 has direct relation with the biased signaling (Zheng, Chu, et al., 2008). Therefore, in this chapter, the methods used to study receptor phosphorylation, receptor palmitoylation, and receptor membrane distribution are described in detail.

2. POSTTRANSLATION MODIFICATION OF GPCR WITH BIASED SIGNALING

The methods described below were modified from their original formats to fit into our studies. One principle for the modification was to avoid the use of radioactive materials. In the following sections, these protocols would be described with OPRM1. Thus when applying these protocols with other GPCRs, necessary modifications should be considered.

2.1. The phosphorylation of OPRM1

The previous radioactive protocol to detect protein phosphorylation normally involves incubating the cells with the ^{32}P -ATP and detects the ^{32}P -labeled protein after incubation. Although the half-life of ^{32}P is short, ~14 days, large quantity of ^{32}P -ATP and great care is needed during operation. Our protocol is less sensitive than the radioactive one but is sufficient for semiquantification. In the subsequent paragraphs, we describe the methods used in our laboratory to detect the phosphorylation of OPRM1, which can also be easily adapted for other GPCRs.

Briefly, the protocol involves the immunoprecipitation of OPRM1 from cell lysate, and the precipitated receptor will be subjected for immunoblotting. The major phosphorylation sites of OPRM1 are S363, T370, and T375, and T375 is more significant than the other two (El Kouhen et al., 2001). Thus, the total phosphorylation was quantified by using antibody against phosphorylated amino acid (Invitrogen, rabbit anti-phosphorylated proteins, 61-8300). The phosphorylation on T375 was quantified by using antibody against phosphorylated T375 (Cell signaling, phospho-OPRM1 S375, 3451).

2.1.1 Materials required

1. *Cells expressed OPRM1.* For high level of OPRM1 exogenously expressed, the human embryonic kidney (HEK) 293 cells stably expressed OPRM1 at about 5 pmol/mg protein is used. For OPRM1 endogenously expressed, the primary cultures from rat hippocampal neurons generated as described by Liao et al. (2007) and Liao, Lin, Law, & Loh (2005) are used.
2. *The cell lysis buffer.* The lysis buffer includes proteinase inhibitors and phosphatase inhibitors in the RIPA buffer (Jiang et al., 2001). The buffer contains 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.25% sodium deoxycholate, 0.1% Nonidet P-40, 0.1% Triton X-100, 50 mM NaF, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 50 mM sodium pyrophosphate, 10 mM sodium vanadate, and 1× protease inhibitor cocktail (Roche, Indianapolis, IN).
3. *Antibodies.* HA antibody (Covance, HA.11 monoantibody, MMS 101R-1000) is used to detect the HA fused at the N-terminal of OPRM1. The antibodies generated in our laboratory in University of Minnesota or Dr. Li-Yuan Liu-Chen's laboratory in Temple University using the last 15 amino acids at the carboxyl terminus as the antigen are used to detect OPRM1 directly. Antibody against phosphorylated amino acid (Invitrogen, rabbit anti-phosphorylated proteins, 61-8300) and antibody against phosphorylated T375 (Cell signaling, phospho-OPRM1 S375, 3451) are used to determine receptor phosphorylation.
4. *Others.* Protein G agarose (Invitrogen, 15920010) and other instruments/reagents for immunoprecipitation and immunoblotting, such as electrophoresis systems and PVDF membrane, are supplied by BioRad (Hercules, CA).

2.1.2 Procedures and cautions

1. After various treatments as desired, place the cell culture dishes/flasks on ice and wash them with PBS at 4 °C twice. Add the lysis buffer at 4 °C (0.7 ml/100 mm dish) to the cells and immediately use cell-scraper to collect the cells into a 1.5-ml eppendorf tube.
2. Rotate the tube in 4 °C for 10 min to achieve complete lysis. Centrifuge the tube at 12,000 rpm, 4 °C for 5 min and collect the resulted supernatant for next step.

Caution 1: Remove the medium and the PBS used for washing completely.

Caution 2: Make sure the sample stay on the ice or in the cold room during all the procedures because the low temperature is critical to keep receptor phosphorylation at its original state.

Caution 3: Control the time used to treat the cells exactly, especially when the time course is studied, as the onset of GPCR phosphorylation is rapid (Tobin, Butcher, & Kong, 2008).

3. Incubate the supernatant with antibody against OPRM1 (1:500) or HA antibody (1:1000) at 4 °C for 5 h with rotation.
4. Add the Protein G agarose (50 µl) into the sample and incubate at 4 °C over night with rotation.
5. Centrifuge the tube at 12,000 rpm 4 °C for 1 min. Use the supernatant to determine the relative protein concentrations in each sample. Wash the beads with lysis buffer for three times and collect the beads through centrifugation (12,000 rpm, 4 °C, 1 min).

Caution 4: Prewash the Protein G agarose with lysis buffer for three times, as ethanol is included in the storage buffer.

Caution 5: Take great care when washing the beads. Remove the supernatants completely but do not make contact with the beads.

6. Incubate the washed beads with loading buffer at 65 °C for 5 min. Collect the supernatant with centrifugation (12,000 rpm, 4 °C, 1 min). Determine receptor phosphorylation by using the antibody mentioned above and the immunoblotting protocol described previously (Zheng, Loh, & Law, 2008).

Caution 6: Use the protein concentrations collected in step (5) to control the amounts of supernatants loaded, in order to make the results comparable among different samples.

Caution 7: Prepare the sample with loading buffer and heating them at 65 °C, but not at 95 °C, is critical for the immunoblotting with OPRM1 and other GPCRs. The higher temperature will result in oligomerization of the receptor protein.

Caution 8: As the protocol works with the sample that normally is at about 0.5 ml in volume, it is important to have no residues during washing and to collect all supernatant after all centrifugation steps.

2.1.3 Advantages and disadvantages—The current protocol is a simple modification of immunoprecipitation and immunoblotting, thus is not difficult to follow. The major advantage of this protocol is that semiquantification for receptor phosphorylation can be carried out without the use of radioactivity. However, the protocol requires great care during sample processing and protein quantification is required to ensure comparable results among different samples. When compared to the traditional radioactive protocol, current one is less sensitive but easier to follow.

The three major phosphorylation sites of OPRM1 (Ser363, Thr370, and Ser375) are differentially activated by agonists (Chu, Zheng, Loh, & Law, 2008; Doll et al., 2011). Ser363 is constitutively phosphorylated in the absence of agonist. Based on the less abilities of morphine to induce phosphorylation on the other two sites and the faster rate of Ser375 phosphorylation than that of Thr370 phosphorylation, Ser375 has been suggested as the primary site of agonist-biased phosphorylation (Doll et al., 2011; Grecksch et al., 2011).

Thus, receptor phosphorylation can also be studied with current protocol and the phosphosite-specific antibodies (Doll et al., 2011).

2.2. The palmitoylation of OPRM1

Palmitoylation is a covalent attachment of palmitic acid to cysteine residues of membrane proteins. Sequence alignment has identified cysteine residues in the carboxy termini as potential palmitoylation sites in about 78% of the 74 GPCRs examined (Probst, Snyder, Schuster, Brosius, & Sealfon, 1992). However, these cysteines are not the only palmitoylation sites (Chen, Shahabi, Xu, & Liu-Chen, 1998; Hawtin, Tobin, Patel, & Wheatley, 2001). The protocol to detect palmitoylation of OPRM1 is based on the report by Drisdell et al. (Drisdel, Alexander, Sayeed, & Green, 2006). Briefly, the protocol blocks the free cysteines on the receptor precipitated with Protein G agarose. After the palmitic acids are removed from the palmitoylated cysteines, biotins are attached to the depalmitoylated cysteines, the palmitoylation of receptor is determined by measuring the amounts of biotin attached to the receptor. As reported originally by Drisdell et al., this protocol has a similar if not higher sensitivity than the traditional protocol using ^3H labeled palmitic acids (Drisdel et al., 2006).

2.2.1 Materials required

1. *N*-ethylmaleimide (NEM), hydroxylamine, and 1-biotinamido-4-[4 - (maleimidomethyl) cyclohexane carboxamido] butane (btn-BMCC) are used to block free cysteines, to remove the covalently linked palmitic acids, and to link biotin to the depalmitoylated cysteines, respectively.
2. Alkaline phosphatase-conjugated streptavidin is used to determine the level of biotin covalently attached to the receptor reflecting receptor palmitoylation.

2.2.2 Procedures and cautions

1. Use the first five steps described in Section 2.1.2 to collect the beads. Incubate the collected beads with 50 mM NEM (dissolved in 0.5 ml lysis buffer) for 3 h with rotation at 4 °C.
2. Wash the beads with lysis buffer for three times and incubate them with 1 M hydroxylamine (dissolved in 0.5 ml lysis buffer) for 3 h with rotation at 4 °C.
3. Wash the beads with lysis buffer for three times and incubate them with 40 μM btn-BMCC (dissolved in 0.5 ml lysis buffer) overnight with rotation at 4 °C.
4. Wash the beads with lysis buffer for three times and incubate them with loading buffer at 65 °C for 5 min. Collect the supernatant with centrifugation (12,000 rpm, 4 °C, 1 min) and load into gel for immunoblotting. Semiquantify receptor palmitoylation by measuring the amounts of biotin attached to the receptor.

Caution 9: Separate the beads from supernatant with centrifugation (12,000 rpm, 4 °C, 1 min).

Caution 10: Normalize the amounts of biotin attached to the receptor against the protein concentrations determined in the supernatant collected before using NEM.

Caution 11: Use 2-bromopalmitate to treat the cells for 12 h as negative control.

Caution 12: Dissolve the three chemicals used in the current protocol in lysis buffer freshly before the incubation.

Caution 13: Omit one of the three incubation steps (1–3) to provide the additional control for the experiments.

2.2.3 Advantages and disadvantages—The current protocol has been reported to have higher sensitive than the radioactive protocol (Drissdel et al., 2006). However, current protocol has five steps of incubation if immunoprecipitation were counted. Thus great care needs to be taken to keep the beads intact during washing and incubation.

3. MEMBRANE DISTRIBUTION OF GPCR WITH BIASED SIGNALING

3.1. Introduction to membrane distribution of GPCR

Lipid raft microdomains are dynamic plasma membrane microdomains containing high levels of cholesterol and sphingolipids (Helms & Zurzolo, 2004; Mayor & Rao, 2004). There are accumulating reports in support of the essential roles of the lipid raft microdomains localization played in GPCR signaling. Extracting cholesterol with methyl- β -cyclodextrin disrupts the lipid raft microdomains and attenuates the signaling of several GPCRs (Monastyrskaya, Hostettler, Buergi, & Draeger, 2005; Navratil et al., 2003; Zhang, Tetrault, Wang, Loh, & Law, 2006). In addition, our previous experiments have demonstrated that distribution of GPCR on cell membrane affects the biased signaling (Zheng, Chu, et al., 2008). Lipid raft microdomains are essential for the adenylyl cyclase (AC) inhibition induced by either morphine or etorphine. However, morphine-, but not etorphine-, induced ERK phosphorylation is influenced by the disruption of lipid raft microdomains (Zheng, Chu, et al., 2008).

3.2. Differential protocols to determine the membrane distribution of GPCRs

Currently, there are several methods that can be used to determine the membrane distributions of GPCRs. Triton X-100, Brij 98, Nonidet P40, and other detergents were used to extract whole cells, and lipid raft micro-domains were then separated from other fractions by centrifugation on sucrose density gradient (Brown & Rose, 1992; Schuck, Honsho, Ekroos, Shevchenko, & Simons, 2003). As the use of detergent may lead to some artifacts (Macdonald & Pike, 2005; Mayor & Maxfield, 1995), detergent-free methods were developed to purify the lipid rafts. Sodium carbonate buffer (pH 11) and isotonic buffer containing EDTA were used to separate lipid rafts by different groups (Damaj et al., 1996; Smart, Ying, Mineo, & Anderson, 1995). Based on these two protocols, we formulated a new method to study the membrane distribution of GPCRs.

3.3. Our protocol to determine the membrane distribution of GPCRs

Briefly, the purified cell membrane is mixed with sodium carbonate buffer and sonicated. The resulted samples are centrifuged with a 5–30% continuous sucrose gradient. The target proteins in each fraction are concentrated with trichloroacetic acid (TCA) precipitation and redissolved to be quantified with immunoblotting.

3.3.1 Materials required

1. Instrument (GradientStation, Biocomp, Fredericton, NB, Canada) to prepare the 5–30% continuous sucrose gradient. Ultracentrifuge (Optima 80XL, SW41 rotor, Beckman Coulter, Brea, CA) to separate the lipid raft microdomains with continuous sucrose gradient.
2. Chemicals for cell membrane preparation and TCA precipitation.

3.3.2 Procedures and cautions

1. Collect cells from one 150-mm dish into 10 ml homogenizing buffer (0.32 *M* sucrose and 10 *mM* HEPES, pH 7.7) and homogenize for 10 strokes with a glass Dounce homogenizer (Wheaton, 40 ml), pestle A.
2. Centrifuge the crude lysate at $1000 \times g$ for 10 min at 4 °C. Collect the supernatant and rehomogenize the pellet until the final pellet appears translucent. Collect the pellet after centrifuging the supernatants at $100,000 \times g$ for 60 min at 4 °C.

Caution 14: Do not stop rehomogenization until the pellet appeared translucent. This normally required homogenization three times and centrifugation.

3. Resuspend the pellet in 0.7 ml 500 *mM* sodium carbonate and further homogenize by passing through needles (22 gauge) for 10 times. Use sonicator equipped with a microprobe (Heat Systems–Ultrasonics) to break up the purified membrane (three 10-s bursts at setting four).

Caution 15: Keep the sonication time with different samples at similar level. The sonication should be sufficient to make the majority of membrane into vesicles with diameter less than 50 nm, as the size of lipid raft has been suggested to be close to this dimension (Edidin, 2003). The sonication step should be optimized when the different instrument is used.

4. Mix the resulted lysate with equal volumes of 80% sucrose [in morpholinoethanesulfonic acid-buffered saline (MBS), pH 6.8]. The total volume is about 1.4 ml.
5. Prepare the 5% and 30% sucrose solution in MBS containing 250 *mM* sodium carbonate. 7 ml of each solution is required for each sample.

Caution 16: The sucrose solution should be prepared with volumetric flask.

6. Formulate the continuous sucrose gradient from the 5% and 30% sucrose solutions with GradientStation (BioComp Fredericton, NB, Canada) in an ultracentrifugation tubes (Beckman, 13.2 mL, 14 × 89 mm). Place the mixed lysate from step 4) at the bottom of the tube. The total volume is about 12.5 ml.

Caution 17: Put 6 ml 30% sucrose in the ultracentrifugation tube first and then add about 6 ml 5% sucrose solution on the top. Place the sample at the bottom of the tube after preparation of the continuous gradient.

7. Centrifuge the gradient at 32,000 rpm for 16 h in a SW41 rotor at 4 °C.
8. Collect 12 fractions (1 ml each) using the Gradient Station.

Caution 18: Make sure the fractions are not mixed with others. In addition, about 0.5 ml residue will be left in the tube after collecting 12 fractions.

9. Add 0.25 ml 100% TCA into each fraction to achieve a final concentration of 20% TCA. Keep the sample on ice for 10 min and then centrifuge at 12,000 rpm, 4 °C for 10 min.

Caution 19: When adding TCA into the sample, it will react with sodium carbonate to release CO₂. Thus, TCA should be added slowly to avoid rapid release of CO₂ and possible lost of sample.

10. Wash the resulted pellet with 0.5 ml acetone twice. Mix the pellet with acetone with vortex, keep on ice for 10 min, and then centrifuge at 12,000 rpm, 4 °C for 10 min.
11. Keep the pellet at room temperature for 10 min to dry the residue acetone. Redissolve the resulted pellet in the loading buffer for immunoblotting analysis. The amount of target protein was determined in each fraction with appropriate antibody.

Caution 20: Majority of the protein is located in the last two fractions. Thus, it will be difficult to dissolve the pellet in the volume of loading buffer as described. However, as the lipid raft microdomains and the non-raft microdomains are in the fourth and sixth fraction (Zheng, Chu, et al., 2008), the last two fractions can be omitted during immunoblotting analysis.

3.3.3 Advantages and disadvantages—The current protocol has more steps than some simple protocols reported by other laboratories (Macdonald & Pike, 2005). It takes nearly 2 days to process cells to loading samples for the immunoblotting and required multiple centrifugations. However, current protocol first purifies the cell membrane to reduce the influence from other membrane structures and also eliminates the possible artifacts resulted from the use of detergents. Finally, by using TCA to concentrate the target proteins after continuous sucrose gradient, the target proteins with lower expression can also be detected.

4. POSSIBLE INTERACTION BETWEEN THESE THREE METHODS

The receptor phosphorylation and palmitoylation have been suggested to influence the membrane distribution of the receptor (Zheng, Loh, et al., 2010). In the final section of current chapter, we discuss the relationship between posttranscriptional modifications and biased signaling.

One of the well-studied biased signaling of OPRM1 is the ERK phosphorylation. The two agonists, morphine and etorphine, induce ERK phosphorylation via PKC-pathway and β -arrestin-pathway, respectively, while both of the agonists induce AC inhibition through G_{i2} . There are other differences between these two OPRM1 agonists. (1) Morphine has lower ability than etorphine to induce receptor phosphorylation, β -arrestin recruitment, and receptor internalization (Keith et al., 1996; Kohout et al., 2004; Zhang et al., 1998). (2) OPRM1 locates in lipid raft microdomains in the absence of agonist. Etorphine, but not morphine, treatment induces the translocation of OPRM1 out of lipid raft microdomains (Zheng, Chu, et al., 2008a). (3) Morphine induces receptor desensitization on G protein-coupled inwardly rectifying potassium channel and intracellular calcium release via PKC-pathway, while etorphine uses β -arrestin-pathway (Chu, Zheng, Zhang, Loh, & Law, 2010; Chu et al., 2008; Johnson et al., 2006). (4) The two agonists have different effects on the downstream transcriptional factors activation and the stability of dendritic spine of rat hippocampal neurons (Zheng, Chu, Zeng, Loh, & Law, 2010; Zheng, Zeng, Chu, et al., 2010; Zheng, Zeng, Zhang, et al., 2010).

Based on the previous investigation on biased signaling, we propose a model to explain these observations (Fig. 18.1). Our model suggests that the biased signaling is resulted from the different abilities of the two agonists to induce receptor phosphorylation. As indicated in Fig. 18.1, in the absence of agonist, OPRM1 is anchored to lipid raft microdomains through the interaction with G_{i2} (Zheng, Chu, et al., 2008). When agonist, not matter whether it is morphine or etorphine, is used to activate the receptor, G_{i2} is, in turn, activated and AC inhibition, possibly mediated by AC V/VI, is observed (Avidor-Reiss, Nevo, Saya,

Bayewitch, & Vogel, 1997; Matsuoka, Suzuki, Defer, Nakanishi, & Hanoune, 1997; Mons & Cooper, 1994). Thus, the two agonists induce similar AC inhibition. After inducing AC inhibition, the differences between the two agonists are revealed. On the one hand, because receptor phosphorylation increases the affinity of receptor to β -arrestin, but decreases the affinity of receptor to G_{i2} (Lefkowitz, 1998), morphine–receptor complex can still bind to G_{i2} and remain within the lipid raft microdomains (Zheng, Loh, et al., 2010). The continued activation of G_{i2} leads to the activation of downstream kinase, PKC as in the case of OPRM1. Increase in PKC activity leads to the ERK phosphorylation. The phosphorylated ERK activated by PKC will remain in the cytosol and induce the subsequent activation of p90RSK and CREB (Frodin & Gammeltuft, 1999; Hauge & Frodin, 2006; Zheng, Loh, et al., 2008). The PKC also mediates receptor desensitization by inducing G_{i2} phosphorylation (Chu et al., 2010).

On the other hand, etorphine induces high level of receptor phosphorylation, which facilitates β -arrestin to compete with G_{i2} for the overlapped binding sites on the receptor (Gurevich & Gurevich, 2006; Lu, Zeng, Liu, & Ceng, 2000). The high affinity of etorphine–receptor complex for β -arrestin leads to the recruitment of β -arrestin from cytosol to cell membrane (Kohout et al., 2004). Binding of β -arrestin to receptor complex uncouples G_{i2} , which desensitizes G_{i2} -mediated signaling and induces the translocation of OPRM1 out of lipid raft microdomains (Lefkowitz & Shenoy, 2005; Lefkowitz & Whalen, 2004; Zheng, Chu, et al., 2008). Binding of β -arrestin to receptor complex also initiates the signaling transduction through β -arrestin-pathway, such as the β -arrestin-mediated ERK phosphorylation (Caunt et al., 2006; Jafri et al., 2006; Wang, Lu, Zhao, & Limbird, 2006). As β -arrestin can interact with phosphorylated ERK and translocate into the nucleus (Ma & Pei, 2007), the Elk-1 in nucleus may be activated (Janknecht, Zinck, Ernst, & Nordheim, 1994). The difference in the transcriptional factors activated by the two agonists is the reason why the two agonists have distinct influence on the microRNA-190 expression and the stability of dendritic spine in hippocampal neurons (Zheng, Zeng, Zhang, et al., 2010; Zheng et al., 2011).

Cholesterol–palmitoyl interaction has been identified in the dimer inter-face of β_2 -adrenergic receptor crystal structure, which suggests the possible roles played by receptor palmitoylation in dimerization and G protein coupling (Milligan, Canals, Pediani, Ellis, & Lopez-Gimenez, 2006; Rasmussen et al., 2007). As both cholesterol and G protein coupling interacts with biased signaling, palmitoylation can play important role in regulating biased signaling.

5. FUTURE DIRECTION OF BIASED SIGNALING

Traditionally, the agonist-biased signaling is used to explain the different functions of agonists, such as the differences in the tolerance induced by OPRM1 agonists (Duttaroy & Yoburn, 1995; Grecksch et al., 2011). As discussed in Section 4, the effects of agonists on receptor phosphorylation and membrane distribution can be used to predict the agonist-biased signaling. Thus protocols described above are helpful in identifying the agonist with higher efficacies and fewer side effects. In addition, manipulating these post-translation modifications as reported with OPRM1 phosphorylation (Zheng et al., 2011) can control the agonist-biased signaling, leading to different agonist-dependent transcription regulation, as demonstrated with our miRNA studies.

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ABBREVIATIONS

AC	adenylyl cyclase
Btm-BMCC	1-biotinamido-4-[4 -(maleimidomethyl) cyclohexane carboxamido] butane
GPCR	G protein-coupled receptor
MBS	morpholinoethanesulfonic acid-buffered saline
NEM	<i>N</i> -ethylmaleimide
OPRM1	μ -opioid receptor
TCA	trichloroacetic acid

References

- Ahn S, Shenoy SK, Wei H, Lefkowitz RJ. Differential kinetic and spatial patterns of beta-arrestin and G protein-mediated ERK activation by the angiotensin II receptor. *The Journal of Biological Chemistry*. 2004; 279:35518–35525. [PubMed: 15205453]
- Avidor-Reiss T, Nevo I, Saya D, Bayewitch M, Vogel Z. Opiate-induced adenylyl cyclase superactivation is isozyme-specific. *The Journal of Biological Chemistry*. 1997; 272:5040–5047. [PubMed: 9030567]
- 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]
- Caunt CJ, Finch AR, Sedgley KR, Oakley L, Luttrell LM, McArdle CA. Arrestin-mediated ERK activation by gonadotropin-releasing hormone receptors: Receptor-specific activation mechanisms and compartmentalization. *The Journal of Biological Chemistry*. 2006; 281:2701–2710. [PubMed: 16314413]
- Chen C, Shahabi V, Xu W, Liu-Chen LY. Palmitoylation of the rat mu opioid receptor. *FEBS Letters*. 1998; 441:148–152. [PubMed: 9877183]
- Cherezov V, Rosenbaum DM, Hanson MA, Rasmussen SG, Thian FS, Kobilka TS, et al. High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. *Science*. 2007; 318:1258–1265. [PubMed: 17962520]
- Chu J, Zheng H, Loh HH, Law PY. Morphine-induced mu-opioid receptor rapid desensitization is independent of receptor phosphorylation and beta-arrestins. *Cellular Signalling*. 2008; 20:1616–1624. [PubMed: 18558479]
- Chu J, Zheng H, Zhang Y, Loh HH, Law PY. Agonist-dependent mu-opioid receptor signaling can lead to heterologous desensitization. *Cellular Signalling*. 2010; 22:684–696. [PubMed: 20043990]
- Damaj BB, McColl SR, Neote K, Songqing N, Ogborn KT, Hebert CA, et al. Identification of G-protein binding sites of the human interleukin-8 receptors by functional mapping of the intracellular loops. *The FASEB Journal*. 1996; 10:1426–1434.
- Doll C, Konietzko J, Poll F, Koch T, Holtt V, Schulz S. Agonist-selective patterns of micro-opioid receptor phosphorylation revealed by phosphosite-specific antibodies. *British Journal of Pharmacology*. 2011; 164:298–307. [PubMed: 21449911]
- Drisdell RC, Alexander JK, Sayeed A, Green WN. Assays of protein palmitoylation. *Methods*. 2006; 40:127–134. [PubMed: 17012024]
- Duttaroy A, Yoburn BC. The effect of intrinsic efficacy on opioid tolerance. *Anesthesiology*. 1995; 82:1226–1236. [PubMed: 7741298]
- Edidin M. The state of lipid rafts: From model membranes to cells. *Annual Review of Biophysics and Biomolecular Structure*. 2003; 32:257–283.
- El Kouhen R, Burd AL, Erickson-Herbrandson LJ, Chang CY, Law PY, Loh HH. Phosphorylation of Ser363, Thr370, and Ser375 residues within the carboxyl tail differentially regulates mu-opioid

- receptor internalization. *The Journal of Biological Chemistry*. 2001; 276:12774–12780. [PubMed: 11278523]
- Elortza F, Nuhse TS, Foster LJ, Stensballe A, Peck SC, Jensen ON. Proteomic analysis of glycosylphosphatidylinositol-anchored membrane proteins. *Molecular & Cellular Proteomics*. 2003; 2:1261–1270. [PubMed: 14517339]
- Foster LJ, De Hoog CL, Mann M. Unbiased quantitative proteomics of lipid rafts reveals high specificity for signaling factors. *Proceedings of the National Academy of Sciences of the United States of America*. 2003; 100:5813–5818. [PubMed: 12724530]
- Frodin M, Gammeltoft S. Role and regulation of 90 kDa ribosomal S6 kinase (RSK) in signal transduction. *Molecular and Cellular Endocrinology*. 1999; 151:65–77. [PubMed: 10411321]
- Gesty-Palmer D, Chen M, Reiter E, Ahn S, Nelson CD, Wang S, et al. Distinct beta-arrestin- and G protein-dependent pathways for parathyroid hormone receptor-stimulated ERK1/2 activation. *The Journal of Biological Chemistry*. 2006; 281:10856–10864. [PubMed: 16492667]
- Grecksch G, Just S, Pierstorff C, Imhof AK, Gluck L, Doll C, et al. Analgesic tolerance to high-efficacy agonists but not to morphine is diminished in phosphorylation-deficient S375A {micro}-opioid receptor knock-in mice. *The Journal of Neuroscience*. 2011; 31:13890–13896. [PubMed: 21957251]
- Gurevich VV, Gurevich EV. The structural basis of arrestin-mediated regulation of G-protein-coupled receptors. *Pharmacology & Therapeutics*. 2006; 110:465–502. [PubMed: 16460808]
- Hauge C, Frodin M. RSK and MSK in MAP kinase signalling. *Journal of Cell Science*. 2006; 119:3021–3023. [PubMed: 16868029]
- Hawtin SR, Tobin AB, Patel S, Wheatley M. Palmitoylation of the vaso-pressin V1a receptor reveals different conformational requirements for signaling, agonist-induced receptor phosphorylation, and sequestration. *The Journal of Biological Chemistry*. 2001; 276:38139–38146. [PubMed: 11466323]
- Helms JB, Zurzolo C. Lipids as targeting signals: Lipid rafts and intracellular trafficking. *Traffic*. 2004; 5:247–254. [PubMed: 15030566]
- Jafri F, El-Shewy HM, Lee MH, Kelly M, Luttrell DK, Luttrell LM. Constitutive ERK1/2 activation by a chimeric neurokinin 1 receptor-beta-arrestin1 fusion protein. Probing the composition and function of the G protein-coupled receptor “signalsome”. *The Journal of Biological Chemistry*. 2006; 281:19346–19357. [PubMed: 16670094]
- Janknecht R, Zinck R, Ernst WH, Nordheim A. Functional dissection of the transcription factor Elk-1. *Oncogene*. 1994; 9:1273–1278. [PubMed: 8134131]
- Jiang A, Lehti K, Wang X, Weiss SJ, Keski-Oja J, Pei D. Regulation of membrane-type matrix metalloproteinase 1 activity by dynamin-mediated endocytosis. *Proceedings of the National Academy of Sciences of the United States of America*. 2001; 98:13693–13698. [PubMed: 11698655]
- Johnson EA, Oldfield S, Braksator E, Gonzalez-Cuello A, Couch D, Hall KJ, et al. Agonist-selective mechanisms of mu-opioid receptor desensitization in human embryonic kidney 293 cells. *Molecular Pharmacology*. 2006; 70:676–685. [PubMed: 16682505]
- Keith DE, Murray SR, Zaki PA, Chu PC, Lissin DV, Kang L, et al. Morphine activates opioid receptors without causing their rapid internalization. *The Journal of Biological Chemistry*. 1996; 271:19021–19024. [PubMed: 8702570]
- Kenakin T. Agonist-receptor efficacy. I: Mechanisms of efficacy and receptor promiscuity. *Trends in Pharmacological Sciences*. 1995a; 16:188–192. [PubMed: 7652927]
- Kenakin T. Agonist-receptor efficacy. II. Agonist trafficking of receptor signals. *Trends in Pharmacological Sciences*. 1995b; 16:232–238. [PubMed: 7667897]
- Kohout TA, Nicholas SL, Perry SJ, Reinhart G, Junger S, Struthers RS. Differential desensitization, receptor phosphorylation, beta-arrestin recruitment, and ERK1/2 activation by the two endogenous ligands for the CC chemokine receptor 7. *The Journal of Biological Chemistry*. 2004; 279:23214–23222. [PubMed: 15054093]
- Lefkowitz RJ. G protein-coupled receptors. III. New roles for receptor kinases and beta-arrestins in receptor signaling and desensitization. *The Journal of Biological Chemistry*. 1998; 273:18677–18680. [PubMed: 9668034]

- Lefkowitz RJ, Shenoy SK. Transduction of receptor signals by beta-arrestins. *Science*. 2005; 308:512–517. [PubMed: 15845844]
- Lefkowitz RJ, Whalen EJ. Beta-arrestins: Traffic cops of cell signaling. *Current Opinion in Cell Biology*. 2004; 16:162–168. [PubMed: 15196559]
- Liao D, Grigoriants OO, Wang W, Wiens K, Loh HH, Law PY. Distinct effects of individual opioids on the morphology of spines depend upon the internalization of mu opioid receptors. *Molecular and Cellular Neuroscience*. 2007; 35:456–469. [PubMed: 17513124]
- Liao D, Lin H, Law PY, Loh HH. Mu-opioid receptors modulate the stability of dendritic spines. *Proceedings of the National Academy of Sciences of the United States of America*. 2005; 102:1725–1730. [PubMed: 15659552]
- Linder ME, Middleton P, Hepler JR, Taussig R, Gilman AG, Mumby SM. Lipid modifications of G proteins: Alpha subunits are palmitoylated. *Proceedings of the National Academy of Sciences of the United States of America*. 1993; 90:3675–3679. [PubMed: 8475115]
- Lu L, Zeng S, Liu D, Ceng X. Inhibition of the amygdala and hippocampal calcium/calmodulin-dependent protein kinase II attenuates the dependence and relapse to morphine differently in rats. *Neuroscience Letters*. 2000; 291:191–195. [PubMed: 10984639]
- Ma L, Pei G. Beta-arrestin signaling and regulation of transcription. *Journal of Cell Science*. 2007; 120:213–218. [PubMed: 17215450]
- Macdonald JL, Pike LJ. A simplified method for the preparation of detergent-free lipid rafts. *Journal of Lipid Research*. 2005; 46:1061–1067. [PubMed: 15722565]
- Matsuoka I, Suzuki Y, Defer N, Nakanishi H, Hanoune J. Differential expression of type I, II, and V adenylyl cyclase gene in the postnatal developing rat brain. *Journal of Neurochemistry*. 1997; 68:498–506. [PubMed: 9003034]
- Mayor S, Maxfield FR. Insolubility and redistribution of GPI-anchored proteins at the cell surface after detergent treatment. *Molecular Biology of the Cell*. 1995; 6:929–944. [PubMed: 7579703]
- Mayor S, Rao M. Rafts: Scale-dependent, active lipid organization at the cell surface. *Traffic*. 2004; 5:231–240. [PubMed: 15030564]
- Milligan G, Canals M, Pediani JD, Ellis J, Lopez-Gimenez JF. The role of GPCR dimerisation/oligomerisation in receptor signalling. *Ernst Schering Foundation Symposium Proceedings*. 2006; 2:145–161. [PubMed: 17703581]
- Monastyrskaya K, Hostettler A, Buergi S, Draeger A. The NK1 receptor localizes to the plasma membrane microdomains, and its activation is dependent on lipid raft integrity. *The Journal of Biological Chemistry*. 2005; 280:7135–7146. [PubMed: 15590676]
- Mons N, Cooper DM. Selective expression of one Ca(2+)-inhibitable adenylyl cyclase in dopaminergically innervated rat brain regions. *Brain Research. Molecular Brain Research*. 1994; 22:236–244. [PubMed: 8015383]
- Navratil AM, Bliss SP, Berghorn KA, Haughian JM, Farmerie TA, Graham JK, et al. Constitutive localization of the gonadotropin-releasing hormone (GnRH) receptor to low density membrane microdomains is necessary for GnRH signaling to ERK. *The Journal of Biological Chemistry*. 2003; 278:31593–31602. [PubMed: 12791688]
- Nebi T, Pestonjamas KN, Leszyk JD, Crowley JL, Oh SW, Luna EJ. Proteomic analysis of a detergent-resistant membrane skeleton from neutrophil plasma membranes. *The Journal of Biological Chemistry*. 2002; 277:43399–43409. [PubMed: 12202484]
- Oldham WM, Hamm HE. Structural basis of function in heterotrimeric G proteins. *Quarterly Reviews of Biophysics*. 2006; 39:117–166. [PubMed: 16923326]
- Probst WC, Snyder LA, Schuster DI, Brosius J, Sealfon SC. Sequence alignment of the G-protein coupled receptor superfamily. *DNA and Cell Biology*. 1992; 11:1–20. [PubMed: 1310857]
- Rasmussen SG, Choi HJ, Rosenbaum DM, Kobilka TS, Thian FS, Edwards PC, et al. Crystal structure of the human beta2 adrenergic G-protein-coupled receptor. *Nature*. 2007; 450:383–387. [PubMed: 17952055]
- Schuck S, Honsho M, Ekroos K, Shevchenko A, Simons K. Resistance of cell membranes to different detergents. *Proceedings of the National Academy of Sciences of the United States of America*. 2003; 100:5795–5800. [PubMed: 12721375]

- Smart EJ, Ying YS, Mineo C, Anderson RG. A detergent-free method for purifying caveolae membrane from tissue culture cells. *Proceedings of the National Academy of Sciences of the United States of America*. 1995; 92:10104–10108. [PubMed: 7479734]
- Tobin AB, Butcher AJ, Kong KC. Location, location, location... site-specific GPCR phosphorylation offers a mechanism for cell-type-specific signalling. *Trends in Pharmacological Sciences*. 2008; 29:413–420. [PubMed: 18606460]
- Urban JD, Clarke WP, von Zastrow M, Nichols DE, Kobilka B, Weinstein H, et al. Functional selectivity and classical concepts of quantitative pharmacology. *The Journal of Pharmacology and Experimental Therapeutics*. 2007; 320:1–13. [PubMed: 16803859]
- Violin JD, Lefkowitz RJ. Beta-arrestin-biased ligands at seven-transmembrane receptors. *Trends in Pharmacological Sciences*. 2007; 28:416–422. [PubMed: 17644195]
- Wang Q, Lu R, Zhao J, Limbird LE. Arrestin serves as a molecular switch, linking endogenous alpha2-adrenergic receptor to SRC-dependent, but not SRC-independent, ERK activation. *The Journal of Biological Chemistry*. 2006; 281:25948–25955. [PubMed: 16809338]
- Zhang J, Ferguson SS, Barak LS, Bodduluri SR, Laporte SA, Law PY, et al. Role for G protein-coupled receptor kinase in agonist-specific regulation of mu-opioid receptor responsiveness. *Proceedings of the National Academy of Sciences of the United States of America*. 1998; 95:7157–7162. [PubMed: 9618555]
- Zhang L, Tetrault J, Wang W, Loh HH, Law PY. Short- and long-term regulation of adenylyl cyclase activity by delta-opioid receptor are mediated by Gα_{i2} in neuroblastoma N2A cells. *Molecular Pharmacology*. 2006; 69:1810–1819. [PubMed: 16525132]
- Zheng H, Chu J, Qiu Y, Loh HH, Law PY. Agonist-selective signaling is determined by the receptor location within the membrane domains. *Proceedings of the National Academy of Sciences of the United States of America*. 2008; 105:9421–9426. [PubMed: 18599439]
- Zheng H, Chu J, Zeng Y, Loh HH, Law PY. Yin Yang 1 phosphorylation contributes to the differential effects of mu-opioid receptor agonists on microRNA-190 expression. *The Journal of Biological Chemistry*. 2010; 285:21994–22002. [PubMed: 20457614]
- Zheng H, Chu J, Zhang Y, Loh HH, Law PY. Modulating micro-opioid receptor phosphorylation switches agonist-dependent signaling as reflected in PKCε activation and dendritic spine stability. *The Journal of Biological Chemistry*. 2011; 286:12724–12733. [PubMed: 21292762]
- Zheng H, Loh HH, Law PY. {beta}-Arrestin-dependent {micro}-opioid receptor-activated extracellular signal-regulated kinases (ERKs) translocate to nucleus in contrast to G protein-dependent ERK activation. *Molecular Pharmacology*. 2008; 73:178–190. [PubMed: 17947509]
- Zheng H, Loh HH, Law PY. Agonist-selective signaling of G protein-coupled receptor: Mechanisms and implications. *IUBMB Life*. 2010; 62:112–119. [PubMed: 20058265]
- Zheng H, Zeng Y, Chu J, Kam AY, Loh HH, Law PY. Modulations of NeuroD activity contribute to the differential effects of morphine and fentanyl on dendritic spine stability. *The Journal of Neuroscience*. 2010; 30:8102–8110. [PubMed: 20554861]
- Zheng H, Zeng Y, Zhang X, Chu J, Loh HH, Law PY. {micro}-Opioid receptor agonists differentially regulate the expression of miR-190 and NeuroD. *Molecular Pharmacology*. 2010; 77:102–109. [PubMed: 19854889]

Figure 18.1.
The schematic demonstration of the biased signaling with OPRM1.