QUANTIFICATION OF CELL SURFACE MU AND DELTA OPIOID RECEPTOR AND ELUCIDATION OF FACTORS GOVERNING SURFACE RECEPTOR POOL

A DISSERTATION
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

Jonathan Alexander Hareid

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

Robert P. Elde, Advisor

October, 2009

Acknowledgements

My success in the pharmacology graduate program would not have been possible without the support and guidance of many people. While that is true for any Ph.D. student, it is especially so for me since I was a joint J.D./Ph.D. student and approached my graduate studies with a unique set of issues and a somewhat different intellectual bent.

My Ph.D. adviser, Professor and Dean Robert Elde, is a remarkable person. He can turn on a dime from being a successful administrator and running the College of Biological Sciences to attending a lab meeting and directing a discussion of scientific issues from a paper or experiment. He was a good role model for me, since I aspire to think both like a scientist and a lawyer. Having matriculated into the pharmacology program after my first year of law school and with little laboratory experience, I slowly but surely made the transition from law student to graduate student, largely with Dr. Elde's encouragement.

That transition was greatly assisted by Drs. Maureen Riedl and Frank Williams, who trained me in the practical aspects of working in a lab and provided constant training and guidance in laboratory techniques, experimental design, and interpreting results. Drs. Therissa Libby and Joe Fullmer, who were graduate students in the lab when I began and for several years thereafter, were also helpful in this regard. Also, nothing would happen in the Elde lab without Galina Kalyuzhnaya, who keeps everything running smoothly by promptly acting on requests for everything from ordering supplies to cleaning coverslips to making phosphate-buffered saline.

While they are too numerous to mention individually, the faculty in the departments of Biochemistry, Physiology, Neuroscience, and of course Pharmacology provided excellent teaching in the continuum of knowledge that is the biomedical sciences in my first few years. My other committee members, Professors Horace Loh, Stan Thayer, and Paul Letourneau, have contributed valuable time and insight into guiding my thesis project as it took shape.

I wish to acknowledge Professor Susan Wolf for creating the pioneering Joint Degree Program in Law, Health & the Life Sciences through which I pursued my dual degrees. After ten-plus years, some outstanding conferences and scholarship, and many successful alumni, the experiment is a proven success. I owe special thanks to Professor Colin Campbell of Pharmacology, who has served at various times as the pharmacology director of graduate studies and associate director of the Joint Degree Program. He has taught me in several pharmacology courses, in the Joint Degree Program seminar each year, and he has provided not only continuous intellectual stimulation but also guidance in the niceties and politics of such things as picking a thesis adviser and simultaneously managing my commitments as a graduate and law student. He, Professor Susan Wolf, and the office staff of Pharmacology, Neuroscience, and the Joint Degree Program have all helped me work through the various administrative and bureaucratic aspects of being a dual degree student in this great university.

Last but certainly not least, I wish to thank my family, including my parents Allen and Rae Ann Hareid, my brother Josh, and the newer additions to the family, Josh's wife Cristina, and their children Louise and Caleb. As the past several years presented me with stresses and challenges, they have been a constant source of support and love.

The complexity of the enterprise I have undertaken has had the unintended consequence of dividing my energies and attention, so that I find myself knowing many of the people I've mentioned (and others to whom I'm not doing justice) less well than I like and they deserve. Nevertheless, it is the personalities and people more than anything else that will stay with me after this experience, and I shall consider them all, now and forever, friends.

Abstract

The mu, delta, and kappa opioid receptors (MOR, DOR, and KOR) mediate the effects of the opioid class of drugs. As with all G-protein coupled receptors, opioid receptors must be installed in the plasma membrane of cells to be functional, and an understanding of the factors governing the quantities of cell surface receptor is important for understanding opioid drug action and solving some of the problems associated with these drugs, such as tolerance and dependence. To explore factors governing the quantity of cell surface receptor, we used nerve growth factor differentiated PC12 cells, which have neuron-like qualities, expressing epitope-tagged DOR and MOR. We developed a method of quantifying cell surface receptor, total cell receptor, and surface-to-total ratios in individual cells, based on separately labeling cell surface and total cell receptor with fluorescent antibodies, acquiring images using fluorescence microscopy, and analyzing images to separately quantify surface and total fluorescence for each cell using Image J software. This method is efficient, avoids some of the technical problems with other methods of surface receptor quantification, and enables quantification of large populations of individual cells, so that cell-to-cell variation can be studied.

Using this method, we confirmed our observation that DOR exists in the plasma membrane in greater quantity than MOR. In PC12 cells regardless of nerve growth factor differentiation, and also in HEK293 cells, we found that DOR had a larger surface-to-total ratio on average than MOR did. This indicates the existence of receptor-specific mechanisms for membrane trafficking and adds another type of differential subcellular targeting of DOR and MOR to several others that have been previously described. We also examined the mathematical relationship between cell surface and total cell receptor

for both DOR and MOR. We found little relationship between the quantities of surface and total receptor for either receptor. However, for both DOR and MOR we found that as the quantity of total cell receptor increased, surface-to-total ratios decreased on average, suggesting that the mechanisms for receptor installation into the plasma membrane are saturable.

Consistent with existing literature, we found that the regulated secretory pathway is an important factor in the cell surface delivery of DOR but not MOR. We confirmed the previous report that depolarization using potassium chloride resulted in rapid installation of DOR into the plasma membrane as measured by an increase in DOR surface-to-total ratio, and we found additionally that this same stimulus did not result in an increase in MOR surface-to-total ratio. We also found that co-expression of the neuropeptide precursor pre-pro-tachykinin caused an increase in DOR but not MOR surface-to-total ratio on average, providing additional evidence for the role of pre-pro-tachykinin in DOR cell surface targeting, a notion that has been challenged recently.

We found that 30 minutes of treatment with the DOR agonist deltorphin caused not only direct internalization of DOR, as expected based on the literature, but also a longer-lasting decrease in the cell surface quantity of DOR. In particular, while the level of cell surface DOR should recover within 12 hours after complete internalization by agonist, based on normal membrane trafficking kinetics that we measured using pulse chase experiments, we found that DOR surface-to-total ratios remained depressed for up to 24 hours after deltorphin treatment, with significant recovery measured only after 48 hours. This indicates that DOR agonists can cause a lasting change in membrane trafficking kinetics which results in low levels of cell surface DOR for some time after agonist exposure. Since the efficacy of DOR agonists is related to the level of surface receptor as suggested by recent evidence *in vivo*, this longer-lasting agonist effect on receptor membrane trafficking may be a component of acute tolerance.

Various types of functional interactions between DOR and MOR have been described in the literature, and we explored the possibility of agonist-induced trafficking interactions by using PC12 cells co-expressing DOR and MOR and our surface receptor quantification method to measure the surface quantity of both receptors in individual cells. We found that 30 minutes of treatment with the DOR-selective agonist deltorphin caused a rapid increase in the quantity of cell surface MOR in PC12 cells expressing both receptors, but not in cells expressing MOR alone. By contrast, treatment with the MOR-selective agonist DAMGO for 30 minutes did not cause a rapid increase in cell surface DOR in co-expressing cells, although existing literature reports that such an increase can occur with longer MOR agonist treatment. Thus we have shown that agonist treatment at a GPCR can lead to rapid installation of a functionally related receptor, and this is the first demonstration we are aware of that DOR agonist treatment can cause plasma membrane installation of MOR. We also found a positive linear relationship between cell surface DOR and cell surface MOR in co-transfected cells. Deltorphin treatment increased this positive trend, while DAMGO treatment decreased it. This is further evidence for receptor interactions, suggesting that agonist treatment at one receptor can either induce installation of a related receptor or that interactions can modify the ability of an agonist to internalize receptor.

Table of Contents

List of Figures

Chapter 1

Introduction and Background

The Opioid Drugs and Their Uses and Problems

Opioids are one of the two main classes of analgesic drugs in use today, the other class being the non-steroidal anti-inflammatory drugs (NSAIDs). The term "opioid" refers broadly to both the opiates and the endogenous peptides they mimic. Opiates include natural products such as morphine, derived from the opium poppy, as well as many semi-synthetic and synthetic congeners. Endogenous opioid peptides are produced in the body and include endorphins, enkephalins, dynorphins and endomorphins (Gutstein and Akil, 2001).

Opioid analgesics are used therapeutically in the treatment of moderate to severe pain. Some opiates such as heroin are used recreationally for their euphoric effects. Opiates are also used therapeutically for other purposes such as to prevent diarrhea.

The opioid analgesics as a class have two major problems: tolerance and dependence. Tolerance is a loss of drug efficacy over time, resulting in a need for increased dose to produce the same drug effect. Dependence is a set of physiological changes that cause adverse effects upon cessation of use of a drug. Tolerance and dependence are physiological factors that contribute to addiction, which is a behavioral pattern characterized by compulsive use of a drug and overwhelming involvement in procuring and using it. Opioid addiction can result from recreational use of the drugs or from their use in pain therapy. Addiction is a major societal problem costing billions of dollars each year. Even in non-addicted individuals, tolerance and dependence are major liabilities that reduce the effectiveness of opioid drug therapy and its potential for pain relief. Hence, there is a need for greater scientific knowledge of the mechanisms behind the drugs' effects, including tolerance and dependence. Such knowledge may lead to improved opioid drugs and therapies.

Opioid Receptors

Like most other drugs, opioids bind to receptors that mediate their effects. Three opioid receptors have been cloned, the mu opioid receptor (MOR), delta opioid receptor (DOR), and kappa opioid receptor (KOR). All three belong to the superfamily of G protein-coupled receptors (GPCRs), so-called because binding of drug to a receptor activates an adjacent protein called a G-protein, which in turn activates more cellular signaling machinery to produce a biological effect. Because opioid drug action is mediated by these receptors, knowledge of how the receptors function is an important part of understanding the effects of the drugs.

Since GPCRs must be installed in the plasma membrane to be functional, an understanding of GPCR pharmacology requires knowledge of the factors that govern the presence of GPCRs in the plasma membrane. Much research on opioid receptors has focused on the processes of receptor desensitization, internalization, degradation, and recycling, because these processes are thought to be involved in drug tolerance, albeit in poorly understood ways (Christie, 2008). These processes all involve receptors that have already been installed in the plasma membrane. Comparatively little is known about the trafficking of newly synthesized opioid receptors prior to insertion in the plasma membrane.

Differential Targeting of MOR and DOR

It has been widely observed that opioid receptors are differentially targeted in neurons. The cloning of the three opioid receptors in the early 1990's (Evans et al., 1992; Kieffer et al., 1992; Chen et al., 1993; Kong et al., 1994; Meng et al., 1993; Minami et al., 1993; Thompson et al., 1993; Wang et al., 1993; Yasuda et al., 1993) enabled the generation of antibodies against the receptors and subsequent determination by immunohistochemical methods of the distribution of the receptors throughout the central nervous system (Elde et al., 1995). This extensive mapping yielded some generalizations about the sub-cellular localization of opioid receptors in neurons: DOR is found almost exclusively in axons, while MOR and KOR are found primarily, although not exclusively, in cell bodies and dendrites. Moreover, confocal and electron microscopy revealed that while MOR and KOR exist primarily in the plasma membrane of cell bodies and dendrites, DOR seems to exist primarily in intracellular vesicles within axons (Arvidsson et al., 1995a; Arvidsson et al., 1995b; Arvidsson et al., 1995c; Cheng et al., 1995; Cheng et al., 1996; Cheng et al., 1997; Commons et al., 1999; Dado et al., 1993; Drake et al., 1996; Nomura et al., 1996; Svingos et al., 1996; Svingos et al., 1999; van Bockstaele et al., 1997; Wang et al., 1998). These generalizations about subcellular targeting to different domains have been nuanced by later findings; for instance, that MOR is found on axons in certain neurons (Browning et al., 2004; Pickel et al., 2004). Recently, DOR-EGFP knock-in mice have been made, and a large portion of DOR in cells from these mice is in the plasma membrane rather than in intracellular stores (Scherrer et al., 2006), although questions remain regarding whether fluorescent-protein-tagged DOR targets the same way as endogenous DOR and hence to what extent this mouse model is valid for studying the behavior of DOR. Although the targeting patterns of DOR and MOR are complex and may defy simple generalizations, differential targeting of these receptors is well established; it has been shown, for instance, that DOR is found primarily in intracellular vesicles in the same neurons in which endogenously co-expressed MOR is found primarily on the cell surface (Wang and Pickel, 2001). Clearly, the targeting of the receptors is not random, which raises the question of the factors influencing initial receptor trafficking.

Relationship Between Neuropeptide Trafficking and Receptor Trafficking

Closely related to the idea that certain receptors exist primarily in intracellular stores is the concept of co-transport of receptor and neuropeptide along axons. Axonally targeted opioid receptors are frequently associated with large dense-core vesicles (LDCVs) that transport neuropeptides along axons (Cheng et al., 1995; Shuster et al., 1999). In dorsal root ganglia neurons, for example, DOR co-localizes with substance P and calcitonin gene-related peptide (CGRP), two neuropeptides secreted by these neurons, and DOR is associated with LDCVs that carry these neuropeptides (Zhang et al., 1998). A similar relationship exists between KOR and vasopressin in the magnocellular neurons of the hypothalamus that secrete this peptide hormone; KOR co-localizes with vasopressin in axons and axon terminals, and KOR is found associated with LDCVs that carry neuropeptide along axons (Shuster et al., 1999). These findings indicate that axonally targeted opioid receptors may be transported and stored with neuropeptides in the regulated secretory pathway. Evidence indicates a direct physical association between DOR and pre-pro-tachykinin in LDCVs in the regulated secretory pathway, and that the DOR transported by this mechanism is required for tolerance to morphine's analgesic effect (Guan et al., 2005).

Activity-dependent Insertion of Receptors into the Plasma Membrane

The regulated secretory pathway, as opposed to the constitutive secretory pathway, carries cargo that is released upon stimulation of the neuron (De Camilli and Jahn, 1990; Jung and Scheller, 1991). Building on the concept of co-transport of receptor and neuropeptide in large secretory vesicles in axons, the relationship between neuronal stimulation and subsequent insertion of opioid receptors into the plasma membrane has been studied (Elde et al., 1995). In the vasopressin-containing magnocellular neurons of the hypothalamus, a physiological stimulus that elicits vasopressin release (salt-loading) leads to a subsequent reduction in proportion of KOR associated with intracellular LDCVs and a concomitant increase in KOR associated with the plasma membrane (Shuster et al., 1999). In the case of DOR, translocation of receptor from intracellular vesicles to plasma membrane has been reported in different neuronal populations following chronic morphine treatment (Cahill et al., 2001); cold water swim stress, a form of behavioral stimulation (Commons, 2003); or agonist activation of surface DORs (Bao et al., 2003). In PC12 cells, treatment with nerve growth factor, which differentiates this cell line into a neuron-like phenotype, induces newly synthesized DOR to be directed to an intracellular vesicular pool, which can be translocated to the plasma membrane with depolarization (Kim and von Zastrow, 2003). Since the receptor must be installed in the plasma membrane to be functional, the large proportion of DOR seen in intracellular vesicles presumably represents a reserve pool of receptor available for mobilization with the appropriate stimulus, just as neuropeptide is maintained in a vesicular reserve pool prior to stimulation.

A receptor installed in the plasma membrane of an axon terminal is likely to be functioning by modifying subsequent release of neurotransmitter or hormone from the synapse. In hypothalamic magnocellular neurons, installation of KOR in axon terminals coupled with vasopressin secretion ostensibly serves as a negative feedback mechanism, because KOR agonists have been shown to inhibit vasopressin release from nerve terminals (Zhao et al., 1988). Surprisingly, it has been reported that in small dorsal root ganglia neurons, agonist activation of DORs induces both CGRP secretion and concomitant insertion of additional DORs, indicating that installation of the receptor is providing positive feedback for further neuropeptide release (Bao et al., 2003). Thus, the phenomena of receptor transport coupled to neuropeptide transport in axons and release of neuropeptide coupled to receptor insertion, have been shown to exist for different receptors and with different neuropeptides, and the insertion of presynaptic receptor can apparently provide either positive or negative feedback. This suggests a more generalized mechanism for regulation of neurochemical signalling in which neuropeptides and receptors are transported and stored in the same vesicles, with stimulation resulting in neuropeptide release causing concomitant insertion of receptors in the plasma membrane at axon terminals, the receptor thereby functioning presynaptically to modify further neuropeptide release.

Relationship Between Cell Surface Receptor and Total Cell Receptor

A GPCR must be installed in the plasma membrane to be functional. The amount of receptor in the membrane is increased by receptor installation and recycling and decreased by internalization. Much research has explored the processes of ligand-induced receptor endocytosis and subsequent trafficking steps, including receptor degradation in lysosomes or recycling to the plasma membrane (Hanyaloglu and von Zastrow, 2007). Less is known about factors influencing initial installation of newly synthesized receptors into the plasma membrane.

One question that is not answered is whether there is a relationship between total cell receptor and receptor in the plasma membrane. The answer to this question bears on several interesting biological questions, such as how tightly coupled the processes of receptor synthesis and installation in the plasma membrane are, whether the amount of receptor in the membrane is the result of fairly passive "flow" from intracellular stores or is more tightly regulated at the installation step, and whether the processes that install receptor in the membrane are saturable. Research on other GPCRs implicates a number of different amino acid structural motifs and chaperone molecules in the delivery of newly synthesized receptor to the cell surface (Tan et al., 2004). This knowledge, coupled with the literature on stimulus-evoked receptor installation already discussed, suggests that the processes of installation are a critical regulatory point for the amount of cell surface DOR and MOR. However, the mathematical relationship between total cell receptor and cell surface receptor has not been widely explored to date, likely because of the lack of established methods for quantifying receptor cell by cell.

MOR and DOR Interactions

A large body of evidence indicates that GPCRs generally, and MOR and DOR specifically, interact. The interactions may take the form of direct physical association such as the formation of heterodimers (Jordan and Devi, 1999), or the interactions can be more functional; for example, agonist binding to one receptor may influence the trafficking of the other. The *in vivo* importance of these interactions is shown by the fact that tolerance to morphine's analgesic effect, which is mediated primarily by MOR (Kieffer, 1999), is abolished in DOR knockout mice (Zhu et al., 1999), clearly implicating DOR in morphine tolerance. Recent evidence indicates an essential role for MOR in DOR-mediated antihyperalgesia (Gendron et al., 2007). Thus, functional interactions between MOR and DOR occur both ways; each receptor can modify the other's effects.

It has been shown that activation of MOR by morphine or other ligands can induce installation of DOR into the plasma membrane of certain neurons (Cahill et al., 2001) (Morinville et al., 2003) (Chieng and Christie, 2009). Moreover, in dorsal root ganglia neurons, the population of cell surface DOR installed by stimulus-dependent release from an intracellular pool of LDCVs has been shown to be critical for the development of tolerance to morphine analgesia (Guan et al., 2005). On the basis of this evidence, it has been proposed that the development of tolerance is positively correlated with the ratio of DOR to MOR on the cell surface (Zhang et al., 2006).

Objectives and Specific Aims

PC12 cells can be differentiated by nerve growth factor (NGF) into a neuronal-like cell. This change is marked by cessation of proliferation and neurite outgrowth (Greene and Tischler, 1976). The differentiation is also characterized by the expression and synthesis of neuronal proteins such as neurofilaments and B-tubulin III; expression of these proteins is correlated with more visible changes such as cessation of proliferation and neurite outgrowth (Schimmelpfeng et al., 2004) (Ohuchi et al., 2002). The cells have a well-developed regulated secretory pathway. Together with the fact that PC12 is a cell line and can be maintained easily in culture, these features make PC12 cells a good model system in which to study intracellular targeting of opioid receptors.

In preliminary studies in PC12 cells we discovered what appeared to be a difference in DOR vs. MOR targeting to the cell membrane. This led us to develop a method of quantifying both total receptor and cell surface receptor in individual cells. By this method, elaborated in the next chapter, we have quantified the targeting difference between DOR and MOR to the cell membrane that we observed in preliminary studies. Moreover, the method can be used to gain insight into many interesting biological questions about the factors governing the surface density of DOR and MOR.

With that background the following specific aims are proposed:

1. Determine the ratio of cell surface to total cell receptor for DOR and MOR in NGF-differentiated PC12 cells.
2. Determine how the difference in DOR and MOR surface-to-total ratios relates to previously reported targeting phenomenon for these receptors.

3. Investigate the long-term effect of agonist treatment on receptor surface-to-total ratios.
4. Investigate the relationship between cell surface DOR and MOR in PC12 cells expressing both receptors, and the possibility of receptor interactions as shown by agonist effects.

Chapter 2

Determination of the Cell Surface-to-Total Ratios of DOR and MOR in Differentiated PC12 Cells

Summary

Differential intracellular targeting between DOR and MOR has been described in the literature. Using immunohistochemistry, we observed a difference in cell surface targeting of DOR and MOR in transiently transfected PC12 cells. We developed a method for quantifying surface and total cell receptor in individual cells, using immunohistochemistry and fluorescence microscopy. Using this method, we confirmed that DOR has a surface-to-total ratio approximately three times greater than MOR in transiently transfected PC12 cells, with or without NGF treatment. As the level of total cell receptor increased, average receptor surface-to-total ratio was reduced. These findings suggest that installation of receptors into the plasma membrane occurs by receptor-specific, saturable mechanisms.

Introduction

Differential targeting of newly synthesized DOR and MOR has been shown in NGF-differentiated PC12 cells. Specifically, NGF treatment in this cell line causes newly synthesized DOR to be directed to an intracellular pool of vesicles, while MOR remains localized on or near the plasma membrane with or without NGF treatment (Kim and von Zastrow, 2003).

In NGF-treated PC12 cells transiently transfected with FLAG epitope-tagged DOR or MOR, we observed an apparent difference in cell surface density of these receptors. Using FLAG antibody before fixation of transfected cells to visualize surface receptor, it appeared that DOR has a greater surface density than MOR does. This is interesting in light of the previous report that NGF treatment in this same cell line causes DOR to be targeted intracellularly. Because visual inspection of surface receptor may fail to account for factors such as cell size and variation in the amount of receptor each transfected cell produces, we sought a way to rigorously quantify cell surface density of transfected DOR and MOR.

Existing techniques for quantifying receptor include Western blotting and autoradiographic ligand-binding studies. Both of these methods, however, have potential pitfalls for selective labeling of cell surface receptor. Western blotting requires homogenization of cell extracts, and it is doubtful to what extent it is possible to obtain a pure cell membrane fraction. Autoradiographic labeling has limitations in resolution that make it difficult to determine whether a labeled receptor is on the plasma membrane or in an intracellular compartment. Also, these methods are only capable of quantifying receptor in populations of cells. We seek to quantify both cell surface receptor and total cell receptor within individual cells, since we believe that cell-to-cell variation within a population of cells may yield interesting insights into receptor biology. Finally, technical problems such as transfection efficiency make it desirable to have a quantification method requiring fewer cells.

For these reasons, we developed a new method of quantifying surface-to-total ratios of receptors in individual cells. The key to the method is separately labeling cell surface receptor and total cell receptor with antibodies. Surface receptor is labeled with antibody directed against an ectodomain epitope prior to fixation. After fixation has made cells permeable to antibodies, total cell receptor is labeled with a different antibody. Fluorescent secondary antibodies enable visualization and imaging of surface and total receptor, and images can then be analyzed using software such as Image J to separately quantify surface and total receptor in individual cells.

Applying this method to our PC12 cells transfected with epitope-tagged DOR or MOR, we determined that DOR has a surface-to-total ratio approximately three times greater than MOR. This was true both with and without NGF treatment in these cells. This indicates that cell surface targeting of the receptors in these cells is governed by different mechanisms than the initial targeting phenomenon described by Kim and von Zastrow (2003). Moreover, by grouping together cells with similar levels of total receptor, we found that surface-to-total ratios decrease at high levels of total receptor, suggesting that the mechanisms responsible for receptor installation in the membrane are saturable.

Methods

Cell Culture

Rat pheochromocytoma (PC12) cells (ATCC, Manassas, VA) were grown in culture medium consisting of 85% DMEM/F12 (Mediatech, Manassas, VA), 10% horse serum (Gibco, Grand Island, NY), 5% fetal bovine serum (Gibco), and 1% penicillin/streptomycin (Gibco). HEK293 cells were a kind gift from Ping-Yee Law at the University of Minnesota, and were maintained in a medium of 90% DMEM (Gibco), 10% fetal bovine serum, and 1% penicillin-streptomycin. Cells were maintained in an incubator at 37°C in 5% CO_2-enriched air. Media was changed every two to three days, and cells were passed every four or five days. For experiments, cells were plated onto poly-L-lysine- and laminin-coated coverslips. Nerve growth factor (NGF, 1 μg/ml, R&D Systems, Minneapolis, MN) was added to the medium to differentiate the PC12 cells. NGF was added to medium upon plating cells onto coverslips and kept in the media throughout culture time (including transfection time), generally three to four days total.

cDNA Constructs and Transfection

N-terminally HA-tagged MOR and DOR constructs, called MORTAG and DORTAG respectively, have been described previously (Afify et al., 1998). These constructs were modified by removing the N-terminal HA sequence and replacing it with a FLAG sequence. Cloning was done by ATG Laboratories (Eden Prairie, MN). A synthetic linker containing the FLAG sequence was cloned into pcDNA3.1(+) to generate pcDNAFLAG. DORTAG and MORTAG were each digested, first by SacI, then by SacII. The fragments containing the DOR and MOR genes were cloned into pcDNAFLAG that had been digested with SacII and EcoRV and gel purified. After transforming competent DH5α E. coli. cells with the ligation, resultant colonies were screened by restriction digest with SacII and XbaI. Colonies containing the predicted restriction pattern for FLAGDOR and FLAGMOR were selected.

For transfection, cells were plated onto poly-L-lysine- and laminin-coated coverslips in a 24-well plate, each well containing 0.5 ml of medium. Plated cells were transfected after two days in culture. Transient transfection was accomplished using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, using 0.8 µg of DNA and 2 µl of Lipofectamine for each well. Transfected cells were generally left in culture for twenty-four hours before fixation and immunocytochemistry.

Antibodies

Polyclonal rabbit anti-FLAG antibody was purchased from Sigma-Aldrich (St. Louis, MO). Fluorescent secondary antibodies were from Jackson ImmunoResearch (West Grove, PA).

Antibodies to the C-termini of DOR and MOR were raised in guinea pigs (Ph.D. Dissertation of Shannon Wright, 2002, University of Minnesota). Peptides with the sequence TACTPSDGPGGGAAA, corresponding to amino acids 358-372 of the cloned mouse delta opioid receptor (DOR1) (Evans et al., 1992; Kieffer et al., 1992), and NHQLENLEAETAPLP, corresponding to amino acids 384-398 of the cloned rat mu opioid receptor (MOR1) (Chen et al., 1993; Thompson et al., 1993) were synthesized using an Applied Biosystems 432 solid-phase peptide synthesizer. The peptides (1 mg/ml) were conjugated to bovine thyroglobulin (40 mg/ml, Sigma-Aldrich) using 7% glutaraldehyde (30 µl/ml, Sigma-Aldrich). For primary immunizations, 1 milligram of the peptide-thyroglobulin conjugate was emulsified with an equal volume of Freund's complete adjuvant (Difco, Detroit, MI) and injected into each of four guinea pigs (Duncan-Hartley, Sasco, Omaha, NE). Secondary immunizations were given every two weeks thereafter using 0.5 milligrams of the peptide-thyroglobulin conjugate emulsified with an equal volume of Freund's incomplete adjuvant. Serum was obtained after the fourth immunization, and thereafter following each subsequent immunization. Animal care was provided by Research Animal Resources at the University of Minnesota, and animal protocols were approved by the University of Minnesota's Institutional Animal Care and Use Committee.

Immunocytochemistry

Transfected cells were rinsed with serum-free medium, then incubated in media containing rabbit anti-FLAG antibody (1:2000, Sigma-Aldrich, St. Louis, MO) at 4°C for thirty minutes to label surface receptor. Because the FLAG epitope was N-terminal on the constructs, the FLAG sequence on receptor installed in the plasma membrane would be extracellular and available for antibody binding prior to fixation and permeabilization. Next, cells were rinsed twice (five to ten minutes each) with serum-free media, then media containing fluorescent secondary antibody (1:200, Jackson ImmunoResearch) was applied to the cells for thirty minutes at 4°C to label primary antibody bound to surface receptor.

Cells were then rinsed briefly in serum-free media, treated with fixative (4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (pH. 6.9)) for twenty minutes at room temperature, and rinsed three times, ten minutes each, in phosphate-buffered saline (PBS, pH 7.2), before incubation with blocking buffer (PBS, 0.3% Triton

X-100, 1% bovine serum albumin, 5% normal donkey serum, 0.01% NaN_3, and 5% Teleostean (fish) gelatin (Sigma-Aldrich) for thirty minutes. Cells were then incubated overnight at 4°C with antibodies raised in guinea pigs to the C-termini of DOR and MOR, 1:1000 in blocking buffer, to label total receptor in transfected cells. Cells were then rinsed three times, ten minutes each, in PBS, before fluorescent secondary antibody was applied (1:200 in blocking buffer, Jackson ImmunoResearch) for one hour at room temperature to label primary antibody bound to total receptor. Finally, cells were rinsed three more times, ten minutes each, in PBS, then coverslips containing cells were mounted onto slides with glycerol and PBS containing 0.1% p-phenylenediamine (Sigma-Aldrich) for imaging.

Imaging and Analysis

Cells were imaged with an Olympus BX50 fluorescence microscope (Olympus, Tokyo, Japan) fitted with a SPOT camera (Diagnostic Instruments, Sterling Heights, MI). Images were collected with a 10x objective to capture several cells in a field and to minimize artifact that may be associated with cell images at higher magnification.

Images were analyzed using Image J software (National Institutes of Health, Bethesda, MD). For each image an automated method was used to set a threshold for positive antibody fluorescence to eliminate background illumination from quantification. Fluorescence was measured by taking the area and average gray value of each fluorescent puncta (comprising cells or parts of cells). To calibrate the gray values reported by Image J to actual fluorescence values, we used InSpeck fluorescent beads (Molecular Probes, Eugene, OR) associated with various fluorescence intensities of the Cy2 and Cy3 fluorophores. We imaged the Inspeck beads in the same manner as the cells, took the average gray value of beads of different fluorescent intensities, and used this to construct a standard curve of fluorescence intensity for each fluorophore.

By convention, we used the Cy3 fluorophore to visualize surface receptor and Cy2 to visualize total cell receptor. We obtained the area and average gray value of all puncta of antibody staining associated with each cell for both Cy2 and Cy3, then interpolated a fluorescence value for each average gray value using the standard curves. We obtained the product of each puncta's area and fluorescence value to obtain total fluorescence per puncta, and got the total fluorescence per cell by summing the values of all associated puncta. We did this for both fluorophores, Cy2 and Cy3, for each cell, and obtained a ratio of Cy3 to Cy2 fluorescence for each cell, corresponding to a ratio of cell surface receptor to total cell receptor.

Results

An Observable Difference in DOR and MOR Cell Surface Receptor

Figure 1 shows high magnification confocal images of typical FLAGDOR- and FLAGMOR-transfected PC12 cells. Surface and total receptor are separately labeled and distinct. The difference in surface receptor between DOR and MOR is striking: the FLAGDOR-transfected cell shows much more FLAG staining than the FLAGMOR-transfected cell does.

Quantification of DOR and MOR Surface-to-Total Ratios

For NGF-differentiated PC12 cells, DOR had an average surface-to-total ratio approximately three times greater than MOR (Figure 2). Specifically, the average surface-to-total ratio for all FLAGDOR-transfected cells studied was 0.027 (+/- 0.005), while that of all FLAGMOR-transfected cells was 0.010 (+/- 0.003).

A similar difference in DOR and MOR surface-to-total ratio was found in un-differentiated (non-NGF treated) PC12 cells (Figure 3). In this group, the average surface-to-total ratio for all FLAGDOR-transfected cells was 0.05 (+/- 0.01), while that of all FLAGMOR-transfected cells was 0.013 (+/- 0.004).

Finally, we examined DOR and MOR surface-to-total ratios in HEK293 cells. As in PC12 cells, we found that DOR had a greater surface-to-total ratio than MOR did. The average surface-to-total ratio for FLAGDOR-transfected cells was 0.033 (+/- 0.007), while that of FLAGMOR-transfected cells was 0.004 (+/- 0.002) (Figure 4).

Relationship between Cell Surface and Total Cell Receptor

Having separately quantified surface and total receptor for each cell, we examined the mathematical relationship between surface and total receptor. Figure 5 shows scatter plots of total versus surface receptor for FLAGDOR- and FLAGMOR-transfected PC12 cells. Large cell-to-cell variation in both total and surface receptor is evident. The plots reveal that there is little mathematical relationship between surface and total receptor for either DOR or MOR. While the trend lines suggest a weakly positive relationship between total and surface receptor, the R^2 values are very small.

We investigated whether there was an upper limit to surface receptor level. We separated PC12 cells into three groups of equal size based on total receptor level for both DOR and MOR, so that low, moderate, and high-expressing cells of each receptor were grouped together. Low expressers had a range of total receptor from 6 to 490 (in units of calibrated fluorescence reported by Image J), moderate expressers ranged from 493 to 1500, and high expressers ranged from 1630 to 10095. We found that surface-to-total ratios of both receptors decreased as total receptor increased: average surface-to-total ratio was lower in medium expressers than in low expressers and lower still in high expressers (Figure 6). Looking simply at average surface receptor, as opposed to surface-to-total ratios , we found that surface receptor increased slightly (statistical significance was borderline) from low to medium expressers but showed almost no difference between medium and high expressers (Figure 6). Again, this result held for both receptors.

Discussion

Differential intracellular targeting of DOR and MOR has been described in the literature. For example, it has been shown that DOR is found intracellularly in the same cells in which endogenously expressed MOR is found primarily on the cell surface (Wang and Pickel, 2001). This may be accounted for by differential sorting of newly synthesized receptor; it has been found that NGF treatment in PC12 cells, which induces this cell type to adopt neuron-like properties, causes newly synthesized DOR to be directed to an intracellular pool that can be mobilized to the cell surface upon stimulation, while MOR in these same cells is targeted to the cell surface regardless of NGF treatment (Kim and von Zastrow, 2003).

Using immunocytochemistry to separately label cell surface and total cell receptor, we observed a robust difference in surface receptor between DOR and MOR in PC12 cells. To rigorously characterize this difference, we developed a new method to quantify both surface and total receptor in individual cells. This method avoids the need to homogenize cell extracts required for Western blotting, giving greater confidence that surface receptor can be labeled separately from total receptor. The method is convenient, fairly high-throughput, and well adapted to situations in which the number of cells available for study is limited by practical constraints such as transfection efficiency. More importantly, the method enables study of cell-to-cell variation and the mathematical relationship between total and surface receptor, which may yield information regarding the mechanisms governing receptor surface quantity.

Using this method, we confirmed that in both NFG-treated and non-NGF-treated PC12 cells, DOR had a greater surface-to-total ratio than MOR did. NGF-treated PC12 cells have neuron-like properties and are often used as a model to study neuronal phenomena. The fact that NGF did not change this pattern indicates that differential membrane trafficking is not confined to neurons or neuron-like cells. PC12 cells, derived from rat pheochromocytomas, are inherently secretory cells regardless of NGF treatment. However, the fact that HEK cells, a simple epithelial cell type, showed a similar disparity in DOR and MOR surface-to-total ratio indicates that this phenomenon is not limited to cells with a well-developed regulated secretory pathway either. Instead, the difference must result from a sorting mechanism different from those previously described.

The amount of receptor in the plasma membrane represents the dynamic result of the processes of installation, internalization, and recycling back to the membrane of internalized receptor. Our results indicate that DOR and MOR are handled differently by at least one of these processes. Additional experiments, such as using drugs to block recycling, will be required to determine how and if each process contributes to the difference.

Because DOR and MOR are structurally similar receptors, our results indicate that the mechanisms involved must have a high degree of receptor specificity. Extensive research has revealed heterogeneity and complexity in the processes of internalization and recycling, involving both receptor-specific and shared sorting proteins (Hanyaloglu and von Zastrow, 2008). In the case of DOR and MOR, it has already been shown that differences in the C-terminus as well as a di-leucine motif in the third intracellular loop contribute to the tendency of MOR to recycle upon agonist-induced internalization, while DOR tends to be degraded in lysosomes (Wang et al., 2003). Initial installation in the membrane is less well understood, but studies of other GPCRs indicate that the process involves structural motifs and a host of accessory and chaperone molecules, enabling significant receptor specificity in installation (Tan et al., 2004). Different modes of receptor installation have been shown in studies using the β-adrenergic receptor (Yudowski et al., 2006); different insertion events result in transient or more lasting receptor installation, and it would be interesting to explore whether these different insertion modes are involved in the differential membrane targeting of DOR and MOR. Future studies using proteomic approaches may reveal the ensemble of proteins involved in DOR and MOR installation into the membrane, and any protein identified that is specific to only one receptor is a likely candidate for contributing to the differential membrane targeting effect.

Kim and von Zastrow (2003) reported that DOR is targeted intracellularly upon NGF treatment in stably transfected PC12 cells, while MOR is targeted primarily to the membrane regardless of NGF treatment (Kim and von Zastrow, 2003). However, Kim and von Zastrow found that NGF treatment did not significantly change the amount of cell surface DOR as measured by flow cytometry; the intracellular reserve pool of DOR evidently came from post-Golgi sorting of newly synthesized DOR. We found that NGF treatment did not significantly change the difference in surface-to-total ratio between DOR and MOR that we measured. Taken together, these findings indicate that NGF treatment is a factor in post-Golgi sorting of newly synthesized receptor but is not a factor in receptor installation in the plasma membrane. We used transient transfection and generally got abundant total cellular receptor staining for both receptors, as opposed to the stable transfection used by Kim and von Zastrow. Importantly, we found that potassium chloride stimulation increased the surface-to-total ratio of DOR but not MOR

(Chapter 3), which is consistent with Kim and von Zastrow's own results and their central claim that DOR is directed to an intracellular pool that can be mobilized to the cell surface upon stimulation.

Because we measured a significant quantity of surface DOR even without any type of stimulation to induce regulated insertion of receptor, a large portion of surface DOR is evidently installed in a constitutive manner. Indeed, while many groups have reported that DOR occurs mainly intracellularly within neurons, others have shown significant quantities of cell surface DOR in neuronal cells (Scherrer et al., 2006). Additional factors such as receptor glycosylation may complicate DOR intracellular targeting and account for the conflicting results. What we have shown is that in a direct comparison between DOR and MOR surface-to-total ratio in a neuron-like cell type expressing abundant quantities of each receptor, DOR exists in the membrane in a larger quantity than MOR, suggesting receptor-specific membrane installation mechanisms.

Our investigation into the relationship between total cell receptor and cell surface receptor provides some hints about the mechanisms responsible for receptor installation into the membrane. First, the fact that there is little relationship between the quantities of total and surface receptor suggest that receptor installation does not result from passive "flow" from intracellular stores; if that were the case, one would expect to find that the quantity of surface receptor increased fairly consistently with that of total receptor. Instead, we found little relationship between total and surface receptor, meaning that intracellular receptor is not free to move down a concentration gradient into the plasma membrane, but rather that the quantity of cell surface receptor is regulated by one or more mechanisms that result in widely varying surface-to-total ratios from cell to cell.

Moreover, these mechanisms are saturable. We found that at higher levels of total cell receptor, the receptor surface-to-total ratio tended to decrease, and that the quantity of surface receptor did not differ significantly from medium- to high-expressing cells. This is consistent with the involvement of specific accessory molecules in receptor installation that can only handle so much cargo at once.

Our findings demonstrate the power of our quantification method. The mathematical relationship between total cell receptor and cell surface receptor provides clues about the cellular mechanisms responsible for receptor installation. Because the method is capable of capturing cell-to-cell differences, it could be used to study variation within a population of cells to agonist effects. By studying DOR and MOR, we have found that surface receptor quantity is governed by receptor-specific, saturable mechanisms.

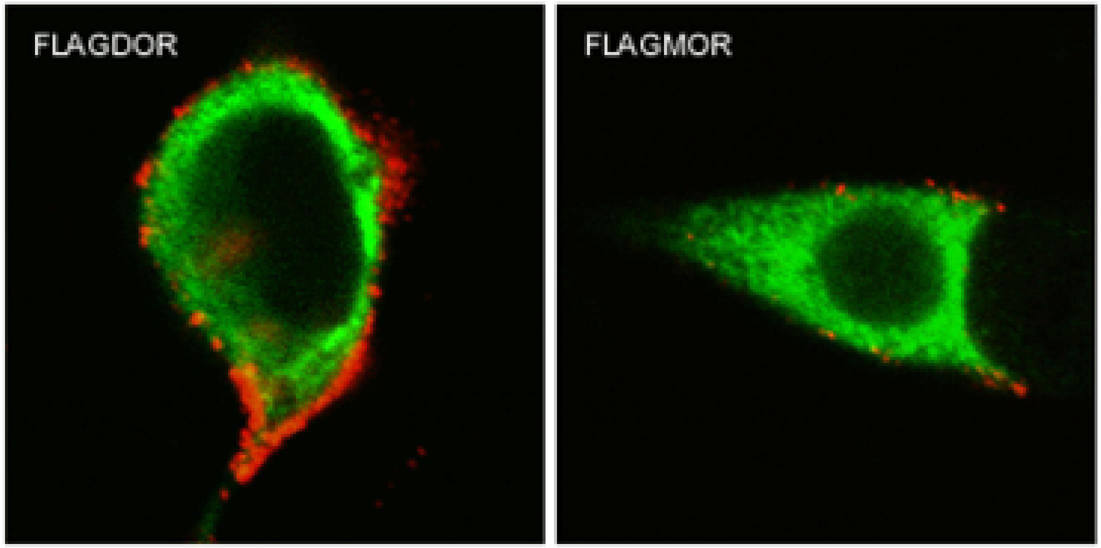

Figure 1. Visualization of Cell Surface and Total Cell Receptor by Immunocytochemistry. Cells are typical FLAGDOR- and FLAGMOR-transfected cells. The red corresponds to FLAG antibody staining, done before fixation to label surface receptor. The green corresponds to C-terminal DOR and MOR antibodies, done after fixation to label total receptor. Images were taken by confocal microscopy with a 60x objective.

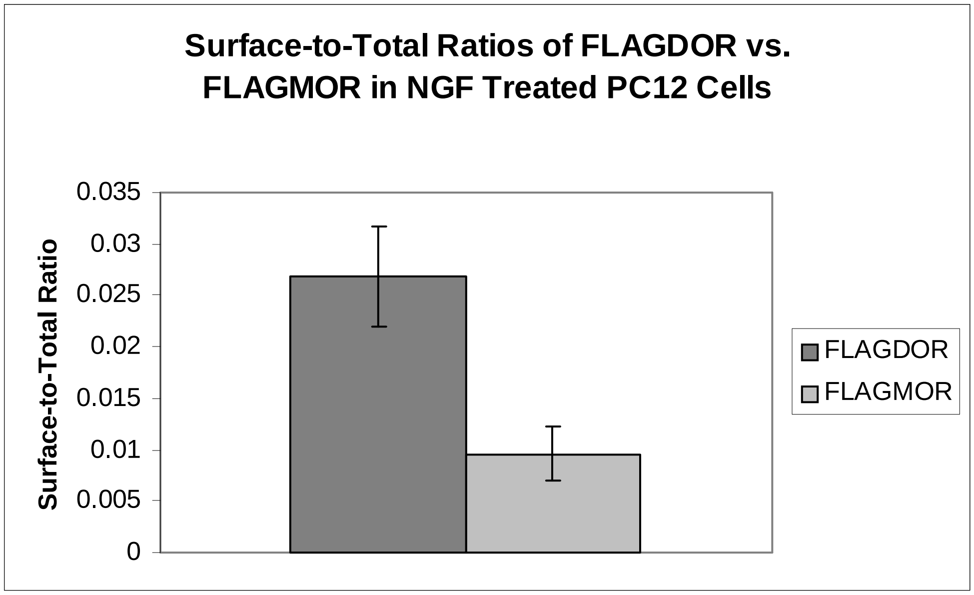

Figure 2. Surface-to-Total Ratios of FLAGDOR vs. FLAGMOR in NGF-treated PC12 cells. The average surface-to-total ratio for all FLAGDOR-transfected cells studied was 0.027 (+/- 0.005), while that of all FLAGMOR-transfected cells was 0.010 (+/- 0.003). Error bars represent +/- SEM for each group. Data is from seven different experiments, $p<0.05$ by t-test.

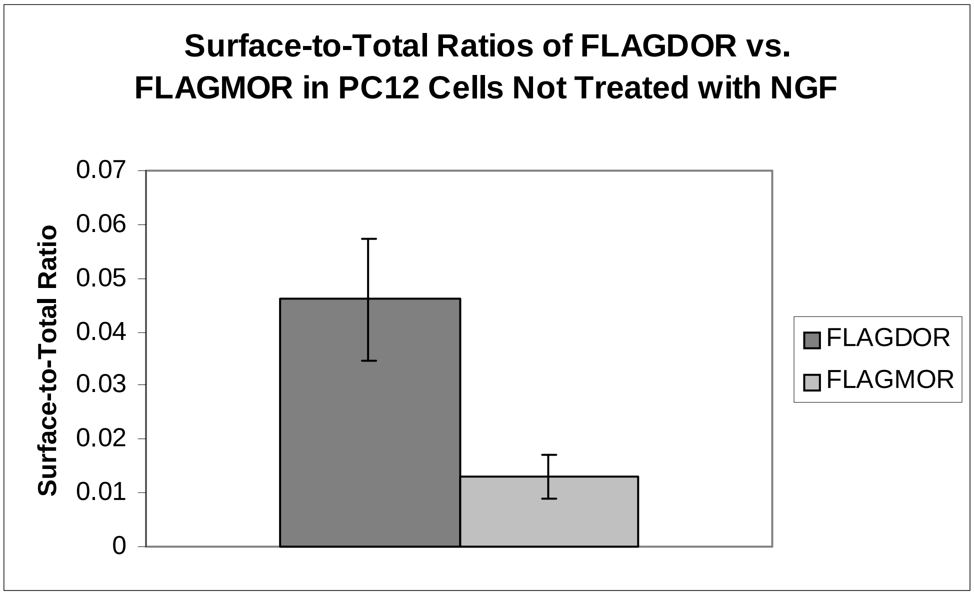

Figure 3. Surface-to-Total Ratios of FLAGDOR vs. FLAGMOR in PC12 Cells Not Treated with NGF. The average surface-to-total ratio for all FLAGDOR-transfected cells was 0.05 (+/- 0.01), while that of all FLAGMOR-transfected cells was 0.013 (+/- 0.004). Error bars represent +/- SEM for each group. Data is from five different experiments, $p<0.05$ by t-test.

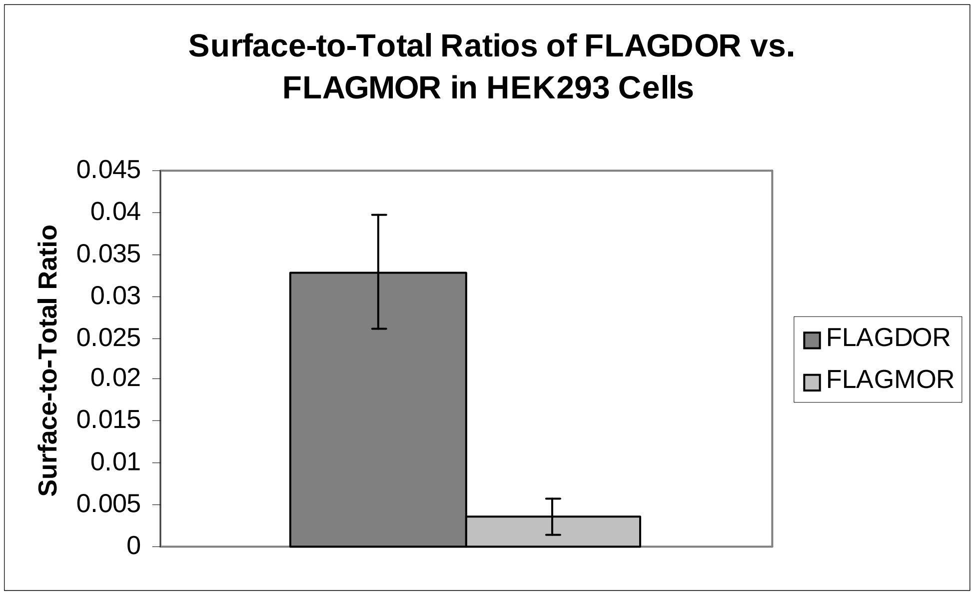

Figure 4. Surface-to-Total Ratios of FLAGDOR vs. FLAGMOR in HEK293 Cells. The average surface-to-total ratio for FLAGDOR-transfected cells was 0.033 (+/- 0.007), while that of FLAGMOR-transfected cells was 0.004 (+/- 0.002). Error bars represent +/- SEM for each group. Data is from four different experiments, $p<0.05$ by t-test.

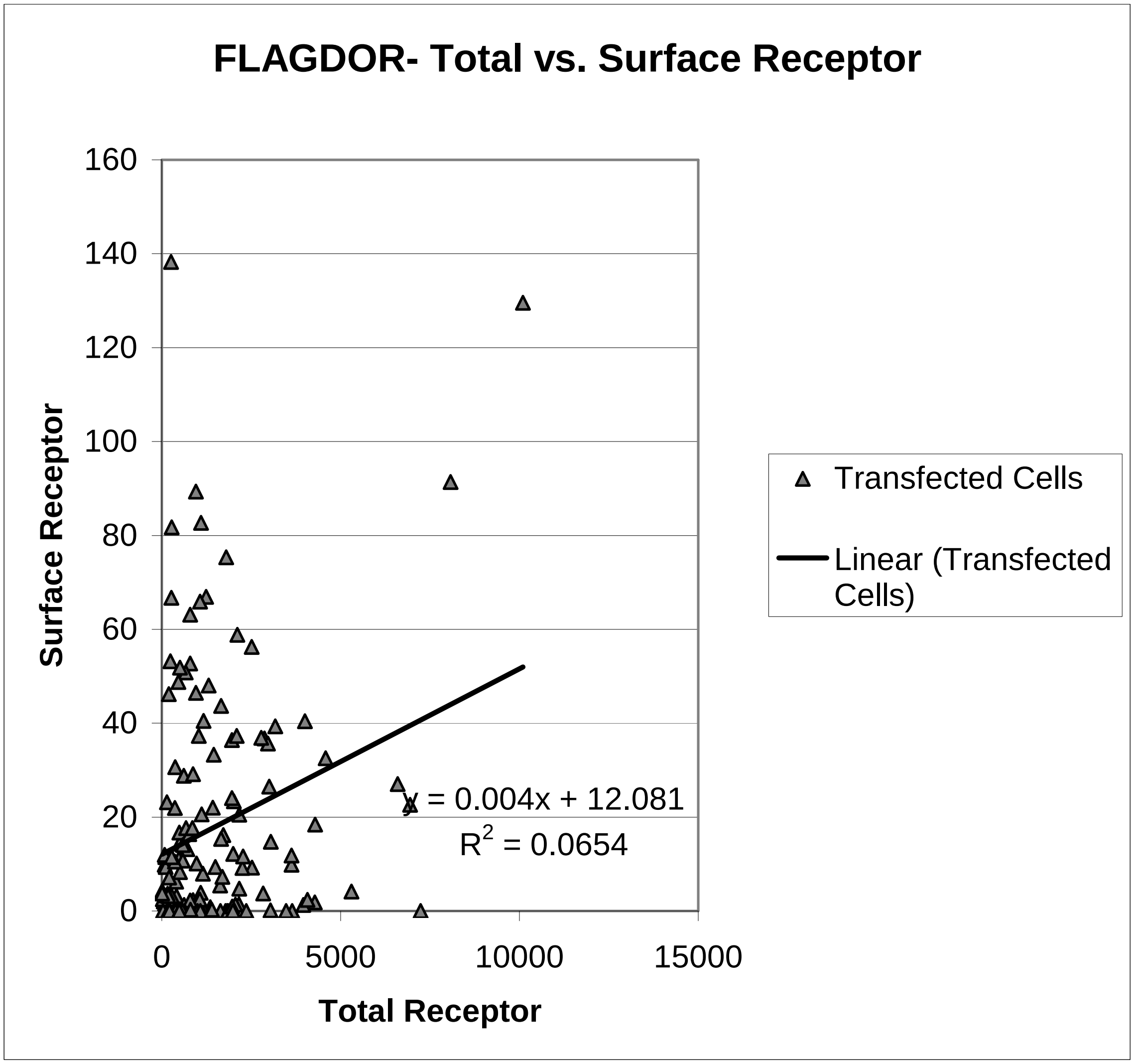

Figure 5a. Relationship between Cell Surface and Total Cell Receptor- FLAGDOR. Scatter plot showing total and surface receptor for each FLAGDOR-transfected PC12 cell.

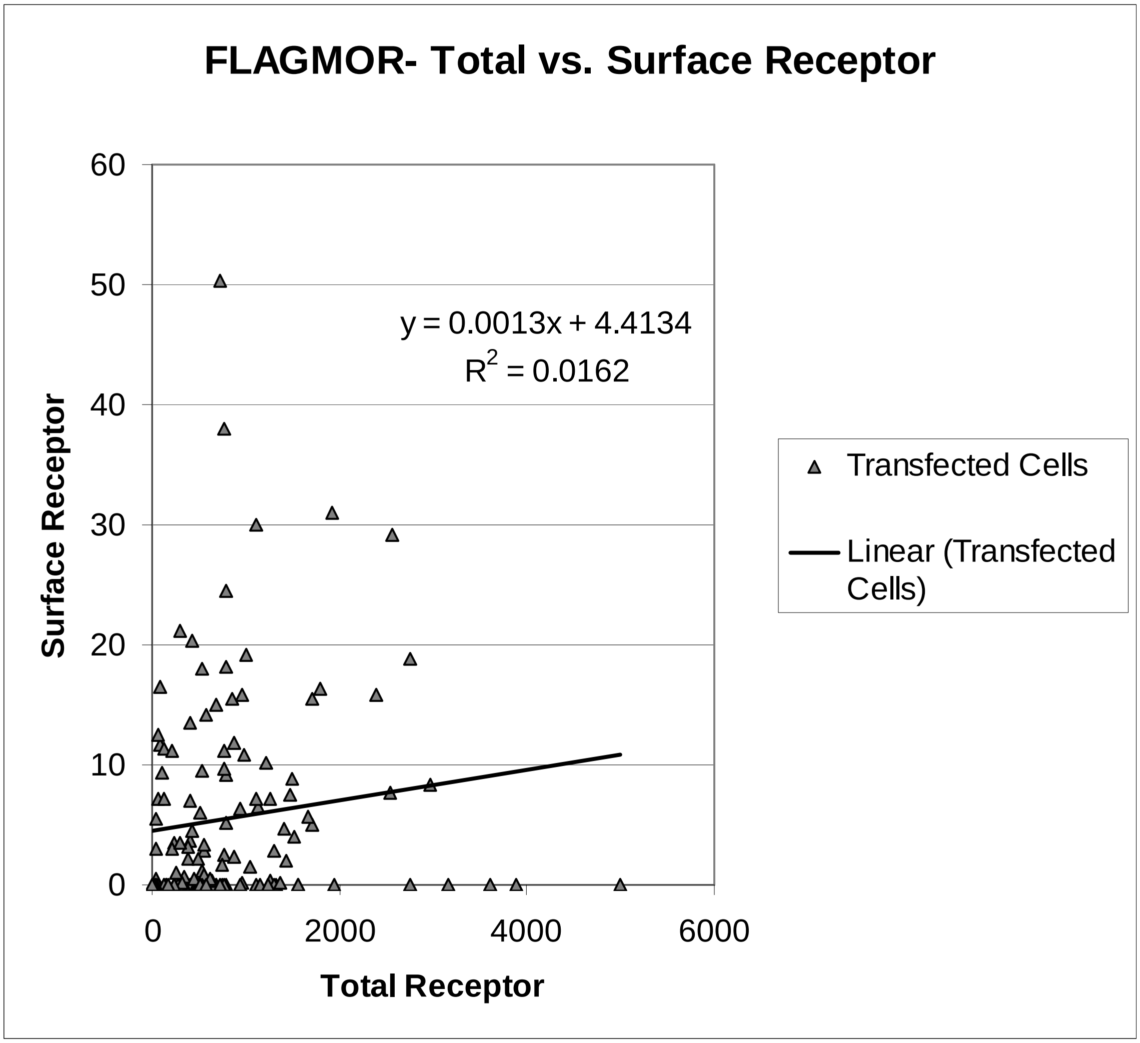

Figure 5b. Relationship between Cell Surface and Total Cell Receptor-FLAGMOR. Scatter plot showing total and surface receptor for each FLAGMOR-transfected PC12 cell.

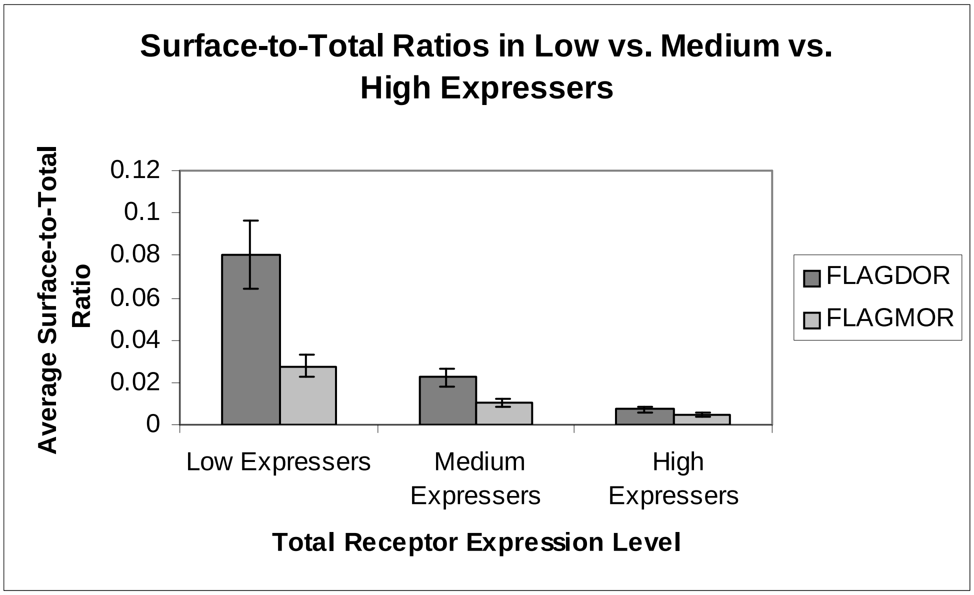

Figure 6a. Surface-to-Total Ratios for Different Receptor Expression Profiles. Transfected PC12 cells were sorted by total cell receptor and put into three groups of equal size corresponding to low, medium, and high expressers. Low expressers had a range of total receptor from 6 to 490 (in units of calibrated fluorescence reported by Image J), moderate expressers ranged from 493 to 1500, and high expressers ranged from 1630 to 10095. Error bars represent +/- SEM for each group.

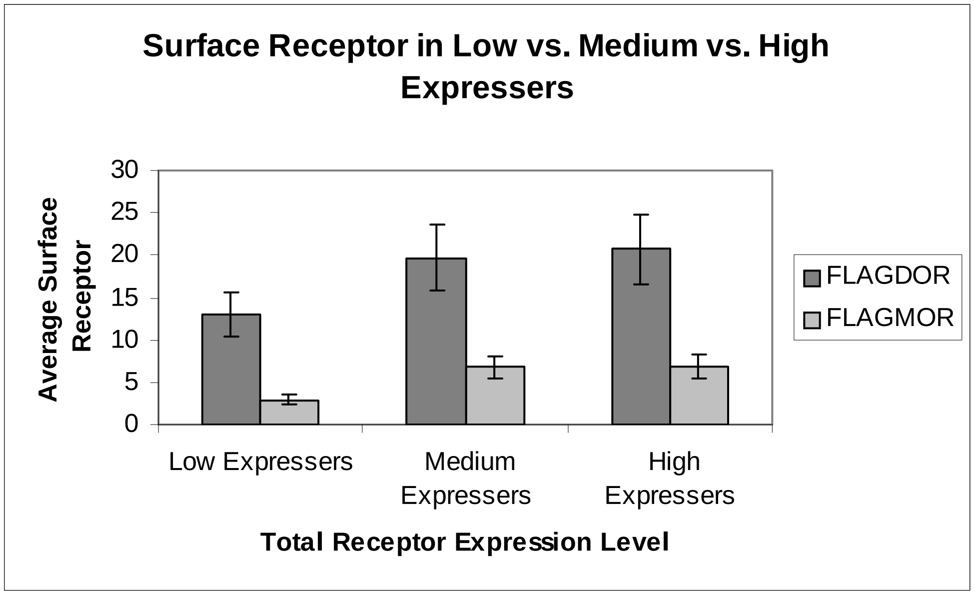

Figure 6b. Surface Receptor for Different Receptor Expression Profiles.

Transfected PC12 cells were sorted by total cell receptor and put into three groups of equal size corresponding to low, medium, and high expressers. Low expressers had a range of total receptor from 6 to 490 (in units of calibrated fluorescence reported by Image J), moderate expressers ranged from 493 to 1500, and high expressers ranged from 1630 to 10095. Error bars represent +/- SEM for each group.

Chapter 3

The Regulated Secretory Pathway: A Major Factor in the Quantity of Cell Surface DOR but not MOR

Summary

The literature has shown a role for the regulated secretory pathway in trafficking of newly synthesized DOR to the plasma membrane. A similar role has not been reported for MOR. Specifically, DOR has been shown to be directed to an intracellular pool of vesicles that are translocated to the cell surface upon stimulation. This intracellular DOR has been shown to exist in direct physical association with the Substance P precursor pre-pro-tachykinin in the regulated secretory pathway. Using NGF-differentiated PC12 cells transiently transfected with DOR or MOR and applying our surface receptor quantification method, we investigated the effects of depolarization and co-expression of pre-pro-tachykinin on DOR and MOR surface-to-total ratios. We found that these factors increased the surface density of DOR but not MOR, consistent with the literature on DOR and the regulated secretory pathway. Hence, in a model system with abundant quantities of receptor, we have verified that the regulated secretory pathway is a significant factor in the quantity of cell surface DOR but not MOR, providing a means for robust, rapid, and receptor-specific control.

Introduction

The regulated secretory pathway is involved in more than its well-established role in the secretion of various neurotransmitters and hormones. The literature has clearly shown that certain GPCRs are delivered to the cell surface via regulated exocytosis. For example, activation of synaptic NMDA receptors leads to rapid redistribution of AMPA receptors from intracellular stores into dendritic spines, which may play a role in long-term potentiation (Shi et al., 1999). The hormone insulin causes rapid delivery of NMDA receptors to the cell surface of neurons via exocytosis (Skeberdis et al., 2001). Regulated ecocytosis has been shown to be important for cell surface delivery of GPCRs as well, notably opioid receptors. Because receptors (except for nuclear receptors) must be on the cell surface to be functional, the regulated secretory pathway is an important determinant of the availability of these receptors to respond to drugs.

A classic demonstration of the role the regulated secretory pathway plays in receptor trafficking is the work by Shuster et al. (1999). Immunohistochemistry and ultrastructural analysis were used to show that the kappa opioid receptor (KOR) colocalized with the neuropeptide hormone vasopressin in cell bodies, axons, and axon terminals of the posterior pituitary, and that KOR was associated with the large secretory vesicles that transport and store vasopressin. A stimulus that would lead to vasopressin release (salt-loading) in vivo caused a translocation of KOR from this vesicular pool to the plasma membrane.

A variety of evidence demonstrates that the regulated secretory pathway is critical for the delivery of functional DOR to the cell surface. First, DOR is often prominent in neuropeptide-positive axon terminals and is often associated with large secretory granules

(Cheng et al., 1995) (Zhang et al., 1998). Second, in small dorsal root ganglia neurons, the DOR agonist deltorphin has been shown to elicit translocation of DORs from intracellular large secretory granules to the plasma membrane, comcomitant with release of calcitonin gene-related peptide (CGRP) (Bao et al., 2003). Third, it has been shown in PC12 cells that exogenously expressed DOR is targeted to an intracellular pool of vesicles upon NGF treatment, and that this population of DORs can be mobilized to the cell surface by potassium chloride stimulation (Kim and von Zastrow, 2003). Finally, Guan et al. (2005) showed a physical association between DOR and the Substance P precursor, pre-pro-tachykinin, that pre-pro-tachykinin was both necessary and sufficient to direct DOR to large secretory granules, that pre-pro-tachykinin was important for delivery of DORs to the cell membrane of small dorsal root ganglia neurons, and that the population of DORs delivered in this manner was essential for DOR-mediated spinal analgesia and morphine tolerance.

These findings regarding the role of pre-pro-tachykinin in DOR delivery to the cell surface have been challenged recently by Scherrer et al. (2009). Using DOReGFP knockin mice, this group found that DOR and Substance P were not expressed in the same primary afferent fibers, and that DOR was targeted to the cell membrane in significant quantities without Substance P.

MOR, in contrast to DOR, seems to be delivered to the cell surface by the constitutive secretory pathway (Zhang et al., 1998). We therefore sought to use NGF-treated PC12 cells transfected with DOR or MOR and our quantification method (see Chapter 2) to test the effects of cellular depolarization and pre-pro-tachykinin co-expression on cell surface DOR and MOR. We will determine if these effects are confined to DOR or can be observed with MOR also, and how robust the effects are in a model system expressing large quantities of receptor.

Methods

We used NGF-differentiated PC12 cells transiently transfected with DOR or MOR and our surface receptor quantification method (see Chapter 2). Briefly, PC12 cells were plated and treated with NGF for two days. At this point, cells were transfected with FLAGDOR or FLAGMOR, and with pre-pro-tachykinin where appropriate, using Lipofectamine 2000. After twenty-four more hours in culture with NGF, FLAG antibody was applied to label surface receptor, cells were fixed, and antibodies to DOR or MOR were then used to label total cell receptor. Cells were imaged with fluorescence microscopy and quantified using Image J.

For pre-pro-tachykinin transfection, we obtained the human pre-pro-tachykinin gene in expression-ready form from Invitrogen's (Carlsbad, CA) Genestorm collection of human clones (clone ID RG001694, catalog # HK1000). We simply co-transfected pre-pro-tachykinin with FLAGDOR or FLAGMOR using Lipofectamine 2000, doubling the amount of Lipofectamine reagent used to account for the increased amount of DNA. The pre-pro-tackykinin clone came with a V5HRP tag, so pre-pro-tachykinin production was verified in co-transfected cells by immunocytochemistry following fixation, using a mouse V5HRP antibody (1:500, Invitrogen).

For stimulation experiments, we elicited depolarization in the same manner used by Kim and von Zastrow (2003). Just prior to application of FLAG antibody, transfected PC12 cells were treated with normal medium supplemented with 55 mM potassium chloride (KCl) and 2 mM calcium chloride ($CaCl_2$) for thirty minutes.

Results

We found that stimulation by depolarization resulted in a three-fold increase in surface-to-total ratio of DOR but not of MOR. The average surface-to-total ratio for non-stimulated FLAGDOR-transfected cells was 0.036 (+/- 0.006), while that of FLAGDOR-transfected cells stimulated by depolarization was 0.12 (+/- 0.02) (Figure 1). The average surface-to-total ratio for non-stimulated FLAGMOR-transfected cells (Figure 2) was 0.018 (+/- 0.003), while that of FLAGMOR-transfected cells stimulated by depolarization was 0.017 (+/- 0.003) (Figure 2).

Similarly, we found that co-expression of pre-pro-tachykinin with FLAGDOR or FLAGMOR resulted in a three-fold increase in surface-to-total ratio of DOR but not of MOR. The average surface-to-total ratio for FLAGDOR-transfected cells alone was 0.033 (+/- 0.005), while that of cells co-expressing FLAGDOR and pre-pro-tachykinin was 0.12 (+/- 0.02) (Figure 3). The average surface-to-total ratio of FLAGMOR-transfected cells alone was 0.019 (+/- 0.004), while that of cells co-expressing FLAGMOR and pre-pro-tachykinin was 0.023 (+/- 0.005) (Figure 4).

Discussion

Our results provide additional support for the hypothesis that the regulated secretory pathway is involved in DOR trafficking. We confirmed Kim and von Zastrow's finding that stimulation by depolarization with potassium chloride induces a translocation of DOR from intracellular stores to the cell surface. Kim and von Zastrow reported a fivefold increase in cell surface DOR as measured by biotinylation of surface receptor and Western blotting. Using the exact same treatment as Kim and von Zastrow to induce depolarization, we measured a three-fold increase in DOR surface-to-total ratio in treated cells compared to untreated cells using our quantification method. Our transfection produced robust DOR synthesis and seemed to result in greater DOR both intracellularly and on the cell surface than in Kim and von Zastrow's cells, based on comparison of our cell images with theirs (see Chapter 2). Since we nevertheless measured a comparable effect of translocation of DOR to the cell surface, this indicates that the cellular machinery involved in stimulus-induced translocation is capable of handling large amounts of receptor in a short time. To the extent that this finding holds true for neurons *in vivo*, it may be important for physiological states such as pain or opiate tolerance that have been shown to involve an increase in surface DOR.

Similarly, we provide confirmation for the finding of Guan et al. (2005) on the role of the Substance P precursor peptide pre-pro-tachykinin in DOR surface targeting. Guan et al. reported a physical association between DOR and pre-pro-tachykinin, that pre-pro-tachykinin was both necessary and sufficient to direct DOR to large secretory granules, that pre-pro-tachykinin was important for delivery of DORs to the cell membrane of small dorsal root ganglia neurons, and that the population of DORs delivered in this manner was essential for DOR-mediated spinal analgesia and morphine tolerance. Both immunoblotting and autoradigraphic analysis of the DOR agonist deltorphin binding sites showed a reduction in surface DOR in the dorsal horn of the spinal cord in pre-pro-tachykinin knockout mice in the work of Guan et al. The role of pre-pro-tachykinin in DOR cell surface delivery has recently been questioned by Scherrer et al. (2009), who contend that interactions are unlikely because DOR and Substance P are not found in proximity in the nerve fibers of their DOReGFP knockin mice. However, consistent with a role for pre-pro-tackykinin in DOR trafficking, we measured an approximate threefold increase in DOR surface-to-total ratio when PC12 cells were co-transfected with pre-pro-tachykinin. The fact that we observed this effect in a cell line shows that it is not confined just to special populations of neurons. Guan et al. showed that pre-pro-tachykinin is both necessary and sufficient to direct DOR to large secretory granules. Because PC12 cells are inherently secretory cells and produce copious large secretory granules, it may be hypothesized that co-transfection of pre-pro-tachykinin directed a significant quantity of cellular DOR in our PC12 cells to large secretory granules, and that this led to plasma membrane installation of a large quantity of DOR that otherwise would not have been installed in the membrane. Moreover, our finding indicates that the stoichiometry between DOR and pre-pro-tachykinin within the cell is an important determinant of cell surface DOR quantity, suggesting that an increase in cell surface DOR will result when pre-pro-tachykinin is elevated. It is interesting that this occurred in our system without any sort of external stimulus. It may be that NGF treatment is resulting in the production of endogenous neurochemicals that induce exocytosis.

Although pre-pro-tachykinin may be necessary to direct DOR to large secretory granules, it is not essential for all DOR insertion into the plasma membrane. We observed a significant quantity of cell surface DOR without any type of stimulation, consistent with Scherrer et al. (2009). Moreover, pre-pro-tachykinin is not essential for depolarization-induced exocytosis of DOR, since we measured an increase in surface DOR with potassium chloride stimulation but without pre-pro-tachykinin co-transfection. Kim and von Zastrow noted that the NGF-induced intracellular pool of DOR was not co-localized with secretogranin, a marker for large secretory granules. These findings suggest that regulated exocytosis of DOR may occur by more than one mechanism and involve more than one vesicle type.

Thus, while we are in agreement with Scherrer et al. that significant quantities of DOR can be delivered to the plasma membrane in a constitutive manner, we have confirmed a role for both pre-pro-tachykinin and activity-dependent insertion in DOR cell surface targeting. Morever, to the extent that the cell lines used by Guan et al., Kim and von Zastrow, and us are not an ideal way to simulate the trafficking of native DOR *in vivo*, it is doubtful whether the DOReGFP mice are either, especially given the report that the GFP tag interferes with ordinary DOR subcellular targeting (Wang et al. 2008). And while Scherrer et al. report that DOR and Substance P do not occur in the same afferent fibers in the DOReGFP mice, Guan et al. provided evidence of a direct physical interaction between the Substance P domain of pre-pro-tachykinin and the third luminal domain of DOR. Guan et al. found that pre-pro-tachykinin gene deletion resulted in reduced DOR levels in laminae I and II of the spinal cord, and we found that pre-pro-tachykinin co-expression could triple the amount of DOR installed in the plasma membrane of PC12 cells. These effects were not found for MOR, although the two receptors are structurally similar. These findings, together with ultrastructural and immunohistochemical evidence (Cheng et al., 1995; Zhang et al., 1998) from tissue, point to a role for pre-pro-tachykinin in DOR subcellular targeting *in vivo* since it seems unlikely that these highly specific interactions exist purely by chance.

Neither stimulation by depolarization nor pre-pro-tachykinin co-expression could increase the surface-to-total ratio of MOR, in contrast to DOR. While Kim and von Zastrow did not report the effect of stimulation on MOR, Guan et al. found that pre-pro-tachykinin knockout mice did not display a difference in MOR surface density. These findings are consistent with the idea that MOR, as opposed to DOR, is targeted primarily to the constitutive secretory pathway. The regulated secretory pathway's role in GPCR surface delivery is evidently very receptor-specific.

Hence, in a model system with abundant quantities of receptor, we have verified that the regulated secretory pathway is an important factor in cell surface DOR quantity, providing robust, rapid, and receptor-specific control. Since Guan et al. provided evidence that DOR delivered in this manner is critical for the development of morphine tolerance, this implies that blocking or otherwise disabling this population of DOR in cells expressing significant pre-pro-tackykinin, such as dorsal root ganglia neurons, is a potential strategy to overcome the problem of tolerance in opioid analgesia.

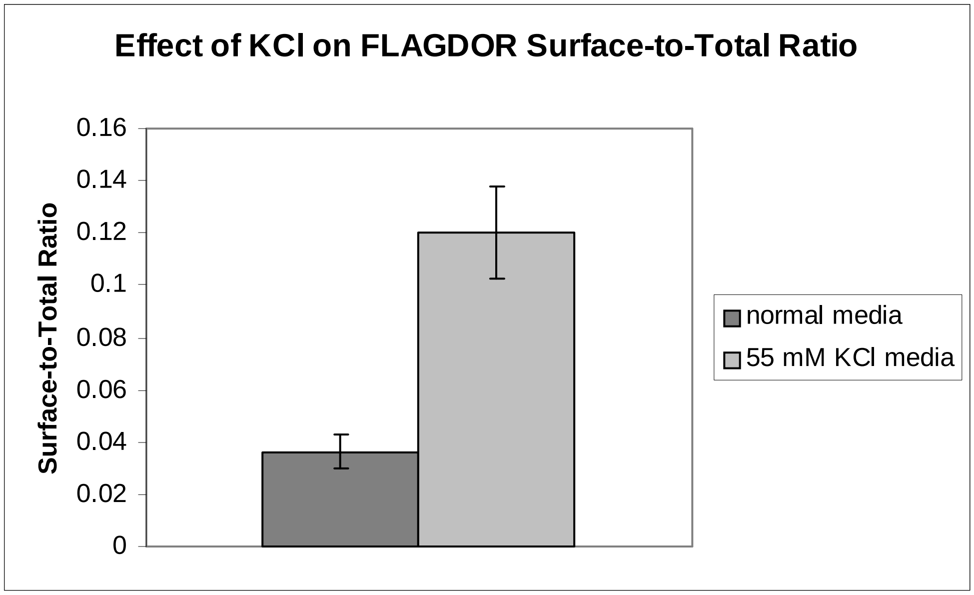

Figure 1. Potassium Chloride Depolarization Increases FLAGDOR Surface-to-Total Ratio. The average surface-to-total ratio for non-stimulated FLAGDOR-transfected cells was 0.036 (+/- 0.006), while that of FLAGDOR-transfected cells stimulated by 55 mM KCl depolarization was 0.12 (+/- 0.02). Error bars represent +/- SEM for each group. Data is from eight different experiments, $p<0.05$ by t-test.

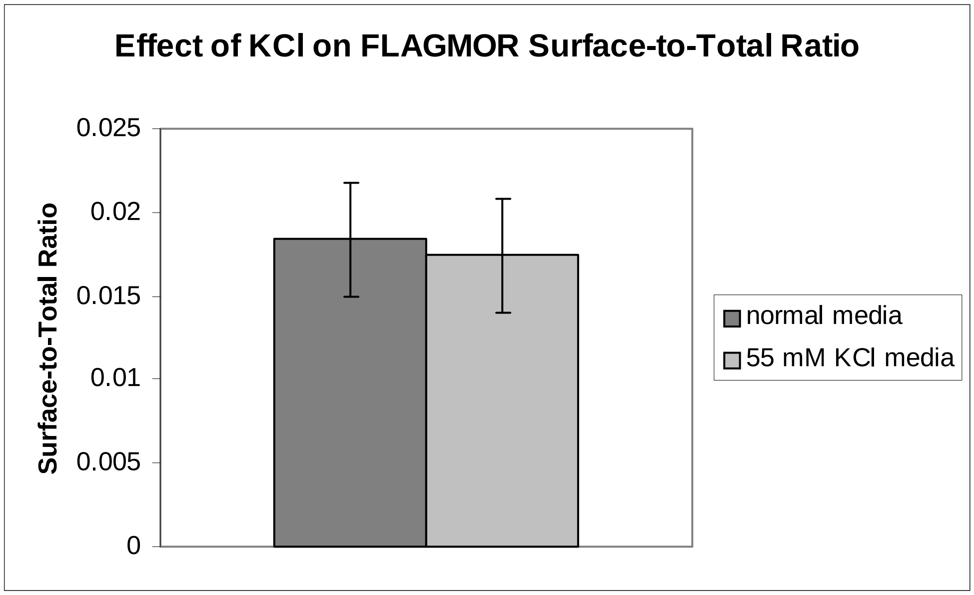

Figure 2. Potassium Chloride Depolarization Has No Significant Effect on FLAGMOR Surface-to-Total Ratio. The average surface-to-total ratio for non-stimulated FLAGMOR-transfected cells (Figure 2) was 0.018 (+/- 0.003), while that of FLAGMOR-transfected cells stimulated by 55 mM KCl depolarization was 0.017 (+/- 0.003). Error bars represent +/- SEM for each group. Data is from eight different experiments.

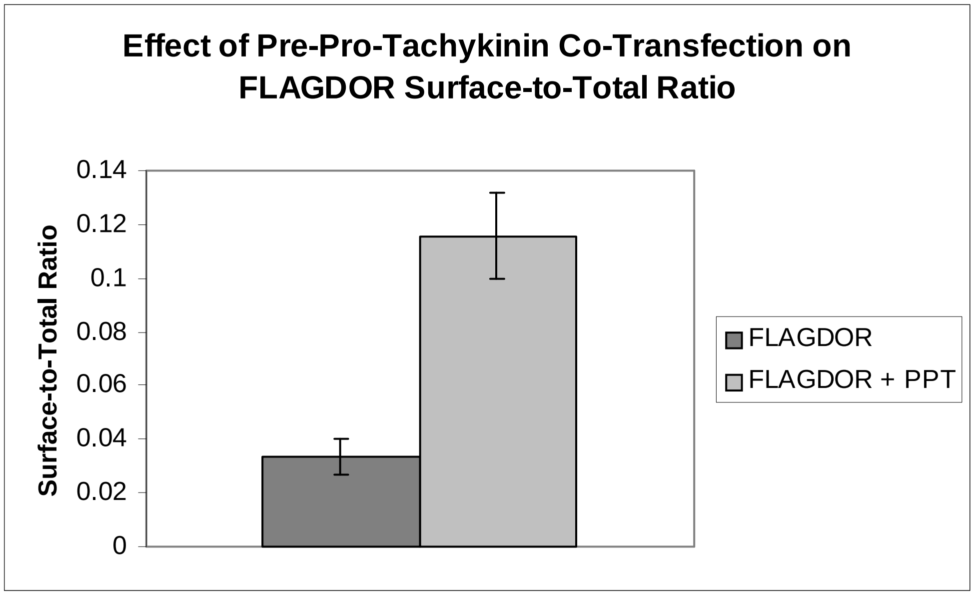

Figure 3. Pre-Pro-Tachykinin Co-Expression Increases FLAGDOR Surface-to-Total Ratio. The average surface-to-total ratio for FLAGDOR-transfected cells alone was 0.033 (+/- 0.005), while that of cells co-expressing FLAGDOR and pre-pro-tachykinin was 0.12 (+/- 0.02). Error bars represent +/- SEM for each group. Data is from nine different experiments, $p<0.05$ by t-test.

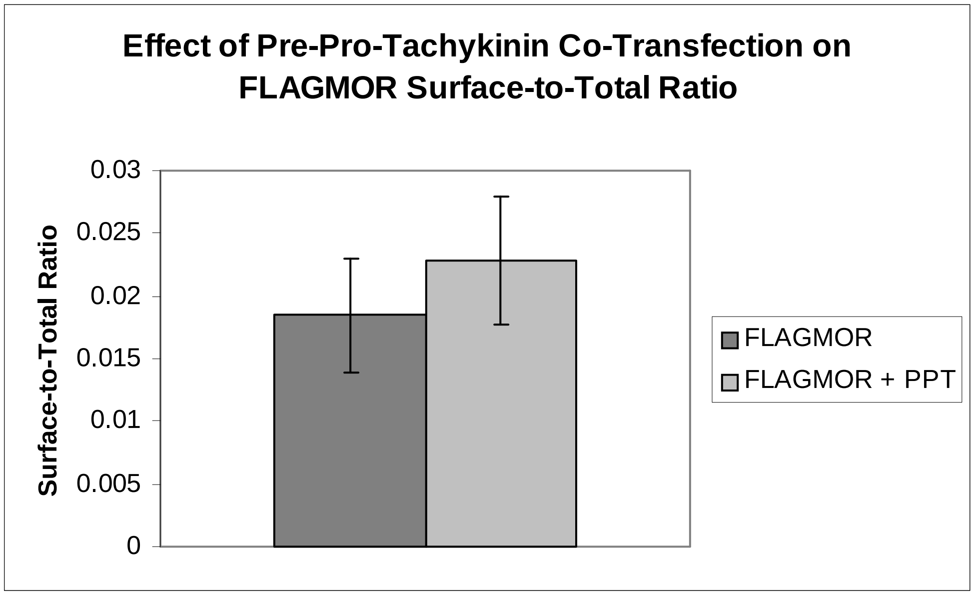

Figure 4. Pre-pro-Tachykinin Co-Expression Does Not Significantly Increase FLAGMOR Surface-to-Total Ratio. The average surface-to-total ratio of FLAGMOR-transfected cells alone was 0.019 (+/- 0.004), while that of cells co-expressing FLAGMOR and pre-pro-tachykinin was 0.023 (+/- 0.005). Error bars represent +/- SEM for each group. Data is from eight different experiments.

Chapter 4

The Lasting Effect of Agonist Treatment on DOR Surface-to-Total Ratio

Summary

Down-regulation of DOR and MOR has been described for neurons *in vivo* following opioid drug treatment. While this may be partially because agonist treatment causes direct internalization of cell surface GPCRs through well-characterized processes, we hypothesized that agonist treatment also results in a reduction in cell surface installation of newly synthesized receptors. We tested this hypothesis using NGF-differentiated PC12 cells stably transfected with DOR and applied our surface receptor quantification method. We determined that the time required for constitutive internalization of all cell surface DOR is between 6 and 12 hours. Because we verified that baseline cell surface DOR did not significantly change during the period in culture over which experiments were conducted, the rate of installation of DOR into the plasma membrane must be about equal to the rate of internalization. Hence, between 6 and 12 hours should also be sufficient after agonist internalization to restore cell surface DOR to baseline level. However, we found that 30 minutes of 100 nM deltorphin treatment caused DOR surface-to-total ratio to be depressed for up to 24 hours following the agonist treatment, with significant recovery of surface DOR measured only at 48 hours after agonist treatment. Because agonist treatment reduces the level of cell surface DOR for longer than would be expected based on the normal kinetics of the receptor, we conclude that agonist treatment results not only in direct internalization but also a longer-lasting change in receptor trafficking kinetics, which may be among the mechanisms responsible for acute tolerance.

Introduction

Prolonged or repeated administration of drugs such as morphine, cocaine, or ethanol causes down-regulation, or decrease in receptor number, of MOR and DOR in brain areas such as the nucleus accumbens and striatum, although the extent of down-regulation depends on the drug and cell type. For example, while chronic ethanol consumption down-regulates MOR in both the nucleus accumbens and striatum, morphine, a drug that actually binds to MOR, shows little ability to down-regulate MOR in these brain areas after single or repeated administration (Turchan et al., 1999), although morphine binding to MOR still produces analgesic tolerance. However, morphine, which also binds to DOR, could down-regulate DOR after chronic administration. Down-regulation of DOR and MOR are involved in drug tolerance and withdrawal to many drugs, although the example of morphine and MOR shows that the relationship between down-regulation and tolerance is not always a simple one.

The most familiar mechanism of GPCR down-regulation is agonist-induced endocytosis (Hanyaloglu and von Zastrow, 2008). GPCR kinases (GRKs) phosphorylate agonist-activated receptors, which promotes binding of β-arrestins to receptor. β-arrestin binding promotes internalization (endocytosis) of receptor into clathrin-coated pits. Considerable evidence implicates agonist-induced endocytosis in opiate tolerance (Christie, 2008). Specifically, both short-term and long-term tolerance to morphine are greatly reduced in β-arrestin knockout mice (Bohn et al., 2000). In the case of DOR, very recent evidence using DOReGFP knockin mice showed that treatment with an agonist that strongly promoted DOR internalization blunted analgesic response to subsequent administration of agonist for several hours, but treatment with a non-internalizing DOR agonist did not reduce the analgesic effect of subsequent agonist administration, strongly indicating that agonist-induced down-regulation is responsible for acute tolerance, at least in the case of DOR agonists (Pradhan et al., 2009).

However, just as the mechanisms of opiate tolerance are very complex, involving changes at different levels of organization of the nervous system (Christie, 2008), the mechanisms involved in receptor down-regulation may also involve more than just agonist-induced endocytosis. We hypothesized that agonist treatment leads to receptor down-regulation not only by direct internalization, but also by causing a longer-lasting change in the kinetics of receptor membrane trafficking. We tested this hypothesis using PC12 cells stably transfected with FLAGDOR and our surface receptor quantification method (see Chapter 2).

Testing the hypothesis involves a technical difficulty: how to determine with certainty that a reduction in measured cell surface receptor results from changed kinetics and not simply from agonist-induced endocytosis. Our solution was to show that surface receptor levels remained depressed for longer than would be expected following complete internalization by agonist. If cell surface receptor is in a steady state, the rate of receptor installation in the membrane (by either installation of newly synthesized receptor or recycling of previously internalized receptor) must equal the rate of constitutive internalization. Using our antibody-based methods, we were able to determine the rate of constitutive internalization by labeling cell surface FLAGDOR with anti-FLAG antibody, then varying the amount of time before fluorescent secondary antibody was applied. As anti-FLAG antibody bound to receptor internalizes, it becomes unavailable for binding by the secondary antibody, and hence the rate of constitutive internalization can be measured as the rate at which FLAG signal diminishes. Moreover, using our surface receptor quantification method, we verified that cell surface FLAGDOR was in the steady state during the period in culture over which experiments were performed, so we knew indirectly from the rate of internalization what the rate of installation should be when the receptor followed normal kinetics. Finally, by applying the DOR-selective agonist deltorphin for thirty-minute administrations at varying intervals before antibody labeling of surface receptor, we found that cell surface DOR remained depressed following deltorphin treatment for far longer than would be expected based on normal kinetics.

Methods

We used PC12 cells stably transfected with FLAGDOR for these experiments. To generate stably transfected cells, wild-type PC12 cells were transiently transfected with FLAGDOR using Lipofectamine 2000 as described previously (see Chapter 2). Isolation and maintenance of stably transfected colonies were carried out using the same culture conditions described for wild-type PC12 cells (see Chapter 2), but with the addition of geniticin (G418) (1 mg/ml, Gibco, Grand Island, NY) to the media.

For experiments, stably transfected PC12 cells were plated onto poly-L-lysine- and laminin-coated coverslips and maintained in culture for three days with continuous NGF treatment. Deltorphin (100 nM, Sigma-Aldrich, St. Louis, MO) was added to the media at appropriate times for 30 minutes, after which cells were rinsed and normal media was restored for the remainder of culture time. We determined in preliminary studies that this concentration of deltorphin was necessary for optimal internalization. Control cells received the rinse but no deltorphin treatment. Deltorphin treatment occurred at 48 hours, 24 hours, 12 hours, 6 hours, or 0 hours before antibody labeling of surface receptor, corresponding to 24 hours, 48 hours, 60 hours, 66 hours, and 72 hours in culture, respectively. For "pulse chase" experiments, rabbit anti-FLAG antibody (1:2000, Sigma-Aldrich) was added to the media at appropriate times for 30 minutes, then cells were rinsed and normal media was restored. Treatment with anti-FLAG antibody in these experiments occurred at 12 hours and 6 hours before the end of the culture period, corresponding to 60 and 66 hours in culture, respectively.

After 72 hours in culture, we applied our protocol for antibody labeling of surface and total cell receptor, imaging, and quantification using Image J as previously described (see Chapter 2). Cells pre-treated with anti-FLAG antibody for pulse chase experiments merely sat in serum-free media while other cells were incubated with anti-FLAG antibody in the usual course of the protocol, while all cells received Cy3 anti-rabbit antibody (1:200, Jackson Immunoresearch, West Grove, PA) at the usual point.

Results

Determination of Baseline FLAGDOR Surface-to-Total Ratios at Starting and End Points of Experimental Period

To determine if cell surface FLAGDOR was in the steady state for the period in culture over which experiments were conducted, we measured FLAGDOR surface-to-total ratios at the starting point (24 hours in culture) and end point of this period (72 hours in culture). No experiments were conducted before 24 hours in culture. We found that the average surface-to-total ratio at the starting point was 0.15 (+/- 0.02), while that at the end point was 0.23 (+/- 0.04) (Figure 1). This increase is not statistically significant (p=0.134 by t-test). This indicates that baseline surface FLAGDOR is at least in the steady state, or perhaps increasing a bit over the experimental period. Hence, the rate of receptor installation into the membrane must be equal or greater than the rate of constitutive internalization.

Time Course for Constitutive Internalization of FLAGDOR

We measured the rate of constitutive internalization by a variation on pulse chase experiments. Specifically, we labeled cells in culture with anti-FLAG antibody at the usual concentration at 0, 6, and 12-hour intervals before we applied the fluorescent secondary antibody. As anti-FLAG antibody bound to receptor internalized, it became unavailable for binding by the secondary antibody, and hence the rate of constitutive internalization could be measured as the rate at which FLAG signal diminished. We found that with no delay between application of primary and secondary antibody to label surface receptor, the average surface-to-total ratio was 0.23 (+/- 0.04). With 6 hours delay, the average surface-to-total ratio was 0.16 (+/- 0.03), while with 12 hours delay, the average surface-to-total ratio was 0.012 (+/- 0.006) (Figure 2). Hence, surface DOR is approximately halved after about 6 hours and is almost gone by 12 hours. Since we have verified that surface DOR is in the steady state, 12 hours or less should be sufficient after complete internalization by agonist to fully restore surface DOR to baseline levels if receptor kinetics are unchanged by agonist.

The Long-term Effect of Acute DOR Agonist Treatment

We applied 100 nM deltorphin for 30 minutes at 6, 12, 24, and 48 hour intervals before the culture end point (and our antibody staining protocol) at 72 hours in culture. At the same time as each drug treatment, we applied a media rinse only to another coverslip to use as a control for any effects the media change may have on FLAGDOR surface-to-total ratios. For cells receiving media change only at 6 hours, 12 hours, 24 hours, and 48 hours before the culture end point, the average surface-to-total ratios were 0.26 (+/- 0.04), 0.25 (+/- 0.06), 0.20 (+/- 0.03), and 0.21 (+/- 0.03), respectively. For cells receiving 30 minutes of 100 nM deltorphin treatment at 6 hours, 12 hours, 24 hours, and 48 hours before the end point, the average surface-to-total ratios were 0.021 (+/- 0.010), 0.003 (+/- 0.001), 0.020 (+/- 0.007), and 0.17 (+/- 0.04), respectively (Figure 3). Following deltorphin treatment, cell surface FLAGDOR remains quite low for up to 24 hours, with significant recovery as compared to control being measured only at 48 hours after deltorphin treatment. Since normal kinetics would yield complete recovery to baseline levels in 12 hours or less, we conclude that deltorphin treatment has a longer-lasting effect on receptor trafficking kinetics that keeps surface receptor levels low for 24 hours or so.

Discussion

It has been observed that DOR does not recycle back to the membrane following agonist-induced endocytosis, but rather tends to be sent to lysosomes and degraded (Ko et al,. 1999). This non-recycling nature of DOR has often been associated with the receptor's slow rate of recovery following agonist internalization. However, we have shown that cell surface DOR remains depressed for longer than would be expected even accounting for the time that would be required for complete recovery of receptor level based on normal kinetics. Thus, we have demonstrated that deltorphin treatment fundamentally changes the kinetics of DOR membrane trafficking to depress cell surface DOR for an extended period of time, roughly 24 hours. This property may result from a decrease in cell surface targeting of newly synthesized receptor, a reduced rate of recycling or installation events, or an increased rate of internalization and degradation. Additional experiments using drugs such as brefeldin A, which blocks vesicle traffic, concanavalin A, which blocks internalization, or the proteasome inhibitor ZLLL should help to elucidate the mechanistic details of this slowed DOR trafficking by indicating the relative contributions of installation, internalization, and recycling to normal and altered receptor kinetics.

Although agonist-induced endocytosis is the best understood mechanism of GPCR down-regulation, there are other reports that agonists alter the kinetics of receptor membrane trafficking. The down-regulation of MOR by agonist was studied in neuroblastoma (N2A) cells (Afify 2002). It was found that the MOR-selective agonist DAMGO produced an increased rate of MOR turnover caused by both increased receptor degradation and decreased receptor biosynthesis. More recently, two distinct modes of receptor insertion were actually observed by total internal reflection fluorescence microscopy for the β2 adrenergic receptor: a transient insertion mode that resulted in rapid lateral dispersion of receptors after insertion, and a more persistent insertion mode that retained inserted receptors in surface-accessible domains for a longer period of time (Yudowski et al., 2006). Treatment with the adenergic agonist isoproterenol resulted in a decrease in transient insertion events and increase in persistent insertion events. These insertion events were on the order of seconds and milliseconds, but it is reasonable to expect that the cumulative effect of one mode or another dominating over time could make a big difference in the amount of receptor installed.

Unlike the instances in these reports, however, the effect we observed lasted for up to 24 hours after drug was withdrawn. A longer-lasting change in receptor trafficking kinetics is a potential contributor to drug tolerance. Indeed, recent evidence using DOReGFP knockin mice strongly indicate that the efficacy of DOR agonists is blunted by internalization of the receptor by previous agonist exposure (Pradhan et al., 2009). In these experiments, pre-treatment of the mice with the highly internalizing agonist SNC80 caused significant internalization of DOR, while pre-treatment with a different agonist, AR-M100390, caused little internalization. The SNC-80-treated mice showed no analgesic response to either agonist 4 hours after pre-treatment, but agonist responsiveness was restored after 24 hours. By contrast, the mice treated with the non-internalizing agonist AR-M100390 retained full analgesic responsiveness to subsequent agonist administration at 4 hours. It is intriguing to compare the time frames of this effect and the one we observed. In both cases the effect lasted several hours, although in our case we still observed significantly reduced cell surface DOR compared to control at

24 hours after agonist treatment, while Pradhan et al. report complete recovery of agonist efficacy at 24 hours. However, the surface-to-total ratio we measured at 24 hours is higher than that at 12 hours, suggesting cell surface DOR had started to recover. It could be that only a small recovery of surface receptor level is necessary to restore agonist efficacy because of the spare receptor effect, the ability of only a small portion of total receptor to produce a full agonist effect. It would be very interesting to correlate restoration of agonist efficacy with recovery of cell surface DOR in SNC80 pre-treated DOReGFP knockin mice. However, since there is evidence that the GFP tag interferes with ordinary DOR subcellular targeting (Wang et al. 2008), it should be ascertained that these mice display similar DOR agonist response profiles to wild-type mice.

In summary, we have shown that down-regulation of cell surface DOR following agonist exposure results from not just direct internalization but also a change in receptor membrane trafficking kinetics. This change, which may be a component of acute tolerance, should be investigated further to determine the biological mechanisms responsible. Understanding these mechanisms may lead the way to new drugs or strategies to improve opioid drug therapy.

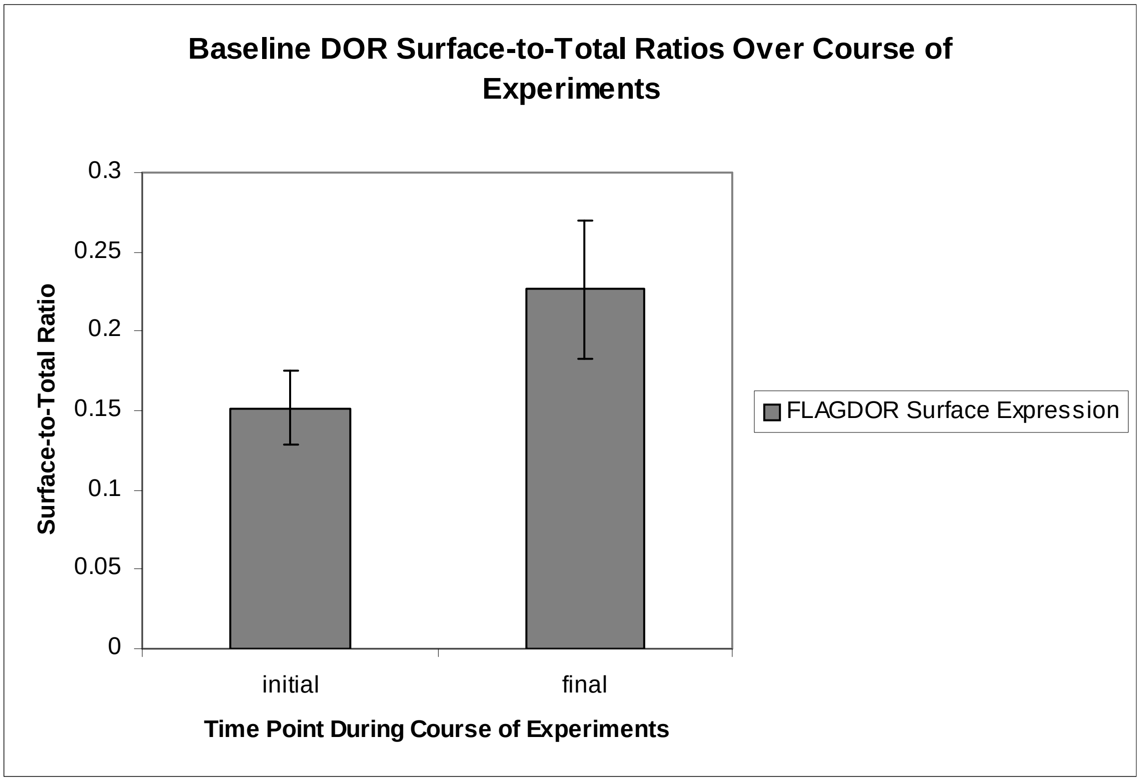

Figure 1. Baseline Surface-to-Total Ratios of FLAGDOR at the Starting Point and End Point of the Experimental Period in Culture. The average surface-to-total ratio at the starting point of the experimental period in culture (24 hours in culture after plating) was 0.15 (+/- 0.02), while that at the end point of the experimental period (72 hours in culture after plating) was 0.23 (+/- 0.04). Error bars represent +/- SEM for each group. Data is from three different experiments, p=0.134 by t-test.

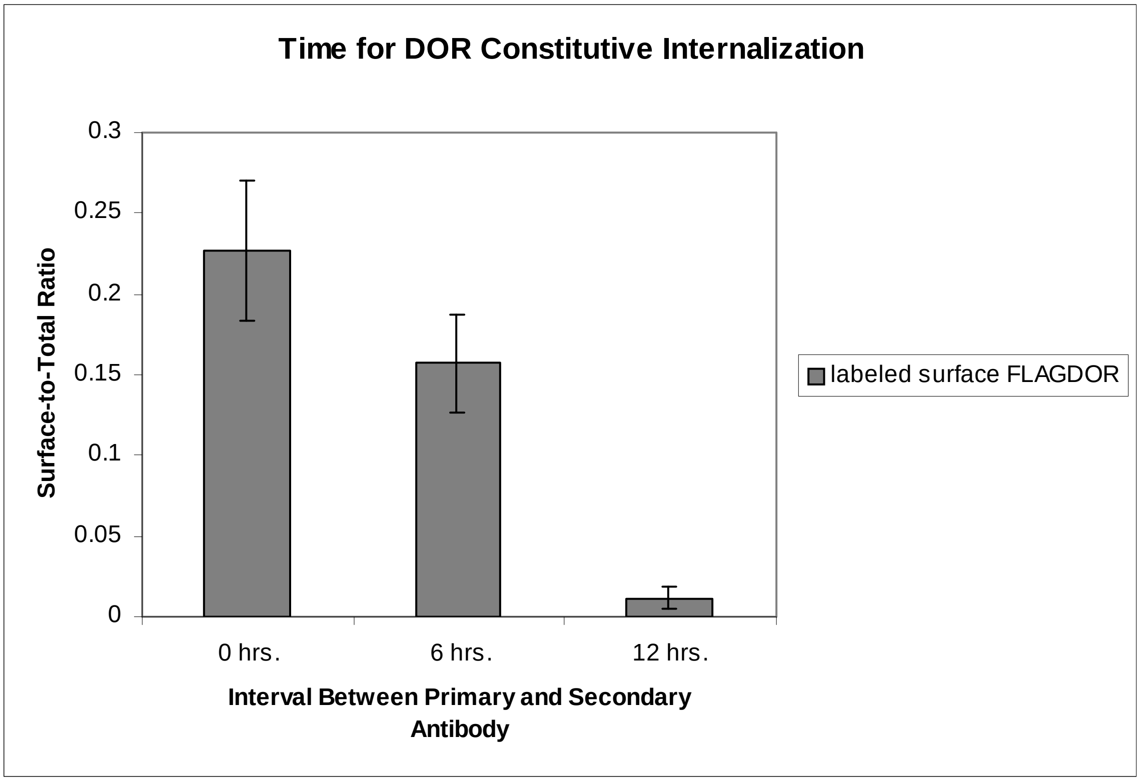

Figure 2. Time Course for Constitutive Internalization of FLAGDOR. With no delay between application of primary and secondary antibody to label surface receptor, the average surface-to-total ratio was 0.23 (+/- 0.04). With 6 hours delay, the average surface-to-total ratio was 0.16 (+/- 0.03), while with 12 hours delay, the average surface-to-total ratio was 0.012 (+/- 0.006). Error bars represent +/- SEM for each group. Data is from three different experiments.

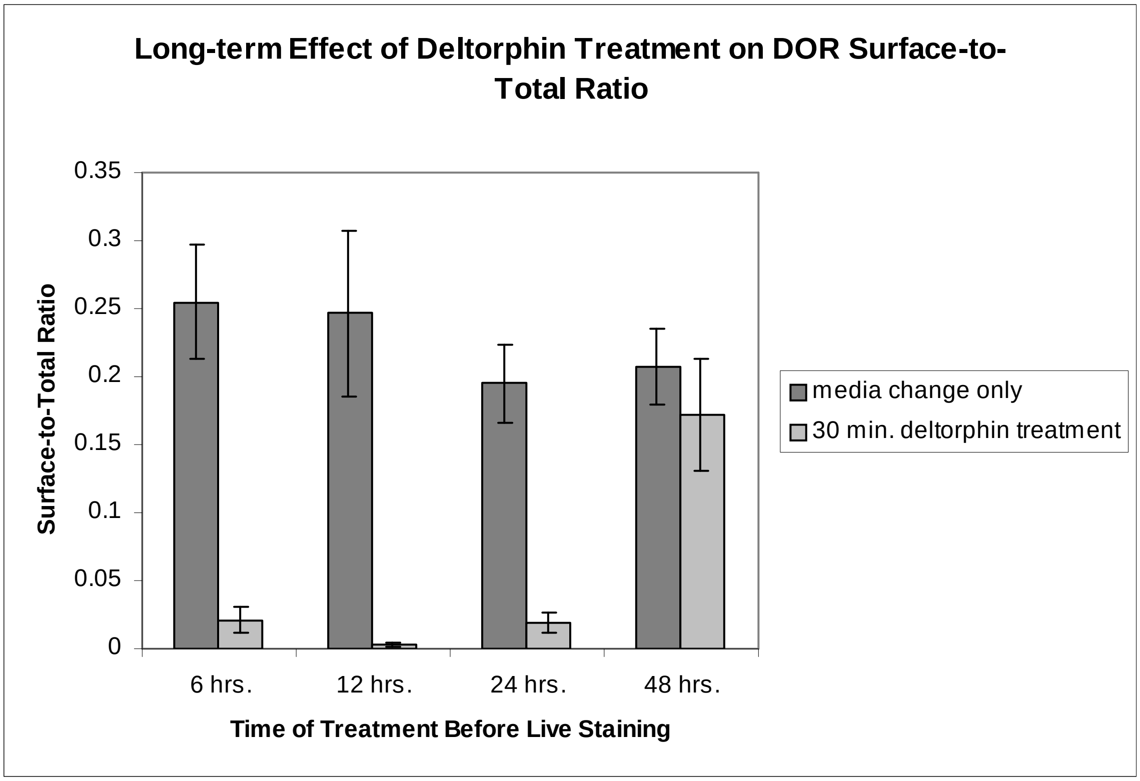

Figure 3. Long-term Effect of Deltorphin Treatment on FLAGDOR Surface-to-Total Ratio. For cells receiving media change only at 6 hours, 12 hours, 24 hours, and 48 hours before live staining, the average surface-to-total ratios were 0.26 (+/- 0.04), 0.25 (+/- 0.06), 0.20 (+/- 0.03), and 0.21 (+/- 0.03), respectively. For cells receiving 30 minutes of 100 nM deltorphin treatment at 6 hours, 12 hours, 24 hours, and 48 hours before live staining, the average surface-to-total ratios were 0.021 (+/- 0.010), 0.003 (+/- 0.001), 0.020 (+/- 0.007), and 0.17 (+/- 0.04), respectively. Error bars represent +/- SEM for each group. Data is from three different experiments.

Chapter 5

Relationship Between Cell Surface DOR and MOR and Agonist Effects in Co-Expressing PC12 Cells

Summary

Interactions between DOR and MOR have been described in the literature. Because our method enables quantification of surface receptor in individual cells, we sought to determine how short-term treatment (30 minutes) with agonist selective for one receptor would affect the other receptor's presence in the plasma membrane. The DOR-selective agonist deltorphin, in addition to internalizing DOR, caused a significant increase in surface MOR in co-expressing cells, an effect that was not seen with deltorphin treatment in cells expressing only MOR. This suggests that agonist activation of DOR results in installation of MOR into the plasma membrane. By contrast, the MOR-selective agonist DAMGO internalized MOR in co-expressing cells, but the increase in surface DOR measured did not reach statistical significance. This suggests that agonist binding to DOR can cause a rapid installation of MOR into the membrane. This is in contrast to MOR, for which chronic agonist treatment has been reported to cause installation of DOR into the plasma membrane, an effect that we did not observe to a significant extent with short-term MOR agonist treatment. This suggests the existence of different modes of agonist-induced receptor installation, one rapid and one slower. We also found a positive linear relationship between cell surface DOR and cell surface MOR in co-transfected cells. Deltorphin treatment increased this positive trend, while DAMGO treatment decreased it. This is further evidence for receptor interactions, suggesting that agonist treatment at one receptor can either induce installation of a related receptor or that interactions can modify the ability of an agonist to internalize receptor.

Introduction

A large body of evidence indicates that GPCRs generally, and MOR and DOR specifically, interact. The interactions may take the form of direct physical association such as the formation of heterodimers (Jordan and Devi, 1999), or the interaction may be more functional; for example, agonist binding at one receptor may influence the behavior of the other. The importance of these interactions is shown by the fact that tolerance to morphine's analgesic effect, which is mediated primarily by MOR (Kieffer, 1999), is abolished in DOR knockout mice (Zhu et al., 1999), clearly implicating DOR in morphine tolerance. Recent evidence indicates an essential role for MOR in DOR-mediated antihyperalgesia (Gendron et al., 2007). Thus, functional interactions between MOR and DOR occur both ways; each receptor can modify the other's effects.

It has been shown that activation of MOR by morphine or other ligands can induce installation of DOR into the plasma membrane of certain neurons (Cahill et al., 2001) (Morinville et al., 2003) (Chieng and Christie, 2009). Moreover, in dorsal root ganglia neurons, the population of cell surface DOR installed by stimulus-dependent release from an intracellular pool of LDCVs has been shown to be critical for the development of tolerance to morphine analgesia (Guan et al., 2005). On the basis of this evidence, it has been proposed that the development of tolerance is positively correlated with the ratio of DOR to MOR on the cell surface (Zhang et al., 2006).

We sought to use PC12 cells transiently co-transfected with DOR and MOR to explore the effects of agonist on the trafficking of functionally related receptors. On the basis of the literature describing interactions between DOR and MOR, we hypothesized that agonist binding at one receptor would lead to cell surface installation of the other receptor.

Methods

We used NGF-differentiated PC12 cells transiently transfected with DOR or MOR and our surface receptor quantification method (see Chapter 2), with certain modifications. Wild-type PC12 cells were plated and treated with NGF for two days. At this point, cells were transfected with FLAGDOR, HAMOR, or both, using Lipofectamine 2000. After twenty-four more hours in culture with NGF, polyclonal rabbit anti-FLAG antibody (1:2000, Sigma-Aldrich, St. Louis, MO) and/or monoclonal mouse HA.11 clone 16B12 (1:2000, Covance, Emeryville, CA) was applied to label surface receptor, Cy3 anti-rabbit and/or Cy2 anti-mouse fluorescent secondary antibodies (1:200, Jackson Immunoresearch, West Grove, PA) was applied to enable visualization of labeled surface receptor, cells were fixed, and antibodies to DOR or MOR were then used to label total cell receptor (in singly transfected cells only, since in doubly transfected cells, both fluorophores characterized for our quantification protocol, Cy2 and Cy3, were already used to label surface receptor). Labeling of surface receptor with primary and secondary antibodies and rinses in between were carried out at 37°C instead of 4°C as in other experiments, since we found that this was necessary to obtain robust HA staining. For this reason, exposure time to antibodies was twenty minutes instead of thirty minutes to minimize the effect of ongoing receptor trafficking at the warmer temperature, although we have found in the past that the temperature at which live staining is conducted makes little difference in surface receptor quantified. Cells were imaged with fluorescence microscopy and quantified using Image J.

Where appropriate, the MOR-selective agonist DAMGO (1 μM, Sigma-Aldrich) or the DOR-selective agonist deltorphin (100 nM, Sigma-Aldrich) were added to culture medium for thirty minutes , then removed just prior to rinsing and antibody labeling of surface receptor at the end of the culture period. We found in previous work that these doses were optimal to obtain significant internalization of receptor by its cognate ligand.

Results

Singly Transfected Cells As Controls

To verify that any agonist effects we observed were not the result of promiscuous binding of agonists, we quantified average surface FLAGDOR and HAMOR in PC12 cells singly transfected with these receptors in the presence and absence of agonist. As Figure 1 shows, in FLAGDOR-transfected cells the average surface FLAGDOR in the absence of agonist was 72 (+/- 10), after 30 minutes of 100 nM deltorphin was 14 (+/- 3), and after 30 minutes of 1 μM DAMGO was 56 (+/- 5). In HAMOR-transfected cells (Figure 2), the average surface HAMOR was 136 (+/- 21), after 30 minutes of 100 nM deltorphin was 133 (+/- 25), and after 30 minutes of 1 μM DAMGO was 73 (+/- 14). Hence, each agonist caused significant internalization of its cognate receptor but did not significantly affect the cell surface quantity of the other receptor.

Agonist Effects in Co-Transfected Cells

We quantified cell surface FLAGDOR and HAMOR in PC12 cells co-transfected with these receptors in the absence and presence of agonists. In the case of FLAGDOR, the average surface receptor in the absence of agonist was 154 (+/- 9), after 30 minutes of 100 nM deltorphin treatment was 78 (+/- 8), and after 30 minutes of 1 μM DAMGO was 170 (+/- 11). The increase in cell surface FLAGDOR in the presence of DAMGO did not reach statistical significance (p=0.251 by t-test).

In the case of HAMOR, the DOR-selective agonist deltorphin caused a significant increase in cell surface HAMOR in co-expressing cells, unlike in cells expressing only HAMOR. The average surface receptor in the absence of agonist was 229 (+/- 14), after 30 minutes of 100 nM deltorphin treatment was 394 (+/- 31), and after 30 minutes of 1 μM DAMGO was 189 (+/- 19). The increase in cell surface HAMOR with deltorphin treatment was statistically significant ($p<0.00001$ by t-test).

Relationship between Cell Surface MOR and DOR

To visualize the mathematical relationship between cell surface DOR and MOR, we made scatter plots of co-transfected cells with surface FLAGDOR on the x-axis and surface HAMOR on the y-axis, in the presence and absence of agonist (Figures 5,6, and 7). In all cases the trend is that as cell surface DOR increases, cell surface MOR increases too, as reflected by the positive slope of the trendlines obtained by linear regression. The R^2 values for these trendlines are not very high, indicating significant scatter. Deltorphin treatment increases the slope of the trendline, while DAMGO treatment decreases the slope.

Discussion

The literature provides many reports of interactions between DOR and MOR. This concept has been challenged recently by Scherrer et al. (2009), who found that the receptors occur almost exclusively in different primary afferent fibers in DOReGFP knockin mice. The subcellular targeting properties of the DOR in these mice is open to question, since it has been found that large fluorescent protein tags such as GFP can interfere with the normal cellular targeting of DOR (Wang et al., 2008). So although many studies of receptor interactions have involved highly artificial conditions such as cell lines expressing abundant quantities of transfected receptor, DOReGFP knockin mice are highly questionable as an approach for avoiding these artifactual effects. Moreover, *in vivo* evidence for interactions is shown by the finding that morphine tolerance is abolished in DOR knockout mice (Zhu et al., 1999), although Scherrer et al. could not replicate that finding. We found additional evidence for interactions, although with some surprises.

First, we verified that agonist action at one receptor can cause additional targeting and installation of the other receptor to the plasma membrane. It is well documented that morphine or other MOR agonists binding to MOR can induce DOR insertion (Cahill et al., 2001) (Morinville et al.,2003) (Chieng and Christie, 2009). We are not aware of reports that binding to DOR of a DOR agonist can induce plasma membrane insertion of MOR.

However, the latter is exactly what we found. In cells co-transfected with FLAGDOR and HAMOR, treatment with the DOR agonist deltorphin caused a significant increase in surface HAMOR, an effect that was not found in cells transfected with HAMOR alone and treated with deltorphin. This raises the question of whether this effect is an artifact of our experimental system, PC12 cells transiently transfected to produce copious quantities of receptor, or whether up-regulation of MOR by agonist action at DOR is a real phenomenon in vivo with physiological relevance. The places to look to answer this question include cells such as dorsal root ganglia neurons, dorsal horn neurons, and periaqueductal grey neurons, where DOR is known to be elevated under certain conditions (Zhang et al., 2006). Since DOR has been shown to be essential for development of tolerance to morphine (Zhu et al., 1999), which works primarily at MOR, it would be odd if agonist activation of DOR led to insertion of additional MOR. However, interactions between DOR and MOR could be more complex than is currently understood, and it is plausible that in certain cell types DOR activation does lead to MOR up-regulation.

Although agonist binding to MOR has been shown to cause plasma membrane insertion of DOR in certain cell types (Cahill et al., 2001) (Morinville et al.,2003) (Chieng and Christie, 2009), we did not measure a statistically significant increase in surface FLAGDOR upon DAMGO treatment in co-transfected cells. However, Morinville et al. reported that morphine treatment of 48 hours was necessary to enhance DOR antinociception in the dorsal horn of the spinal cord. The work by Chieng and Christie similarly reported that chronic morphine treatment was necessary to induce DOR insertion in periaqueductal grey neurons. So a likely explanation for our result seems to be that the time of our DAMGO treatment, 30 minutes, was insufficient to elicit a measurable insertion of DOR. This is interesting given the obviously faster insertion of HAMOR that we found upon 30 minutes of deltorphin treatment. Also, DOR can be inserted into the plasma membrane rapidly following some types of stimuli, such as 30 minutes of potassium chloride depolarization of NGF-treated PC12 cells (Kim and von Zastrow, 2003), and in large quantities in proportion to the total receptor in the cell (Chapter 3). Evidently, there are different modes of agonist-induced receptor insertion, and the mechanisms involved should be investigated further.

We also examined the mathematical relationship between cell surface DOR and cell surface MOR in co-transfected cells, with and without agonist treatment. We did this by making scatter plots of surface DOR vs. surface MOR, with DOR on the x-axis and MOR on the y-axis. Linear regression revealed a positive relationship between surface DOR and surface MOR; as DOR increases, so does MOR, although the correlation as indicated by R^2 values is not very strong. While this relationship is consistent with receptor interactions such as heterodimers or a trafficking relationship, it may merely reflect that cellular trafficking machinery tends to install similar amounts of DOR and MOR on the membrane, since the receptors are structurally similar. However, evidence for interactions is provided by the fact that agonist treatment changed the relationship between DOR and MOR. Deltorphin treatment increased the slope of the trendline, while DAMGO treatment decreased it. This means that the quantity of surface MOR became more sensitive to the quantity of surface DOR with deltorphin treatment, and less sensitive with DAMGO treatment. The former may be because deltorphin treatment induced installation of MOR, as previously discussed, and cells with more DOR for deltorphin binding accounted for the bulk of the overall increase in surface MOR. Alternatively, it may be that physical interaction with MOR stabilized DOR against internalization by deltorphin, so that cells with more DOR tended to be the ones with more MOR. Consistent with this interpretation, it has been found that expression of MOR is necessary for DOR-mediated anti-hyperalgesia by DOR agonists, and it was suggested by the investigators that MOR may stabilize DOR while the latter interacts with agonist (Gendron et al., 2007). By the same token, the decrease in the positive trend between surface DOR and surface MOR with DAMGO treatment may mean that interaction with DOR makes MOR more susceptible to agonist-induced internalization. Receptor interactions could stabilize against internalization in some cases but enhance internalization in others.

In summary, we have found evidence for a rapid insertion of MOR into the plasma membrane following agonist activation of DOR, a novel finding that should be investigated further. We have also found evidence consistent with a role for interactions between MOR and DOR in mediating agonist response at these receptors.

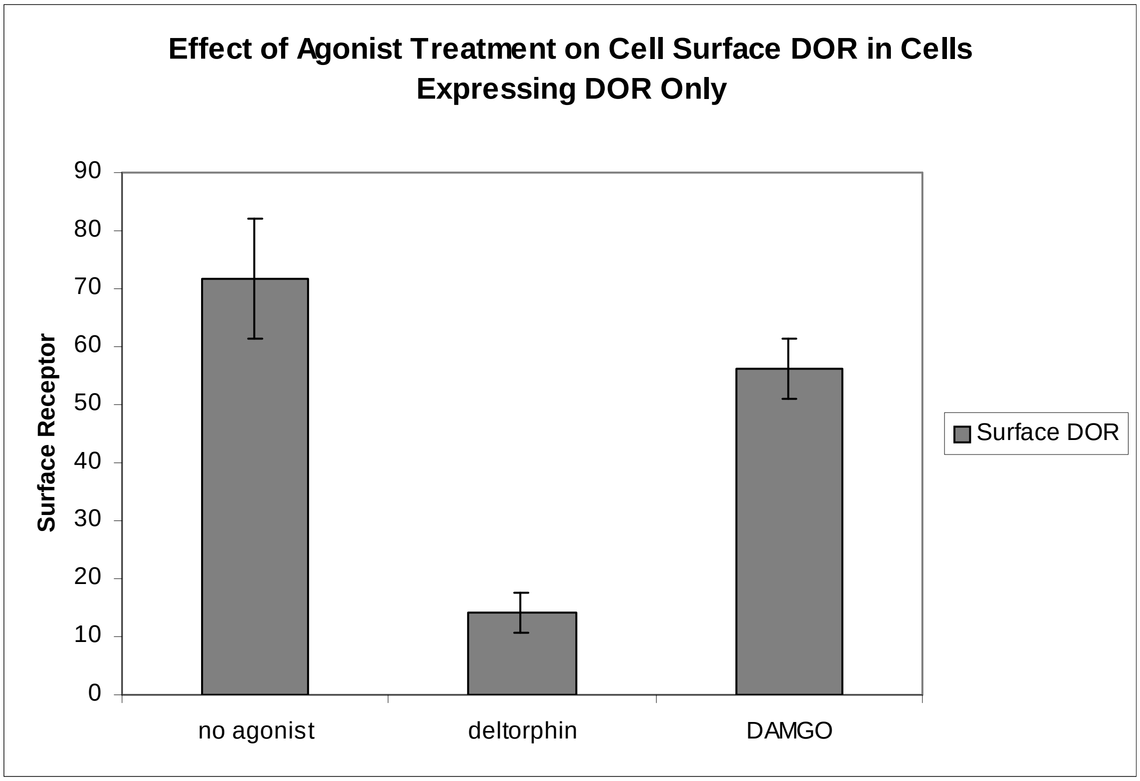

Figure 1. Effect of Agonist Treatment on Cell Surface FLAGDOR in Cells Expressing FLAGDOR Only. The average surface receptor in the absence of agonist was 72 (+/- 10), after 30 minutes of 100 nM deltorphin was 14 (+/- 3), and after 30 minutes of 1 μM DAMGO was 56 (+/- 5). Error bars represent +/- SEM for each group. Data is from three different experiments.

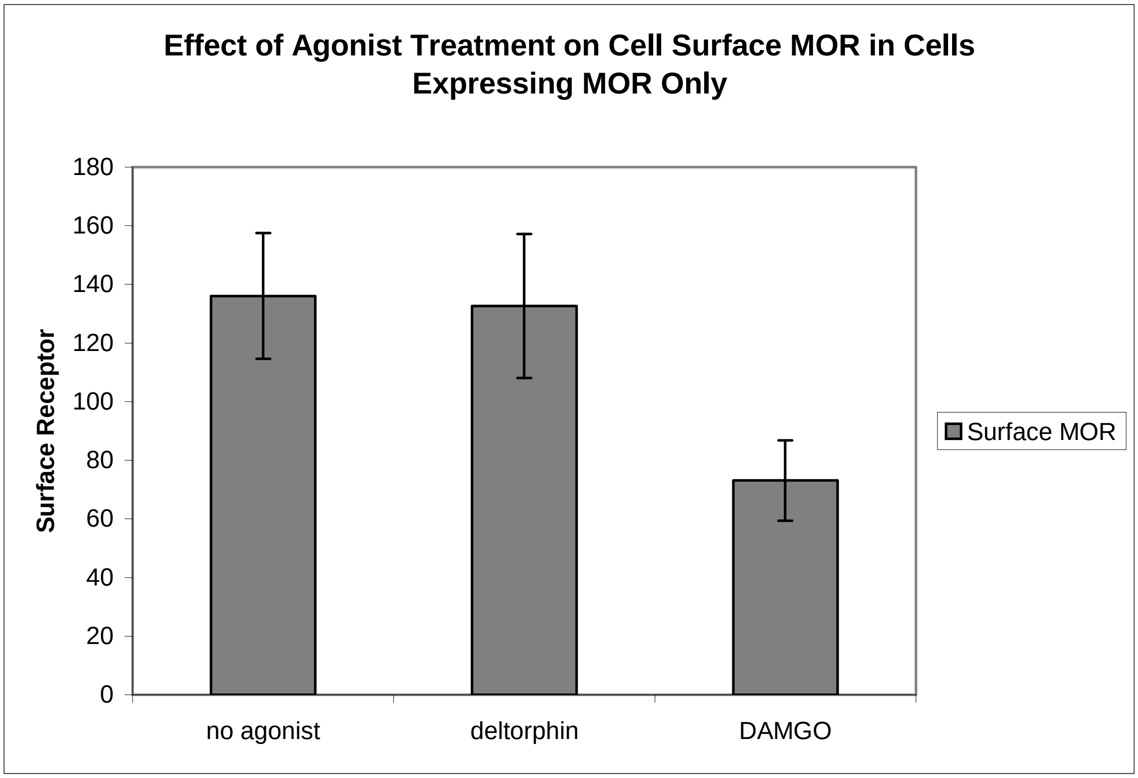

Figure 2. Effect of Agonist Treatment on Cell Surface HAMOR in Cells Expressing HAMOR Only. The average surface receptor in the absence of agonist was 136 (+/- 21), after 30 minutes of 100 nM deltorphin was 133 (+/- 25), and after 30 minutes of 1 μM DAMGO was 73 (+/- 14). Error bars represent +/- SEM for each group. Data is from three different experiments.

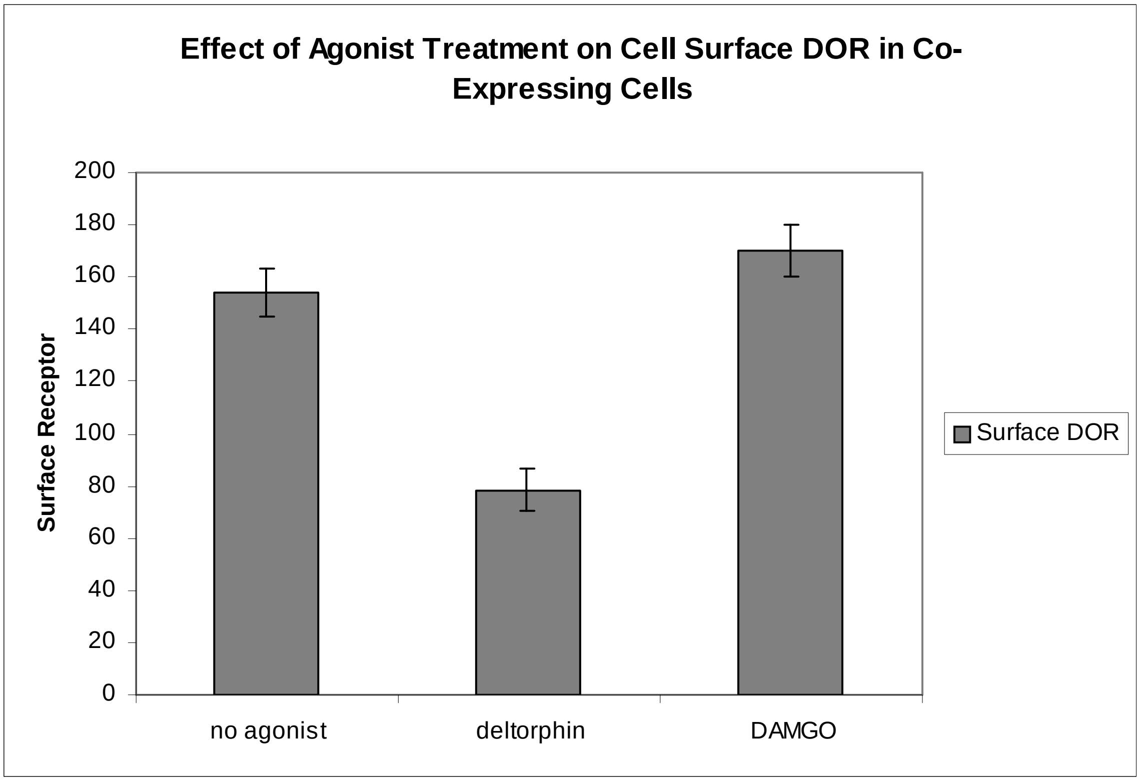

Figure 3. Effect of Agonist Treatment on Cell Surface FLAGDOR in Co-Expressing Cells. The average surface receptor in the absence of agonist was 154 (+/- 9), after 30 minutes of 100 nM deltorphin treatment was 78 (+/- 8), and after 30 minutes of 1 μM DAMGO was 170 (+/- 11). Error bars represent +/- SEM for each group. Data is from three different experiments.

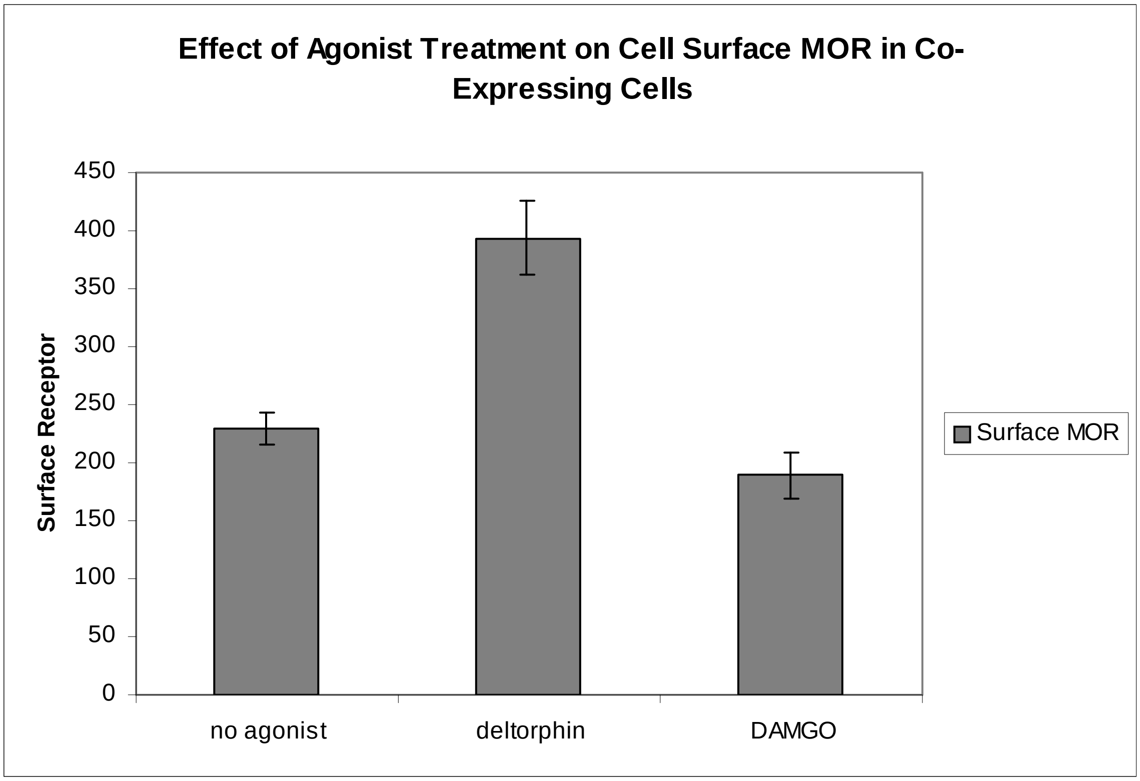

Figure 4. Effect of Agonist Treatment on Cell Surface HAMOR in Co-Expressing Cells. The average surface receptor in the absence of agonist was 229 (+/- 14), after 30 minutes of 100 nM deltorphin treatment was 394 (+/- 31), and after 30 minutes of 1 μM DAMGO was 189 (+/- 19). Error bars represent +/- SEM for each group. Data is from three different experiments.

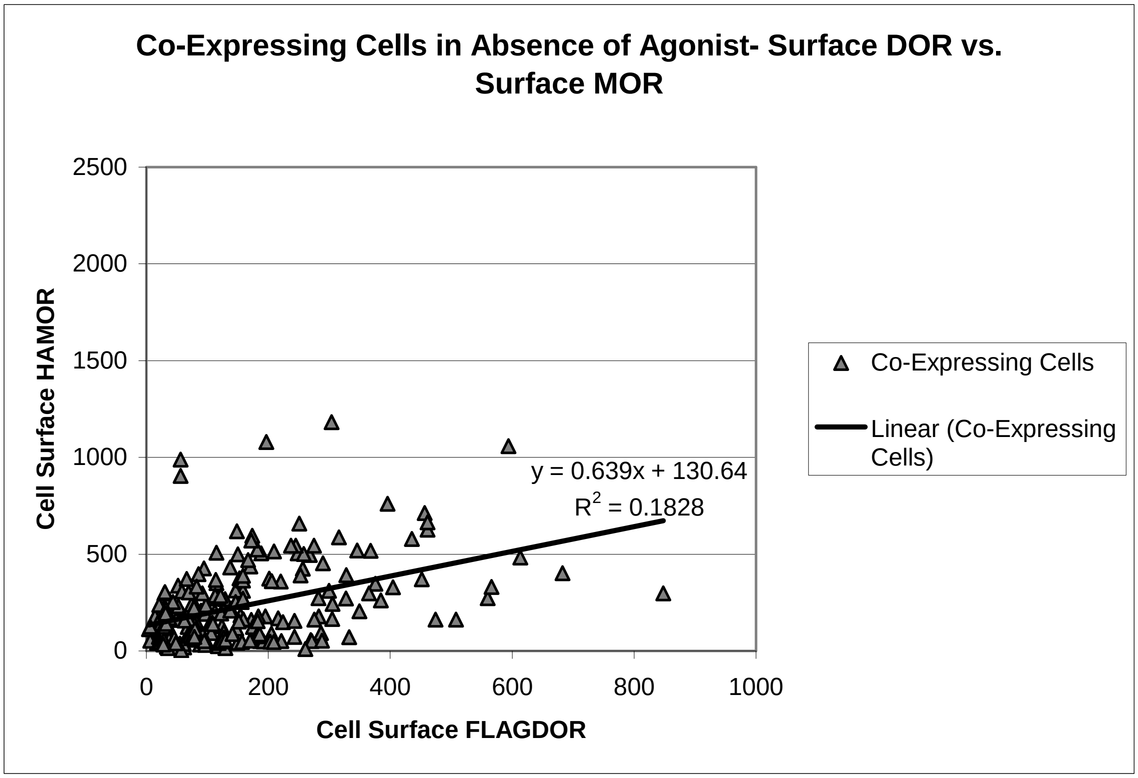

Figure 5. Relationship between Cell Surface FLAGDOR and HAMOR in Co-Expressing Cells in Absence of Agonist. Scatter plot showing cell surface FLAGDOR and HAMOR for each co-expressing cell. Data is from three different experiments.

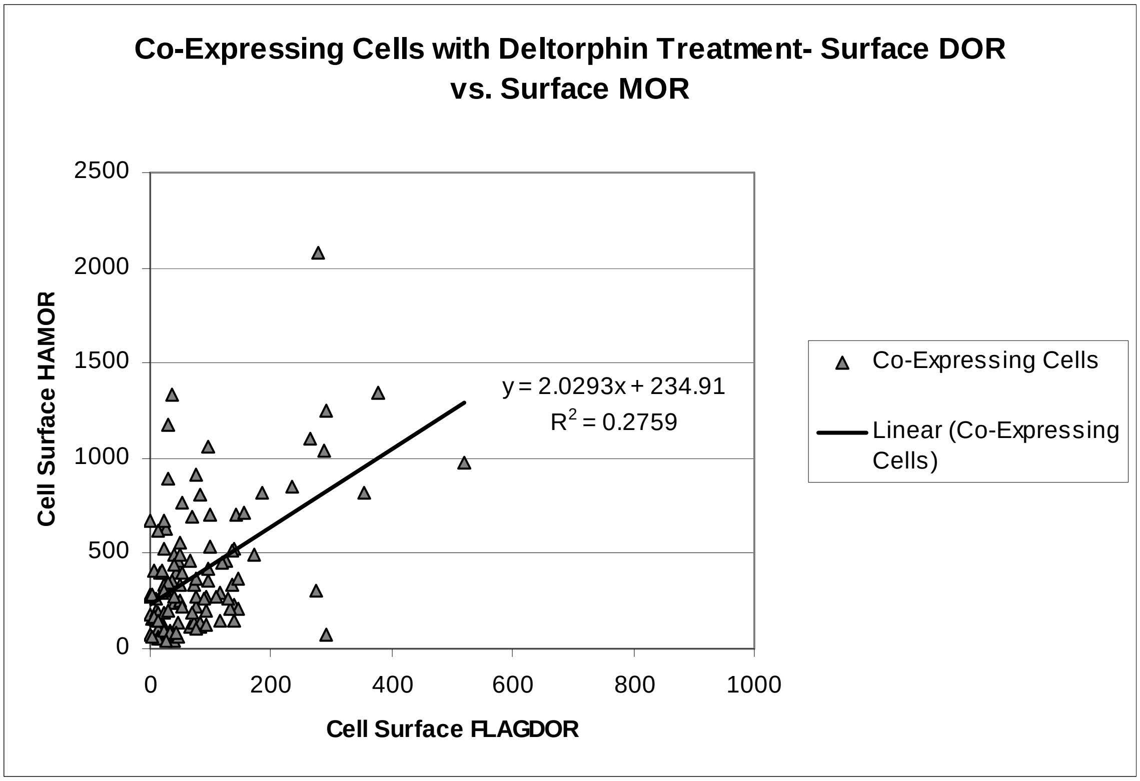

Figure 6. Relationship between Cell Surface FLAGDOR and HAMOR in Co-Expressing Cells with 30 Minutes of 100 nM Deltorphin Treatment. Scatter plot showing cell surface FLAGDOR and HAMOR for each co-expressing cell. Data is from three different experiments.

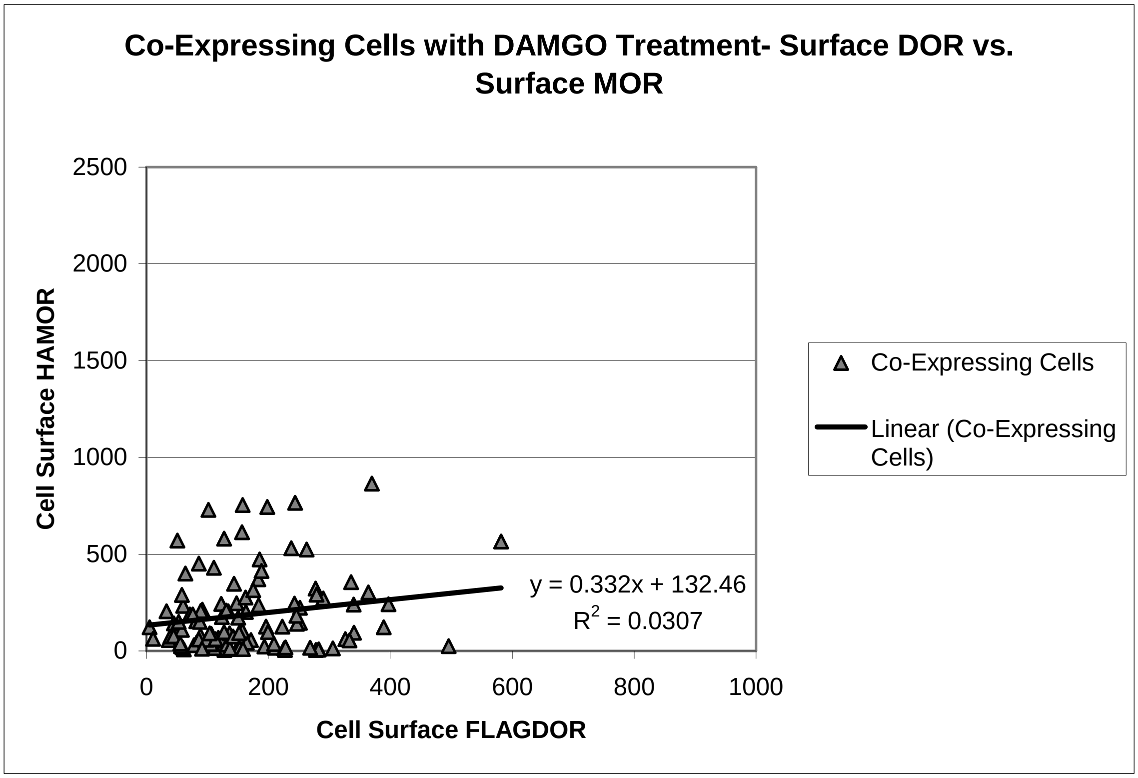

Figure 7. Relationship between Cell Surface FLAGDOR and HAMOR in Co-Expressing Cells with 30 Minutes of 1 μM DAMGO Treatment. Scatter plot showing cell surface FLAGDOR and HAMOR for each co-expressing cell. Data is from three different experiments.

References

Afify EA (2002) Turnover of mu-opioid receptors in neuroblastoma cells. Brain Res Mol Brain Res 106:83-87.

Afify EA, Law PY, Riedl M, Elde R, Loh HH (1998) Role of carboxyl terminus of mu- and delta-opioid receptor in agonist-induced down-regulation. Brain Res Mol Brain Res 54:24-34.

Arvidsson U, Dado RJ, Riedl M, Lee JH, Law PY, Loh HH, Elde R, Wessendorf MW (1995) Delta (δ)-opioid receptor immunoreactivity: distribution in brain stem and spinal cord and relationship to biogenic amines and enkephalin. J Neurosci 15:1215-1235.

Arvidsson U, Riedl M, Chakrabarti S, Lee JH, Nakano A, Dado R, Loh HH, Law PY, Wessendorf MW, Elde R (1995) Distribution and targeting of a μ-opioid receptor (MOR1) in brain and spinal cord. J Neurosci 15:3328-3341.

Arvidsson U, Riedl M, Chakrabarti S, Vulchanova L, Lee JH, Nakano A, Lin X, Loh HH, Law PY, Wessendorf MW, Elde R (1995) The κ-opioid receptor (KOR1) is primarily postsynaptic: combined immunohistochemical localization of the receptor and endogenous opioids. Proc Natl Acad Sci USA 92:5062-5066.

Bao L, Jin SX, Zhang C, Wang LH, Xu ZZ, Zhang FX, Wang LC, Ning FS, Cai HJ, Guan JS, Xiao HS, Xu ZQ, He C, Hokfelt T, Zhou Z, Zhang X (2003) Activation of delta opioid receptors induces receptor insertion and neuropeptide secretion. Neuron 37:121-133.

Bohn LM, Gainetdinov RR, Lin FT, Lefkowitz RJ, Caron MG (2000) μ-Opioid receptor desensitization by β-arrestin-2 determines morphine tolerance but not dependence. Nature 408:720-723.

Browning KN, Kalyuzhny AE, Travagli RA (2004) Mu-opioid receptor trafficking on inhibitory synapses in the rat brainstem. J Neurosci 24:7344-7352.

Cahill CM, Morinville A, Lee MC, Vincent JP, Collier B, Beaudet A (2001) Prolonged morphine treatment targets delta opioid receptors to neuronal plasma membranes and enhances delta-mediated antinociception. J Neurosci 21:7598-7607.

Chen Y, Mestek A, Liu J, Hurley JA, Yu L (1993) Molecular cloning and functional expression of a μ-opioid receptor from rat brain. Mol Pharmacol 44:8-12.

Cheng PY, Liu-Chen LY, Chen C, Pickel VM (1996) Immunolabeling of mu opioid receptors in the rat nucleus of the solitary tract: extrasynaptic plasmalemmal localization and association with Leu5-enkephalin. J Comp Neurol 371:522-536.

Cheng PY, Liu-Chen LY, Pickel VM (1997) Dual ultrastructural immunocytochemical labeling of mu and delta opioid receptors in the superficial layers of the rat cervical spinal cord. Brain Res 778:367-380.

Cheng PY, Svingos AL, Wang H, Clarke CL, Jenab S, Beczkowska IW, Inturrisi CE, Pickel VM (1995) Ultrastructural immunolabeling shows prominent presynaptic vesicular localization of delta-opioid receptor within both enkephalin- and nonenkephalin-containing axon terminals in the superficial layers of the rat cervical spinal cord. J Neurosci 15:5976-5988.

Chieng B, Christie MJ (2009) Chronic morphine treatment induces functional delta-opioid receptors in amygdala neurons that project to periaqueductal grey. Neuropharm 57:430-437.

Christie MJ (2008) Cellular neuroadaptations to chronic opioids: tolerance, withdrawal, and addiction. Br J Pharmacol 154:384-396.

Commons KG (2003) Translocation of presynaptic delta opioid receptors in the ventrolateral periaqueductal gray after swim stress. J Comp Neurol 464:197-207.

Commons KG, van Bockstaele EJ, Pfaff DW (1999) Frequent localization of mu opioid and NMDA-type glutamate receptors at postsynaptic sites in periaqueductal gray neurons. J Comp Neurol 408:549-559.

Dado RJ, Law PY, Loh HH, Elde R (1993) Immunofluorescent identification of a delta (δ)-opioid receptor on primary afferent nerve terminals. Neuroreport 5:341-344.

De Camilli P, Jahn R (1990) Pathways to regulated exocytosis in neurons. Annu Rev Physiol 52:625-645.

Drake CT, Patterson TA, Simmons ML, Chavkin C, Milner TA (1996) Kappa opioid receptor-like immunoreactivity in guinea pig brain: ultrastructural localization in presynaptic terminals in hippocampal formation. J Comp Neurol 370:377-395.

Duvernay MT, Zhou F, Wu G (2004) A conserved motif for the transport of G protein-coupled receptors from the endoplasmic reticulum to the cell surface. J Biol Chem 279:30741-30750.

Elde R, Arvidsson U, Riedl M, Vulchanova L, Lee JH, Dado R, Nakano A, Chakrabarti S, Zhang X, Loh HH, Law PY, Hokfelt T, Wessendorf M (1995) Distribution of neuropeptide receptors: new views of peptidergic neurotransmission made possible by antibodies to opioid receptors. Annals of the New York Academy of Sciences 757:390-404.

Evans CJ, Keith DE, Morrison H, Magendzo K, Edwards RH (1992) Cloning of a delta opioid receptor by functional expression. Science 258:1952-1955.

Gendron L, Pintar JE, Chavkin C (2007) Essential role of mu opioid receptor in the regulation of delta opioid receptor-mediated antihyperalgesia. Neurosci 150:807-817.

Greene LA, Tischler AS (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc Natl Acad Sci USA 73:2424-2428.

Guan JS, Xu ZZ, Gao H, He SQ, Ma GQ, Sun T, Wang LH, Zhang ZN, Lena I, Kitchen I, Elde R, Zimmer A, He C, Pei G, Bao L, Zhang X (2005) Interaction with vesicle luminal protachykinin regulates surface expression of δ-opioid receptors and opioid analgesia. Cell 122:619-631.

Gutstein HB, Akil H (2001) Opioid analgesics. *In* Goodman & Gilman's: The Pharmacological Basis of Therapeutics, Tenth Edition (2001). Editors: Joel G. Hardman & Lee E. Limbird. 569-619 pp.

Hanyaloglu AC, von Zastrow M (2007) Regulation of GPCRs by membrane trafficking and its potential implications. Annu Rev Pharmacol Toxicol 48:19.1-19.32.

Jordan BA, Devi LA (1999) G-protein-coupled receptor heterodimerization modulates receptor function. Nature 399:697-700.

Jung LJ, Scheller RH (1991) Peptide processing and targeting in the neuronal secretory pathway. Science 251:1330-1335.

Kieffer BL (1999) Opioids: first lessons from knockout mice. Trends in Pharmacol Sci 20:19-26.

Kieffer BL, Befort K, Gaveriaux-Ruff C, Hirth CG (1992) The delta-opioid receptor:

isolation of a cDNA by expression cloning and pharmacological characterization. Proc Natl Acad Sci USA 89:12048-12052.

Kim K, von Zastrow M (2003) Neurotrophin-regulated sorting of opioid receptors in the biosynthetic pathway of neurosecretory cells. J Neurosci 23:2075-2085.

Ko JL, Arvidsson U, Williams FG, Law PY, Elde R, Loh HH (1999) Visualization of time-dependent redistribution of delta-opioid receptors in neuronal cells during prolonged agonist exposure. Brain Res Mol Brain Res 69:171-185.

Kong H, Raynor K, Yano H, Takeda J, Bell GI, Reisine T (1994) Agonists and antagonists bind to different domains of the cloned κ opioid receptor. Proc Natl Acad Sci USA 91:8042-8046.

Meng F, Xie GX, Thompson RC, Mansour A, Goldstein A, Watson SJ, Akil H (1993) Cloning and pharmacological characterization of a rat kappa opioid receptor. Proc Natl Acad Sci USA 90:9954-9958.

Minami M, Toya T, Katao Y, Maekawa K, Nakamura S, Onogi T, Kaneko S, Satoh M (1993) Cloning and expression of cDNA for the rat κ-opioid receptor. FEBS Lett 329:291-295.

Morinville A, Cahill CM, Esdaile MJ, Aibak H, Collier B, Kieffer BL, Beaudet A (2003) Regulation of delta-opioid receptor trafficking via mu-opioid receptor stimulation: evidence from mu-opioid receptor knock-out mice. J Neurosci 23:4888-4898.

Nomura S, Ding YQ, Kaneko T, Li JL, Mizuno N (1996) Localization of mu-opioid receptor-like immunoreactivity in the central components of the vagus nerve: a light and electron microscope study in the rat. Neuroscience 73:277-286.

Ohuchi T, Maruoka S, Sakudo A, Arai T (2002) Assay-based quantitative analysis of

PC12 cell differentiation. J Neurosci Methods 118:1-8.

Pickel VM, Chan J, Kash TL, Rodriguez JJ, MacKie K (2004) Compartment-specific localization of cannabinoid 1 (CB1) and mu-opioid receptors in rat nucleus accumbens. Neuroscience 127:101-112.

Pradhan AAA, Becker JAJ, Scherrer G, Tryoen-Toth P, Filliol D, Matifas A, Massotte D, Gaveriaux-Ruff C, Kieffer BL (2009) *In vivo* delta opioid receptor internalization controls behavioral effects of agonists. PLoS ONE 4:1-11.

Scherrer G, Imamachi N, Cao YQ, Contet C, Mennicken F, O'Donnell D, Kieffer BL, Basbaum AI (2009) Dissociation of the opioid receptor mechanisms that control mechanical and heat pain. Cell 137:1148-1159.

Scherrer G, Tryoen-Toth P, Filliol D, Matifas A, Laustriat D, Cao YQ, Basbaum AI, Dierich A, Vonesh JL, Gaveriaux-Ruff C, Kieffer BL (2006) Knockin mice expressing fluorescent δ-opioid receptors uncover G protein-coupled receptor dynamics *in vivo.* Proc Natl Acad Sci USA 103:9691-9696.

Schimmelpfeng J, Weibezahn KF, Dertinger H (2004) Quantification of NGF-dependent neuronal differentiation of PC-12 cells by means of neurofilament-L mRNA expression and neuronal outgrowth. J Neurosci Methods 139:299-306.

Shi SH, Hayashi Y, Petralia R, Zaman S, Wenthold R, Svoboda K, Malinow R (1999) Rapid spine delivery and redistribution of AMPA receptors after synaptic NMDA receptor activation. Science 284:1811-1816.

Shuster SJ, Riedl M, Li X, Vulchanova L, Elde R (1999) Stimulus-dependent translocation of κ opioid receptors to the plasma membrane. J Neurosci 19:2658-2664.

Skeberdis VA, Lan JY, Zheng X, Zukin RS, Bennett MVL (2001) Insulin promotes rapid delivery of N-methyl-D-aspartate receptors to the cell surface by exocytosis. Proc Natl Acad Sci USA 98:3561-3566.

Svingos AL, Colago EE, Pickel VM (1999) Cellular sites for dynorphin activation of kappa-opioid receptors in the rat nucleus accumbens shell. J Neurosci 19:1804-1813.

Svingos AL, Moriwaki A, Wang JB, Uhl GR, Pickel VM (1996) Ultrastructural immunocytochemical localization of mu-opioid receptors in rat nucleus accumbens-extrasynaptic plasmalemmal distribution and association with leu(5)-enkephalin. J Neurosci 16:4162-4173.

Tan CM, Brady AE, Nickols HH, Wang Q, Limbird LE (2004) Membrane trafficking of G protein-coupled receptors. 44:559-609.

Thompson RC, Mansour A, Akil H, Watson SJ (1993) Cloning and pharmacological characterization of a rat mu opioid receptor. Neuron 11:903-913.

Turchan J, Przewlocka B, Toth G, Lason W, Borsodi A, Przewlocki R (1999) The effect of repeated administration of morphine, cocaine and ethanol on mu and delta opioid receptor density in the nucleus accumbens and striatum of the rat. Neurosci 91:971-977.

van Bockstaele EJ, Commons K, Pickel VM (1997) Delta-opioid receptor is present in presynaptic axon terminals in the rat nucleus locus coeruleus: relationships with methionine5-enkephalin. J Comp Neurol 388:575-586.

von Zastrow M (2003) Mechanisms regulating membrane trafficking of G protein-coupled receptors in the endocytic pathway. Life Sci 74:217-224.

von Zastrow M, Svingos A, Haberstock-Debic H, Evans C (2003) Regulated endocytosis of opioid receptors: cellular mechanisms and proposed roles in physiological adaptation to opiate drugs. Current Opinion in Neurobiology 13:348-353.

Wang HB, Guan JS, Bao L, Zhang X (2008) Distinct subcellular distribution of delta-opioid receptor fused with various tags in PC12 cells. Neurochem Res 33:2028-2034.

Wang W, Loh HH, Law PY (2003) The intracellular trafficking of opioid receptors directed by carboxyl tail and a di-leucine motif in Neuro2A cells. J Biol Chem 278:36848-36858.

Wang QP, Ochiai H, Guan JL, Nakai Y (1998) Ultrastructural localization of mu-1 opioid receptor in the dorsal raphe nucleus of the rat. Synapse 29:240-247.

Wang H, Pickel VM (2001) Preferential cytoplasmic localization of delta-opioid receptors in rat striatal patches: comparison with plasmalemmal mu-opioid receptors. J Neurosci 21:3242-3250.

Wang JB, Imai Y, Eppler CM, Gregor P, Spivak CE, Uhl GR (1993) Mu opiate receptor: cDNA cloning and expression. Proc Natl Acad Sci USA 90:10230-10234.

Wessendorf MW, Elde RP (1985) Characterization of an immunofluorescence technique for the demonstration of coexisting neurotransmitters within nerve fibers and terminals. J Histochem Cytochem 33:984-994.

Yasuda K, Raynor K, Kong H, Breder CD, Takeda J, Reisine T, Bell GI (1993) Cloning and functional comparison of κ and δ opioid receptors from mouse brain. Proc Natl Acad Sci USA 90:6736-6740.

Yudowski GA, Puthenveedu MA, von Zastrow M (2006) Distinct modes of regulated receptor insertion to the somatodendritic plasma membrane. Nature Neurosci 9:622-627.

Zhang X, Bao L, Guan JS (2006) Role of delivery and trafficking of δ-opioid peptide receptors in opioid analgesia and tolerance. Trends in Pharmacol Sci 27:324-329.

Zhang X, Bao L, Arvidsson U, Elde R, Hokfelt T (1998) Localization and regulation of the delta-opioid receptor in dorsal root ganglia and spinal cord of the rat and monkey: evidence for association with the membrane of large dense-core vesicles. Neuroscience 82:1225-1242.

Zhao BG, Chapman C, Bicknell RJ (1988) Functional κ-opioid receptors on oxytocin and vasopressin nerve terminals isolated from the rat neurohypophysis. Brain Res 462:62-66.

Zhu Y, King MA, Schuller AG, Nitsche JF, Riedl M, Elde RP, Unterwald E, Pasternak GW, Pintar JE (1999) Retention of supraspinal delta-like analgesia and loss of morphine tolerance in delta opioid receptor knockout mice. Neuron 24:243-252.