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Regulation of Mu Opioid Receptor Trafficking, Signaling, and Recycling in Neurons

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Regulation of Mu Opioid Receptor Trafficking, Signaling, and Recycling in Neurons

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Y. Joy Yu provided the experimentation and manuscript preparation of the greater majority of the following dissertation, which constitutes a substantial and comparable contribution to that of other dissertations in the greater Molecular and Cellular Neurobiology fields. Collaborative contributions from others are acknowledged on the title pages of the relevant chapters.

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- Y. Joy Yu

Regulation of Mu Opioid Receptor Trafficking, Signaling and Recycling

ABSTRACT

Mu opioid receptors (MOR) expressed in the central nervous system mediate a wide range of physiological effects ranging from analgesia to addiction. Much remains unknown as to how these receptors are regulated by drugs at the neuronal level. This was addressed using both epitope-tagged and endogenous MORs expressed in multiple neuronal populations. In contrast to observations of MOR trafficking in heterologous cell lines, morphine drove robust and rapid internalization of MORs in medium spiny neurons of the striatum. Both trafficking and signaling of MORs can be significantly altered by co-activation of another G-protein coupled receptor (GPCR), the neurokinin1 receptor (NK1R). Co-activation of NK1Rs expressed on the same neuron significantly diminished the extent to which opioid drugs were able to drive MOR endocytosis. Both mutational disruption of high affinity βarrestin binding to NK1Rs and overexpression of βarrestin prevented this cell autonomous and non-reciprocal inhibition of MOR endocytosis, suggesting that competition for cytoplasmic βarrestins plays a major role in NK1R-mediated inhibition of MOR endocytosis. Additionally, this interaction produced a reduced opioid-induced desensitization of adenylyl cyclase signaling in striatal neurons. After internalization, rapid and efficient recycling of MORs in neurons was found to depend on a specific sorting sequence in the cytoplasmic tail of the receptor, which was

previously identified to drive MOR recycling in non-neural cells. Although this sorting sequence is functionally interchangeable with the recycling sequence of another signaling receptor, the β2 adrenergic receptor (β2AR), the kinetics of exocytic insertion into the plasma membrane during recycling differed between the two receptors. These results identify a novel function of sequence-directed recycling in mediating rapid local delivery of signaling receptors to the somatodendritic plasma membrane.

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CHAPTER 1: INTRODUCTION

G-protein coupled receptors (GPCRs) are a large superfamily of seven-transmembrane integral membrane proteins that include the majority of signaling receptors expressed in the central nervous system (CNS). They have become the most important class of drug targets in modern pharmacology. These signaling receptors respond to a vast array of sensory and chemical stimuli including light, odor, taste, and hormones. In the CNS, neuropeptides and neurotransmitters are of the most abundant of these chemical stimuli. In fact, over 90% of nonsensory GPCRs are expressed in the brain, where they play a wide variety of roles from chemosensory recognition to complex behavioral traits (Vassilatis et al., 2003; Gainetdinov et al., 2004). Neuronal activity leading to the release and binding of these specific neuromodulators onto different GPCRs plays a critical role in the maintenance of normal brain function.

One important group of GPCRs present in the CNS is the family of opioid receptors. There are three pharmacologically distinct types of mammalian opioid receptors (mu, delta, and kappa) and they are broadly expressed throughout the nervous system (Gray et al., 2006). Opioid receptors mediate a wide range of physiological effects in the nervous system. This family of receptors belongs to the class A (rhodopsin) family of G_i/G_o protein-coupled receptors. Opioid receptors are activated through the binding of both endogenous opioid neuropeptides and also exogenous opiate drugs. In the CNS, endogenous opioid peptides include enkephalins, endorphins, and dynorphins derived from four different precursor proteins. Opioid receptors are also activated by exogenously

administered opioid drugs, which are used and abused most widely for both their analgesic and rewarding properties. That activation of these receptors by opioid drugs leads to reward, tolerance, and withdrawal processes promoting opioid addiction greatly undermines their therapeutic utility. What remains a fascinating area of study is why some of these exogenous drugs, such as morphine and heroin, have such a high propensity for tolerance and addiction. An important component to our understanding of how opioid drugs produce such a wide array of complex physiology stems from how these receptors function at the cellular level in the nervous system.

The efficient transduction of neurochemical signals from the extracellular environment into the cell entails a highly regulated and complex series of events. When an opioid agonist binds, the receptor undergoes a change in conformation, which makes them more readily accessible to heterotrimeric G_i-proteins. The activated and dissociated α- and $\beta\gamma$-subunits of the G_i-proteins trigger a number of signaling cascades within the cell, including inhibition of adenylyl cyclase, activation of a potassium conductance, inhibition of calcium conductance, and inhibition of neurotransmitter release (Connor and Christie 1999). Some of the earliest observations providing direct evidence between receptor activation and G-protein coupling comes from both studies of hepatic adenylate cyclase activity and the purified muscarinic receptor (Rendell et al., 1977; Haga et al., 1985). The activated receptor is rapidly phosphorylated by G-protein receptor kinases (GRKs), and this receptor modification stimulates the binding of non-visual (β)-arrestins to the receptor (Ferguson 2001). Arrestin act as a scaffold to recruit AP-2 and clathrin and promotes endocytosis of receptors into the cell (Goodman et al., 1996; Goodman et

al., 1998). In the acidic environment of the early endosome, the ligand dissociates and the receptor is dephosphorylated. Opioid receptors are sorted either predominantly into the lysosome for degradation (e.g., delta) or returned back to the cell surface (e.g., mu) or "recycled", and primed for another round of signal transduction (Koch et al., 1998; Li et al., 1999; Tsao and von Zastrow 2000.) A more detailed discussion of the mechanisms underlying these processes will be discussed below.

Mu opioid receptor function in the central nervous system

The mu opioid receptor (MOR) is a particularly important member of the opioid family because it mediates physiological effects of both endogenous opioid neuropeptides and alkaloid drugs such as morphine. Of the three opioid receptor types, MOR is considered to be primarily responsible for mediating antinociception, euphoria, and development of addiction to analgesics such as morphine. This was first established through the finding that morphine's analgesic addictive, and withdrawal properties are abolished in mice lacking the mu opioid receptor (Matthes et al., 1996). Further, it provides a direct link between morphine's ability to activate MORs and the therapeutic and adverse downstream effects induced by this widely used drug.

Although opioid drugs are powerful and effective analgesics, they can create profound adaptive changes to the nervous system that both limit their therapeutic value and can be come highly addictive. Early studies revealed that prolonged treatment with morphine or the synthetic opioid etorphine produced adaptations within individual neuroblastoma cells, suggesting the existence of cellular correlates of physiological tolerance and

dependence (Sharma et al., 1977). While we now know that the cells used in this seminal work (NG108-15 cells) express primarily delta opioid receptors, signaling via mu opioid receptors is also extensively regulated in native neurons (Chavkin and Goldstein 1984). In the CNS, MORs are expressed in numerous brain regions critical for reward processing, including the striatum, ventral tegmental area (VTA), and amygdala (Wise 1996; Koob et al., 2001; Nestler 2001; Poulin et al., 2006). Blockade of MORs in the nucleus accumbens of the striatum and in the VTA attenuates heroin self-administration and conditioned place preference of morphine administration (see Shippenberg and Elmer 1998 for review). Lesions in the nucleus accumbens also disrupt both opiate and psychostimulant self-administration, suggesting that the rewarding effects of both types of drugs are mediated by a similar neural circuitry (Zito et al., 1985). These receptors are also highly expressed in the locus coeruleus (LC) and periacqueductal gray (PAG), where they have been suggested to play a role in opioid withdrawal behaviors (Bozarth 1994; Maldonado 1997).

Agonist-mediated MOR internalization and desensitization

Although opiate-induced effects in the nervous system is complicated by interactions between neural circuits, a fundamental way to approach our understanding of the effect of opioids starts with the study of cellular and molecular mechanisms underlying MOR regulation. An essential part of this regulation is at the level of receptor activation and subsequent membrane trafficking of receptors into the endocytic pathway. Regulated endocytosis of receptors is the primary means by which neurons can modulate the number of receptors available for activation. Expression of the cloned mu opioid receptor

in numerous heterologous cell lines has vastly increased our understanding of how these receptors function on a cellular level.

Numerous groups have found that individual opiate ligands can differ dramatically in their effects on the subcellular localization of MORs, over various time scales and in both non-neural and neural cells (Arden et al., 1995; Keith et al., 1996; Sternini et al., 1996; Evans CJ 2000). In particular, there is considerable debate regarding morphine's ability to drive rapid MOR endocytosis and desensitization to the same degree compared to other opioid agonists (Keith et al., 1996; Zhang et al., 1996; Yu et al., 1997; Keith et al., 1998; Bohn et al., 2000; Connor et al., 2004; Dang and Williams 2005; Chu et al., 2008). Highly efficacious mu opioid agonists such as etorphine and the synthetic [D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin (DAMGO) can drive rapid endocytosis of MORs in a cultured fibroblastic cell model, whereas morphine induced little detectable MOR internalization (Keith et al., 1996; Whistler et al., 1999). However, morphine was able to drive internalization of an engineered chimeric MOR with the carboxyl terminus of the delta opioid receptor, suggesting that this distal cytoplasmic portion of the MOR is at least partially responsible for the lack of morphine-induced internalization (Whistler et al., 1999).

After agonist activation, several mechanisms exist for turning off receptor signaling and MOR desensitization. Ligand-activated GPCR desensitization processes were primarily elucidated from early studies of the β2-adrenergic receptor in non-neural systems (Benovic et al., 1987; Bouvier et al., 1988; Pippig et al., 1993; Ferguson 2001), and have

proven to be relevant to many other GPCRs including opioid receptors in neurons (Gainetdinov et al., 2004). Briefly, binding of agonist drives a change in receptor conformation, which allows for phosphorylation by GRKs at serine and threonine residues in the carboxyl terminus of the receptor (Krupnick and Benovic 1998). Subsequent association of β-arrestins with the phosphorylated receptor uncouples it from its G-protein and thus prevents further GPCR signaling (i.e, desensitization) and promotes clathrin-mediated endocytosis (Goodman et al., 1996; Goodman et al., 1998; Ferguson et al., 1998).

One of the theories behind why morphine drives such poor internalization of MORs is that the activated receptor may elude traditional GRK-mediated phosphorylation and desensitization via β-arrestin (Whistler and von Zastrow 1998; Zhang et al., 1998). It has been hypothesized that the lack of morphine-mediated MOR internalization leads to prolonged signaling and in turn, an enhanced susceptibility to long-term changes in neural plasticity associated with morphine addiction and abuse (Whistler et al., 1999). However, acute morphine treatment has been reported to cause desensitization in LC neurons, and the rate of morphine desensitization was increased in animals chronically treated with morphine (Dang and Williams 2005). This suggests that the mechanism behind morphine-mediated tolerance could be derived from an increased rate of desensitization after prolonged morphine treatment (Dang and Williams 2004). Additionally, recent studies have indicated that acute morphine treatment is capable of driving rapid internalization of both recombinant and endogenous MORs in nucleus accumbens neurons (Haberstock-Debic et al., 2003). Similar observations were also

observed with further characterization and analysis, the results of which are presented in Chapter 2. Together, these findings suggest that at least in some physiologically relevant CNS neurons, the extent of receptor endocytosis alone could not account for differences observed with morphine tolerance and abuse.

Although the GRK- and β-arrestin-dependent mechanisms for desensitization may be the most thoroughly studied, other mechanisms for MOR desensitization have also been described. Opioid receptor desensitization can also occur by GRK- and β-arrestin-independent processes. A mutant MOR missing its putative agonist-induced phosphorylation sites in the cytoplasmic tail preserved its ability to internalize and desensitize despite the loss of agonist-induced phosphorylation and loss of β-arrestin translocation to the activated receptor (Qiu et al., 2003). This observation is consistent with the model that receptor phosphorylation can help facilitate, but is not required, for internalization of MOR. However, there is evidence that β-arrestin can bind to unphosphorylated agonist-occupied receptors, suggesting that the mutant truncated MOR may merely bind β-arrestin with reduced efficiency (Gurevich et al., 1995). MOR phosphorylation can also occur independent of GRKs in a ligand-dependent manner, providing another mechanism for opioid-induced MOR desensitization. Inhibition of protein kinase C (PKC) activity, but not GRK2, reduced MOR desensitization by morphine. In contrast, DAMGO-mediated desensitization was affected by expression of a dominant-negative GRK2, but not by inhibition of PKC activity (Johnson et al., 2006). This suggests that morphine and DAMGO, in addition to their differences in driving MOR internalization, also diverge in mechanisms of desensitization. Bailey et al., (2009)

also reports on morphine-induced desensitization that is mediated by a PKC-dependent mechanism, although their chronic treatment protocol is more consistent with the time course of downregulation of receptors (such as can occur by lysosomal proteolysis and a reduction of receptor expression at the cell surface) rather than acute desensitization. Further downstream, activation of the MAPK pathway has been suggested to be required for MOR desensitization to occur (Polakiewicz et al., 1998; Tan et al., 2003). Additionally, mechanisms of heterologous MOR desensitization have been reported to occur in the CNS, both via other GPCR activation and also at the level of downstream effectors (Fiorillo and Williams 1996; Blanchet and Luscher 2002; Tan et al., 2003).

Signaling consequences of MOR activation and implications for tolerance

A well-studied downstream signaling consequence of MOR activation is inhibition of adenylyl cyclase activity and cAMP production (Brandt et al., 1976; Sharma et al., 1975). Under chronic exposure to opioids, cellular adaptations can occur leading to tolerance and withdrawal. One of the most well-characterized of these mechanisms is the compensatory upregulation of the cAMP signaling pathway observed after agonist removal (Sharma et al., 1975). This increase in adenylyl cyclase activity is viewed as a homeostatic response to chronic exposure to opiates and is considered a hallmark of opiate tolerance and withdrawal (for a review see Williams et al., 2001). Cellular tolerance is primarily a result of several mechanisms related to receptor trafficking. The first is the uncoupling of the activated receptor from its effectors (i.e., desensitization) and a possible decrease in receptor number after prolonged drug treatment (i.e., downregulation). Another mechanism involves adaptive cellular changes associated with

prolonged signaling in response due to chronic opioid treatment, (e.g., superactivation of the cAMP pathway) with prolonged morphine exposure. Chronic morphine treatment has been shown to produce this compensatory upregulation in both cultured cells and animals (Sharma et al., 1975; Avidor-Reiss et al., 1996; Bonci and Williams 1997; Chieng and Williams 1998). These two forms of cellular tolerance may be intricately linked, where failure in receptor internalization would lead to prolonged receptor activation, downstream signaling, and compensatory upregulation of cellular responses. An interesting study showing this in vivo found that chronic high doses of etorphine produced tolerance via downregulation of receptors, whereas low doses of etorphine did not produce tolerance (Stafford et al., 2001). In contrast, chronic morphine treatment resulted in tolerance without receptor downregulation, but instead presumably due to adaptive compensatory mechanisms (Stafford et al., 2001). This phenomenon has been suggested to be a result of morphine's inability to produce robust MOR internalization, which leads to chronic receptor activation, cellular adaptations, and thus potentially contributing to morphine's apparently increased propensity to produce tolerance (Whistler et al., 1999; Finn and Whistler 2001; Koch et al., 2005). This has led to the hypothesis that agonist activity and receptor endocytosis have opposing effects on receptor-mediated signaling (Whistler et al., 1999). Termed "RAVE" (Relative Activity Versus Endocytosis), this hypothesis argues that opioid drugs that have a high ability to activate potassium currents (i.e., Relative Activity) but lower ability to drive endocytosis (i.e., Versus Endocytosis) would activate downstream effectors for a prolonged period due to lack of endocytosis, and thus lead to cellular adaptations. In contrast, drugs that have a low RAVE ratio (ex. DAMGO, etorphine) would be able to uncouple from their

effectors, remove receptors from the cell surface via endocytosis, and would not drive compensatory mechanisms such as cAMP superactivation (Whistler et al., 1999). However, this theory of morphine-induced tolerance may be more complex than previously thought due to studies demonstrating morphine's ability to drive robust internalization in nucleus accumbens striatal neurons in vivo and its ability to produce desensitization in LC neurons (Haberstock-Debic et al., 2003; Dang and Williams 2005; Arrtamangkul et al., 2008). Thus, it remains unclear whether morphine-mediated MOR trafficking per se is the predominant cause of opioid tolerance. Differences in cellular environment could be an important factor in contributing to MOR trafficking as it relates to tolerance.

Opioid receptor interactions with other GPCRs

In order to appreciate how MORs function in native tissue, we must take into consideration interactions that may be occurring between opioid receptors and other neurochemical systems. There is considerable evidence indicating that the opioid system can interact with other GPCR systems in the nervous system to modulate behaviors such as drug seeking, tolerance, reward, and withdrawal. Given its role in mediating addiction and reward in general, it is not surprising that the mesolimbic dopamine system is intimately linked to the motivational aspects of heroin-taking behavior (Johnson and North 1992; Shippenberg and Elmer 1998). Additionally, mice lacking the D2 dopamine receptor showed a lack of place-preference to morphine's rewarding effects, without changes in expression of morphine withdrawal or food reward detection (Maldonado et al., 1997). Highlighted by genetic deletion studies in mice, other neurochemical

interactions with the opioid system are somewhat more intriguing. Both deletion of the cannabinoid 1 receptor (CB1) and CB1 receptor antagonists can prevent morphine self-administration and conditioned reinforcement (Ledent et al., 1999). In orexin knock out mice, morphine dependence is significantly attenuated, supporting its role in opiate-mediated physiology (Georgescu et al., 2003). Deletion of MOR itself has also provided evidence of cross talk between receptor systems. The finding that MOR knock out animals no longer self-administer ethanol, but instead show aversion to it, provides evidence that these receptors may play a critical role in the rewarding aspects of ethanol (Roberts et al., 2000). Similarly, nicotine's rewarding effects, in addition to antinociception and dependence, were also abolished in MOR knock out mice (Berrendero et al., 2002).

One of the more striking examples of how other GPCR systems can modulate opioid function comes from studies of neurokinin 1 receptor (NK1R) knock out mice. First reported by Murtra et al., (2000), $NK1R^{-/-}$ mice no longer find morphine rewarding and showed a reduced physical response to opiate withdrawal, compared to wild type mice. This loss in reward detection was specific to morphine since these mice still displayed normal expected preferences to psychostimulants and natural reward such as food (Murtra et al., 2000; Ripley et al., 2002). Interestingly, the central analgesic actions of morphine remained present in $NK1R^{-/-}$ mice, further emphasizing that NK1Rs play a specific role in opiate reward mechanisms (Ripley et al., 2002).

In the CNS, MORs and NK1Rs are co-expressed in several different brain regions, many of which are relevant to reward processing and opioid physiology including the ventral

striatum, amygdala, and locus coeruleus (Pickel et al., 2000; Gadd et al., 2003; Nakaya et al., 1994; Jabourian et al., 2005; Poulin et al., 2006). Their co-expression on the same neurons in some of these brain regions provides the opportunity for possible cell autonomous regulation of receptor function. Indeed, there is evidence in HEK 293 cells that MORs and NK1Rs can heterodimerize (Pfeiffer et al., 2003). This physical interaction was reported to dramatically alter the internalization and resensitization of both receptors. Specifically, either DAMGO or the NK1R agonist substance P was able to drive cross-phosphorylation of receptors and co-internalization of MOR-NK1R heterodimers. NK1Rs have a higher binding affinity for β-arrestin compared to MOR and as a result, the NK1R-arrestin complex is internalized together into endosomes after agonist-mediated endocytosis (Oakley et al., 1999; Oakley 2001). Thus, co-internalization with MOR resulted in a delay in MOR resensitization due to prolonged interaction with β-arrestin that is not normally observed with MOR activation alone (Pfieffer et al., 2003). This study provided evidence that physical interaction between the two receptors is one possible means by which other GPCRs can modulate normal opioid receptor function.

It is plausible that in the CNS, co-expression a variety of different receptors on the same neuron allows for the almost certain possibility that heterologous interactions may be affecting normal function of receptors. Both homo- and hetero-oligomerization of GPCRs have been described at length and shown to have implications for receptor trafficking, signaling, and pharmacology (Devi 2001; Gomes et al., 2002; Lee et al., 2003). Cross regulation between different GPCRs have also been shown at the level of endocytic

regulatory machinery (Klein et al., 2001; Schmidlin et al., 2002; Terrillon et al., 2004). However, most of these previous reports, including the NK1R-MOR heterodimerization study, were primarily conducted in non-neural cells. Whether MOR trafficking and signaling can be affected by NK1R activation in physiologically relevant CNS neurons will be addressed in Chapter 3. More generally, these previous studies highlight the fact that of co-expression of different receptors complicates conventional receptor trafficking and must be taken into consideration when studying agonist-mediated opioid receptor trafficking and signaling.

Post-endocytic trafficking of MORs

GPCRs differ in their post-endocytic sorting fates after internalization, where some receptors are recycled back to the plasma membrane while others are sorted to lysosomes for degradation (for reviews see Hanyaloglu and von Zastrow 2007 and Marchese et al., 2008). This divergence may be first initiated at the level of the plasma membrane with receptor modification via phosphorylation on serine and threonine residues present in the cytoplasmic tail and third intracellular loop of the receptor. For example, mutations of the distal portion of the β2AR tail can disrupt PDZ domain-mediated interaction with cytoplasmic sorting proteins to not only inhibit recycling to also re-directs internalized receptors to lysosomes for degradation (Cao et al., 1999; Cong et al., 2001). Conversely, replacing the delta opioid receptor cytoplasmic tail, which is primarily sorted to lysosomes after internalization, with the β2AR "recycling" cytoplasmic sequence is sufficient to promote recycling of internalized receptors (Tsao and von Zastrow 1999; Gage et al., 2001).

In the opioid receptor family, MORs undergo efficient recycling back to the plasma membrane following endocytosis in both neural and non-neural cells (Law and Loh 1999; Finn and Whistler 2001). Recycling of receptors back to the cell surface is associated with the functional recovery of signaling and is important for maintaining physiological responsiveness to opioids (Koch et al., 1998). While rapid endocytosis of MOR has been demonstrated in CNS neurons (Keith et al, 1998; Trafton and Basbaum 2004; Haberstock-Debic et al., 2003; Mills et al., 2004; Martini and Whistler 2007; Arttamangkul et al., 2008), relatively little is known about post-endocytic trafficking of opioid receptors in the nervous system. Recycling has been shown to occur in enteric neurons (Minnis et al., 2003) and locus coeruleus slice preparations expressing transgenic Flag-MORs (Arttamangkul et al., 2008). Additionally, ligand-induced internalization of MOR in myenteric neurons is reversible within several hours following ligand removal (Sternini et al., 1996).

In non-neural cell models, this post-endocytic trafficking itinerary is determined by a specific sorting mechanism that recognizes a discrete cytoplasmic sequence present in the carboxyl-terminal cytoplasmic tail of the MOR (Law and Loh 1999; Tsao and von Zastrow 2000; Tanowitz and von Zastrow 2003; Hanyaloglu and von Zastrow 2007). First described with β2ARs, cytoplasmic recycling sequences have been shown to direct efficient recycling of a number of different GPCRs (Tsao and von Zastrow 2000; Tanowitz and von Zastrow 2003; Gage et al., 2005; Vargas and von Zastrow 2004). However, previous studies have only shown sequence-mediated recycling of GPCRs in

non-neural cells. We are beginning to understand the specific cellular mechanisms underlying MOR recycling in neurons and these findings will be discussed in Chapter 4.

Recycling of MOR and rapid membrane trafficking events

Endocytosis and recycling of MORs occurs within minutes after agonist exposure, leading to rapid receptor turnover in the plasma membrane. A fundamental issue of membrane receptor trafficking in general is how receptors are quickly delivered back to the membrane at the right time and place. This is particularly important in polarized neuronal cells where compartment-specific distribution of receptors play a critical role in neuronal function (Horton and Ehlers 2003). The vast majority of what we know about GPCR recycling in both heterologous cell lines and neurons is derived from studies cells using immunocytochemical and biochemical methods (von Zastrow 2003). Although these previous studies have helped us to understand important cellular and molecular processes mediating receptor recycling, it was not until recently that we have started to learn about the temporal aspects of recycling in neurons.

Recent studies using total internal fluorescence (TIRF) microscopy to study discrete recycling events in live neurons has helped gain significant insight into how and where receptor recycling is occurring. TIRF microscopy allows for rapid imaging of single exocytic vesicular events occurring in the plasma membrane with a high degree of temporal resolution. This form of imaging utilizes the optical phenomenon of total internal reflection, which generates an evanescent field of illumination that selectively excites fluorophores within ~100nm of cell surface, thus allowing selective visualization

of discrete events happening at or near the plasma membrane. Additionally, imaging in the TIRF field also allows for maximal signal-to-noise ratio and minimizes phototoxicity (Steyer et al., 2001). Time-lapse imaging of individual exocytic recycling events in live neurons was first observed with β2ARs fused at the amino-terminal extracellular domain to a green fluorescent protein variant superecliptic pHluorin (SpH) (Yudowski et al., 2006). This SpH epitope is highly fluorescent when exposed to the extracellular environment with a neutral and its fluorescence is rapidly and reversibly quenched in the acidic environment of the endocytic pathway (Miesenbock et al., 1998; Sankaranarayanan et al., 2000). Paired with rapid TIRF imaging, single vesicular SpH-β2AR recycling events were observed throughout the somatodendritic plasma membrane of hippocampal neurons, where they exhibited distinct kinetic modes of receptor dispersion after insertion into the plasma membrane (Yudowski et al., 2006). While most events were transient (i.e., lasting < 1 sec.), a sub-population of insertion events persisted at the cell surface for an extended period of time before dispersing. These distinct kinetic modes were also observed with AMPA receptors in neurons (Yudowski et al., 2007). The recycling of behavior of MOR was found to be distinct from that of previously described signaling receptors, and these results will be presented and further discussed in Chapter 4.

Here, several novel findings underlying the regulation and function of MORs in CNS neurons are presented through studies of the cellular mechanisms underlying the activation, trafficking, and post-endocytic sorting of MORs in physiologically relevant neurons. Briefly: [1)] Thorough analysis of both endogenous and recombinant MOR in striatal medium spiny neurons reveal robust internalization of MORs after acute

morphine treatment in both cell bodies and dendrites, suggesting that morphine's ability to drive receptor internalization could be dependent on cellular background. [2] Heterologous regulation of MOR trafficking and signaling were observed in neurons of the striatum, amygdala, and locus coeruleus where co-activation of NK1Rs inhibited MOR endocytosis in response to DAMGO. This inhibition affected not only trafficking but also desensitization of MOR, suggesting that MOR function can be significantly altered by co-activated receptors expressed on the same neuron. [3] A cytoplasmic MOR recycling sequence functions to mediate rapid and efficient receptor recycling in multiple populations of CNS neurons and determines the kinetics of exocytic recycling events. More generally, GPCR recycling sequences may play an important role in maintaining proper spatiotemporal pattern of receptor distribution under conditions of rapid receptor and membrane turnover.

CHAPTER 2: MORPHINE PROMOTES RAPID, ARRESTIN-DEPENDENT ENDOCYTOSIS OF MU OPIOID RECEPTORS IN STRIATAL NEURONS

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ABSTRACT

Morphine activates mu opioid receptors (MOR) without promoting their rapid endocytosis in a number of cell types. A previous study suggested that morphine can drive rapid redistribution of MOR in the Nucleus Accumbens, but it was not possible in this *in vivo* study to identify a specific membrane trafficking pathway affected by morphine, to exclude possible indirect actions of morphine via opiate-regulated neural circuitry, or to define the mechanism of this morphine-dependent regulation. In the present study we have addressed these questions using dissociated primary cultures of rat striatal neurons as a model system. Morphine promoted a rapid redistribution of both endogenous and recombinant MOR within 30 minutes after drug addition to the culture medium. This effect was mediated by rapid endocytosis and occurred in a cell-autonomous manner, as indicated by its detection in cells plated at low density and in cultures in which depolarization was blocked by tetrodotoxin. Morphine-induced endocytosis of MOR was quantitatively similar to that induced by the enkephalin analogue DAMGO, and endocytosis induced by both ligands was inhibited by a dominant-negative mutant version of arrestin 3 (beta-arrestin-2). These results extend previous *in vivo* results and indicate that morphine is indeed capable of driving rapid

endocytosis of mu opioid receptors in an important subset of opiate-responsive CNS neurons. They also suggest a cellular mechanism by which beta-arrestins may modulate the physiological effects of morphine *in vivo*.

INTRODUCTION

Morphine mediates most of its physiological effects by activating the mu opioid receptor (MOR), a G protein-coupled receptor which is also activated by natively produced opioid neuropeptides (Kieffer, 1999; Evans, 2000). A fundamental mechanism of opioid receptor regulation involves rapid endocytosis of receptors via clathrin-coated pits (Keith et al., 1996; Chu et al., 1997). Endocytosis has been proposed to contribute both to attenuated opioid signaling after acute agonist activation and to the rapid recovery of opioid responsiveness after agonist removal (Qiu et al., 2003). The latter role is thought to be particularly important (Koch et al., 1998) although endocytosis can also promote receptor down-regulation leading to a prolonged reduction of cellular opioid responsiveness (Afify et al., 1998; Tsao and von Zastrow, 2000). Hence, endocytosis of opioid receptors is an important regulatory process with potentially diverse functional consequences.

While various opioid peptide agonists promote rapid endocytosis of opioid receptors, morphine-activated receptors are relatively resistant to this regulatory process (Keith et al., 1996; Alvarez et al., 2002; Borgland, 2001). Endocytosis of opioid receptors is regulated by phosphorylation and non-visual (beta-) arrestins (Zhang et al., 1998; Whistler and von Zastrow, 1998). Morphine-activated receptors are generally phosphorylated to a smaller degree (Yu et al., 1997; Zhang et al., 1998) or with slower kinetics (Schultz et al., 2004) than receptors activated by opioid peptide agonist, contributing to distinct functional consequences (Koch et al., 2001).

Much of what is known about opioid receptor traffic has been learned using heterologous cell models. With some exceptions (e.g., Kim and von Zastrow, 2003), similar processes occur in neurons (Bushell et al., 2002; Whistler et al., 1999; Keith et al., 1998; Trafton et al., 2000; Sternini et al., 1996; Alvarez et al., 2002; Borgland, 2001; Connor et al., 2004). Studies in heterologous cells indicate that morphine-induced endocytosis of MOR can be greatly enhanced by over-expressing G-protein coupled receptor kinases (GRKs) or arrestins (Zhang et al., 1998; Whistler and von Zastrow, 1998). However we are not aware of previous evidence that morphine promotes rapid endocytosis of opioid receptors to a comparable degree as opioid peptide agonists in cells expressing these regulatory proteins at endogenous levels.

We previously observed that acute morphine injection *in vivo* produced a rapid increase in intracellular MOR immunoreactivity visualized in medium spiny neurons (Haberstock-Debic et al., 2003). However, in this *in vivo* study we were unable to elucidate any mechanisms underlying this unexpected regulatory effect of morphine, including whether this effect is mediated by regulation of MOR in endocytic or biosynthetic membrane pathways (Haberstock-Debic et al., 2003; Kim and von Zastrow, 2003), or whether this regulatory effect is a direct action of morphine or an indirect consequence of endogenous enkephalin release (Shiomi et al., 1981). Here we have investigated these questions using primary cultures of dissociated rat striatal neurons, a preparation which is thought to be largely representative of the major GABAergic medium spiny neurons present in the Nucleus Accumbens (Heimer et al., 1982).

MATERIALS AND METHODS

DNA constructs. A FLAG (DYKDDDD) epitope-tagged version of the cloned murine mu (MOR1) (Kaufman et al., 1995) opioid receptor (gift of Dr. Chris Evans, UCLA) was cloned into pCAGGS/SE vector, a chicken β-actin-promoter driven expression vector (Niwa et al., 1991). A beta-arrestin-2 (arrestin-3) - GFP construct (gift of Dr. Marc Caron, Duke University) was excised using SacI/DraIII sites from the vector described previously (Barak et al., 1997) and cloned in pCAGGS/SE vector using a DraIIII/EcoRI linker/adapter (+linker GTGAACCTTAAGATGGCGAG; -linker AATTCTCGCCATCTTAAGGTTCACGTA). C-terminal fragment of this construct (residues 319-418), which has been shown previously to have potent dominant negative activity on arrestin-promoted endocytosis (Krupnick et al., 1997), was obtained (gift of Dr. Jeffrey Benovic, Thomas Jefferson University), and a hemagglutinin (HA) epitope tag (YPYDVPDYA) was added to the N-terminus and the tagged construct was cloned into the pCAGGS/SE vector. All sequences were confirmed by dideoxynucleotide sequencing (UCSF Genetics Core Facility).

Cell cultures and transfections. *Primary striatal neuronal culture*. Brains were aseptically isolated from E17-18 rat embryos taken from pregnant Sprague-Dawley rats, and the striatum (caudate-putamen and nucleus accumbens) was dissected out based on the criteria of Ventmiglia and Lindsay (1998). Dissected cells were dissociated in 1X trypsin/EDTA solution (GIBCO) for 20 min before 1mL trypsin inhibitor was added for 5 minutes at room temperature prior to gentle mechanical trituration using a glass pipet. All washing steps, as well as the trituration of tissue, were done in DMEM + 10% FCS

(Gibco). Transfections were carried out using either a cationic lipid transfection reagent (Effectene, Qiagen) or electroporation (Rat Neuron Nucleofector^{TM} system, Amaxa Biosystems). When cationic lipid was used for transfection, the dissociated cells were plated out on poly-D-lysine-coated glass coverslips into 24-well plates and cultured in Neurobasal medium supplemented with B27, L-glutamate. On day 4-6 after culture was prepared, cells were transfected and maintained in culture for 12-14 days. Neurons were transfected in 24-well plates with pCAGGS/SE plasmid containing FLAG-tagged mu opioid receptor or cotransfected with pCAGGS/SE containing GFP-beta-arrestin-2 or HA-tagged dominant negative beta-arrestin-2. When electroporation was used for transfection, neurons were transfected immediately after dissociation and plated out on poly-L-lysine-coated glass coverslips previously washed in 70% nitric acid and rinsed over two days. Poly-L-lysine was prepared in 0.1 M sodium borate buffer (pH 8.5). Electroporation was conducted using $5x10^6$ dissociated cells, 2 μg of plasmid DNA and 100 μl of Rat Neuron Nucleofector Solution. After transfection, cells were kept for 10 min in pre-warmed RPMI media to recover before they were transferred into 24-well plates and cultured in DMEM + 10% FCS on day one, then in Neurobasal medium supplemented with B27, L-glutamate starting on day 2. Immunochemical staining procedures were carried out on cultures between days 7 –10 after plating.

Immunocytochemical procedures

Staining for endogenous MOR. For immunocytochemical localization of endogenous MOR, striatal neurons grown on poly-D-lysine-coated glass coverslips were treated 30 min with 10 μM morphine or DAMGO or left untreated before fixation (4%

paraformaldehyde in PBS, 4˚C x 20 minutes). Specimens were permeabilized and blocked using 0.3% Triton X-100, 1%BSA in PBS for 60 min. Staining for MOR immunoreacitivity was performed using an affinity-purified rabbit polyclonal antibody raised against the carboxyl-terminal cytoplasmic domain (amino acids 384-398) of rat MOR1 (1:500; ImmunoStar Inc., Hudson, WI). Primary antibody incubation was carried out for 36 h at 4^{o}C in 0.3% Triton X-100, 1%BSA in PBS. Specimens were washed over several hours with multiple changes of ice-cold Tris-buffered saline (TBS, pH 7.4), then incubated with goat ant-rabbit antibody conjugated with the cyanine dye Cy3 (3 μg/ml; Jackson ImmunoResearch, West Grove, PA) in 0.3% TritonX-100, 0.5% BSA in PBS for 45 min at room temperature. Specimens were again washed extensively in TBS and mounted to glass slides (using Fluormount antifade reagent) prior to examination by fluorescence microscopy.

Staining protocol for antibody "feeding" experiments. To specifically visualize internalization of FLAG-MOR from the cell surface, M1 anti-FLAG monoclonal (3.5 μg/ml; Sigma) was added to the culture medium of FLAG-MOR transfected striatal neurons and incubated for 30 min to label surface receptors. Neurons were then incubated for an additional 30 min in the presence of the indicated ligand. In some experiments, specimens were quickly washed three times at the end of this incubation prior to fixation in PBS lacking Ca^{2+} and Mg^{2+} and supplemented with 0.04% EDTA to dissociate ("strip") the M1 anti-FLAG antibody bound to residual surface receptors remaining in the plasma membrane (thereby leaving antibody bound only to the internalized receptors). Specimens were then fixed in 4% paraformaldehyde in PBS,

washed with ice-cold TBS and endocytosed antibody was detected by incubating permeabilized cells with Cy3-conjugated goat anti-mouse antibody (3 μg/ml; Jackson ImmunoResearch, West Grove, PA) prepared in 0.1% saponin, 2%BSA dissolved in PBS. Specimens were washed extensively in TBS and mounted as described above for fluorescence microscopy. The number of neurons displaying peripheral (membrane) receptor localization or punctate, endocytosed receptor localization were counted using coded slides and data were accumulated from 20-60 cells per animal. Results of cell counts are expressed as means ± SD. Statistical analysis of differences between control (untreated) and experimental (drug-treated) groups was performed using Student's *t* test.

Colocalization of FLAG-MOR with beta-arrestin, endosome markers and MAP2. Dual staining for FLAG-tagged MOR and HA-tagged mutant beta-arrestin-2 in the same neurons was performed by first "feeding" neurons with a rabbit polyclonal anti-FLAG (1 μg/ml; Sigma) for 30 min, as described above, adding the indicated ligand for an additional 30 min, and fixing with 4% paraformaldehyde dissolved in PBS. Specimens were then washed in cold TBS, permeabilized and blocked using 0.1% saponin, 2% BSA in PBS and then incubated with HA.11 mouse monoclonal anti-HA (5 μg /ml; Covance, Princeton, NJ) prepared in the same buffer. Bound HA.11 and FLAG antibodies were visualized using fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (3 μg/ml; Jackson ImmunoResearch) and Cy3-conjugated goat anti-rabbit antibody (3 μg/ml; Jackson ImmunoResearch), respectively. In experiments in which dual localization of FLAG-MOR and MAP2 was performed, specimens were fixed with 4% paraformaldehyde in PBS, washed in cold TBS, permeabilized and blocked as indicated

above and incubated with a rabbit polyclonal anti-FLAG (Sigma) and mouse monoclonal anti-MAP2 (16 μg/ml; Sigma) prepared in 0.1% saponin, 2%BSA in PBS. Specimens were then washed three times with ice-cold TBS and incubated with a Cy3-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch) and FITC-conjugated goat anti-mouse antibody (Jackson ImmunoResearch) diluted in 0.1%saponin, 2%BSA in PBS. Stained specimens were mounted using Fluormount and examined by epifluorescence microscopy using a Nikon (Melville, NY) Diaphot microscope equipped with a 60x / NA 1.4 objective, mercury arc lamp illumination, and standard dichroic filter sets (Omega Optical). Images were collected using a cooled CCD camera (Princeton Instruments) interfaced to an Apple Macintosh computer using IPLab (Scanalytics) software.

For simultaneous detection of FLAG-MOR and endocytosed transferrin, specimens were serum-starved for 60 min and incubated with Alexa488-conjugated diferric transferrin (50μg/ml; Molecular Probes) in serum-free medium for 30 min. Surface FLAG-MOR was labeled by incubating with 1 μg/ml Alexa594-conjugated M1 anti-FLAG monoclonal (prepared by standard methods using Alexa-fluor 594 N-hydroxysuccinimide ester, Molecular Probes), cells were incubated under the indicated conditions for 30 min, and then fixed using 4% paraformaldehyde dissolved in PBS. Dual staining for LAMP1 and FLAG-MOR was performed by surface labeling FLAG-MORs with Alexa 594 -conjugated M1 as above, incubating under the indicated conditions for 30 min and fixing using 4% paraformaldehyde dissolved in PBS. Fixed specimens were washed 3 times in ice-cold TBS, permeabilized and blocked using 0.1% saponin, 2%BSA in PBS and then incubated with anti-LAMP1 monoclonal antibody (0.2 μg/ml; Stressgen

Biotechnologies) dissolved in the same buffer for 60 min. Specimens were then washed extensively with TBS and Alexa488-conjugated goat-anti mouse IgG_1 (4 μg/ml; Molecular Probes) dissolved in 0.1% saponin, 2% BSA in PBS was applied for 30 min to detect LAMP1 immunoreactivity. Specimens were then mounted to glass slides and examined using a Zeiss LSM510 confocal laser microscope using a 63X / NA 1.4 objective and a pinhole diameter of 1 Airy disc. For all colocalization experiments, manufacturer-recommended settings were used and control imaging of single-labeled specimens was carried out to confirm lack of detectable bleedthrough between channels.

Dual color visualization and quantitative analysis of MOR internalization

Ratiometric internalization assay using differentially labeled primary antibodies. Coverslips were incubated for 30 min in media containing 2μg/ml Alexa594-conjugated M1 anti-FLAG antibody to label surface receptors, incubated with 10μM morphine, 10 μM DAMGO or left untreated (control) for an additional 30 min. Alexa594-M1 bound to residual surface (not internalized) receptors was "stripped" by washing three times rapidly with ice-cold PBS without Ca^{2+} and Mg^{2+} and supplemented with 0.04% EDTA, thereby leaving Alexa594-M1 antibody bound only to the internalized receptors. Neurons were then fixed in 4% paraformaldehyde in PBS without permeabilization, washed in cold TBS and incubated with 2μg/ml Alexa488-conjugated M1 anti-FLAG antibody (prepared by standard methods using Alexa-fluor 488 N-hydroxysuccinimide ester, Molecular Probes) using nonpermeabilized conditions (2%BSA in PBS without saponin), to label only the residual surface pool of FLAG-MOR. Epifluorescence microscopy was

performed using an inverted microscope fitted with Nikon PlanApo 10x / NA0.5 or Plan 20X / NA0.5 dry objectives for low magnification viewing, and a Nikon PlanApo 60x / NA 1.4 oil objective for high magnification viewing. To quantify receptor internalization, cell bodies were selected at random using 20X/NA0.5 dry objectives and outlined using IPLab software to calculate integrated intensity of Alexa594 and Alexa488 fluorescence signals separately. Background signals for each channel (obtained from mean determinations from control labeling of untransfected neurons) were scaled to the integrated area of each measurement and subtracted. Another control was carried out in which transfected neurons were incubated in the absence of agonist (control condition) and labeled simultaneously with Alexa488 –M1 and Alexa594-M1, both at 2 μg/ml, to determine the ratio between specific Alexa488 and Alexa594 signal intensities when bound in similar amount. This ratio was used to obtain normalized green and red signal intensities from background-subtracted Alexa488 and Alexa594 determinations, respectively. The percentage of internalized receptors was then estimated by: % internalized = [red / (green + red)] x 100. 20-30 neuronal cell bodies were analyzed per animal, derived from four animals per condition, and mean and standard deviation of the calculated ratios were determined. Statistical analysis of differences between control (untreated) and experimental (drug-treated) groups was performed using Student's *t* test.

Ratiometric internalization assay using differentially labeled primary and secondary antibodies. To further evaluate ligand effects on FLAG-MOR internalization, an additional assay was devised that does not depend on antibody "stripping." First, specimens were incubated with Alexa594-conjugated M1 for 30 min to label surface

FLAG-MOR and then incubated under the indicated ligand conditions for an additional 30 min. Cells were then fixed without permeabilization with 4% paraformaldehyde dissolved in PBS. Specimens were washed in ice-cold TBS and incubated with Alexa488 donkey-anti mouse IgG (Molecular Probes) for 45 min under nonpermeabilized conditions (2%BSA in PBS without saponin) to label only primary antibodies bound to FLAG-MOR present in the plasma membrane. Internalized receptors were therefore labeled only with one fluorochrome (Alexa594) and residual surface receptors were labeled with both Alexa488 and Alexa594. Imaging of cell body regions was carried out and background was subtracted as described above from Alexa488 and Alexa594 values separately to yield net green and red signals, respectively. The percentage of receptors internalized in response to the indicated agonist was then calculated from the background-subtracted Alexa488 (green) and Alexa594 (red) values according to following formula: % internalized = [1 –(green/red ratio in agonist treated) / (green/red ratio in nontreated)] x 100. In each experiment, 50 neuronal bodies were analyzed for each condition. The results shown were compiled from two experiments representing separate animals and culture preparations. Statistical analysis of differences between control (untreated) and experimental (drug-treated) groups was performed using Student's *t* test.

Immunoblotting procedures. Rat striatal neurons were grown in 6-well plates for 7 – 10 days before they were lysed in cold lysis buffer (0.1% Triton X-100, 10mM Tris, 150 mM NaCl, 1mM $CaCl_2$ and 25 mM KCl) containing protease inhibitors (100_l/ml leupeptin, aprotinin, pepstatin and 1mM pefabloc) for 30 min gently rocking at +4^{o}C.

HEK293 cells were also cultured in 6-well plates and lysed using the same method. Cell extracts were obtained by centrifugation of detergent lysates in a microcentrifuge (14,000 rpm for 15 min). The supernatant from this spin was taken as clarified extract and a volume of extract corresponding to 40 μg of total protein from each extract (determined the Bradford protein assay reagent marketed by BioRad, with BSA as standard) was separated by SDS-PAGE. Resolved proteins were transferred to nitrocellulose membranes (Micron Separations, Inc.). Detection of GRK2 was carried out by incubating the blots with rabbit polyclonal GRK2 antibody (0.4 μg/ml; Santa Cruz Biotechnology) for 60 min, washing in TBST (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, and 0.1% (v/v) Tween 20), and incubating with donkey anti-rabbit antibody conjugated to horseradish peroxidase for 30 min (Amersham). Immunoreactive proteins bands were detected using Super Signal (Pierce) and Kodak XAR film. For detection of beta-arrestin, striatal neurons and HEK293 cells were cultured in 6-well plate and cell extracts were prepared as above. 40 μg of total protein from each extract was separated by SDS-PAGE, transferred to nitrocellulose membrane and detection was carried out using antiserum raised against a sequence (residues 384-397) in beta-arrestin-2 that is conserved in beta-arrestin-1 (1 μg/ml; Affinity Bioreagents) for 60 min. After washing in TBST (as above) blots were incubated with donkey anti-rabbit antibody conjugated to horseradish peroxidase for 30 min (Amersham) and immunoreactive protein bands were detected using Super Signal (Pierce) and Kodak XAR film.

RESULTS

Both morphine and DAMGO promote rapid redistribution of endogenous MOR immunoreactivity in dissociated striatal neurons

Whereas morphine fails to produce rapid effects on MOR localization in heterologous cell models or in several neural cell types examined *in situ* and in culture (e.g., Keith et al., 1996; Trafton et al., 2000; Keith et al., 1998; Whistler et al., 1999; Sternini et al., 1996; Bushell et al., 2002), a pronounced change in the subcellular localization of MOR was observed in the intact Nucleus Accumbens within 30 minutes after systemic administration of morphine (Haberstock-Debic et al., 2003). To determine whether a similar effect of morphine could also be observed in cultured neurons, we examined MOR localization in primary cultures of rat striatal neurons. A large fraction of these neurons express endogenous MOR and are arguably more representative of Nucleus Accumbens neurons than the (largely excitatory) hippocampal cultures used by others and us in previous studies (e.g., Bushell et al., 2002; Whistler et al., 1999). Consistent with this, a significant fraction (~ 60 %) of neurons present in these cultures were labeled with a previously described antiserum recognizing the carboxyl-terminal cytoplasmic domain of MOR1 (Arvidsson et al., 1995). In cells cultured in the absence of opioid agonist, endogenous MOR immunoreactivity was visualized over the entire surface of the cell, consistent with localization of a major fraction of opioid receptors in the neuronal plasma membrane (Fig. 1, left panel). A pronounced redistribution of endogenous MOR was observed within 30 minutes after addition of DAMGO (10 μM) to the culture medium, where MOR immunoreactivity was visualized in a punctate pattern consistent

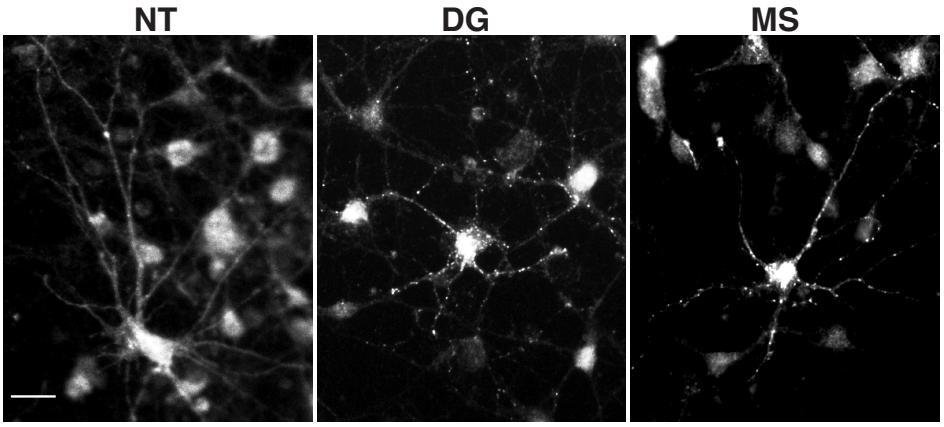

Figure 1. Both morphine and DAMGO produce rapid redistribution of endogenous MOR in cultured striatal neurons. Endogenous MOR distribution was visualized in untransfected striatal neurons by anti-MOR immunostaining under permeabilized conditions. Representative epifluorescence images show MOR distribution in the untreated condition (NT), after 30 min treatment with 10 μM DAMGO (DG), or after 30 min treatment with 10 μM morphine (MS). In the NT condition, MOR were located at the plasma membrane, while morphine (MS) and DAMGO (DG) induced significant receptor redistribution visualized as puncta staining. Size bar 10 μm.

with surface clustering and / or internalization of receptors (Fig.1, middle panel). A closely similar punctate pattern of MOR immunoreactivity was observed in cultures incubated in the presence of morphine (10 μM) for 30 minutes (Fig.1, right panel). Similar results were observed in the majority (>70%) of neurons in which endogenous MOR immunoreactivity was detected.

Morphine-dependent redistribution of MOR is mediated by rapid endocytosis

To specifically examine redistribution of MOR present in the plasma membrane, we utilized a previously described FLAG epitope-tagged version of MOR1 that allows selective labeling of recombinant receptors accessible to antibodies at the cell surface. FLAG-MOR labeled by monoclonal antibody in non-permeabilized cells was distributed over the plasma membrane of transfected neurons (Fig. 2A, left panel), consistent with the appearance of endogenous immunoreactivity in permeabilized cells (Fig. 1). DAMGO induced a rapid redistribution of surface-labeled MOR from a relatively smooth distribution to a punctate localization throughout the neurons, consistent with DAMGO-induced redistribution of MOR observed previously in other neurons (Fig. 2A, middle panel and insets). Morphine also induced a rapid and pronounced redistribution of surface-labeled FLAG-MOR (Fig. 2A, right panel), consistent with the endogenous localization data (Fig. 1) but contrasting with its failure to drive detectable receptor redistribution in several other cell types. The reproducibility of these effects was confirmed by quantification of the percentage of transfected neurons in which punctate localization of FLAG-MOR was observed (numbers below panel A).

Punctate redistribution of surface-labeled MOR could reflect surface clustering of opioid receptors and/or their endocytosis. To specifically determine if morphine promotes rapid endocytosis of MOR in these neurons, residual antibody-labeled receptors remaining in the plasma membrane were selectively "stripped" by chelating extracellular calcium after incubation in the absence or presence of agonist. In cells incubated in the absence of opioid agonist, nearly all of the anti-FLAG monoclonal was dissociated by the EDTA strip, consistent with receptors remaining predominantly in the plasma membrane in the absence of agonist activation (Fig. 2B, left panel). Within 30 minutes after addition of 10 μM DAMGO to the culture medium, punctate FLAG immunoreactivity was readily apparent and was resistant to EDTA strip (Fig. 2B, middle panel). A similar pool of "protected" receptors was observed after acute morphine exposure (10 μM, 30 min) (Fig. 2B, right panel). Internalized FLAG-MOR observed in morphine-exposed neurons overlapped significantly with endocytosed transferrin, a marker of early and recycling endosomes (Maxfield and McGraw, 2004) (Fig. 2C, yellow represent colocalization). In contrast there was no detectable colocalization with the lysosome marker LAMP1 (Fig. 2D). Similar results were observed following DAMGO-induced internalization of FLAG-MOR (data not shown), suggesting that both morphine and DAMGO induce rapid endocytosis of FLAG-MOR primarily to early and / or recycling endosomes under these conditions.

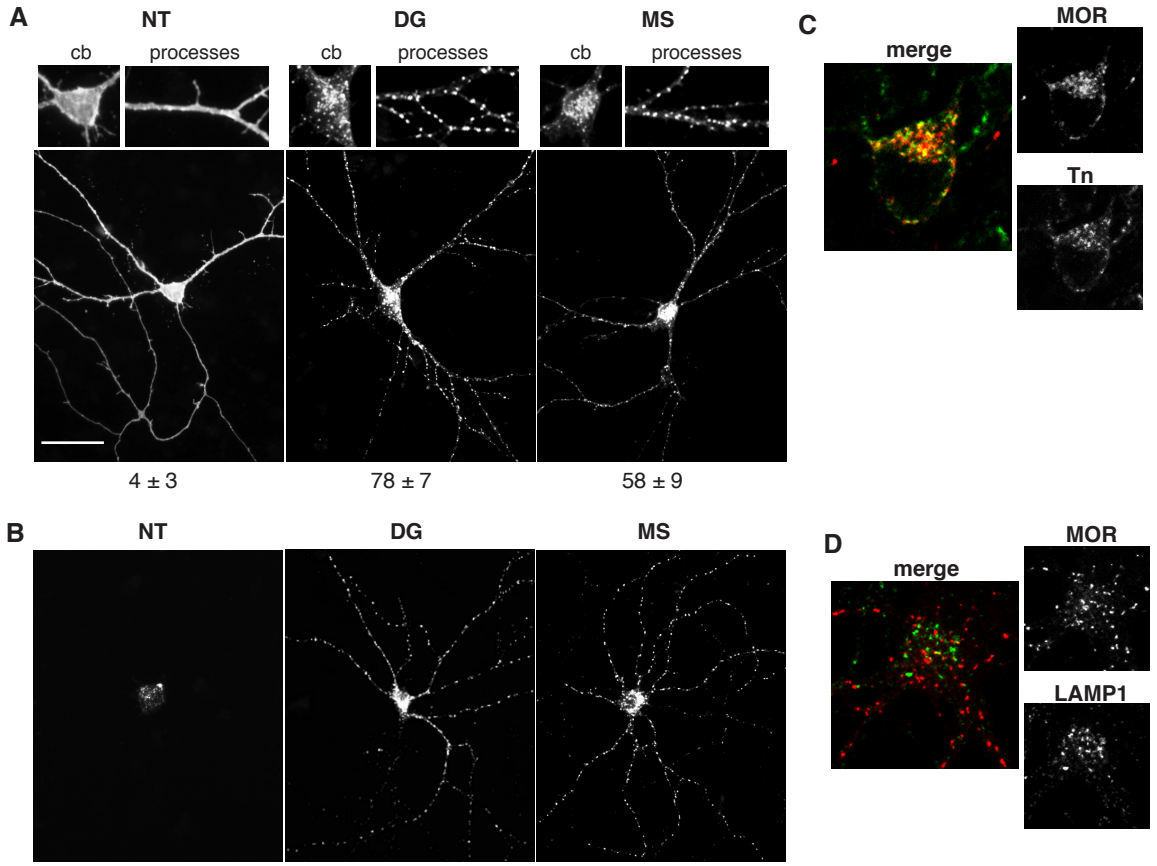

Figure 2. Both morphine and DAMGO induce rapid internalization of MOR in striatal neurons. Panel A: FLAG-MOR present in the plasma membrane of transfected striatal neurons (Effectene) was specifically labeled in intact cells using M1 antibody. Panel A shows representative epifluorescence images of surface-labeled MOR obtained after 30 minutes incubation in the absence of agonist (NT), in the presence of 10 μM DAMGO (DG), or in the presence of 10 μM morphine (MS). Insets above images show typical examples of MOR localization in cell bodies (cb) and processes under the indicated conditions. Both DAMGO and morphine produced substantial redistribution of surface-labeled MOR. Panel B: FLAG-MOR was surface-labeled and treated with agonists as in Panel A. Antibody bound to receptors remaining in the plasma membrane was stripped (after the 30 minute incubation period) using an EDTA wash, so that only internalized receptors were visualized. Little internalized MOR was observed in the untreated condition, whereas both DAMGO and morphine produced significant internalization of FLAG-MOR. Panel C: Striatal neurons expressing FLAG-MOR were briefly serum-starved, then fed with Alexa488-transferrin (Tn) and Alexa594-M1 antibody to label transferrin receptor and FLAG-MOR, respectively, prior to treatment with morphine (30 min, 10 mM). Representative examples of merged and unmerged confocal images are shown; FLAG-MOR (red) and transferrin (green). Yellow indicates colocalization. Panel D: Striatal neurons expressing FLAG-MOR were fed with Alexa594-M1 antibody to label the surface MOR prior to treatment with agonist morphine (30 min, 10 μM). Cells were fixed and stained with mouse anti-LAMP1 antibody. An Alexa488-conjugated goat anti-mouse IgG1 antibody was used to visualize LAMP1. Representative merged and unmerged images are shown; FLAG-MOR (red) and LAMP1 (green). Absence of yellow indicates no colocalization. Size bar 20 μm.

Morphine-induced endocytosis of MOR is a direct and specific consequence of opioid receptor activation in receptor-expressing neurons

The opiate antagonist naloxone (10 μM) did not promote MOR endocytosis by itself and completely blocked the effects on MOR localization of both DAMGO and morphine (Fig. 3A), confirming that endocytosis induced by both agonists is specifically mediated by opioid receptor activation. A concern in the previous *in vivo* studies (Haberstock-Debic et al., 2003) was that morphine-induced redistribution of receptors could represent an indirect opioid effect via endogenous neuropeptide release. Indeed, a significant fraction of neurons in the Nucleus Accumbens contain vesicular pools of enkephalin, which is a strongly promotes MOR endocytosis in cultured cells (Keith et al., 1998) and is released in response to neuronal depolarization (Shiomi et al., 1981). To investigate this possibility in striatal cultures, specimens were preincubated for 15 min with a high concentration (2μM) of the sodium channel blocker tetrodotoxin (TTX) to prevent neuronal depolarization. The indicated opioid ligand was then added in the continuous presence of TTX for an additional 30 min. Rapid redistribution of FLAG-MOR, both in response to DAMGO and morphine, was clearly evident in the presence of TTX (Fig. 3B). Together, these results suggest that rapid endocytosis of MOR in striatal neurons can occur as a direct and specific consequence of receptor activation by morphine.

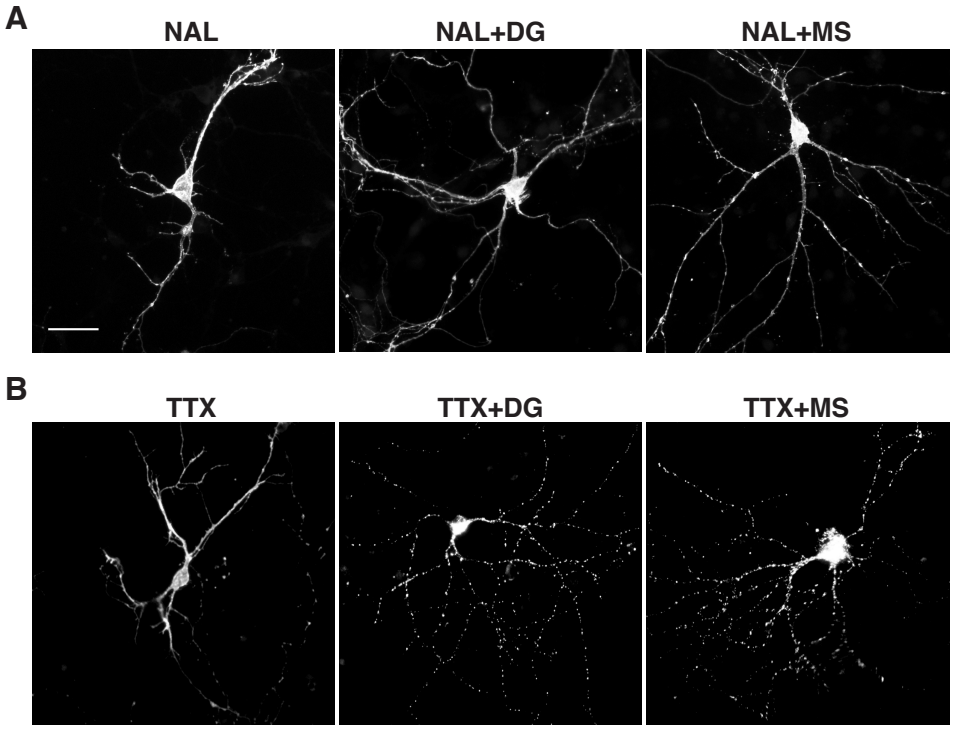

Figure 3. Morphine and DAMGO-induced endocytosis of MOR requires receptor activation but not depolarization of neurons. Panel A: Transfected striatal neurons (Effectene) were surface-labeled for FLAG-MOR, pretreated for 15 minutes with the opiate antagonist naloxone (10 μM), and then incubated for an additional 30 minutes with naloxone only (NAL), naloxone together with 10 μM DAMGO (NAL + DG) or naloxone together with 10 μM morphine (NAL + MS). Naloxone alone did not produce detectable redistribution of surface-labeled FLAG-MOR, and inhibited receptor redistribution induced by both DAMGO and morphine. Panel B: Transfected striatal neurons were surface-labeled for FLAG-MOR, pretreated for 15 minutes with 2 μM tetrodotoxin, and then incubated for an additional 30 minutes with tetrodotoxin alone (TTX), tetrodotoxin together with 10 μM DAMGO (TTX + DG) or tetrodotoxin together with 10 μM morphine (TTX + MS). Tetrodotoxin did not produce detectable redistribution of surface-labeled MOR and did not prevent receptor redistribution induced by either DAMGO or morphine. Size bar 20 μm.

Morphine and DAMGO induce rapid internalization of MOR in all neuronal compartments except proximal axons

In order to quantify the amount of FLAG-MOR internalization produced in striatal neurons, cultures were transfected using cationic lipid and surface-localized FLAG-MOR was specifically labeled with Alexa594-conjugated M1 monoclonal antibody, incubations were carried out in the absence or presence of the indicated opioid for 30 min, and the calcium-dependence of M1 binding was used to selectively dissociate ("strip") Alexa594-M1 conjugate from receptors remaining in the plasma membrane. Cells were then fixed and surface-accessible receptors were labeled with Alexa488-conjugated M1 monoclonal antibody. These experiments confirmed the ability of both DAMGO and morphine to induce rapid internalization of FLAG-MOR, as indicated by the pronounced increase of red (endocytosed) FLAG-MOR relative to surface (green) FLAG-MOR observed within 30 min after addition of either agonist to the culture medium (Fig. 4A-C). Quantification of fractional receptor internalization in multiple, randomly selected neurons was then carried out using fluorescence ratio imaging (see Materials and Methods). These experiments confirmed the reproducibility of the morphine effect, and indicated that morphine can drive significant rapid endocytosis of FLAG-MOR in striatal neurons (Fig. 4D).

While pronounced morphine-induced internalization of FLAG-MOR was observed in the vast majority of transfected striatal neurons, there was potential concern about sample bias because in our hands the cationic lipid method results in transfection of only a small fraction (<1%) of cultured neurons. Furthermore, while we and others have extensively

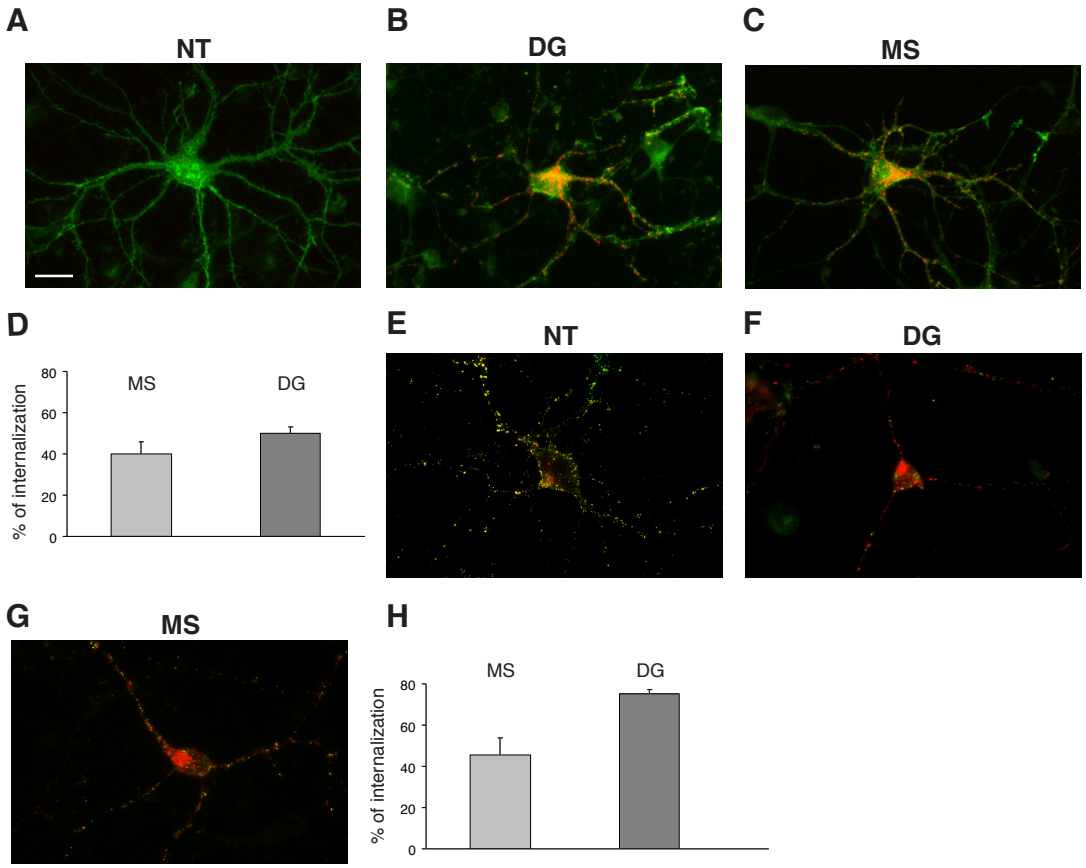

Figure 4. Quantification of DAMGO and morphine-induced internalization using dual color ratiometric staining. Panels A - D: FLAG-MOR transfected striatal neurons (Effectene) were first surface-labeled for FLAG-MOR with Alexa594-M1 antibody, then incubated for 30 min in the absence of agonist (NT), presence of 10 μM DAMGO (DG) or presence of 10 μM morphine (MS). Alexa594-M1 antibody was stripped from the residual surface FLAG-MOR using EDTA wash and cells surface receptors were then labeled under non-permeabilized conditions with Alexa488-M1. This resulted in selective labeling of internalized (red) and surface (green) FLAG-MOR. Representative images from the indicated conditions are presented in panels A-C, respectively. Quantitative fluorescence microscopy was used to calculate the percentage of FLAG-MOR internalization produced by DAMGO or morphine, as described in Materials and Methods (panel D). Bars represent mean internalization determined from 20-30 cell bodies selected at random in the neuronal cultures, averaged over four independent cultures (each prepared from a separate litter). Error bars represent the standard deviation across the determinations. Panels E - H FLAG-MOR transfected striatal neurons (Nucleofector) were surface labeled for FLAG-MOR with Alexa594-M1 antibody, then incubated for 30 min without agonist (NT) or with 10 μM DAMGO or 10 μM morphine. Cells were fixed and under nonpermeabilized conditions, then residual surface receptors were labeled with Alexa488 donkey-anti mouse secondary antibody. Representative images are presented in panels E-G. Quantitative fluorescence microscopy was used to calculate percentage of FLAG-MOR internalization as described in Materials and Methods. Bars represent mean internalization determined from 50 cell bodies, averaged over two independent cultures prepared from different litters. Error bars represent the standard deviation across individual determinations. Size bar 5 μm.

utilized the calcium dependence of the M1 antibody for surface "stripping" in various cell types, there was still some concern about possible confounding effects of this manipulation on receptor trafficking when applied to striatal neurons. To address these concerns we devised a second approach to quantify FLAG-MOR internalization using electroporation, which in our hands transfects a relatively large proportion (20 – 30%) of neurons in the culture preparation, and combined this with an immunochemical labeling method that does not require calcium depletion of the culture medium. In this method, surface-accessible FLAG-MOR was specifically labeled with Alexa594-M1 conjugate, and ligand incubations were carried out as described above. Instead of "stripping" residual labeled surface receptors after ligand incubation, neurons were fixed under non-permeabilizing conditions and residual surface receptors (labeled already with Alexa594-M1) were selectively detected using an Alexa488-conjugated anti-mouse secondary antibody. Dual color fluorescence imaging was then conducted to visualize internalized (red) and surface (red + green) FLAG-MOR. Representative images of control (NT), morphine (MS) and DAMGO (DG) treated conditions are shown (Fig. 4E –G). Quantification of these data by ratiometric analysis (see Materials and Methods) confirmed the ability of morphine to induce pronounced rapid internalization of FLAG-MOR, although in these experiments morphine did so to a somewhat smaller degree than DAMGO (Fig. 4H).

In the previous *in vivo* study (Haberstock-Debic et al., 2003), morphine was observed to induce a rapid redistribution of MOR selectively in dendrites relative to the cell body and axon. However, in the cultured striatal neurons fluorescence ratio imaging suggested that

morphine produced significant internalization of FLAG-MOR in cell bodies as well as in multiple processes extending from the cell body (Fig. 4B and C). To investigate this observation further, dual label studies were performed using MAP2 as a somatodendritic marker (Fig. 5). As expected FLAG-MOR was localized in a relatively smooth distribution over the entire surface of neurons incubated in the absence of opioid agonist (Fig. 5A). Following exposure to 10 μM morphine for 30 minutes, FLAG-MOR was localized in a punctate pattern indicative of endocytosed receptors in MAP2-positive dendrites and the cell body (Fig. 5B). We also observed significant morphine-induced redistribution of FLAG-MOR in axons defined by lack of MAP2 immunoreactivity (Fig. 5B, details - arrow indicates a representative axon).

In neurons cultured at sufficiently low density, the axon was unambiguously identified by morphological criteria (and confirmed by lack of MAP2 co-staining, not shown). In these neurons, fluorescence ratio imaging confirmed that morphine produced a rapid accumulation of internalized FLAG-MOR in axons (Fig. 6A, arrow indicates axon of a representative neuron). However, internalized FLAG-MOR (Fig. 6A, left panel) was visualized preferentially in distal portions (d) of the axon but not in the proximal segment (p), even though surface FLAG-MOR (Fig. 6A, middle panel) was readily detected in the plasma membrane of the proximal axon. The selective internalization of FLAG-MOR in distal but not proximal axons contrasted with the visualization of internalized receptors throughout distal and proximal dendrites in the same neurons, as emphasized in the same neuron in the merged color image (Fig. 6A, right panel).

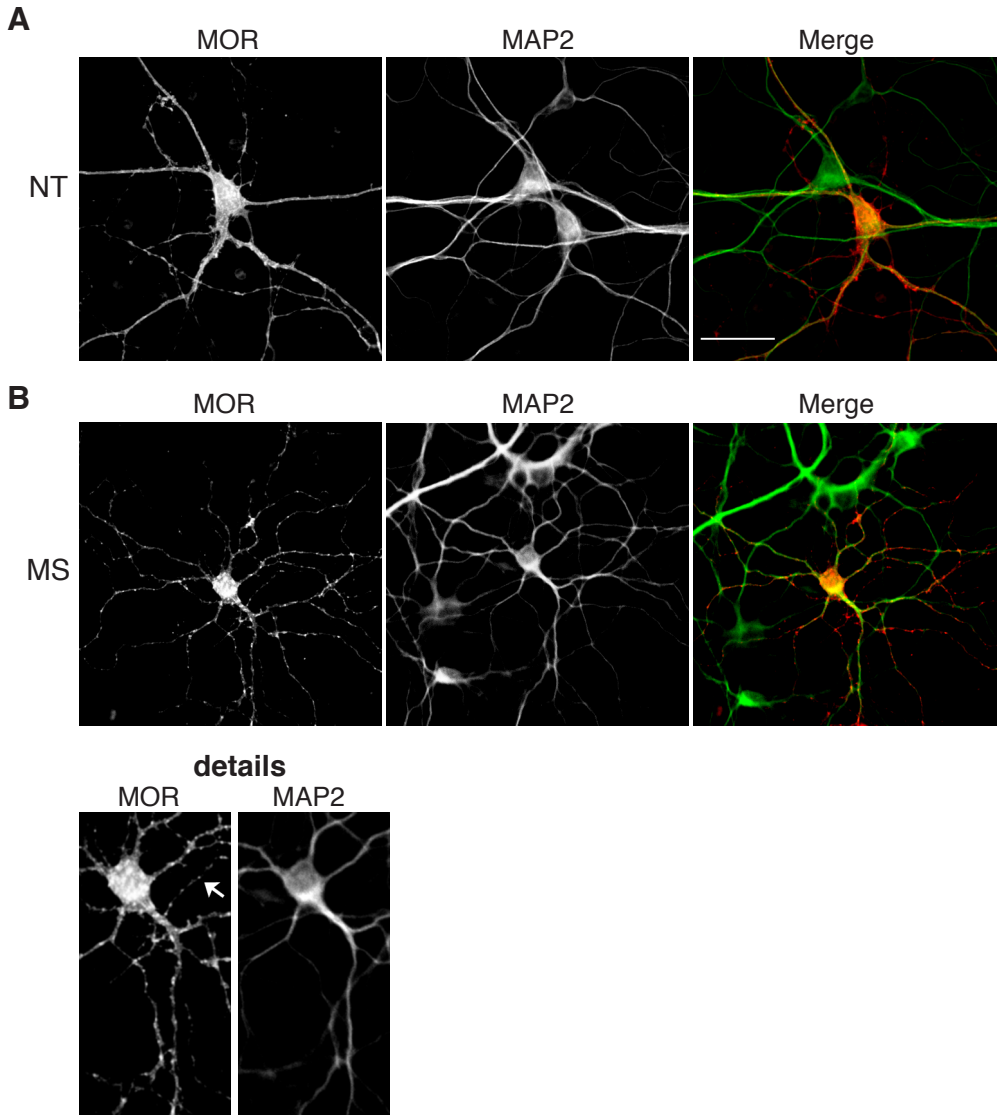

Figure 5. Morphine-induced internalization of MOR is observed both in the somatodendritic compartment and axon. FLAG-MOR transfected striatal neurons (Effectene) were fixed, permeabilized and double-stained with anti-FLAG rabbit polyclonal antibody to label receptors and an anti-MAP2 mouse monoclonal antibody to mark the somatodendritic compartment. A Cy3-conjugated goat anti-rabbit antibody was used to visualized MOR, and FITC-goat anti-mouse antibody to visualized MAP2. Panel A: Representative images from neurons incubated in the absence of agonist (NT). FLAG-MOR was observed in a smooth distribution consistent with plasma membrane localization in both axonal (MAP2 negative) and somatodentritic (MAP2 positive) compartments. Panel B: Representative images from neurons incubated for 30 minutes in the presence of 10 μM morphine (MS) before fixation. FLAG-MOR was observed in a punctate pattern consistent with internalized receptors in both MAP2 –positive and –negative compartments. The detail shows increased magnification emphasizing punctate FLAG-MOR in a representative axon (arrow). Size bar 20 μm.

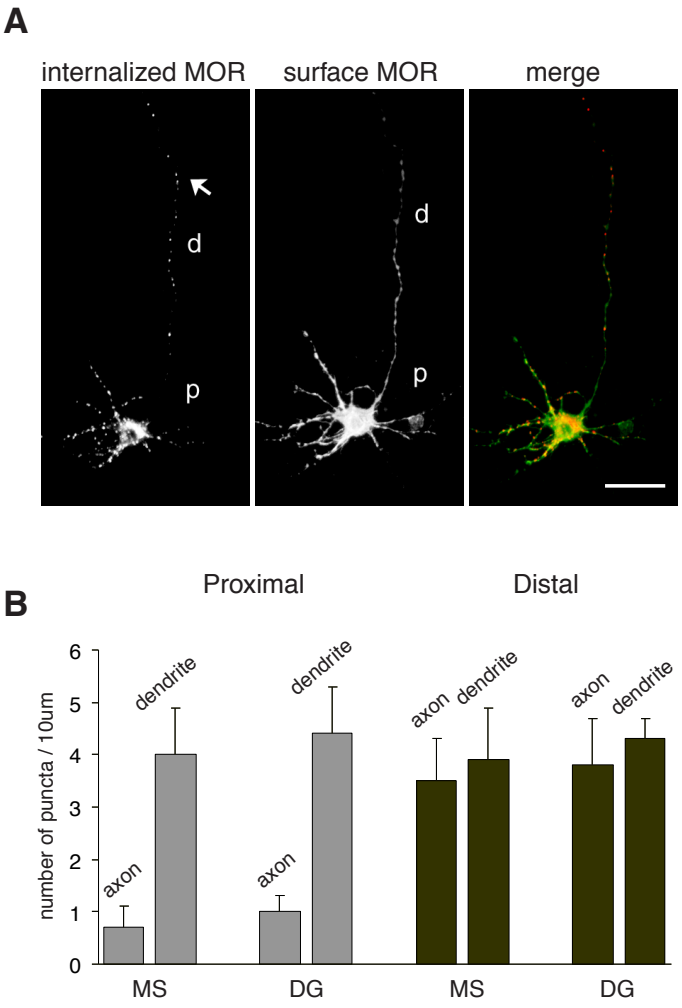

Figure 6. Reduced MOR internalization in the proximal axon. Transfected striatal neurons (Effectene) were labeled as in Figure 4 to detect internalized FLAG-MOR (Alexa594-M1) and surface FLAG-MOR (Alexa488-M1) after incubation of neurons for 30 minutes with 10 μM morphine (MS) or 10 μM DAMGO (DG). Panel A: Immunofluorescence images of a representative morphine-treated neuron (arrow indicates axon). Internalized MOR was found in the distal (d) but not proximal (p) part of the axon (left image). Surface MOR was observed in all compartments, including the proximal (p) and distal (d) axon (middle image). Size bar 10mm. Panel B: Quantification of density of internalized puncta in proximal and distal parts of axon and dendrites after MS and DAMGO treatment. The proximal portion was defined as 0-15 mm from cell body and the distal portion as 15-50 mm from the cell body. Measurements were done on 20 cells per culture preparation, and three culture preparations (representing three separate litters) were analyzed per condition. Bars represent mean number of puncta per 10 μm and error bars represent standard deviation.

To quantify these observations, and to compare the localization of internalized MOR produced by morphine relative to DAMGO, punctate structures containing endocytosed FLAG-MOR were counted in the axon and dendrites of multiple striatal neurons selected at random in the culture preparation. This analysis confirmed that little internalized FLAG-MOR was present in the proximal portion (defined as 0-15 μm from cell body) of the axon in morphine-treated cells, whereas internalized FLAG-MOR puncta were plentiful in proximal dendrites (Fig 6B, bars 1 and 2). In contrast, internalized FLAG-MOR puncta were plentiful in more distal portions (defined as 15 – 50 μm from the cell body) of both the axon and dendrites of the same neurons (bars 5 and 6). The same effect was observed with DAMGO (Fig. 6B bars 3 and 4; 7 and 8). Taken together, these results indicate that morphine can induce rapid endocytosis of MOR in all neuronal compartments, in contrast to the highly selective effect of morphine on dendritic MOR suggested in studies of receptor localization in the intact Nucleus Accumbens. These results also suggest that MOR present in the proximal segment of the axon is resistant to endocytosis following activation by either agonist.

Morphine-induced internalization of MOR, like internalization induced by DAMGO, is mediated by a beta-arrestin-dependent mechanism

Rapid endocytosis of opioid receptors induced by opioid peptides is mediated by a highly conserved mechanism, in which non-visual (or beta-) arrestins play a key role by linking activated receptors to clathrin-coated pits (Zhang et al., 1998; Whistler et al., 1998). To determine whether this mechanism functions in striatal neurons and is relevant to morphine-induced endocytosis observed in these cells, the ability of a dominant-negative

mutant version of arrestin-3 (beta-arrestin-2) to inhibit FLAG-MOR endocytosis was investigated. This mutant arrestin construct has been shown previously to disrupt arrestin-mediated endocytosis of various GPCRs (Krupnick et al., 1997). As expected, overexpression of HA-tagged mutant beta-arrestin-2 strongly inhibited DAMGO-induced endocytosis of FLAG-MOR in striatal neurons. This was indicated by the failure of surface-labeled FLAG-MOR to undergo detectable DAMGO-induced redistribution in either the cell body (cb) or processes (p) of striatal neurons expressing the mutant arrestin construct (Fig. 7A). In contrast, DAMGO-induced endocytosis of FLAG-MOR was readily detected in adjacent neurons not expressing mutant arrestin (Fig. 7B). However, overexpressing a GFP-tagged wild type beta-arrestin-2 at similar levels (confirmed by beta-arrestin immunoblot, not shown) did not detectably inhibit DAMGO-induced endocytosis of FLAG-MOR, supporting the biochemical specificity of the dominant-negative effect on DAMGO-induced endocytosis of FLAG-MOR (Fig. 7C).

Identical results were obtained when the effect of mutant arrestin expression on FLAG-MOR endocytosis induced by morphine was examined. Overexpression of dominant negative mutant beta-arrestin-2 strongly inhibited morphine-induced redistribution of surface-labeled FLAG-MOR in both the cell body and neuronal processes (Fig. 7D), whereas morphine-induced endocytosis was readily apparent in neurons not expressing mutant arrestin (Fig. 7E). Endocytosis was also observed in processes extending from non-transfected neurons that were in close apposition to those extending from transfected neurons (Fig. 7D, arrow). Furthermore, overexpression of wild type beta-arrestin-2 did not prevent morphine-induced endocytosis of MOR (Fig. 7F). The inhibitory effect of

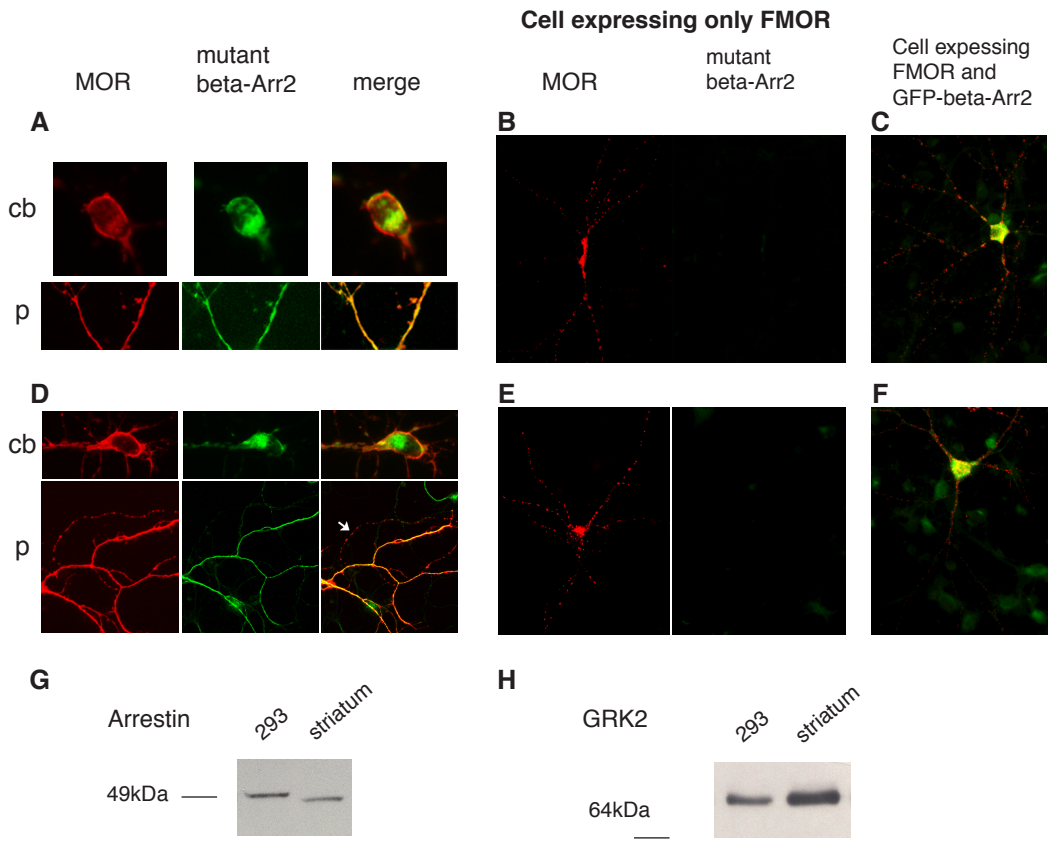

Figure 7. MOR internalization induced by both morphine and DAMGO is beta-arrestin-dependent. Striatal neurons were co-transfected (Effectene) with FLAG-MOR and HA-tagged mutant beta-arrestin-2. FLAG-MOR was surface labeled with M1 anti-FLAG antibody and neurons were treated with 10 μM DAMGO (DG) or 10 μM morphine (MS) for 30 minutes. Neurons were then stained in permeabilized condition with an anti-HA antibody, and bound M1 and HA.11 antibodies were visualized using Cy3-goat anti-mouse antibody and FITC-goat anti-rabbit antibody. Panels A and D show a representative neuron treated with DAMGO or morphine, respectively. Immunofluorescent images show FLAG-MOR (red) and mutant beta-arrestin-2 (green) localization in cell body (cb) and processes (p) in DAMGO (A) or morphine (D) treated conditions. Note that in cells expressing FLAG-MOR (red) and the HA-tagged dominant negative beta-arrestin2 (green) mutant, morphine or DAMGO-induced internalization was blocked as majority of FLAG-MOR is localized to the plasma membrane. An arrow (panel D) shows a process where MS-induced MOR redistribution and mutant arrestin was not expressed. Panels B and E: Representative immunofluorescent images show distribution of FLAG-MOR in cells not expressing the dominant negative beta-arrestin-2 mutant after DAMGO (B) or morphine (E) treatment for 30 minutes. Note that DAMGO or morphine induced MOR redistribution shown as puncta staining that is consistent with internalization. Panels C and F: Images represent distribution of FLAG-MOR and wild type GFP-beta-arrestin-2 in DAMGO (C) or morphine (F) treated neurons. Note that overexpression of wild type GFP -beta-arrestin2 did not influence DAMGO or morphine-induced internalization. Panel G: A representative immunoblot of equal amounts (40 mg cell protein) from HEK293 or striatal neuron cultures probed with the beta-arrestin-1/2 antibody. Panel H: A representative immunoblot from culture extracts probed with the GRK2 antibody.

dominant negative beta-arrestin mutant on ligand-induced endocytosis was significant and observed in all neurons coexpressing FLAG-MOR and dominant negative beta-arrestin (~40% of neurons expressing FLAG-MOR also expressed dominant negative beta arrestin). These results further confirm the importance of arrestins in promoting rapid endocytosis of MOR in response to opioid peptides, and they indicate that the same regulatory mechanism mediates morphine-induced endocytosis in striatal neurons.

The ability of morphine to promote rapid arrestin-depenent endocytosis of MOR in striatal neurons was surprising because morphine does not strongly promote this process in various other cell types, including non-neural HEK293 cells in which opioid effects on MOR trafficking have been studied in some detail. In this cell type it is possible to greatly enhance morphine-induced endocytosis either by over-expressing beta-arrestins or GRK2 (Zhang et al., 1998; Whistler et al., 1998). It has also been suggested that differences in cellular concentrations of these regulatory proteins may dictate the regulatory effects of opioid drugs *in vivo* (Bohn et al., 2004). Thus we considered the possibility that cell type-specific differences in arrestin and / or GRK expression may play a role. To begin to investigate this possibility, we used immunoblotting to compare relative levels of beta-arrestins in extracts prepared from striatal neurons and HEK293 cells. Immunoblotting for beta-arrestins was carried out using an antiserum raised against a sequence (residues 384 – 397) in beta-arrestin-2 (arrestin 3) that is conserved in beta-arrestin-1 (arrestin 2). When equal amounts of cellular extract were loaded and comparatively blotted, total beta-arrestin immunoreactivity detected in striatal neurons did not exceed, and in fact was slightly less than, beta-arrestin immunoreactivity detected

in HEK293 cells (Fig. 7G). However differences were observed in electrophoretic mobility of the major beta-arrestin species detected. The major immunoreactive band in HEK293 cells resolved at an apparent molecular mass of ~50 kDa, consistent with the electrophoretic mobility of beta-arrestin-1 (arrestin 2) (Attramadal et al., 1992). The major immunoreactive band detected in striatal lysates had a slightly increased electrophoretic mobility. With the available reagents we were unable to determine whether this species represents beta-arrestin-2 (arrestin 3) or phosphorylated beta-arrestin-1 (arrestin 2), both of which resolve at a slightly smaller apparent molecular mass than (unphosphorylated) beta-arrestin-1 (Attramadal et al., 1992; Lin et al., 1997). To investigate relative GRK levels we focused on GRK2, which is relatively abundant in both striatum (Erdtmann-Vourliotis et al., 2001) and HEK293 cells (Menard et al., 1997). Immunoblotting using an antiserum raised against a conserved portion of GRK2 (residues 468 – 689) detected a single species with indistinguishable electrophoretic mobility in both extracts. Comparative immunoblotting indicated that GRK2 levels detected in striatum were moderately elevated relative to those detected in HEK293 cells (Fig. 7H). This increase was highly reproducible across multiple culture preparations (n = 4) and was confirmed using a different GRK2 antibody (not shown).

DISCUSSION

The present results demonstrate that morphine promotes rapid redistribution of both endogenously expressed and recombinant MOR1 in striatal neurons, and that this process is mediated by rapid endocytosis of receptors. Using dissociated cell culture and tetrodotoxin to block depolarization, we were able to unambiguously establish that morphine-induced endocytosis is a primary (cell-autonomous) effect of morphine on target neurons, rather than a secondary effect of neural circuitry that could potentially mediate morphine-dependent endocytosis via release of endogenous opioid peptides in the intact CNS (Haberstock-Debic et al., 2003). Furthermore, we were able to demonstrate that both morphine- and DAMGO- induced endocytosis of MOR require beta-arrestins. This observation suggests a fundamental similarity between the regulatory effects of morphine and opioid peptides in a specific population of physiologically relevant CNS neurons.

Our results argue strongly that morphine-activated opioid receptors can be regulated by the GRK/arrestin system in a subset of neurons relevant to opiate responses *in vivo*. This contrasts with the greatly diminished effect of morphine on this regulatory process in heterologous cell models and in several other relevant neuronal populations, including neurons in cortex, gut and spinal cord (Keith et al., 1998; Sternini et al., 1996; Trafton et al., 2000). These observations may be useful for understanding the effects beta-arrestin and GRK mutations on morphine effects in intact animals (Bohn et al., 2000; Terman et

al., 2004), which have so far been difficult to reconcile with the available cell biological data.

An important question for future study is why morphine promotes rapid endocytosis of MOR in striatal neurons to a greater degree than in other cell types. A reasonable possibility, supported by studies of MOR endocytosis in non-neural cell models, is that relevant GRKs and/or beta-arrestins are expressed at particularly high levels in striatal neurons. Based on comparative immunoblot analysis we think that increased expression of total beta-arrestins is an unlikely explanation, as total beta-arrestin immunoreactivity was actually slightly higher in HEK293 cells than in cultured striatal neurons. However, the different electophoretic mobilities observed suggested that there may be qualitative differences in major arrestin species expressed in striatal neurons. One possibility is that the major arrestin expressed in striatal neurons is beta-arrestin-2 (arrestin 3), which has slightly higher electrophoretic mobility than beta-arrestin-1 (Attramadal et al., 1992). An alternative possibility is that this species represents a modified form of beta-arrestin-1, such as the phosphorylated beta-arrestin-1 species reported previously to have increased electrophoretic mobility (Lin et al., 1997). With presently available reagents, we were unable to determine if the observed results reflect preferential expression of beta-arrestin-2 (arrestin 3) in striatal neurons, or a difference in post-translational modification. We presently favor the latter hypothesis, as beta-arrestin-2 appears to be the major arrestin isoform expressed in several brain regions including striatum (Gurevich et al., 2002). Either hypothesis could be functionally significant, as distinct beta-arrestin isoforms have been shown to differ in their ability to promote GPCR endocytosis in heterologous cell

models (Santini et al., 2000) and modification of beta-arrestin-1, both by phosphorylation (Lin et al., 1997) and ubiquitination (Shenoy et al. 2001), affects its endocytic activity. Another possibility is that cell type-specific differences in GRK abundance could play a role in distinguishing MOR regulation in striatal neurons. The GRK isoform that is most effective in mediating morphine-induced desensitization of MOR *in vitro*, GRK3 (Kovoor et al., 1998), is not expressed at detectable levels in striatum (Erdtmann-Vourliotis et al., 2001). However GRK2 is quite abundant in striatum and is capable of promoting morphine-induced desensitization and endocytosis of MOR when overexpressed in HEK293 cells (Zhang et al., 1998). Our immunoblot data indicates that GRK2 is indeed expressed in striatal neurons at higher levels than in HEK293 cells. A potential concern is that GRK2 appears to be expressed at even higher levels in some other brain regions, such as cortex (Erdtmann-Vourliotis et al., 2001), where morphine has not been observed previously to promote rapid endocytosis of MOR (e.g., Keith et al. 1998). Furthermore, our efforts thus far to knock down GRK2 expression in striatal neurons have not revealed a selective effect on morphine-induced endocytosis of MOR (data not shown). Thus further study will be required to define the possible importance of quantitative or qualitative differences in GRK and/or arrestin expression in determining cell type-specific differences in morphine-induced internalization of MOR. It is also conceivable that there are additional protein(s) controlling ligand-specific endocytosis of MOR in neurons, which have not yet been anticipated from studies of non-neural cell models.

Another interesting question for future study is why morphine induces redistribution of MOR selectively in dendrites in the intact Nucleus Accumbens but appears to do so in all membrane domains (except proximal axons) in cultured striatal neurons. It is possible that the GRK / arrestin regulatory machinery is organized into sub-membrane domains in the intact tissue and that this organization is disrupted in dissociated cell culture. It is also conceivable that there are additional features of neuronal organization (perhaps involving receptor regulatory proteins in addition to GRKs and arrestins) that contribute to the specificity of receptor regulation occurring *in vivo*. Thus, defining the relative importance of GRK expression patterns in CNS neurons, and identifying possible additional protein(s) that regulate opioid receptor endocytosis in specific neurons or neuronal membrane sub-domains, is an important direction for further examinations. While the present results focus exclusively on acute regulatory effects and suggest that acutely internalized receptors are localized to early / and or recycling endosomes, it will be interesting in future studies to extend the analysis to more prolonged actions of opioids. For example chronic morphine, but not etorphine, increases total mu-opioid receptor binding in several regions of rat brain including the striatum (Brady et al., 1989; Reddy et al., 1994; Tao et al., 1987). This suggests that additional mechanisms of regulation (such as control of receptor biosynthesis) may play a major role in chronic settings, and emphasizes the potential importance of future studies to determine how MOR is sorted at later times after endocytosis.

The present results provide a fundamental advance in our understanding of opiate drug action by establishing that morphine can promote rapid endocytosis of MOR, and do so

by a similar mechanism as opioid peptides and in a physiologically relevant population of CNS neurons. These results revise our previous view of morphine simply as a "non-internalizing" ligand in the nervous system and suggest that cell type-specific differences in receptor regulation may contribute to determining the physiological actions of opiate drugs *in vivo*.

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CHAPTER 3: NEUROKININ1 RECEPTORS REGULATE MORPHINE-INDUCED ENDOCYTOSIS AND DESENSITIZATION OF MU OPIOID RECEPTORS IN CNS NEURONS

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ABSTRACT

Mu opioid receptors (MORs) are G protein-coupled receptors (GPCRs) that mediate the physiological effects of endogenous opioid neuropeptides and opiate drugs such as morphine. MORs are co-expressed with neurokinin 1 receptors (NK1Rs) in several regions of the central nervous system (CNS) that control opioid dependence and reward. NK1R activation affects opioid reward specifically, however, and the cellular basis for this specificity is unknown. We found that ligand-induced activation of NK1Rs produces a cell autonomous and non-reciprocal inhibition of MOR endocytosis induced by diverse opioids. Studies using epitope-tagged receptors expressed in cultured striatal neurons and a neuroblastoma cell model indicated that this heterologous regulation is mediated by NK1R-dependent sequestration of arrestins on endosome membranes. First, endocytic inhibition mediated by wild type NK1Rs was overcome in cells over-expressing β-arrestin2, a major arrestin isoform expressed in striatum. Second, NK1R activation promoted sequestration of β-arrestin2 on endosomes, whereas MOR activation did not.

Third, heterologous inhibition of MOR endocytosis was prevented by mutational disruption of β-arrestin2 sequestration by NK1Rs. NK1R-mediated regulation of MOR trafficking was associated with reduced opioid-induced desensitization of adenylyl cyclase signaling in striatal neurons. Further, heterologous regulation of MOR trafficking was observed in both amygdala and locus coeruleus neurons that naturally co-express these receptors. These results identify a cell autonomous mechanism that may underlie the highly specific effects of NK1R on opioid signaling and suggest, more generally, that receptor-specific trafficking of arrestins may represent a fundamental mechanism for coordinating distinct GPCR-mediated signals at the level of individual CNS neurons.

INTRODUCTION

G-protein coupled receptors (GPCRs) mediate a wide variety of physiological functions and are the targets of a vast array of both therapeutic and abused drugs. Opioid receptors comprise a subfamily of GPCRs that are activated both by endogenously produced opioid neuropeptides and exogenous alkaloid drugs such as morphine (Evans 2000). The mu opioid receptor (MOR) is the primary target mediating analgesic, euphoric, and reinforcing effects of morphine (Matthes et al., 1996). After exposure to opioid peptides, MOR is phosphorylated and associates with β-arrestin (also called non-visual arrestin), which modulates opioid signaling and functions as an adaptor protein to promote MOR endocytosis via clathrin-coated pits (Keith et al., 1996; Whistler and von Zastrow, 1998; Zhang et al., 1998; Oakley et al., 1999). This series of events is thought to contribute fundamentally to controlling MOR-mediated signaling under conditions of prolonged or repeated ligand exposure (Koch et al., 1998; Qiu et al., 2003). While morphine promotes relatively little regulated endocytosis of MOR compared to opioid peptides in several cell models, it does strongly produce endocytosis in striatal neurons, a brain region important for reward processing (Heimer et al., 1982; Keith et al 1998; Whistler et al., 1999; Bushell et al., 2002; Haberstock-Debic et al., 2003; Trafton and Basbaum, 2004; Haberstock-Debic et al., 2005). Therefore, regulated endocytosis of MOR may play an important role in the actions of endogenous MOR in response to both peptides and drugs such as morphine.

Whereas MORs are major direct targets receptors of opiate drug action, various other GPCRs modulate opioid function in vivo. Of these, the neurokinin 1 receptor (NK1R) has been found to modulate MOR-dependent responses with a remarkably high degree of specificity. Mice lacking NK1Rs are insensitive to the rewarding properties of morphine, while the rewarding effects of food and cocaine are preserved (Murtra et al., 2000; Ripley et al., 2002). The antinociceptive effects of morphine remain intact in NK1R knock-out animals, further supporting the specificity of NK1R function in modulating opiate reward (De Felipe et al, 1998). Moreover, MORs and NK1Rs are highly expressed in several brain regions relevant to reward processing, including the ventral striatum and amygdala (Pickel et al., 2000; Gadd et al., 2003; Nakaya et al., 2004; Jabourian et al., 2005; Poulin et al., 2006).

Given the known physiological interaction between the neurokinin and opioid neurochemical systems, and the co-expression of NK1R and MOR in relevant brain regions, we considered the possibility that these distinct receptor systems might interact functionally at the level of individual neurons. In the present study, we investigated this hypothesis in several cell populations, using both recombinant and natively expressed receptors. Our results identify a cell-autonomous mechanism by which NK1Rs specifically modulate MOR trafficking and signaling, and also suggest a more general principle of heterologous regulation of diverse GPCRs in the CNS.

MATERIALS AND METHODS

Plasmid DNA and mutagenesis

The amino-terminally FLAG (DYKDDDD) epitope-tagged murine mu opioid receptor (F-MOR) was described previously (Keith et al., 1996). The rat NK1 neurokinin receptor (cDNA generously provided by Dr. Nigel Bunnett, University of California, San Francisco, San Francisco, CA) was tagged in its amino-terminal extracellular domain with an HA (YPYDVPDYA) epitope-tag using PCR (HA-NK1R). Truncation at residue 355 was accomplished by introducing a stop codon using PCR (HA-NK1-355x). Tagged receptor constructs were cloned into pCAGGS (Niwa et al., 1991) for expression in cultured neurons, and pcDNA3.1 (Invitrogen) for expression in neuroblastoma 2A cells (N2A). A bovine arrestin 3 (β-arrestin2) construct, tagged with EGFP as described previously (Mundell and Benovic, 2000), was generously provided by Dr. J. Benovic (Thomas Jefferson University). All constructs were confirmed by DNA sequencing (Elim Biopharmaceuticals, San Francisco, CA).

Cell cultures and transfections

Primary striatal neurons and primary amygdala neurons were dissected from embryonic day 17-18 rat embryos taken from pregnant Sprague-Dawley rats. The striatum (caudate-putamen and nucleus accumbens) were dissected based on the criteria of Ventimiglia and Linsday (1998). The amygdala was identified as described by Altman and Bayer (1995). Dissected tissue was dissociated in 1x trypsin/ EDTA solution (Invitrogen, Gaithersburg, MD) for 15 minutes before 1mL of trypsin inhibitor was added for 5 minutes at room

temperature. Cells were washed and triturated in DMEM plus 10% fetal calf serum (FCS; Invitrogen). Mechanical trituration of tissue was performed using a glass pipette. Neuronal transfections were performed using electroporation (rat neuron nucleofector system; Amaxa Biosystems, Gaithersburg, MD) immediately after dissociation and plated on poly-L-lysine-coated (1mg/mL in 0.1 M sodium borate buffer, pH 8.5) glass coverslips washed previously in 70% nitric acid and rinsed over 2-3 days. Electroporation was conducted using 5 x 10^6 dissociated cells, 3 μg of plasmid DNA, and 100 μL of rat neuron nucleofector solution. After transfection, neurons were kept in pre-warmed RPMI media (Cell culture facility, University of California San Francisco, San Francisco, CA) for 10 minutes for recovery before being transferred onto 24-well plates. Media on the cells was replaced with Gibco Neurobasal media (Invitrogen) supplemented with B27 (Gibco) and L-glutamine 24 hours post transfection. Neurons were maintained 7-10 days in culture before assaying.

Neuroblastoma 2A cells were obtained from University of San Francisco cell culture facility and maintained in Gibco DMEM (Invitrogen) media supplemented with 10% FCS and penicillin/streptomycin. Cells were plated on poly-D-lysine coated (50μg/mL in water) glass coverslips onto 24-well plates for immunocytochemistry and 6 cm dishes for biotinylation assays. Transfections were performed using a cationic lipid transfection reagent (Effectene; Qiagen, Hilden, Germany). Cells were transfected when they were at 50% confluency and assayed 24 hours post transfection.

Immunocytochemistry and quantitative analysis of FLAG-MOR internalization. F-MOR and HA-NK1R distribution was visualized after dual immunocytochemical labeling with antibodies recognizing the distinct epitope tags. Rabbit anti-FLAG polyclonal antibody (0.2μg/mL; Sigma, St. Louis, MO) and mouse anti-HA (5μg/mL; Covance) antibodies were added to the culture medium of transfected cells, and surface labeling was carried out for 30 minutes before an additional 30 minute incubation in the presence or absence of the indicated ligand(s). After quick washes with either TBS (for neurons) or PBS (for N2A cells), cells were fixed with 4% paraformaldehyde dissolved in Ca^{2+}-free PBS supplemented with 5% sucrose for 15 minutes. Specimens were permeabilized and blocked for 20 minutes in a solution containing either 0.1% saponin (for neurons) or 0.1% Triton X-100 (for N2A cells), and 2% BSA dissolved in the appropriate buffered saline. Secondary labeling was carried out using Alexa594 goat anti-rabbit IgG (for F-MOR) and Alexa488 donkey anti-mouse IgG (for HA-NK1R) antibodies (2μg/mL; Molecular Probes, Invitrogen), prepared in the same blocking solution and incubated for 30 minutes. Cells were then washed extensively with the appropriate buffered saline and mounted onto glass slides for fluorescence microscopy. Epifluorescence microscopy was carried out using a Nikon (Melville, NY) Diaphot microscope equipped with a 60x/numerical aperture (NA) 1.4 objective, mercury arc lamp illumination and standard dichroic filter sets (Omega Optical, Brattleboro, VT). Images were collected with a CCD camera (Princeton Instruments, Trenton, NJ) and analyzed using MetaMorph® software (Molecular Devices, Sunnyvale, CA). Confocal microscopy was carried out with a Zeiss (Oberkochen, Germany) LSM510 instrument, equipped with a 63x/NA 1.4 objective and using a pinhole diameter of 1 Airy disc.

To quantify ligand effects observed with epifluorescence microscopy, we used a previously described assay to assess ratiometric staining of surface and internalized receptors (Haberstock-Debic et al., 2005). In brief, cells were incubated with Alexa594 (Molecular Probes)-conjugated M1 anti-FLAG monoclonal antibody (5μg/mL; Sigma, St. Louis, MO) for 30 minutes (to selectively label F-MOR present in the plasma membrane) and then incubated in the absence or presence of the indicated ligand(s) for an additional 30 minutes. Cells were fixed and incubated with Alexa488-conjugated donkey anti-mouse IgG (Molecular Probes) for 30 minutes under nonpermeabilized conditions (2% BSA in TBS) to selectively detect only those labeled F-MORs remaining at the plasma membrane. Therefore, internalized receptors were labeled with only Alexa594, while surface F-MORs were labeled with both Alexa594 and Alexa488. The percentage of receptor internalization induced by the indicated agonist exposure was calculated from background-subtracted Alexa594 (red) and Alexa488 (green) fluorescence intensity values according to the following formula: % internalized = [1-(green/red ratio in agonist treated)/(green/red ratio in untreated)] x 100. For each experiment, 50 neuronal cell bodies or N2A cells were analyzed per condition. Results were compiled from five experiments for each cell type (and four for the N2A truncated HA-NK1R experiments). For experiments in primary cultures, these represented separate animals and culture preparations. Statistical analysis of differences between experimental groups was performed using unpaired Student's *t*-test.

Colocalization of receptors with β-arrestin2-EGFP. Colocalization of HA-NK1R and HA-NK1 355x with β-arrestin2-EGFP (in both localization and over-expression

experiments) was obtained through a similar staining protocol as described above, except that rat anti-HA (5μg/mL; Roche) was used as primary antibody and Alexa594 conjugated anti-rat IgG (Molecular Probes) was used as secondary antibody. Triple localization of F-MOR, HA-NK1R, and β-arrestin2-EGFP was carried out using Alexa594 conjugated to M1 anti-FLAG and rat anti-HA primary antibodies, and HA-NK1R was visualized with Alexa647 goat anti-rat secondary antibody (Molecular Probes). Cells were imaged using the same confocal microscopy settings as described above. For colocalization experiments, control imaging of single-labeled cells was performed to confirm lack of detectable bleed through between channels.

Immunocytochemistry analysis of endogenous receptors. For immunocytochemical localization of endogenous receptors, cultured amygdala neurons (grown ~7 days in culture on poly-L-lysine coated glass coverslips) were incubated with 10μM of both DAMGO and substance P for 30 minutes followed by a 10 minute fixation in ice-cold methanol. Cells were blocked in PBS containing 5% goat serum, 0.3% Tween-20, and 0.1% Triton X-100 in PBS for 45 minutes. Staining for endogenous MOR and NK1R was performed using an affinity-purified rabbit antibody recognizing the carboxyl terminus of MOR1 (Keith et al, 1998) and a guinea pig receptor antibody recognizing the distinct carboxyl-terminus of NK1 (1:5000; AB15810, Millipore), respectively. Permeabilized specimens were co-incubated with both antibodies at 25˚C for 2.5 hrs and then extensively washed in PBS after primary incubation. Redistribution of receptors was visualized after secondary labeling with Alexa594-conjugated goat anti-rabbit IgG (for F-MOR) and Alexa488-conjugated donkey anti-mouse IgG (for HA-NK1R) antibodies

(2μg/mL; Molecular Probes) prepared in the same blocking solution as above and incubated for 60 minutes.

Receptor internalization with surface biotinylation and immunoblotting

N2A cells were grown to 50% confluency on 6 cm dishes and transfected with F-MOR and HA-NK1R, or F-MOR and the truncated HA-NK1R (HA-NK1 355x). Transfected cells were washed with cold Ca^{2+}- and Mg^{2+}-free PBS and then incubated in 0.3mg/mL Sulfo-NHS-SS-biotin (Pierce) in PBS at 4°C for 20 minutes. Cells were washed with TBS and placed in DMEM medium for 10 minutes at 37°C prior to incubation for 30 minutes under the indicated conditions. Cells were then washed on ice with TBS, to remove and quench residual biotinylation reagent, and remaining cell surface biotinylated receptors were stripped at 4°C two times for 15 minutes each using 100mM MESNA (Sigma-Aldrich) in 50mM Tris 8.8, 100mM NaCl, 1mM EDTA, and 0.2% BSA. Stripped cells were then quenched with iodoacetamide buffer (22 mg/mL iodoacetamide in TBS) for 10 minutes at 4°C. Cells were extracted with lysis buffer containing 1mg/mL iodoacetamide in IP buffer (0.2% Triton X-100, 150mM NaCl, 25 mM KCl, 10 mM Tris 7.4) with 0.1mM EDTA and a Complete mini EDTA-free protease inhibitor cocktail tablet (1 tablet/10mL; Roche). Cell debris was removed by centrifugation at 13,000 x g for 15 minutes at 4°C. Extracts containing equal amounts of cellular protein (determined using Coomassie Plus protein assay from Pierce) were incubated with immobilized streptavidin beads (Pierce) on a rotator overnight at 4°C. Protein-bound streptavidin beads were centrifuged at 3,000 x g for 1 minute at 4°C and washed twice with IP buffer. Proteins were eluted and denatured in SDS sample buffer with 10% βmercaptoethanol

and separated by SDS/PAGE. Proteins were transferred to nitrocellulose and biotinylated proteins were detected by immunoblotting with anti-FLAG M1 mouse antibody (5μg/mL; Sigma) followed by anti-mouse IgG horseradish peroxidase conjugate (1:5000, Amersham) and chemiluminescence detection using ECL reagent (Amersham).

Determination of receptor signaling by measurement of cAMP accumulation

Dissociated striatal neurons were co-transfected with F-MOR and HA-NK1R and maintained for 7-10 days in culture before assaying. Prior to agonist treatment, neurons were incubated in media containing the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, Sigma) at a concentration of 1 mM. To assess acute signaling response, neurons were exposed for 3 minutes to 3μM forksolin and 1 mM IBMX, in the presence 10μM morphine, 10μM substance P, or both. To assess MOR desensitization, neurons were preincubated in the presence morphine, substance P or both for 30 minutes. The indicated agonists were then washed out and the ability of neurons to subsequently respond to opiate was assessed by rechallenge for 10 minutes with 100nM morphine in the presence of 3μM forskolin and 1 mM IBMX. After agonist incubations, cells were immediately washed once with ice cold PBS before lysing in 0.1M HCl with 0.1% Triton X-100. Lysates were collected and particulates removed by centrifugation 1000 x g for 5 minutes at room temperature. The concentration of cAMP present in clarified lysates was determined according to the non-acetylated version of the Correlate EIA™ Direct Cyclic AMP Enzyme Immunoassay Kit protocol (Assay Designs, Ann Arbor, MI). Acute signaling response was calculated from ligand-induced inhibition of forskolin-stimulated cAMP production relative to that measured in cells challenged with forskolin alone.

Desensitization was estimated by the decrease in morphine-induced inhibition of cAMP accumulation measured in the rechallenge compared to that measured in cells not previously exposed to opiate. For examination of substance P effects on desensitization, only experiments in which morphine-driven MOR desensitization was reliably detected in the absence of substance P were included in the analysis. In each individual experiment, all manipulations and determinations were carried out in triplicate and averaged. The number of independent experiments included in each comparison is indicated in the text. Statistical analyses of differences among experimental conditions were performed using unpaired *t*-test.

Receptor internalization in neurons from FLAG-MOR-Tg/+, MOR -/- mice

4-6 week old mice were anesthetized with isoflurane and the brain was removed and sliced horizontally (200 μm thickness) using a vibratome (Leica, Nussloch, Germany) in ice-cold artificial cerebro-spinal fluid (ACSF) containing the following (in mM): 126 NaCl, 2.5 KCl, 1.2 MgCl2, 1.2 NaH2PO4, 2.4 CaCl2, 21.4 NaHCO3 and 11 glucose as previously described (Arttamangkul et al., 2008). Slices including the locus coeruleus were allowed to warm up to 34˚C in oxygenated ACSF containing (+) MK-801 (10 μM, Sigma-Aldrich, St. Louis, MO) for 15 minutes and then incubated in a solution containing Alexa 594-conjugated M1 antibody. The tissue was visualized with an upright microscope (Olympus, Center Valley, PA.) equipped with a custom-built two-photon apparatus, as described previously (Arttamangkul et al., 2008). Data were acquired and collected using Scan Image Software (Pologruto et al., 2003). A z-series was collected at 1 μm intervals for 15 μm. Drugs were applied by perfusion. All

experiments were done at 35˚C. Analyses were done off-line using Image J (NIH) software as described in detail previously (Arttamangkul et al., 2008). For the control condition, integrated intensity data were obtained from labeled slices before drug application. This fluorescence intensity was defined as total surface-accessible receptor immunoreactivity (C). Integrated fluorescence intensity measured after drug perfusion, followed by calcium free ACSF containing ethylene glycol-bis(β-aminoethylether) N,N,N',N'-tetraacetic acid (0.5 mM EGTA, 10 min), was defined as internalized receptor immunoreactivity (I). Percentage of internalization was calculated by (I/C) x 100.

RESULTS

Morphine and substance P drive rapid redistribution of mu opioid and neurokinin 1 receptors, respectively, in dissociated striatal neurons

To begin to investigate possible regulatory interactions between mu opioid receptors and neurokinin 1 receptors, we examined the subcellular distribution of receptors when co-expressed in dissociated striatal neurons. Cultured striatal neurons were co-transfected with FLAG-tagged mu opioid receptors (F-MOR) and HA-tagged neurokinin 1 receptors (HA-NK1R), and the localization of each receptor was examined by dual-label epifluorescence microscopy. In the absence of ligand (untreated condition), antibody-labeled F-MOR was observed in a distribution indicative of plasma membrane localization (Fig 1A, top row, left panel). Similar surface localization was also observed with HA-NK1R (middle panel). The overlay image confirmed an overlap in distribution between F-MOR and HA-NK1R on the plasma membrane (right panel). Examination of the cell body at higher magnification emphasized the overlap in F-MOR and HA-NK1R localization (insets). Exposure of cells to 10μM morphine (MS) for 30 minutes produced a substantial redistribution of labeled F-MOR into a punctate intracellular pattern (Fig 1A, middle row, left panel), consistent morphine-induced endocytosis of MOR shown previously in these neurons (Haberstock-Debic et al., 2005). In contrast, morphine did not detectably affect the plasma membrane localization of labeled HA-NK1R (middle panel and overlay). Conversely, activating HA-NK1Rs with substance P (SP) resulted in selective internalization of HA-NK1Rs, while F-MORs remained predominantly in the plasma membrane (Fig 1A, bottom row).

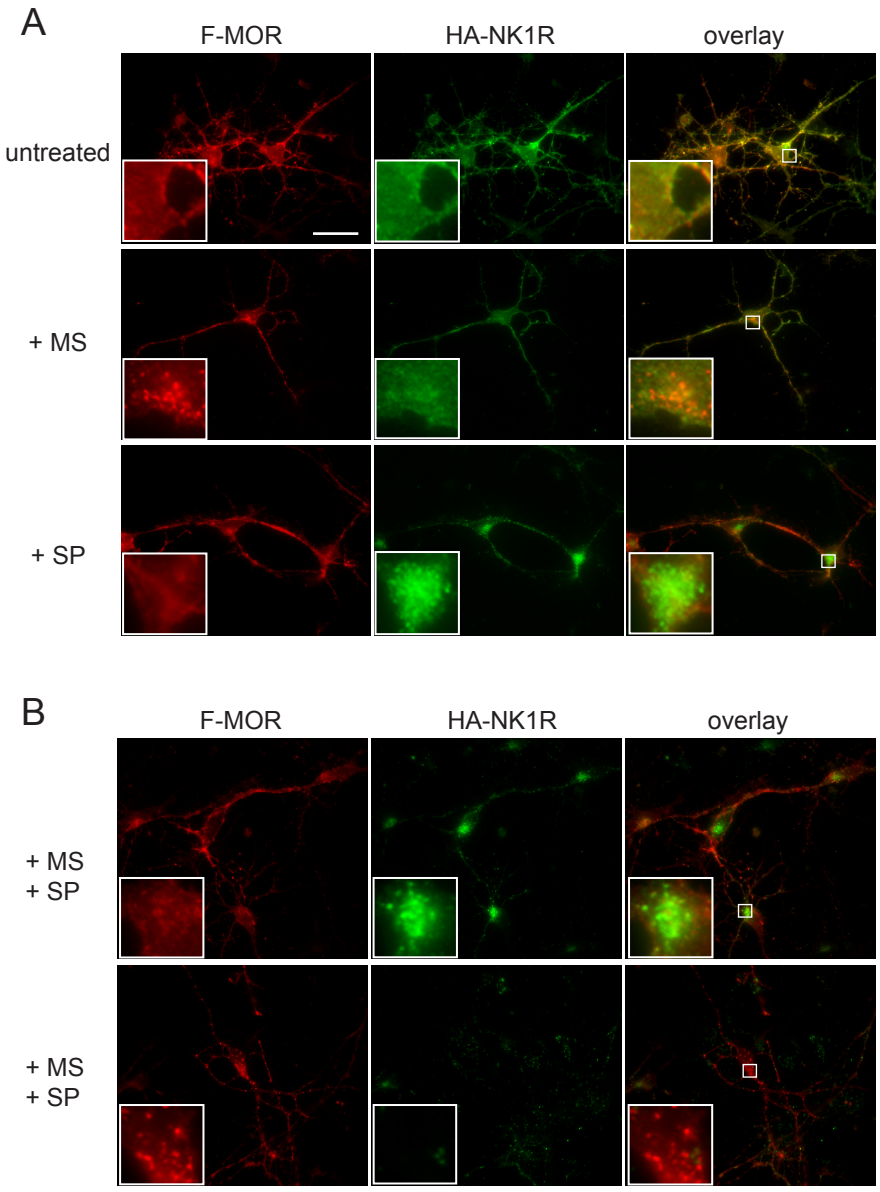


Figure 1. Morphine and substance P drive redistribution of F-MOR and HA-NK1R selectively in striatal neurons. Dual-labeled epifluorescence micrographs showing F-MOR (red) and HA-NK1R (green) distribution in striatal neurons. Insets within images show a higher magnification view of a representative region of the cell body (location indicated by box in overlay image). (A) Incubation with 10 μM morphine (MS, middle row) or 10 μM substance P (SP, bottom row) for 30 minutes produced selective redistribution of F-MOR and HA-NK1R, respectively. (B) Co-incubation with MS and SP prevented F-MOR redistribution while HA-NK1R redistribution was indistinguishable from that exposed to SP alone (top row). In neurons transfected with only F-MOR, addition of SP in the media had no effect on F-MOR redistribution in response to MS (bottom row). Scale bar, 20 μm.

Activation of NK1R via substance P inhibits regulated internalization of MOR

We next examined the effects of activating both receptors by exposing neurons to morphine and substance P simultaneously. Labeled HA-NK1R redistributed rapidly into a punctate pattern in the combined presence of both agonists, indistinguishable from the redistribution observed in neurons exposed to substance P alone (Fig 1B top row, compared to Fig 1A bottom row). Surprisingly, F-MORs co-expressed on these same cells remained localized predominantly in the plasma membrane, in contrast to the pronounced redistribution observed in the presence of morphine alone (Fig 1B top row, compared to Fig 1A, middle row). This selective redistribution of HA-NK1R was highlighted in higher magnification of a representative region of the cell body (inset). To investigate whether this non-reciprocal inhibition of F-MOR redistribution was dependent on the presence of HA-NK1Rs, we identified cells on the same coverslip expressing only F-MOR and not HA-NK1Rs. In these neurons, incubation with both morphine and substance P produced robust redistribution of F-MOR into a punctate pattern, similar to what was observed with morphine alone (Fig 1B, bottom row). Together, these results show that morphine-induced redistribution of F-MOR in neurons is specifically inhibited by simultaneous activation of co-expressed HA-NK1Rs by substance P.

To determine whether the agonist-mediated receptor redistribution observed by immunofluorescence is the result of receptor endocytosis, we used a previously described dual-labeling method to distinguish between surface-localized and internalized MORs (Haberstock-Debic et al., 2005). F-MORs present in the plasma membrane were initially

surface-labeled using Alexa594-conjugated anti-FLAG monoclonal antibody. After 30 minutes of ligand incubation, neurons were fixed and receptors remaining in the plasma membrane were selectively labeled with Alexa488-conjugated secondary antibody (Fig 2A, middle column). Internalized receptors appeared red, whereas plasma membrane-localized receptors labeled with both fluorochromes appeared yellow in the overlay image (Fig 2A). Representative images of striatal neurons revealed that a significant fraction of F-MOR present initially in the plasma membrane internalized following exposure of neurons to morphine for 30 minutes (Fig 2A, top row). In contrast, incubation of neurons with both morphine and substance P resulted in little internalization of F-MOR, demonstrated by an overlap in staining of total receptor and remaining surface receptor (Fig 2A, bottom row). Ratiometric quantification in multiple neurons (as described in Materials and Methods) confirmed the significant inhibitory effect of HA-NK1R activation on regulated endocytosis of F-MOR (Fig 2B, black bars).

The enkephalin analog D-Ala^2-*N*-Me-Phe^4-Glycol^5-enkephalin (DAMGO) is known to drive MOR endocytosis more readily and to a greater degree compared to morphine in many cell types (Keith et al 1998; Whistler et al., 1999; Bushell et al., 2002; Trafton and Basbaum, 2004). Thus we tested whether activation of NK1Rs receptors can also inhibit endocytosis of MOR induced by DAMGO. Incubation of striatal neurons with 10μM DAMGO for 30 minutes produced substantial internalization of F-MOR, which was somewhat larger in absolute magnitude than that induced by morphine (Fig 2B, white bars). Co-application of substance P resulted in a less pronounced, but still significant, inhibition of F-MOR endocytosis. Thus, NK1R-mediated inhibition of MOR trafficking

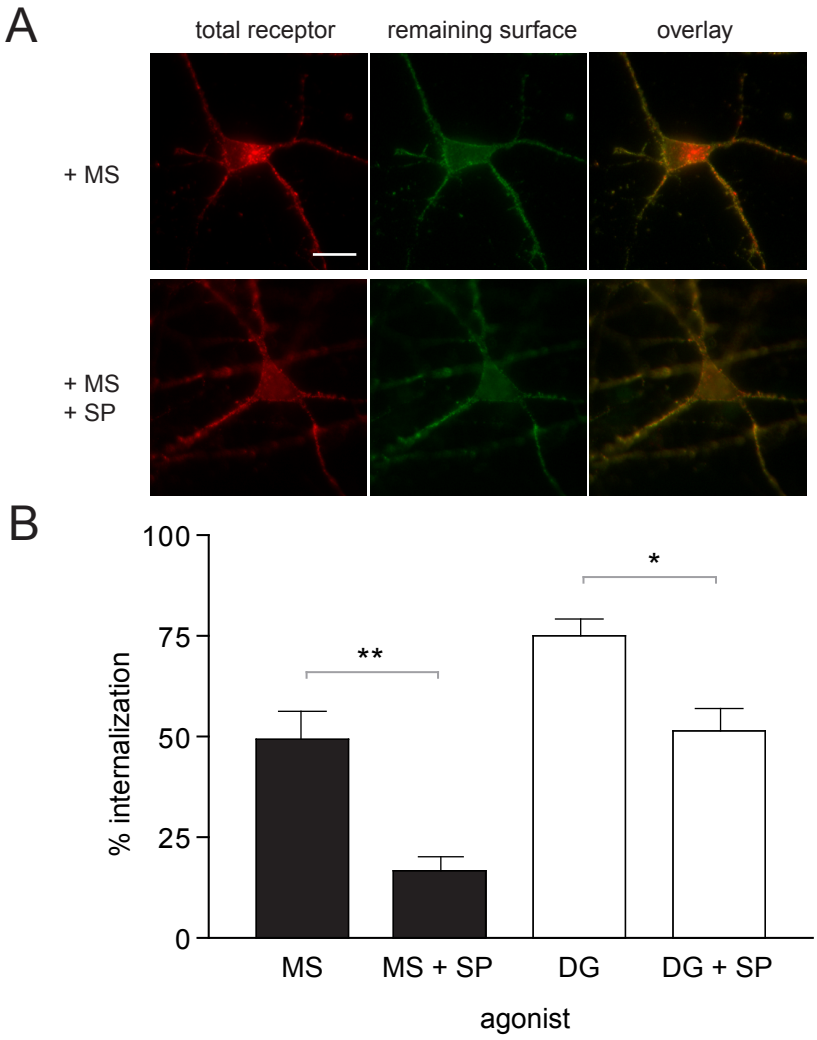


Figure 2. Regulated endocytosis of F-MOR is inhibited by SP-mediated activation of co-expressed HA-NK1R. Striatal neurons were transfected with F-MOR and HA-NK1R. (A) Neurons were first incubated with M1-Alexa594 antibody (red) to label surface F-MORs (first column). After 30 minutes of agonist incubation, neurons were fixed under non-permeabilizing conditions and incubated with Alexa488 (green) to label remaining F-MORs on the plasma membrane (middle column). Morphine treatment resulted in internalized punctate structures of labeled receptors (top left) that are not accessible to the cell surface (top middle). This is emphasized in the overlay image (top right). In neurons incubated with both MS and SP, total and remaining surface receptor labeling overlapped almost completely, indicating a lack of F-MOR internalization (bottom row). (B) A ratio of green (surface receptors after agonist treatment) to red (total receptor) fluorescence intensity was used to determine percent F-MOR internalization when incubated with either MS or DAMGO alone, or in combination with SP (see Materials and Methods for detail). Bar graphs represent mean internalization determined from ~50 cell bodies selected at random in neuronal cultures and averaged over five independent cultures (* $p < 0.01$, ** $p < 0.005$). Scale bar, 10 μm.

is not restricted opiate drugs such as morphine, and can also affect regulated endocytosis induced by opioid peptide.

Regulated MOR endocytosis is inhibited by NK1R activation in N2A cells

We next asked if the pronounced inhibitory effect of NK1R activation on MOR trafficking could be observed in a cultured cell model that is advantageous for mechanistic and biochemical studies. Typically, morphine drives endocytosis of MORs poorly in transformed cell lines (Keith et al., 1996; Alvarez et al., 2002). Nevertheless, by surveying a number of neurosecretory cell types, we found rapid morphine-mediated endocytosis of MORs in mouse-derived Neuro2A (N2A) cells. We first assessed the subcellular translocation of F-MOR and HA-NK1Rs with antibody labeling of cells transiently co-expressing these two receptors under various agonist conditions. Confocal microscopy demonstrated a predominantly plasma membrane distribution of both receptor types in the absence of ligand (Fig 3A, top row). Exposure to 10μM morphine for 30 minutes selectively promoted redistribution of F-MORs to intracellular vesicles, whereas HA-NK1Rs remained predominantly on the cell surface (Fig 3A, second row). Conversely, incubation with substance P resulted in selective internalization of HA-NK1Rs but not F-MORs (Fig 3A, third row). Further replicating the results obtained in striatal neurons, simultaneous incubation of N2A cells with both morphine and substance P resulted in selective internalization of HA-NK1Rs and little visible redistribution of F-MOR from the plasma membrane (Fig 3A, bottom row). Quantification by ratiometric labeling confirmed the inhibitory effect of HA-NK1R activation on morphine-induced endocytosis of F-MORs (Fig 3B, black bars). DAMGO-induced internalization of F-

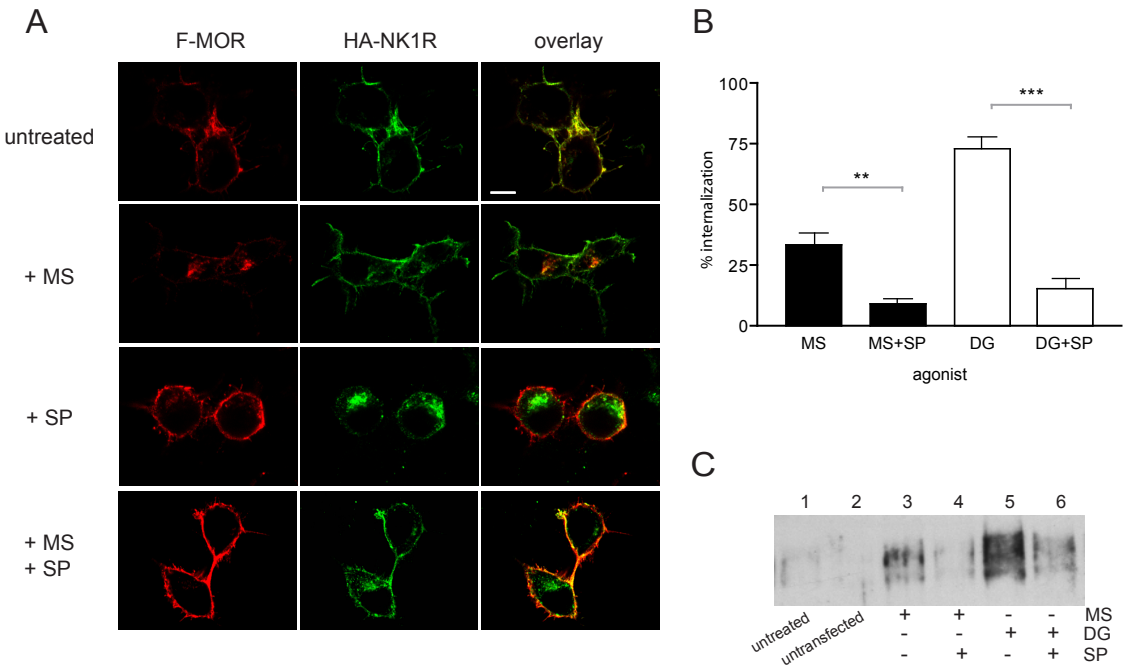


Figure 3. Heterologous regulation of F-MOR endocytosis in mouse neuroblastoma (N2A) cells. (A) Dual-labeled confocal fluorescence micrographs show F-MOR (red) and HA-NK1R (green) transiently expressed in N2A cells. In untreated cells, both F-MOR and HA-NK1R showed a plasma membrane distribution, with minimal labeled receptors present internally in the cell (top row). Incubation with either 10mM MS or 10mM SP for 30 minutes resulted in a selective increase in either labeled F-MORs (second row, first panel) or HA-NK1Rs (third row, middle panel), respectively, present internally in the cell. Simultaneous incubation with MS and SP produced visibly less F-MOR internalization compared to incubation with MS alone (bottom row, first panel). Scale bar, 10 μm. (B) Quantification of ratiometric staining in N2A cells show inhibition of F-MOR internalization in response to both MS and DG when SP is also present. Bar graphs represent mean internalization determined from ~50 cell bodies selected at random in N2A cultures and averaged over five independent transfections (**p<0.005, ***p<0.0005). (C) Isolated biotinylated F-MORs were detected by immunoblotting with anti-FLAG antibody in N2A cells co-expressing both F-MOR and HA-NK1R. Incubation with both SP and MS (lane 4) or DG (lane 6), compared to either MOR agonists alone (lane 3 and 5), for 30 minutes resulted in a decrease in the internalized pool of F-MORs (n= 3 independent experiments).

MOR was also significantly inhibited by HA-NK1R activation in N2A cells, and this was even more pronounced than observed in striatal neurons (Fig 3B, white bars).

To further confirm the HA-NK1R-dependent inhibition of F-MOR endocytosis, we used a biochemical assay to specifically measure the fate of surface-biotinylated receptors in a population of cells co-expressing both F-MOR and HA-NK1R. Cells were surface-biotinylated and then subjected to agonist incubation for 30 minutes. Biotin attached to receptors remaining in the plasma membrane was selectively cleaved using a membrane-impermeant reducing agent and the internalized (still biotinylated) pool of F-MORs was selectively detected following isolation on streptavidin beads. Very little biotinylated signal was detected in cells incubated in the absence of opioid agonist, confirming the efficiency of the cleavage reaction and, as expected, both morphine and DAMGO produced significant internalization of F-MORs (a representative immunoblot is shown in Fig. 3C, compare lane 1 to lanes 3 and 5). F-MOR internalization induced by both opioid agonists was dramatically reduced, essentially to the level of untreated control cells, when cells were incubated in the presence of substance P together with either morphine or DAMGO (Fig 3C, compare lanes 3 and 5 with lanes 4 and 6).

NK1R-dependent inhibition of MOR endocytosis is mediated by receptor-specific trafficking of arrestin to endosomes

What is the mechanism underlying the HA-NK1R-dependent inhibition of MOR endocytosis? Given that NK1R is a G_q-coupled receptor, one possibility is endocytic inhibition could occur as a consequence of G_q-mediated signaling via phospholipase C

(PLC). This hypothesis is potentially consistent with a previous report that protein kinase C (an effector of the G_q-PLC pathway) can inhibit MOR internalization (Ueda et al., 2001), but is contradicted by the recent finding that activation of a distinct G_q-coupled receptor (the 5HT2A serotonin receptor) enhances rather than inhibits MOR internalization (Lopez-Gimenez et al., 2008). Further arguing against this hypothesis, we found that blocking PLC activity in N2A cells using the specific inhibitor U73122 did not prevent NK1R-dependent inhibition of MOR endocytosis (Supplemental Fig. 1). Another hypothesis is that the observed endocytic inhibition is caused by depletion of functional arrestin activity from the cytoplasm by sequestration on the endosome membrane. Whereas many GPCRs (including MOR) rapidly dissociate from arrestins upon endocytosis, a subset of GPCRs (including the NK1R) are capable of driving pronounced sequestration of arrestins on endosomes (Oakley et al., 2001). Further, endosomal sequestration by such GPCRs was shown previously to mediate heterologous inhibition of endocytosis of non-sequestering GPCRs in non-CNS cells (Klein et al. 2001; Schmidlin et al. 2002). To examine whether depletion of arrestin might mediate HA-NK1R-dependent inhibition of MOR endocytosis in neuronal cells, we first asked if increasing cytoplasmic arrestin by over-expression is sufficient to rescue morphine-induced endocytosis of MOR. To do so we focused on arrestin 3 (β-arrestin2), which is the major non-visual arrestin expressed in the striatum (Gurevich et al., 2002). EGFP-tagged β-arrestin2 was transfected into N2A cells co-expressing F-MOR and HA-NK1Rs, and triple color confocal microscopy was used to detect and localize each of the proteins specifically. In contrast to cells expressing arrestins at endogenous levels, co-activation of HA-NK1Rs in cells over-expressing β-arrestin2 failed to inhibit morphine-

induced endocytosis of F-MOR (Fig 4). This was indicated by the ability of both F-MOR and HA-NK1Rs to endocytose rapidly to an overlapping population of endocytic vesicles in cells exposed to both morphine and substance P, and these endocytic vesicles also visibly sequestered EGFP-tagged β-arrestin2 from the cytoplasm.

To determine if arrestin sequestration occurs in N2A cells, we co-expressed HA-NK1R and EGFP-tagged β-arrestin2 and examined arrestin redistribution after substance P treatment. Immunofluorescence analysis confirmed that activation of HA-NK1R for 30 minutes was sufficient to promote rapid and pronounced redistribution of EGFP-tagged β-arrestin2 to receptor-containing endosomes (Fig 5A, left column). This effect was specific to HA-NK1Rs because activation of F-MORs with either morphine or DAMGO alone did not produce detectable sequestration of arrestin on endosomes (not shown). By truncating a distal portion of the HA-NK1R tail (HA-NK1 355x), we were able to disrupt substance P-induced arrestin sequestration on endosomes (Figure 5A, right column). This portion of the NK1R tail was previously shown to be required for endosomal sequestration of arrestins in non-neural cells (Oakley et al., 2001). Importantly, substance P-induced receptor endocytosis was not blocked by this mutation (Fig 5A, bottom right).

We next asked if disrupting arrestin sequestration prevents endocytic inhibition of MORs in cells expressing arrestins at endogenous levels. N2A cells expressing F-MOR and HA-NK1 355x were exposed to their respective agonists individually or in combination. As expected, incubation with morphine or substance P alone selectively promoted endocytosis of F-MORs or HA-NK1Rs, respectively (not shown). However, rapid

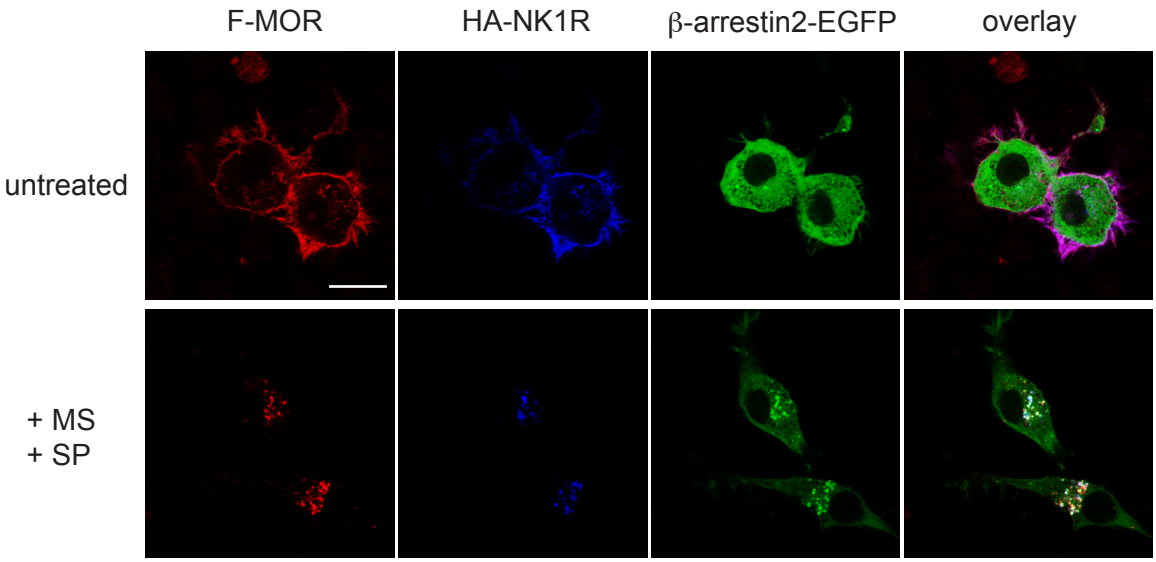


Figure 4. Over-expression of β-arrestin2-EGFP in N2A cells co-expressing F-MOR and HA-NK1R prevents inhibition of F-MOR endocytosis. Confocal fluorescence micrographs of N2A cells co-expressing F-MOR (red), HA-NK1R (blue), and β-arrestin2-EGFP (green). Over-expression of β-arrestin2 resulted in internalization of F-MOR (bottom row, first panel) in response to 30 minutes of 10 μM MS even in the presence of 10 μM SP. Internalization of HA-NK1R was not detectably affected by β-arrestin2 over-expression (bottom row, second panel). Scale bar, 20 μm.

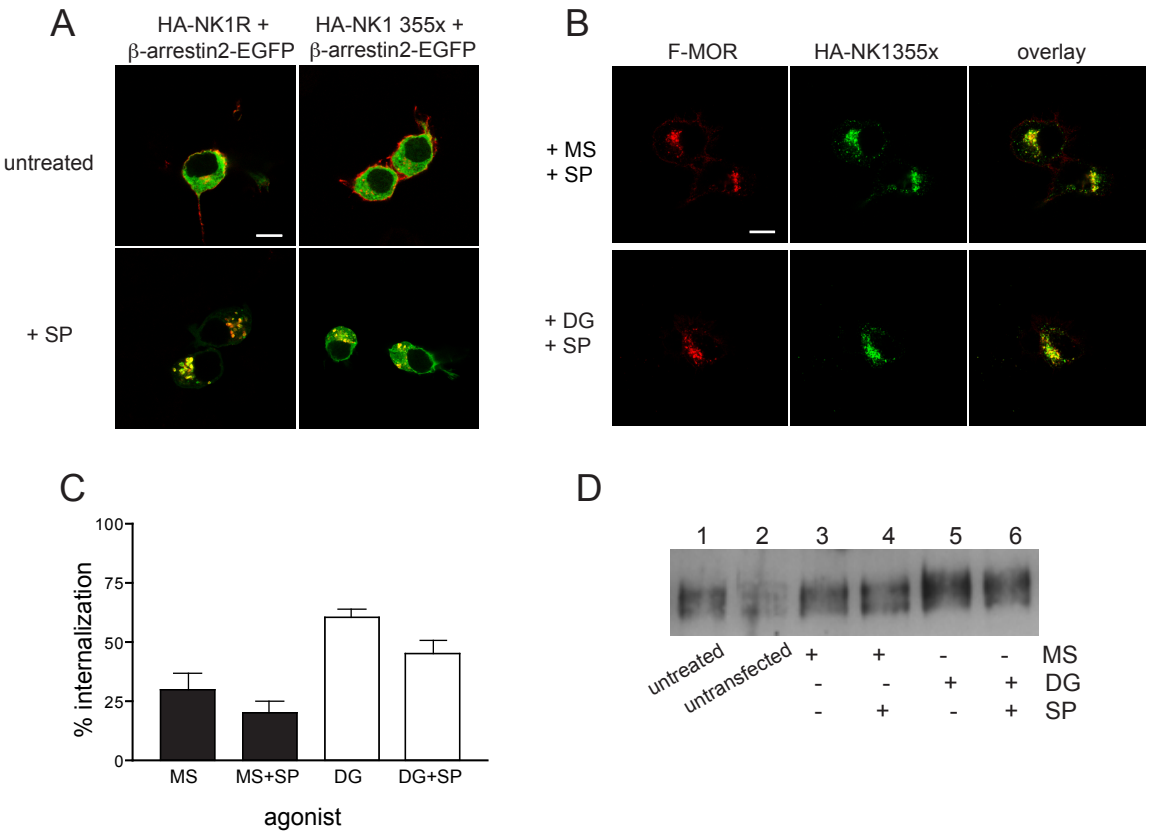


Figure 5. Mutational disruption of NK1R-mediated sequestration of β-arrestin prevents inhibition of F-MOR endocytosis in N2A cells. (A) Confocal micrographs of N2A cells co-expressing either full-length HA-NK1R (left panels, red) or truncated HA-NK1R (right panels, red) with β-arrestin2-EGFP (green). Incubation with 10 μM SP in cells expressing the truncated HA-NK1R (355x) resulted in less co-localization with receptors in endosomal clusters and higher cytoplasmic β-arrestin2-EGFP distribution (left column), compared to β-arrestin2-EGFP distribution in the wild type HA-NK1R under the same conditions (right column). Scale bar, 10 μm. (B) In N2A cells co-expressing F-MOR (red) and HA-NK1 355x receptors (green), both morphine and DAMGO were able to drive internalization of F-MORs even in the presence of substance P. Scale bar, 10 μm. (C) Quantification using ratiometric staining of F-MORs in N2A cells co-expressing HA-NK1 355x revealed that co-incubation with SP produced comparable levels of F-MOR internalization compared to treatment with either MS or DG alone. Bar graphs represent mean internalization determined from ~50 cell bodies selected at random in the N2A cultures and averaged over four independent transfections. (D) Biochemical assay of F-MOR internalization by surface biotinylation confirmed that F-MOR internalization induced by either MS or DG (lanes 3 and 5) was not significantly inhibited by co-application of SP (lanes 4 and 6; n= 3 independent experiments).

endocytosis of both F-MORs and HA-NK1 355x receptors was also observed in cells exposed to both morphine and substance P, in contrast to the endocytic inhibition of MOR produced by co-activation of the wild type HA-NK1R (Fig 5B, top panels). Similarly, incubation with DAMGO also resulted in MOR endocytosis, even in the presence of substance P (Fig 5B, bottom panels).

Ratiometric quantification of F-MOR internalization in N2A cells co-expressing HA-NK1 355x confirmed that the degree of internalization observed in cells exposed to both morphine and substance P was not significantly different from that observed when exposed to morphine alone (Fig 5C, black bars). Similarly, the endocytic inhibitory effect on DAMGO-induced internalization was also greatly reduced (Fig 5C, white bars). Biochemical analysis using surface biotinylation of receptors further confirmed this loss of endocytic inhibition. Addition of substance P to the culture medium did not detectably reduce the internalized pool of F-MORs produced by either morphine or DAMGO (Fig 5D, compare lanes 3 and 5 with lanes 4 and 6). Together, these results strongly suggest that endosomal sequestration of arrestin by activated HA-NK1Rs is both necessary and sufficient to produce the observed inhibition of F-MOR endocytosis in N2A cells.

To determine whether a similar mechanism mediates the NK1R-dependent endocytic inhibitory effect in striatal neurons, we carried out the equivalent experiment in primary neuronal cultures co-expressing F-MOR and HA-NK1 355x. In contrast to the significant inhibition of morphine-induced endocytosis of F-MOR observed in cells produced by activating wild type HA-NK1Rs, substance P-mediated activation of HA-NK1 355x did

not prevent morphine induced endocytosis of F-MOR (Fig 6 top panels, compare to Fig 1B). DAMGO-induced endocytosis of F-MOR was also not detectably impaired by activation of co-expressed HA-NK1 355x receptors (Fig 6, bottom panels).

NK1R-dependent regulation selectively inhibits MOR desensitization, without affecting acute signaling, in opioid-naive neurons

To begin to assess the functional significance of this arrestin-dependent MOR-NK1R regulatory effect, we examined MOR signaling via heterotrimeric G proteins. Because MORs couple primarily to G_i, we used forskolin to stimulate adenylyl cyclase activity and examined MOR signaling via inhibition of forskolin-induced cAMP production in cultured striatal neurons co-expressing F-MORs and HA-NK1Rs. As expected, exposure of neurons to morphine for 3 minutes significantly inhibited cAMP production (Fig 7A). This acute signaling response was not detectably affected by co-activation of HA-NK1Rs with substance P. Further, consistent with HA-NK1R coupling primarily to G_q, substance P-mediated activation of HA-NK1Rs in the absence of opiate did not change cAMP levels detectably from the control (i.e., forskolin only) condition.

To examine whether NK1R-mediated inhibition of MOR endocytosis is associated with an effect on MOR desensitization, we assayed the ability of morphine to inhibit cAMP accumulation in neurons pre-exposed to agonist. Pre-incubation of neurons with morphine for 30 minutes strongly attenuated the ability of a second challenge of morphine to inhibit forskolin-stimulated cAMP accumulation in neurons, compared to the morphine-induced inhibition observed in opiate-naive neurons (Fig 7B, compare bars 2

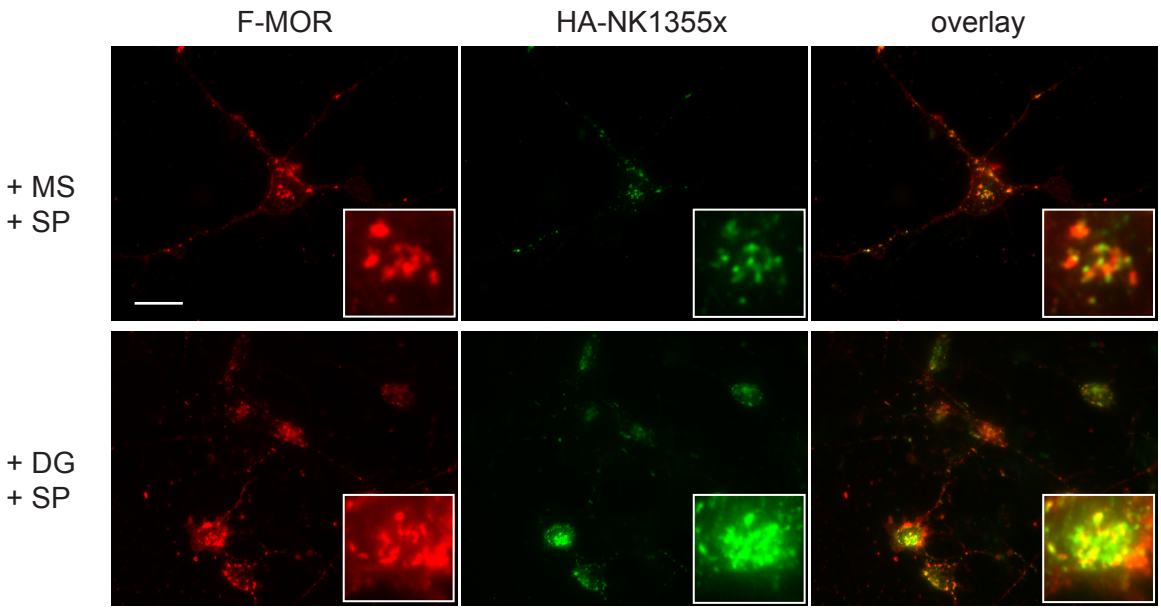


Figure 6. Mutational disruption of NK1R-mediated sequestration of β-arrestin prevents inhibition of F-MOR endocytosis in striatal neurons. Dual-labeled epifluorescence micrographs show F-MOR (red) and HA-NK1R (green) distribution in striatal neurons. Insets within images show magnification of representative receptor distribution. Incubation with 10 μM MS for 30 minutes resulted in a redistribution of F-MORs in striatal neurons even in the presence of 10 μM SP when the truncated version of the NK1R was co-expressed with F-MOR (top row). A similar redistribution of F-MOR was observed when neurons were incubated with both 10 μM DG and SP. Scale bar, 20 μm.

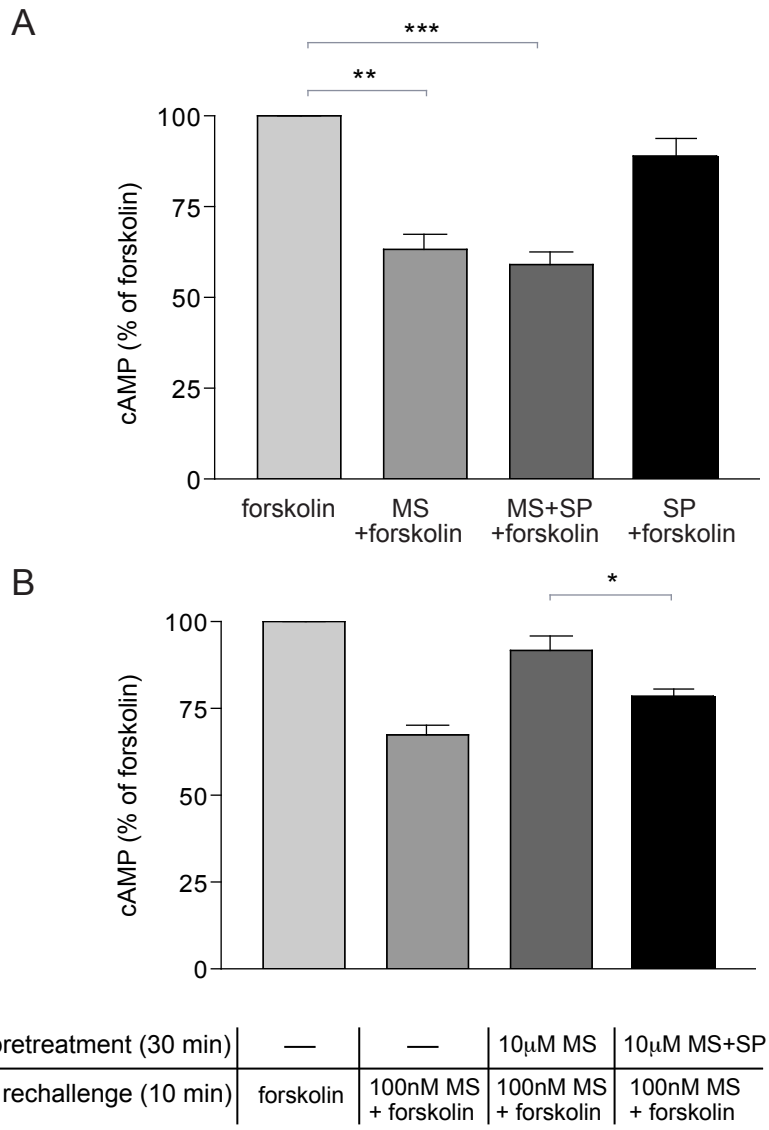


Figure 7. NK1R-mediated inhibition of MOR endocytosis is associated with reduced functional desensitization of opioid-dependent regulation of adenylyl cyclase. Striatal neurons co-expressing F-MOR and HA-NK1R were cultured and assayed for cAMP levels (see Materials and Methods). (A) The amount of cAMP accumulated in response to adenylyl cyclase stimulation with 3 μM forskolin was defined as 100% cAMP production (left bar). Bars represent mean forskolin-stimulated cAMP accumulation observed in the presence of a saturating concentration (10 μM) of morphine (MS), substance P (SP), or both (n=4, ** $p< 0.005$, ***$p< 0.0005$). (B) Neurons were preincubated for 30 minutes in the absence or presence of 10 μM MS alone, or 10 μM MS together with 10 μM SP. After agonist washout neurons were re-challenged for 10 minutes with a sub-saturating concentration (100 nM) of MS together with 3 μM forskolin. Bars represent mean cAMP accumulation measured in the re-challenge period compared to that induced by forskolin alone in previously untreated neurons (n=4, *$p< 0.05$).

and 3 from the left). Interestingly, this morphine-induced attenuation of subsequent opiate response was significantly reduced by inclusion of substance P in the pre-incubation (Fig. 7B, compare bars 3 and 4 from the left). We verified in control experiments that forskolin-stimulated cAMP accumulation measured in the absence of opioid did not differ significantly between the pre-treatment conditions when compared to drug-naïve neurons (Supplemental Fig. 2). Together, these results indicate that NK1R activation does not detectably affect acute opiate signaling, but significantly reduces functional desensitization of MOR signaling produced by opiate pre-exposure.

MOR endocytosis is inhibited by NK1R activation in a neuronal population expressing endogenous receptors

To determine whether a similar inhibition of MOR endocytosis can occur with receptors expressed at endogenous levels, we explored this phenomenon in several populations of neurons where both MOR and NK1Rs are reportedly co-expressed. In our cultures, immunocytochemical staining of dissociated striatal cultures revealed that MOR and NK1Rs were expressed largely in distinct neurons, making this preparation unfavorable for examining heterologous regulation of endogenous receptor trafficking (data not shown).

Another brain region that is known to co-express these receptors and modulate behavioral reward to opioids is the amygdala (Gadd et al., 2003; Nakaya et al., 2004; Poulin et al., 2006). We cultured primary rat amygdala neurons and found that a subset of these neurons were positive for both endogenous MOR and NK1R immunoreactivity, while

others expressed only one but not the other receptor type. In neurons expressing MORs but not NK1Rs, incubation of cultures with both 10 μM DAMGO and substance P for 30 minutes resulted in a pronounced redistribution of MOR immunoreactivity, indicative of rapid internalization of MORs (Fig. 8a, top left). In contrast, redistribution of MORs was substantially reduced in neurons co-expressing both MORs and NK1Rs under the same agonist conditions, while NK1R endocytosis in response to substance P was unaffected (Fig. 8A, bottom row). We verified in control experiments that DAMGO alone stimulated a pronounced redistribution of MOR in both NK1R–positive and –negative neurons (not shown). These results suggest that inhibition of MOR internalization by NK1R activation also occurs in neurons that endogenously express these receptors.

MOR endocytosis is inhibited by NK1R activation in locus coeruleus neurons.

To test if cross-inhibition occurs in another relevant population of CNS neurons, and to obtain quantitative data, we examined the effect of substance P on MOR internalization using locus coeruleus neurons from F-MOR Tg+/-, MOR-/- mice (Arttamangkul et al., 2008). This brain region consists primarily of noradrenergic neurons, which possess a high density of both endogenous MORs and NK1Rs (Cheeseman et al., 1983; Tempel and Zukin, 1987; Nakaya et al., 1994). In slices imaged in the absence of agonist, immunoreactive MOR was localized primarily in the plasma membrane, as indicated by the peripheral staining pattern in 2-photon optical sections (Fig. 8B, top left panel). Because morphine does not cause significant internalization in LC neurons, [Met]^{5}enkephalin (ME) was used in these experiments (Arttamangkul et al., 2008). Bath application of 10μM ME for 15 minutes induced a redistribution of F-MOR receptors to a

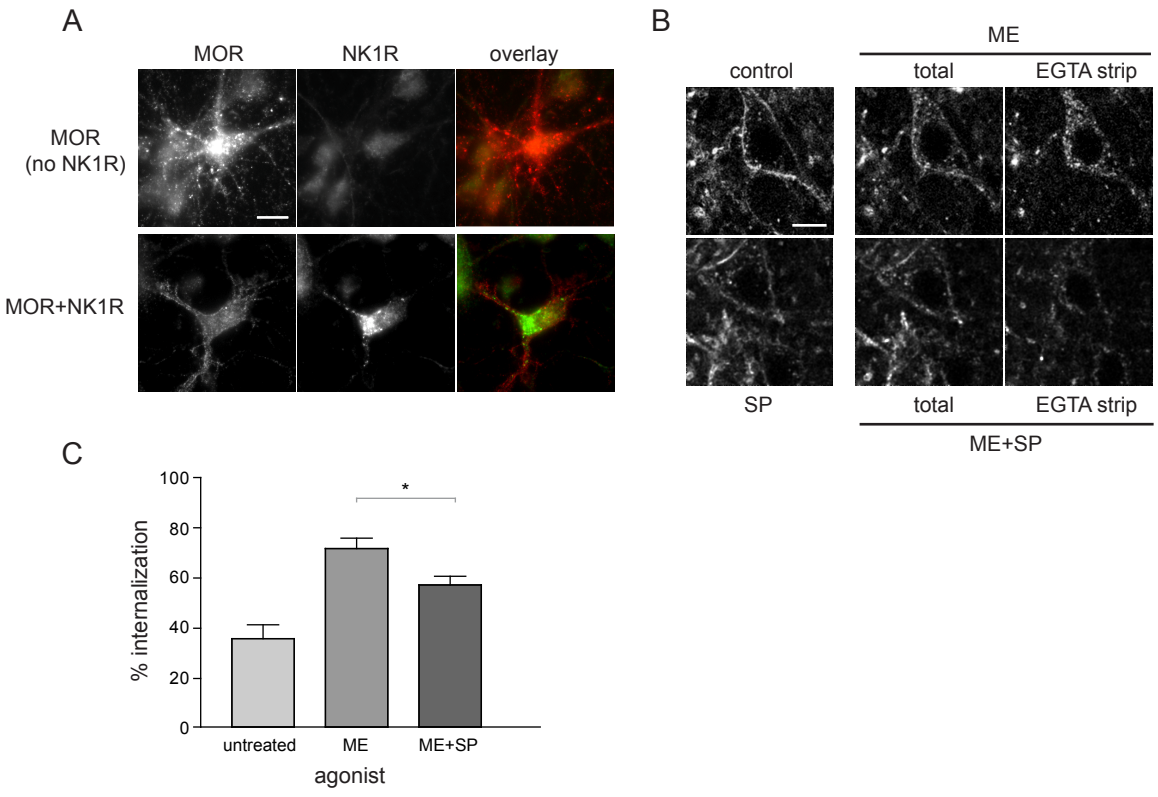
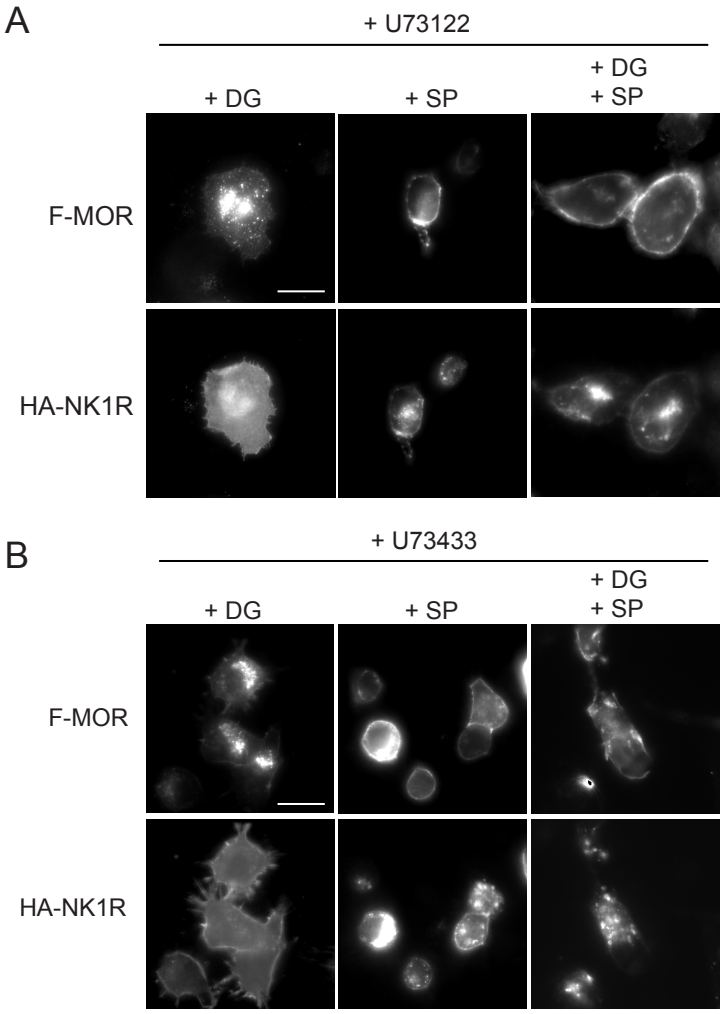
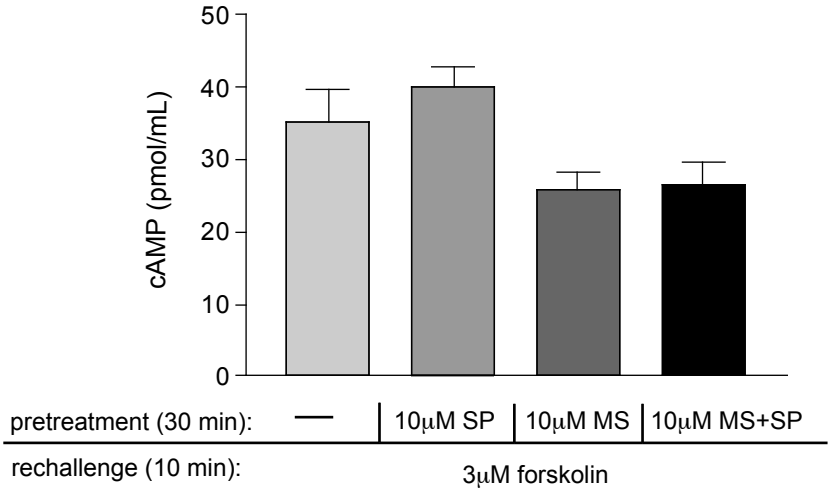


Figure 8. Heterologous inhibition of MOR endocytosis mediated by endogenously expressed NK1Rs. (A) Dual localization of endogenous MORs and NK1Rs was visualized in dissociated amygdala cultures after co-incubation with 10 μM of DAMGO and 10 μM SP for 30 minutes. Pronounced internalization of endogenous MOR was observed in cells not expressing detectable NK1R immunoreactivity (top row), while internalization of MOR was dramatically inhibited in neurons (visualized on the same coverslip) expressing both MOR and NK1R (bottom row). Epifluorescence images shown are representative of 3 independent experiments and were processed in parallel. Scale bar, 10 μm. (B) 2-photon fluorescence imaging of F-MOR trafficking in live locus coeruleus neurons prepared from F-MOR-Tg/+,MOR -/- mice. F-MORs present in the plasma membrane were labeled by incubating acutely prepared brain slices with M1-Alexa594 for 45 minutes (top left). The same neuron was then perfused with 10 μM [Met]5enkephalin (ME) for 15 minutes (top middle). Remaining surface-accessible receptors were then stripped by adding calcium free buffer containing EGTA (top right). The same series of manipulations carried out in the presence of 10 μM SP perfusion (bottom panels). Examples of mid-plane optical sections are shown. (C) Summary of the internalization results obtained by measurement of EGTA-resistant Alexa594 measured in neurons across multiple experiments. ME exposure increased F-MOR uptake from residual levels 36 ± 5% (n = 3) to 71.5 ± 4.4 % (n =15). Application of ME in the presence of SP significantly reduced FLAG-MOR uptake (57.1 ± 3.7%, n=15, *p= 0.01). Each experiment represents imaging of a different brain slice, compiled from dissection of 11 animals. Scale bar, 10 μm.

punctate pattern consistent with regulated endocytosis of MOR (Fig. 8B, top middle panel). This was confirmed by subsequent application of calcium-free EGTA solution, which dissociates antibody from surface-accessible receptors, leaving the internalized receptor pool specifically labeled (Fig. 8B, top right panel). Application of 10μM substance P to slices did not detectably change the plasma membrane localization pattern of labeled MOR (Fig. 8B, bottom left panel), indicating that activating the NK1Rs alone did not promote significant internalization of MOR. Subsequent application of ME, in the continued presence of substance P, produced some redistribution of MOR but the magnitude of this effect was visibly reduced compared to that observed in slices exposed to ME alone (Fig. 8B, middle panels). Consistent with this, the internalized fraction of labeled MOR, which is resistant to the EGTA strip condition, was also visibly reduced in slices exposed to ME in the presence of substance P (Fig. 8B, right panels). Quantification of these results over multiple experiments confirmed that substance P significantly inhibited ME-induced internalization of MOR in locus coeruleus neurons (Fig. 8C).



Supplemental Figure 1. NK1R-mediated inhibition of MOR endocytosis does not require signaling via PLC. N2A cells co-expressing F-MOR and HA-NK1R were pre-incubated for 15 minutes with 10mM of either the PLC inhibitor (U73122; Sigma) or its inactive analog (U73433; Sigma) before adding the indicated agonist(s) and incubating cells for an additional 30 minutes in the continued presence of U73122 or U73433. (A) Representative epifluorescence micrographs showing localization of F-MOR (top row) and HA-NK1R (bottom row) in cells exposed to the active PLC inhibitor U73122 and incubated for 30 minutes in the presence of 10 μM DAMGO (+DG), 10μM substance P (+SP) or both (+DG +SP). The PLC inhibitor did not prevent endocytosis of either receptor induced by its respective agonist when applied alone (compare top and bottom panels of the left and middle columns), nor did it prevent the SP-dependent inhibition of MOR endocytosis (compare top and bottom panels of the right column). (B) The corresponding micrographs from cells incubated in the presence of the inactive analog U73433, confirming that SP-dependent inhibition of MOR endocytosis was indistinguishable from that observed in the presence of the active PLC inhibitor (compare with the corresponding panels in part A of the figure). Scale bar, 20μM.



Supplemental Figure 2. Conditions of agonist pre-incubation used to assess MOR desensitization do not significantly alter forskolin-stimulated cAMP accumulation measured after agonist washout. Striatal neurons co-expressing F-MOR and HA-NK1R were incubated for 30 minutes in the absence or presence of the indicated agonists, washed free of ligand as in Figure 7B, and re-challenged with forskolin alone for 10 minutes to stimulate cAMP accumulation. Pre-incubation with 10 μM SP did not detectably change subsequent forskolin-stimulated cAMP accumulation compared to untreated neurons (n=4, p=0.4). No significant effect was observed after pre-incubation with10 μM morphine alone, compared to no pretreatment (n=5, p=0.1), or pre-incubation with both 10 μM morphine and 10 μM substance P (n=5, p=0.9).

DISCUSSION

The present results demonstrate that NK1R activation mediates a pronounced and cell autonomous inhibition of MOR endocytosis induced by diverse opioid agonists. Whereas either morphine or DAMGO promote rapid endocytosis of MOR in striatal neurons, simultaneous activation of NK1Rs with substance P strongly inhibited this fundamental regulatory process. We identified a transformed neurosecretory cell line in which pronounced morphine-induced endocytosis of MOR also occurs, and observed a similar endocytic inhibition mediated by NK1R activation in these cells. Our results indicate that, in both cell types, inhibition of MOR endocytosis is mediated by NK1R-dependent sequestration of arrestins on endosomes. This cell-autonomous regulatory mechanism was also found to produce a functionally significant attenuation of morphine-induced desensitization of MOR signaling via adenylyl cyclase. Furthermore, heterologous regulation of MOR trafficking was established in two additional populations of CNS neurons that mediate behavioral effects of opiate drugs and was observed in neurons expressing receptors at endogenous levels. Together, these observations strongly suggest that NK1Rs can significantly modulate both trafficking and downstream signaling of MORs in multiple populations of physiologically relevant CNS neurons.

Our findings thus identify a novel mode of functional interaction between MORs and NK1Rs. A previous study of these two GPCRs expressed in a non-neural cell model (HEK293 cells) reported that ligand-induced activation of either MOR or NK1R promoted co-endocytosis of both GPCRs by formation of MOR / NK1R heterodimers (Pfeiffer et al., 2003). Such heterodimer-driven endocytic "dragging" was not clearly

evident in the present study, as opioid and neurokinin agonists selectively promoted endocytosis of MORs and NK1Rs, respectively. Further, several observations argue that the regulatory interaction revealed in the present study cannot be explained by heterodimer formation. First, activation of NK1Rs inhibited, rather than promoted, MOR endocytosis. Second, we did not observe rapid endocytosis of either MOR or NK1R in the absence of the appropriate receptor agonist. Third, the observed cross-regulatory effect was non-reciprocal, as NK1R activation inhibited MOR endocytosis but not vice versa. The present results do not exclude heterodimer-based regulation of opioid receptors by neurokinins, and we are aware that the degree to which various GPCRs form heteromeric complexes varies among cell types (Bouvier, 2001). Our findings argue strongly, however, that the distinct mechanism established in the present study plays a dominant role in mediating functional interaction between MOR and NK1Rs in CNS neurons.

Cell autonomous, non-reciprocal endocytic inhibition has been observed previously among various GPCRs in non-neural cells as well as myenteric neurons (Klein et al., 2001; Schmidlin et al., 2002), and is consistent with the existence of significant differences among individual GPCRs in their ability to mediate arrestin trafficking from the cytoplasm to the endosome membrane (Oakley et al., 1999). NK1Rs, in particular, are able to mediate arrestin sequestration on endosomes in HEK293 cells (Oakley, 2001; Schmidlin et al., 2002). To our knowledge the present results are the first to demonstrate that arrestin sequestration can mediate heterologous regulation of GPCR trafficking in CNS neurons. Second, we show that this links NK1R activation to regulation of MORs in

multiple populations of CNS neurons relevant to the behavioral effects of opiate drugs, and that this regulation can be observed among endogenously expressed receptors. Thus, we believe that arrestin trafficking to endosome membranes likely represents a fundamental mechanism for integrating receptor-mediated signaling effects across distinct GPCRs in diverse neural cell types. Further, this function of arrestin trafficking in signal integration is clearly distinct from the previously proposed role of endosome-associated arrestins in scaffolding signaling complexes on the endosome membrane in response to activation of particular GPCRs (Lefkowitz et al., 2006). Although the actual stoichiometry of non-visual arrestins (or β-arrestins) relative to GPCRs is not clearly established in native cell types, our finding that endogenously expressed receptors mediate significant heterologous inhibition in both amygdala and locus coeruleus neurons suggests that the effective functional activity of β-arrestins is a limiting factor determining endocytic regulation of GPCRs in relevant CNS neurons. This conclusion, in addition to its specific relevance to opioid regulation, suggests a general principle that may underlie regulation between diverse GPCR-mediated signaling processes occurring simultaneously in the same neurons.

It is likely that the ability of NK1R activation to alter morphine's endocytic activity can persist even in the absence of continuous substance P release. Recycling of NK1Rs is reported to be slow in various cell types (Grady et al., 1995; Grady et al., 1996; Wang et al., 2002). Dissociation of the β-arrestin-receptor complex is dependent on dephosphorylation of receptors in endosomes and important for resensitization of internalized NK1Rs (Garland et al., 1996). Prolonged retention of β-arrestins on

endosomes, together with slow recycling of NK1Rs, could thus result in low cytoplasmic levels of this regulatory protein after an initial round of receptor activation. In addition, the lack of MOR desensitization as a result of inhibiting endocytosis alters the normal signaling response to drugs such as morphine. Thus, it is possible that the NK1R-mediated regulation of MOR endocytosis, in addition to its acute trafficking effects, contributes to longer-term plasticity in the regulatory profile of opioid drugs.

We were surprised to find a lack of overlap in MOR and NK1R expression on our dissociated striatal culture preparation. It is possible that this reflects developmental differences between our cultures (which are derived from embryonic rats) and adult animals used in previous studies (Pickel et al., 2000; Jabourian et al., 2005). However, we did observe that MORs and NK1Rs are co-expressed in a significant fraction of cultured amygdala neurons. This brain region is known to be important for mediating conditioned reward processes, expresses both MOR and NK1R, and ablation of NK1Rs in these neurons has previously been reported to reduce morphine reward behavior (Everitt et al., 1991; Gadd et al., 2003). We also observed substance P-induced heterologous inhibition of MOR internalization in an acute slice preparation of locus coeruleus, using a recently developed method that allowed quantitative assessment of MOR endocytosis (Arttamangkul et al., 2008). In addition to the amygdala, NK1R-expressing neurons in the locus coeruleus may also play a role in morphine-driven behaviors (Nestler et al., 1994) and in opiate withdrawal (Redmond and Krystal, 1984; Rasmussen 1990). Therefore, it is conceivable that the cell autonomous regulation established in the present study contributes to NK1R-dependent modulation of opiate

responses evident from study of NK1R-knockout animals and animals in which NK1R function is chemically ablated (Gadd et al, 2003). There is also considerable co-expression of MOR and NK1R in the trigeminal dorsal horn (Aicher et al., 2000). Although we did not examine this region in the present study, and NK1Rs are not essential for morphine antinociception in vivo (De Felipe et al, 1998), it is possible that receptor co-expression could confer NK1R-dependent regulation on opioid signaling in these neurons as well.

The observed inhibitory effect of NK1R activation on MOR endocytosis and desensitization in CNS neurons suggests a novel mechanism by which neurokinin signaling may affect opioid responses in multiple brain regions. Important questions for future study include further examination of the effects of cell autonomous regulation on opioid-mediated neurophysiology and investigation into how interaction between these two neurochemical systems affects functional connectivity between other brain regions. For example, the central nucleus of the amygdala receives reciprocal dopaminergic afferents from the ventral tegmental area and is connected to the core of the nucleus accumbens in the striatum (Ungerstedt, 1971; Wallace et al., 1992). It is conceivable, therefore, that the ability of other GPCRs to alter the trafficking and signaling properties of MORs in response to opiates could have profound effects on neural circuits driving reward behavior. By establishing cell autonomous regulation of MOR by NK1R activation in several neuronal populations, the present study identifies a new principle by which diverse GPCR-linked signaling systems may be coordinately regulated in the CNS.

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CHAPTER 4: SEQUENCE-DIRECTED RECYCLING OF POST-SYNAPTIC SIGNALING RECEPTORS SPECIFIES KINETICALLY DISTINCT MODES OF LOCAL SURFACE DELIVERY

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ABSTRACT

It is increasingly clear that many CNS signaling receptors undergo vesicular turnover in the post-synaptic plasma membrane on a time scale of minutes. Little is known about how signaling receptors are delivered to, or laterally distributed in, the somatodendritic surface so rapidly. We investigated these questions by focusing on the mu opioid receptor (MOR) and beta-2 adrenergic receptor (B2AR), two members of the G protein-coupled receptor (GPCR) superfamily that are rapidly mobile in the somatodendritic plasma membrane yet differ in native surface distribution. Endogenous and recombinant MORs returned to the surface of dendrites with similarly rapid kinetics as the B2AR (within 30 – 45 min), but efficient recycling of each receptor was mediated by a different cytoplasmic sorting sequence. Time-lapse TIR-FM imaging revealed that both sequences drive local exocytic insertion of receptors directly into the soma and throughout the dendritic shaft. Surprisingly, individual sequences imposed markedly different kinetics on the subsequent lateral movement of receptors. Recycling directed by the B2AR-derived sequence resulted in retention of receptors for a variable and sometimes prolonged time period (up to ~30 sec) near the site of local dendritic insertion, whereas

recycling directed by the MOR-derived sequence produced uniformly rapid (<1 sec) lateral dispersion. These results identify a novel function of sequence-directed recycling in mediating rapid local delivery of signaling receptors to the somatodendritic plasma membrane, and show that recycling sequences specifically couple local exocytic insertion to distinct kinetic modes of post-synaptic surface trafficking.

INTRODUCTION

Membrane trafficking of post-synaptic signaling receptors in central nervous system (CNS) neurons is a highly dynamic process. Though initially thought to be relatively static, it is increasingly clear that the post-synaptic plasma membrane contains a large fraction of signaling receptors that exhibit rapid lateral mobility (Choquet and Triller 2003). These receptors are also subject to incredibly specific regulation by vesicular trafficking processes, which plays an important role in the maintenance of spatiotemporal distribution of various signaling receptors under a fluctuating extracelular environment. However, the cellular mechanism underlying the trafficking properties of post-synaptic CNS signaling receptors remain poorly understood.

The endocytic pathway is one a specific place where robust membrane dynamics of CNS signaling receptors are particularly evident. Endocytic trafficking mechanisms are known to contribute to diverse processes of neural adaptation and plasticity by controlling the number of signaling receptors available for functional activation in the post-synaptic plasma membrane (Beattie et al., 2001; Carroll et al., 2001; Malinow and Malenka 2002; Sorkin and von Zastrow, 2002; Sheng and Kim 2002). Following endocytosis, post-synaptic signaling receptors undergo specific sorting between divergent downstream membrane pathways. One particular pathway relevant to the dynamic nature of the post-synaptic membrane is the rapid recycling of receptors back to the somatodendritic surface. This process can mediate an essential turnover of signaling receptors on a very rapid timescale. Recycling of various G protein-coupled receptors (GPCRs), the largest

family of signaling receptors mediating post-synaptic regulation in the CNS, often follows ligand-induced endocytosis and desensitization of receptors. One example of this is the mu opioid receptor (MOR), a GPCR that mediates signaling via endogenously produced opioid neuropeptides and is a major pharmacological target of highly effective yet commonly abused analgesic drugs (Evans 2000). It is thought that the recycling of MOR promotes functional resensitization of cellular opioid signaling and plays a critical role in regulating physiological responsiveness in the setting of chronic or repeated opioid administration (Koch et al., 2005; Martini and Whistler 2007). Thus, membrane dynamics in opioid trafficking can play a major role not only in downstream signaling of MOR, but also influence delivery and distribution of receptors back to the plasma membrane.

In this study, we investigated the fundamental problem of how receptors are replaced in the plasma membrane under conditions where there is rapid receptor turnover. Specifically, we focused on two GPCRs, the MOR and beta-2 adrenergic receptor (β2AR), both of which are capable of agonist-mediated endocytosis in CNS neurons and are distributed non-uniformly at steady state (Aoki et al., 1987; Wang et al., 1997). Our results indicate that MORs, like β2ARs, undergo rapid vesicular trafficking through the recycling pathway in primary striatal neurons where they are natively expressed. Interestingly, we found that this local and rapid delivery process is driven by receptor-specific cytoplasmic sorting sequences, previously observed only in non-neural cell models. Each sequence was found to produce a remarkably different effect on the lateral mobility of receptors following local exocytic insertion. These present results clearly

define a local delivery pathway that is sufficient to explain the remarkable ability of CNS neurons to turn over their receptor post-synaptic receptor complement. We propose that sequence-directed recycling, by linking local exocytic insertion to alternate kinetic modes of surface receptor trafficking, provides a novel means for efficiently generating receptor-specific patterns of surface distribution in the highly dynamic somatodendritic membrane environment.

MATERIALS AND METHODS

cDNA construction. The amino-terminally Flag (DYKDDDD) epitope-tagged versions of the cloned murine mu opioid receptor (MOR), murine mu opioid receptor lacking the last 17 amino acids in the carboxyl terminus (MORΔ17), and murine delta opioid receptor (DOR) were described previously (Keith et al., 1996; Tanowitz and von Zastrow 2003). The MOR and MORΔ17 receptors were sub-cloned into a pCAGG/SE vector at the *SacI/XhoI* sites.

SpH-MOR was generated by fusing the superecliptic pHluorin to the amino terminus of the previously described murine mu opioid receptor (Keith et al., 1996). The amino-terminally tagged SpH-β2AR was previous described (Yudowski et al., 2006). Chimeric SpH-MOR/β2AR constructs were generated by PCR-amplification of either the last 4 or last 10 carboxyl-terminal cytoplasmic residues of the β2AR incorporated into the reverse primer using the MOR cDNA as template. This fragment was then incorporated into SpH-MOR in frame at *5' BamHI* and 3' *XhoI* restriction sites. The chimeric SpH-

β2AR/MOR17 receptor was generated by subcloning a previously described β2AR/MOR17 receptor into SpH-β2AR at *5' HindIII* and *3' EcoRV* restriction sites (Tanowitz and von Zastrow, 2003). All receptors were cloned into a pCAGG/ES vector for expression in neurons. All constructs were confirmed by DNA sequencing (Elim Biopharmaceuticals, San Francisco, CA).

Cell cultures and transfections. Primary striatal, cortical, and hippocampal neurons were dissected from embryonic day 17-18 rat embryos taken from pregnant Sprague-Dawley rats. The striatum (caudate-putamen and nucleus accumbens) were dissected based on the criteria of Ventimiglia and Linsday (1998). The cortex and hippocampus was identified as described by Altman and Bayer (1995). Dissected tissue was dissociated in 1x trypsin/ EDTA solution (Invitrogen, Gaithersburg, MD) for 15 minutes before 1mL of trypsin inhibitor was added for 5 minutes at room temperature. Cells were washed and triturated in DMEM plus 10% fetal calf serum (FCS; Invitrogen). Mechanical trituration of tissue was performed using a glass pipette. Neuronal transfections were performed using electroporation (rat neuron nucleofector system; Amaxa Biosystems, Gaithersburg, MD) immediately after dissociation and plated on poly-L-lysine-coated (1mg/mL in 0.1 M sodium borate buffer, pH 8.5) glass coverslips at a density of 2 x 10^5 neurons per well. Electroporation was conducted using 5 x 10^6 dissociated cells, 3 μg of plasmid DNA, and 100 μL of rat neuron nucleofector solution. After transfection, neurons were kept in pre-warmed RPMI media (Cell culture facility, University of San Francisco, San Francisco, CA) for 10 minutes for recovery before being transferred onto 24-well plates. Media on the cells was replaced with Gibco Neurobasal media (Invitrogen) supplemented with B27

(Gibco) and L-glutamate 24 hours post transfection. Unless otherwise noted, all neurons were maintained 14 days in culture prior to immunocytochemistry and biotinylation assays. For live imaging, striatal neurons were maintained for 7 days in culture.

Immunocytochemistry analysis of endogenous receptor recycling. For immunocytochemical localization of endogenous receptor recycling, cultured striatal neurons (~7 days in culture) were pretreated with 10ug/mL cyclohexamide for 1 hour prior to incubation with 10μM of DAMGO for 30 minutes. Cells were either fixed immediately or incubated for an additional 45 minutes in the absence of agonist and in the presence of media containing 10μM of the opiate antagonist naloxone to prevent subsequent receptor activation and to allow recycling to occur. Cells were then fixed in ice-cold methanol for 10 minutes and blocked in PBS containing 5% goat serum, 0.3% Tween-20, and 0.1% Triton X-100 in PBS for 45 minutes. Staining for endogenous MOR was performed using an affinity-purified rabbit antibody recognizing the carboxyl terminus of MOR1 (Keith et al., 1998). Permeabilized specimens were incubated with the MOR1 antibody (1:1000) at room temperature for 2.5 hours and then extensively washed in PBS. Redistribution of receptors was visualized after secondary labeling with Alexa488-conjugated goat anti-rabbit IgG (2ug/mL; Molecular Probes) prepared in the same blocking solution as above and incubated for 30 minutes. Cells were then washed extensively with the appropriate buffered saline and mounted onto glass slides for fluorescence microscopy. Epifluorescence microscopy was carried out using a Nikon (Melville, NY) Diaphot microscope equipped with a 60x/numerical aperture (NA) 1.4 objective, mercury arc lamp illumination and standard dichroic filter sets (Omega

Optical, Brattleboro, VT). Images were collected with a CCD camera (Princeton Instruments, Trenton, NJ) and analyzed using MetaMorph® software (Molecular Devices, Sunnyvale, CA).

Immunocytochemistry and quantitative analysis of receptor recycling. Dissociated neurons were transfected with Flag-MOR, Flag-MORΔ17, or Flag-DOR. Dual-color antibody labeling of surface Flag-tagged receptors was used to follow the fate of ligand-induced internalization and recycling of receptors. Cells were incubated with Alexa594-conjugated M1 anti-Flag monoclonal antibody (Molecular Probes; 5 μg/ml) for 30 minutes at 37°C to selectively label Flag-tagged receptors present in the plasma membrane. Surface-labeled cells were then incubated in the absence (i.e., untreated) or presence of 10μM DAMGO (for MOR and MORΔ17) or 10μM DADLE (for DOR) for an additional 30 minutes. Agonist was washed out and remaining surface M1 antibody was dissociated ("stripped") with multiple washes using PBS supplemented with 0.04% EDTA. For assessment of internalization, neurons were immediately fixed in 4% paraformaldehyde in PBS for 15 minutes and then quenched in TBS. For neurons used to assess recycling, cells were incubated for an additional 45 minutes in the absence of agonist and in the presence of media containing 10μM naloxone. Neurons were then washed in PBS, fixed, and blocked in 3% BSA in TBS for 1 hour at room temperature. Cells were then incubated with FITC-conjugated anti-mouse antibody (4 ug/mL) for 1 hour in blocking solution to selectively detect only those labeled receptors that were once internalized and had returned back to the cell surface. In order to quantify the extent of recycling observed with epifluorescence microscopy, we used a previously described

assay to assess ratiometric staining of surface and internalized receptors (Tanowitz and von Zastrow 2003). In brief, the percentage of receptors recycled in individual cells following agonist washout was calculated from background-subtracted Alexa594 (red) and FITC (green) fluorescence intensity values. Epifluorescence microscopy was performed using an inverted microscope (Nikon Diaphot) equipped with a Nikon 20x/ numerical aperature (NA) 1.4 objective, mercury arc lamp illumination and standard dichroic filter sets (Omega Optical). Images were collected with a CCD camera (Princeton Instruments). For each experiment, ~30-50 neuronal cell bodies were analyzed per condition. Results were compiled from five experiments for each cell type and represented separate animals and culture preparations. Statistical analysis of differences between experimental groups was performed using unpaired Student's *t*-test.

Receptor recycling with surface biotinylation and immuoblotting Cortical neurons were transfected with either Flag-MOR or Flag-MORΔ17 and cultured in 6-well plates at a density of 6 x 10^6. Transfected cells were washed with cold Ca^{2+}- and Mg^{2+} -free PBS and then incubated with 0.3 mg/ml Sulfo-NHS-SS-biotin (Pierce) in PBS at 4°C for 30 minutes. Neurons were washed with TBS and placed in DMEM for 10 minutes at 37°C before incubation for 30 minutes in 10μM DAMGO. For the control stripping and internalization conditions, cells were washed on ice with TBS to remove and quench residual biotinylation reagent and remaining cell surface biotinylated receptors were stripped at 4°C three times for 15 minutes each using 100mM MESNA (Sigma-Aldrich) in 50mM Tris 8.8, 100mM NaCl, 1mM EDTA, and 0.2% BSA. Stripped cells were then washed with TBS and quenched with iodoacetamide buffer (22 mg/ml iodoacetamide in

TBS) for 10 minutes at 4°C. For the recycling conditions, neurons were returned to media containing 10μM naloxone for 45 minutes. Cells were washed and stripped as previously mentioned. All cells were extracted with lysis buffer containing 1 mg/ml iodoacetamide in IP buffer (0.2% Triton X-100, 150 mM NaCl, 25 mM KCl, 10 mM Tris 7.4) supplemented with 0.1 mM EDTA and a Complete mini EDTA-free protease inhibitor cocktail tablet (1 tablet/10 ml; Roche). Cell debris was removed by centrifugation at 13,000 x g for 15 minutes at 4°C. Extracts containing equal amounts of cellular protein were incubated with immobilized streptavidin beads (Pierce) on a rotator overnight at 4°C. Protein-bound streptavidin beads were centrifuged and washed with IP buffer. Proteins were eluted and denatured in SDS sample buffer with 10% β-mercaptoethanol and separated by SDS/PAGE. Proteins were transferred to nitrocellulose and biotinylated proteins were detected by immunoblotting with anti-Flag M1 mouse antibody (5 μg/mL; Sigma) for 1 hour followed by anti-mouse IgG horseradish peroxidase conjugate (1:5000, Amersham) for 1 hour. Immunoreactive protein bands were detected using Super Signal (Pierce) and quantified by densitometry. Quantification reflects results from 3 independent experiments.

Live imaging of neurons in culture. Cultured striatal neurons were transfected with SpH-tagged receptors by eletroporation (Amaxa Biosystems) immediately after dissociation and plated on 35mm glass bottom dishes (MatTek, Ashland, MA). Neurons were cultured ~7 days before assaying. Imaging was carried out using a Nikon TE-2000E inverted microscope with a 100x 1.49 numerical aperture (NA) TIRF objective. A 488 nm single-line argon ion laser (Melles Griot, Rochester, NY) was used for total internal reflection

fluorescence (TIRF) microscopy illumination modes. Neurons were imaged in conditioned neurobasal medium supplemented with 125 mM NaCl, 2.5 mM Kcl, 2mM $CaCl_2$, 10mM D-glucose, and 30mM HEPES buffer (pH=7.4). Temperature was controlled at 37°C using a thermoelectric stage (Bioscience Tools, San Diego, CA) and an objective warmer (Bioptechs, Butler, PA). Time-lapse sequences were imaged at a continuous rate of 10 frames per second. Insertion events were analyzed using ImageJ64 software.

RESULTS

Mu opioid receptor recycling occurs in distinct populations of primary neurons

Although agonist-mediated recycling of the mu opioid receptor (MOR) has previously been demonstrated in heterologous cells (Tanowitz and von Zastrow 2003), it is unknown whether endogenous MORs can efficiently recycle in CNS neurons in which they are natively expressed. To determine this, we examined endogenous MOR localization in primary cultures of rat striatal neurons, where a significant fraction of these neurons express endogenous MORs (Gray et al., 2006). In the absence of agonist, MOR immunoreactivity was visualized as a smooth plasma membrane distribution over the surface of the neuron (Fig 1A, left). Incubation with 10μM of the enkephalin analog D-Ala^2-*N*-Me-Phe^4-Glycol^5-enkephalin (DAMGO) for 30 minutes produced pronounced redistribution of endogenous MORs into a punctate pattern consistent with internalization of receptors (Fig 1A, middle; Haberstock-Debic et al., 2005). In order to determine whether redistributed MORs could recycle back to the cell surface, agonist was washed out and neurons were allowed to recover for 45 minutes in the absence of DAMGO and in the presence of the opiate antagonist naloxone (10μM) in order to block any residual agonist activity. Under these conditions, MOR expression changed from a punctate pattern back to a plasma membrane distribution (Fig 1A, right). Redistribution of MORs back to the cell surface after agonist-mediated internalization was unaffected by addition of 10μg/mL cyclohexamide (to block new receptor synthesis) in the culture medium for 1 hr. prior to agonist treatment, suggesting that endogenous MORs can engage in rapid and efficient recycling in primary CNS neurons.

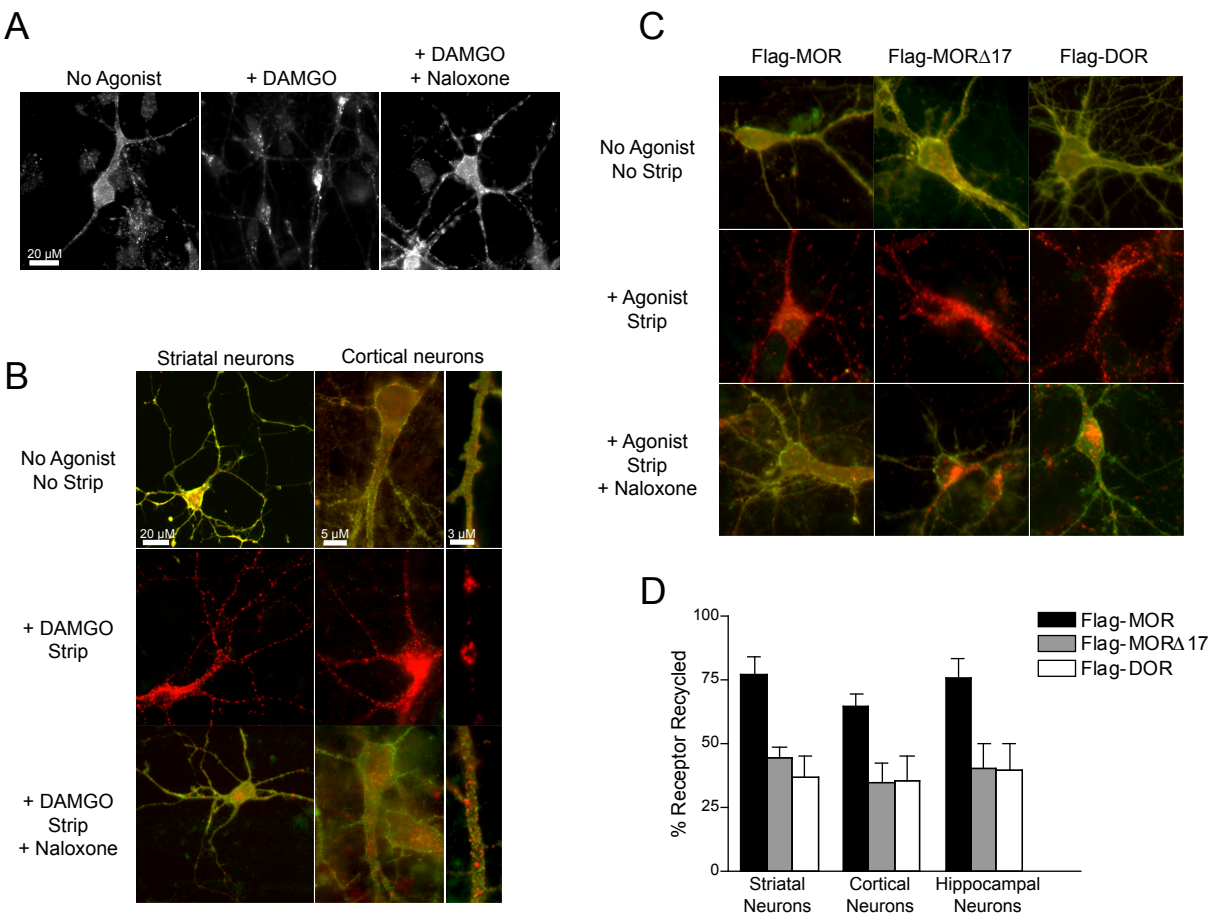

Figure 1. MOR recycling is sequence-dependent and occurs in both the cell body and dendrites of neurons from multiple brain regions. (A) Representative epifluorescence micrographs show localization of endogenous MOR in dissociated striatal cultures. Incubation with 10mM DAMGO for 30 minutes caused a redistribution of receptors. Subsequent washout of agonist and recovery in the presence of 10mM naloxone produced a redistribution of internalized receptors back to the plasma membrane. (B) Striatal and cortical neurons expressing Flag-MOR were labeled with M1-Alexa594 antibody. Incubation with 10 μM DAMGO, followed by stripping of remaining surface M1 antibody with EDTA, shows internalization of receptors in both cell bodies and dendrites of striatal and cortical neurons (middle panels). Incubation with FITC-conjugated anti-mouse antibody identified Flag-MORs that had recycled back to the plasma membrane after recovery in the absence of agonist and presence of 10 μM naloxone for 45 minutes. (C) Dual-label fluorescence antibody labeling compares the extent of recycling between Flag-MOR, Flag-MORΔ17, and Flag-DOR. (D) A ratio of green (surface receptors after agonist treatment followed by washout and recovery in naloxone) to red (total receptor) fluorescence intensity was used to determine percentage of Flag-MOR, Flag-MORΔ17, and Flag-DOR recycling in striatal, cortical and hippocampal neurons. Bar graphs represent mean percent receptor recycled determined from 30-50 cell bodies selected at random in neuronal cultures and averaged over five independent cultures.

MOR recycling occurs in both the cell body and dendrites of multiple neuronal populations

To further investigate recycling of MORs in neurons, we use amino-terminus epitope-tagged recombinant receptors to unambiguously follow the trafficking fate of surface receptors. Primary cultures of striatal and cortical neurons were dissected from embryonic rat brains and transfected by electroporation with Flag-MOR. In order to examine the trafficking of Flag-MORs, we used a previously established dual-color fluorescence labeling technique to distinguish between the internalized and recycled pool of receptors (Tanowitz and von Zastrow 2003). Surface receptors were first labeled with a calcium-dependent anti-Flag antibody (M1) conjugated to Alexa594 (red) that remains associated with receptors in the recycling pathway (Cao et al., 1999), and recycling of this internal receptor pool was identified by surface accessibility with a secondary FITC (green) antibody (see Materials and Methods for details). In the absence of opioid, F-MORs remained localized in the somatodendritic plasma membrane in both striatal and cortical neurons, as indicated by substantial overlap in red and green fluorescence (Fig 1B, top panels). Incubation of neurons with 10μM of the enkephalin analog D-Ala^2-*N*-Me-Phe^4-Glycol^5-enkephalin (DAMGO) for 30 minutes resulted in the endoctyosis of F-MORs. This was observed as a punctate distribution pattern of M1-labeled F-MORs in both striatal and cortical neurons where internalized receptors were resistant to dissociation, or "stripping", of the M1 antibody when calcium was depleted from the extracellular medium with PBS containing EDTA (Fig 1B, middle panels). Internalized receptors were clearly detected both in the cell body and in dendrites using this approach. FITC secondary labeling was undetectable under these conditions, confirming that the

stripping condition effectively dissociated M1 antibody from receptors remaining in the plasma membrane.

In order to test the ability of internalized receptors to recycle back to the plasma membrane, neurons were returned to normal culture medium after stripping and incubated for an additional 45 minutes in the absence of DAMGO and in the presence the opiate antagonist naloxone (10μM) to block any residual agonist activity. Dual-color epifluorescence microscopy revealed a pronounced increase in M1-labeled receptors accessible to extracellular FITC secondary antibody, indicating that a substantial fraction of internalized F-MORs returned to the cell surface after agonist washout (Fig 1B, bottom panels). This nearly complete recycling of F-MORs was observed both in the cell body and dendrites of striatal and cortical neurons.

Recycling of MORs is sequence-dependent in multiple neuronal populations

Efficient recycling of the mu opioid receptor (MOR) after agonist-induced endocytosis has previously been demonstrated to be dependent on a specific sequence present in the distal portion of its carboxyl terminus in non-neural cell models (Tanowitz and von Zastrow 2003). However, it is unknown whether this "recycling signal" also functions in neurons. To address this question, the dual label fluorescence method was used to compare endocytic trafficking of the wild type MOR with that of mutant MOR from which the 17-residue C-terminal recycling sequence was removed by truncation (F-MORΔ17) and with that of the wild type delta opioid receptor (F-DOR) that naturally lacks this sequence (Tsao and von Zastrow 2000). Cultured cortical neurons were

transfected with Flag-MOR, Flag-MORΔ17, or Flag-DOR. Dual-label epifluorescence microscopy revealed a smooth plasma membrane distribution of receptors in untreated cells (Fig 2C, top panels). With 30 minutes of either 10μM DAMGO (for Flag-MOR and Flag-MORΔ17) or 10μM [D-Ala2, D-Leu5] enkephalin (DADLE, for Flag-DOR) incubation, all three receptor types underwent agonist-mediated endocytosis, as indicated by a punctate distribution pattern of labeled receptors. Stripping of remaining surface M1-Alexa594 antibodies with PBS-EDTA revealed comparable receptor internalization between Flag-MOR, Flag-MORΔ17, and Flag-DOR (Fig 1C, middle panels). However, the extent of recycling varied greatly between the three receptors types. Whereas wild type Flag-MORs recycled almost completely when cells were visualized 45 minutes after agonist washout, a considerable fraction of labeled Flag-MORΔ17 mutant receptors remained in endocytic vesicles that were not accessible to extracellular secondary antibody (Fig 1C, bottom panels). A similar deficiency in recycling was observed with Flag-DORs (Fig 1C, bottom right panel). To determine whether recycling occurs in other neuronal types, this dual-label fluorescence assay was also conducted using cultured striatal and hippocampal neurons. Quantification of receptor recycling across multiple experiments by fluorescence imaging using a previously established ratiometric assay (Tanowitz and von Zastrow 2003) confirmed efficient recycling of Flag-MORs in all three neuronal types (Fig 1D). Further, quantification verified that both the mutant Flag-MORΔ17 and Flag-DOR lacking the MOR-derived recycling sequence recycled to a significantly smaller extent.

Biochemical analysis of MOR recycling in neurons

To further test the function of the MOR-derived recycling sequence in neurons, we used a biochemical assay to specifically measure the fate of surface-biotinylated receptors in a population of cortical neurons expressing either Flag-MOR or Flag-MORΔ17. Neurons were surface-biotinylated and then immunoblotted using an anti-Flag antibody after a streptavidin pull-down (Fig 2A). A schematic diagram illustrates the procedure used to follow the fate of biotinylated receptors during both endocytosis and recycling (Fig 2B). After surface biotinylation, cortical neurons expressing either Flag-MOR or Flag-MORΔ17 were incubated with 10μM DAMGO for 30 minutes. Biotin attached to receptors remaining in the plasma membrane was selectively cleaved using a membrane-impermeant reducing agent and the internalized (still biotinylated) pool of Flag-tagged receptors was selectively detected using anti-Flag following isolation on streptavidin beads (Fig 3C, lane 4). Internalization between Flag-MOR and Flag-MORΔ17 was comparable, further confirming that truncation of the MOR recycling sequence does not affect agonist-mediated endocytosis of MOR (Fig 3C lane 3; Fig 3D). In order to examine recycling of these receptors, agonist was then washed out and neurons were incubated with naloxone for 45 minutes (Fig 3C, lane 5). Recycled receptors (which were protected from the initial round of biotin cleavage) underwent a second biotin cleavage and remaining internal receptors (i.e., receptors that had failed to recycle) were isolated on streptavidin beads and immunoblotted with M1 anti-Flag antibody (Fig 3C, lane 6). Compared to Flag-MOR, significantly more Flag-MORΔ17 remained internal, indicating a failure to recycle back to the plasma membrane (Figure 3C, compare lane 6 for Flag-MOR versus Flag-MORΔ17). Quantification of the immuoblot showed a significant

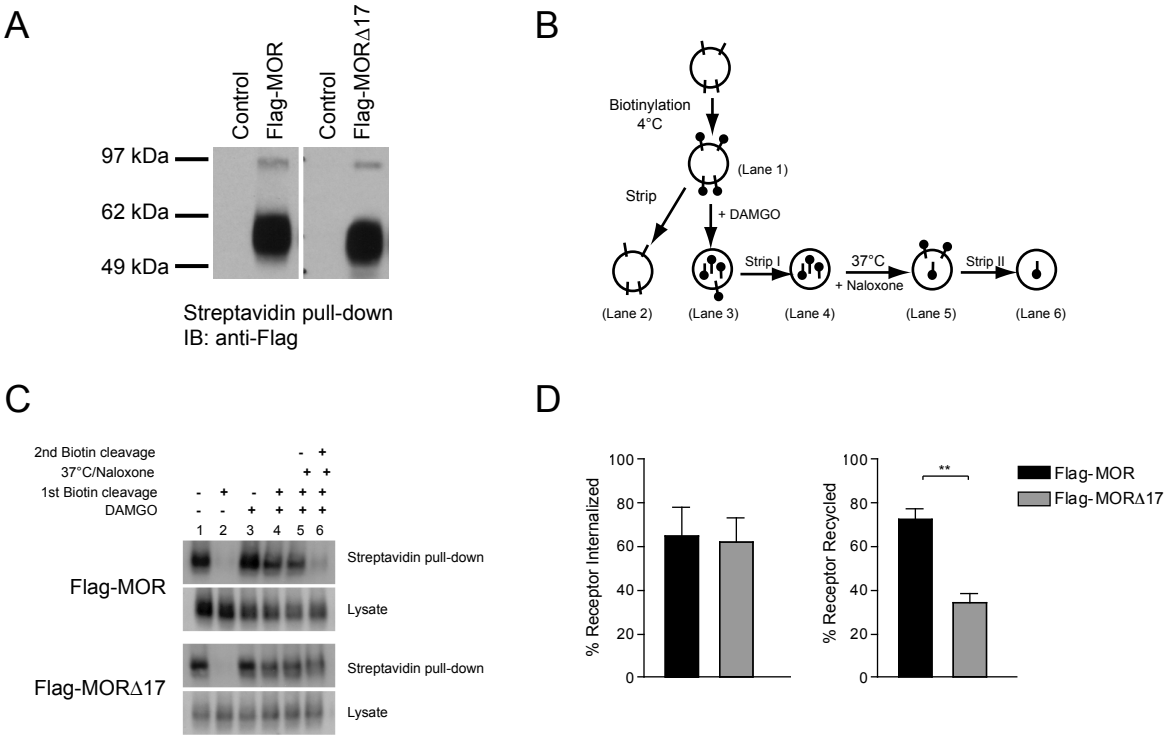

Figure 2. Biochemical analysis establishes that MOR recycling is sequence-dependent in neurons. (A) Surface biotinylation followed by streptavidin pull-down and immublotting of Flag-MOR and Flag-MORΔ17. (B) Schematic of biotinylation recycling assay. The internalized pool of receptors was followed back to the cell's surface (i.e., recycling) through two subsequent surface biotin cleavage strips. (C) Biochemical assay of receptor recycling by surface biotinylation show that although the internalized pools of receptors between Flag-MOR and Flag-MORΔ17 is comparable (lane 4), the pool of internal Flag-MORs after agonist washout and recovery is significantly less, compared to Flag-MORΔ17 (lane 6). (D) Quantification from surface biotinylation experiments. Bar graphs represent mean internalization (left) and mean percent of receptor recycled (right) averaged over three independent experiments (**p< 0.002).

difference in recycling between the wild type and mutant MOR in cortical neurons (p= 0.002; Fig 3D, right panel).

Vesicular fusion events mediate mu opioid receptor recycling in both the cell body and dendrites of striatal neurons

While epifluorescence microscopy and biochemical analysis allowed us to examine MOR recycling in whole populations of neurons, we know little about how and where individual recycling events occur in real time. In order to examine single-event recycling episodes in live neurons, we fused the green fluorescent protein variant superecliptic pHluorin (SpH) to the amino-terminal extracellular domain of the murine mu opioid receptor (SpH-MOR). When exposed to the extracellular environment with a neutral pH of 7.4, SpH is highly fluorescent. However, its fluorescence is rapidly and reversibly quenched in the acidic environment of the endocytic pathway (Miesenbock et al., 1998; Sankaranarayanan et al., 2000). Total internal reflection fluorescence (TIRF) microscopy was used for the rapid imaging of single vesicular events in order to observe exocytic insertion events in conditions for maximal signal-to-noise ratio and minimize phototoxicity (Steyer et al., 2001). This method has previously been shown to resolve discrete vesicular fusion events mediating recycling of another GPCR, the beta2 adrenergic receptor (β2AR), as well as surface delivery of AMPA-type ionotropic glutamate receptors (Yudowski et al., 2006; Yudowski et al., 2007).

A punctate distribution of SpH-tagged receptor fluorescence was observed in TIRF images of DAMGO-exposed neurons, both in the soma and regions of dendritic shaft

domain illuminated by the ~100 nm thick evanescent illumination field (Fig 3A), consistent with endocytic 'clusters' shown previously to represent clathrin-coated pits containing ligand-activated receptors (Puthenveedu and von Zastrow, 2006;Yudowski et al., 2006). These punctate structures were not highly dynamic during the 1 minute imaging episode, as expected because individual receptor-containing coated pits have a surface lifetime of ~ 1 minute (Puthenveedu and von Zastrow, 2006). Rapid serial acquisition (10 Hz) of TIR-FM images revealed distinct bursts of increased SpH-MOR fluorescence intensity on a much faster time scale, visible both in the soma and dendrites of the same neurons (Fig 3A). These abrupt surface events were distinguished from endocytic clusters by their substantially higher brightness and uniformly abrupt appearance within a single 100 msec frame, similar to the individual vesicular fusion events mediating surface insertion of SpH-tagged β2AR and AMPA receptors previously observed in hippocampal neurons (Yudowski et al., 2006; Yudowski et al., 2007). Confirming this, SpH-MOR fusion events (similar to β2AR and AMPAR events described previously) were rapidly quenched by addition to the imaging medium of a membrane-impermeant acidic buffer (0.1M MES pH= 5.5, not shown). Together these observations suggest that SpH-MOR fusion events observed under these conditions represent discrete receptor-containing vesicular fusion events.

Multiple SpH-MOR-containing insertion events were observed during each minute-long imaging episode (16± 4.1 events/ neuron/ minute; n= 19 neurons) and, as observed also for SpH-β_2AR in hippocampal neurons, SpH-MOR insertion events occurred throughout the soma and dendrites. Surface insertion was often followed by visually apparent lateral

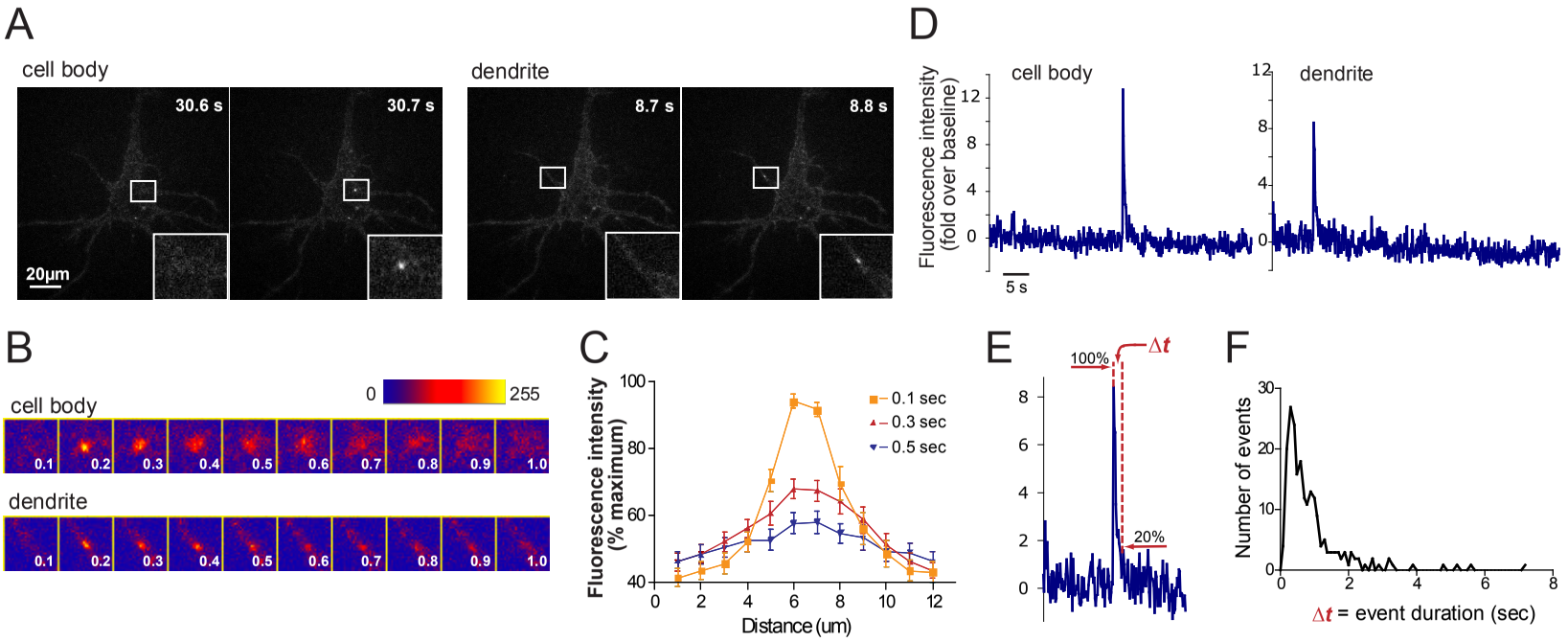

Figure 3. TIRF imaging reveals vesicular insertion of SpH-MOR into the soma and dendrites. Dissociated rat striatal neurons expressing SpH-MOR were incubated in the presence of 10 μM DAMGO for 15 minutes and imaged by rapid TIRF microscopy. (A) Sequential 100-ms exposures show representative vesicular fusion events mediating surface insertion of SpH-MORs into both the cell body (left panels) and dendrite (right panels) of a cultured medium spiny neuron. (B) Detailed view of sequential 100-msec frames showing time course of SpH-MOR fluorescence after insertion into both the soma (top) and dendritic shaft (bottom). Fluorescent gradient scale represents intensity of pixel values ranging from 0 (min) to 255 (max). (C) Fluorescent line scan analysis of multiple SpH-MOR insertion events, demonstrating rapid lateral spread of fluorescence after insertion. Average normalized pixel intensity is plotted as a function of distance in the line scan. Curves representing the frame with the maximum pixel intensity (t= 0.1 sec) and two subsequent 100-ms exposures (t= 0.3 sec and 0.5 sec) are plotted. Error bars represent mean standard error over n= 26 events. (D) Time course of surface fluorescence intensity change measured at the displayed vesicular insertion events, after subtraction of the baseline fluorescence signal representing average receptor number in the plasma membrane and displayed as fold-increase relative to this baseline value. (E) Expanded view of the time course plotted in panel D (right), showing the method used to measure event duration (Δt). (F) Histogram showing the distribution of individual event durations compiled from analysis of 1 min episodes derived from all individual events recognized visually in the soma and dendrites of multiple neurons (n= 307 events).

spread of SpH-MOR in both plasma membrane domains (Fig 3B). Analysis of line scans in sequential 100-ms images across discrete fluorescent events revealed rapid lateral spreading of SpH-MOR fluorescence during the decay of insertion spots (Fig 3C). Parallel experiments conducted using the MRS-deleted SpH-MORΔ17 mutant receptor construct confirmed similar DAMGO-induced concentration of receptors in coated pits but revealed a greatly reduced frequency of visible receptor insertion events (<2 events/ neuron/ minute; n= 12). These observations indicate that the majority of insertion events visualized in our experiments originated from the recycling pathway, further verifying the MRS-dependence of MOR recycling in neurons and support the steady state hypothesis of MOR internalization (Keith et al., 1998). These results directly show that MRS-dependent recycling of MOR occurs rapidly after DAMGO-induced endocytosis and can be observed even in the continuous presence of opioid agonist.

Receptor-specific differences in lateral dispersion after insertion to the somatodendritic surface

We verified that SpH-MOR rapidly dissipated from the site of vesicular insertion by measuring fluorescence intensity as a function of time in a small (3 x 3 pixel) region including the diffraction-limited spot representing the site of abrupt initial appearance (Fig 3D). We then measured the time interval between the appearance of the fluorescent event and the subsequent frame in which the background-subtracted local surface fluorescence intensity dissipated to < 20% of this peak value (Fig 3E). Using this approach, the vast majority of SpH-MOR vesicular insertion events were found to

dissipate within 1 - 2 sec after initial surface insertion, with an average duration of 0.86 ± 0.05 sec (Fig 3F; n= 307 events in 19 neurons).

It has previously been established that SpH-β2AR can exhibit long-lasting persistent events in hippocampal neurons (Yudowski et al., 2006). Because SpH-MOR displayed only transient, and no persistent, plasma membrane insertion events, we next asked whether it is the unique characteristics of the receptor that determines insertion kinetics, or the cellular background in which it is expressed. Therefore, we examined whether SpH-β2AR persistent events can also occur in striatal neurons. Cultured striatal neurons expressing SpH-β2AR were treated with 10μM of the adrenergic agonist isoproterenol for 15 minutes before imaging by TIRF illumination. Multiple insertion events were observed throughout the somatodendritic plasma membrane during serial acquisitions in 100-ms intervals. Although most events were transient in nature, a subset of events lasted noticeably longer than what was observed with SpH-MOR (Fig 4A and B). A representative fluorescence intensity trace show that these events are characterized by the onset of a stable spot of increased fluorescence, followed by a period of its persistence in the plasma membrane before a decline in fluorescence intensity to background levels. Although differing in their duration, both transient and persistent insertion events were similar in fluorescence intensity (data not shown). Similar to what was previously reported in hippocampal neurons (Yudowski et al., 2006), numerous SpH-β2AR insertion events exhibited surface lifetimes in excess of 3 seconds, suggesting that kinetically heterogenous SpH-β2AR recycling events can also occur in striatal neurons (Fig 4B). Remarkably, in contrast, the onset and then dispersion of SpH-MOR insertion events

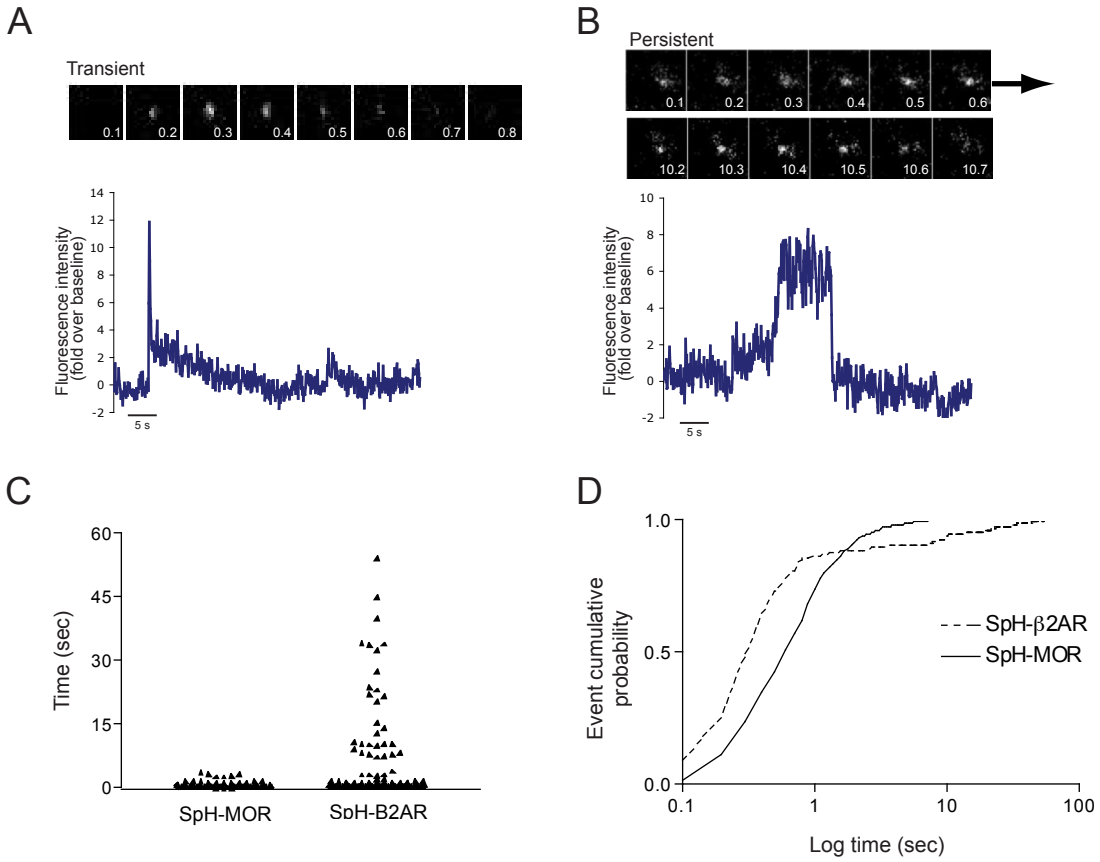

Figure 4. SpH-β2AR insertion events show both transient and persistent insertion kinetics and differ from the kinetics of SpH-MOR insertion events. Dissociated rat striatal neurons were transfected with SpH-β2AR, incubated in the presence of 10 μM isoproterenol for 15 minutes, and imaged by TIRF illumination in 100-ms intervals. (A) Time series show sequential frames of a representative transient SpH-β2AR insertion event (top panels). The time course of fluorescence intensity change of this transient insertion event is shown below. (B) Time series show sequential frames of a representative persistent SpH-β2AR insertion event (top panels). Arrow indicates a gap of 0.6 seconds during which the insertion event remained visible as a discrete spot. The time course of fluorescence intensity change of this persistent event is shown below. (C) Scatter plot comparison of all observed SpH-MOR (n= 307) and SpH-β2AR (n= 300) insertion events plotted according to event duration. Each triangle represents a single plasma membrane insertion event. (D) Cumulative event histogram comparison of all SpH-β2AR and SpH-MOR insertion events shows a difference in

were both shorter in duration and represented a more uniform population (Fig 4C). Cumulative frequency analysis supported a bimodal kinetic distribution of SpH-β2AR insertion events, whereas parallel analysis of SpH-MOR recycling events fit a single population (Fig 4D). Further analysis revealed that events lasting 3 or more seconds comprised 10% of total observed SpH-β2AR insertion events (n= 300 events in 10 neurons), while only 2.6% of SpH-MOR insertion events fell into this category (Fig 6B). Together, these results indicate that the kinetics of insertion events are dictated not by differences in neuronal type where the receptors are expressed, but are rather determined by unique features of the receptor itself.

The cytoplasmic tail of the beta2-adrenergic receptor is sufficient for occurrence of persistent insertion events

Although both MORs and β2ARs undergo sequence-directed recycling, the specific residues between the two recycling signals are distinctly different. In order to address this, we constructed SpH-MOR/β2AR chimeric receptors to investigate whether recycling sequences play a role in kinetics of receptor insertion. It has previously been established that recycling of β2ARs is mediated by a specific amino acid sequence present in the cytoplasmic tail of the receptor (Cao et al., 1999). Specifically, the last 4 amino acids of β2AR cytoplasmic tail contains the PDZ protein-interaction domain and is the minimal sequence to promote sorting of internalized receptors into the rapid recycling pathway (Gage et al., 2005). To determine whether this sequence domain is sufficient for presence of persistent insertion events, we constructed a chimeric receptor where the MOR recycling sequence was replaced with the last 4 amino acids of the β2AR (Fig 5A).

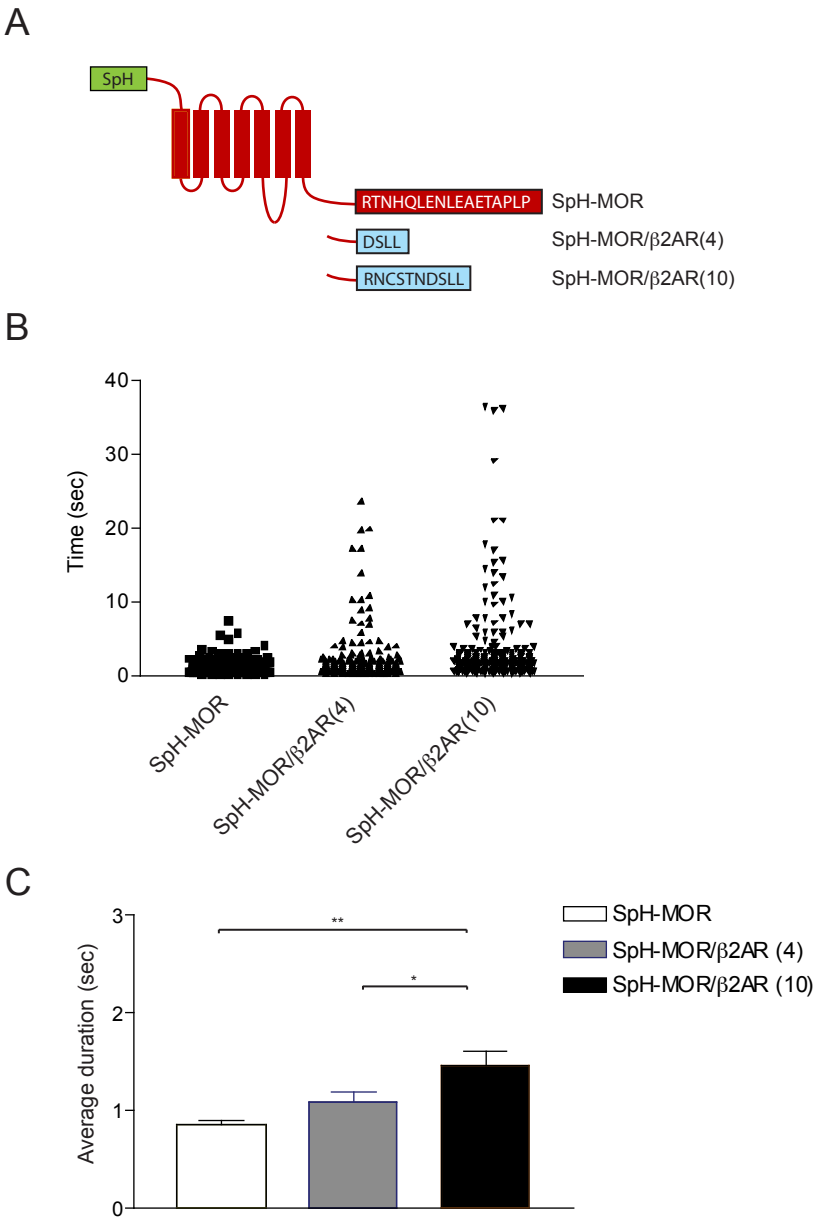

Figure 5. The cytoplasmic tail of the β2AR mediates persistent kinetic insertion events. (A) Schematic of wild-type SpH-MOR and SpH-MOR/β2AR chimeric receptors. For the chimeric receptors, either the last four or the last ten amino acids of the β2AR cytoplasmic tail were substituted for the MOR cytoplasmic tail recycling sequence. (B) Scatter plot diagram compares all observed SpH-MOR, SpH-MOR/β2AR(4), and SpH-MOR/β2AR(10) insertion events plotted according to event duration. (C) Bar graphs represent mean duration of all SpH-MOR (n= 307), SpH-MOR/β2AR(4) (n= 493), and SpH-MOR/β2AR(10) (n= 661) individual insertion events (**p= 0.002, *p= 0.5).

Cultured striatal neurons expressing SpH-MOR/β2AR(4) were treated with 10μM DAMGO for 15 minutes prior to imaging by TIRF illumination in the continuous presence of agonist. Sequential 100-ms exposures revealed frequent insertion events in the plasma membrane of both cell bodies and dendrites of these neurons that were similar in character to the wild type SpH-MOR, demonstrating that the cytoplasmic recycling sequences of distinct GPCRs are functionally interchangeable in neurons. There was a slight increase in the frequency of persistent insertion events compared to wild-type SpH-MOR (Fig 5B), although the average duration of all SpH-MOR/β2AR(4) events was not significantly longer than that of SpH-MOR (Fig 5C; $p= 0.1$).

Structural and biochemical studies indicate that although PDZ interactions are mediated primarily by the last 4 residues of β2AR's cytoplasmic tail, there is evidence that residues upstream of this core motif can enhance additional binding affinity and specificity (Zhang et al., 2006). To investigate whether an extended recycling sequence plays a role in the frequency of persistent insertion events, we engineered a chimeric mu opioid receptor where the MOR recycling sequence was replaced with the last 10 amino acids of β2AR cytoplasmic tail SpH-MOR/β2AR(10) (Fig 5A). Cultured striatal neurons expressing SpH-MOR/β2AR(10) were exposed to 10μM DAMGO for 15 minutes prior to imaging by TIRF illumination in the continuous presence of agonist. Similar to what was previously observed, sequential 100-ms exposures revealed receptor insertion events in both the cell body and dendrites of neurons. A scatter plot diagram of all insertion events shows that SpH-MOR/β2AR(10) exhibited a marked increase in frequency of longer-lasting events, compared to both SpH-MOR and SpH-MOR/β2AR(4) (Fig 5B). This

increase was also revealed in a significantly longer average duration of insertion events (Fig 5C, p= 0.002). Surprisingly, the extended replacement of the β2AR sequence (i.e., 10 residues) produced insertion events that had a significantly longer average duration compared to the chimera with the minimal 4-resude recycling sequence (Fig 5C, p= 0.03). Together, the positive correlation between extent of β2AR recycling sequence substitution and insertion event duration suggests the recycling sequence of the β2AR cytoplasmic tail is sufficient to mediate persistent exocytic insertion events.

The cytoplasmic tail of β2AR is necessary for persistent insertion events

To further investigate the β2AR cytoplasmic tail's role in mediating persistent insertion events, we next asked if the β2AR recycling sequence is necessary for long-lasting events. A chimeric receptor was constructed in which the cytoplasmic tail of the SpH-β2AR was replaced with the MOR recycling sequence (SpH-β2AR/MOR17; Fig 6A). Cultured striatal neurons expressing SpH-β2AR/MOR17 were incubated with 10μM isoproterenol for 15 minutes before imaging by TIRF illumination. Analysis of insertion event duration show that replacing the β2AR cytoplasmic tail with the MOR recycling sequence significantly reduced the average duration of insertion events, compared to wild type SpH-β2AR (Fig 6A; p< 0.0001), suggesting that the extended recycling sequence of the β2AR is required for persistent insertion events.

When all insertion events were grouped either as transient (< 3 seconds) or persistent (≥ 3 seconds), a notably larger proportion of all SpH-MOR/β2AR(10) events were persistent, compared to wild-type SpH-MOR (7.6% vs. 2.6%, Fig 6C). Although not statistically significant, SpH-MOR/β2AR(4) did exhibit noticeably more events lasting 3 seconds or

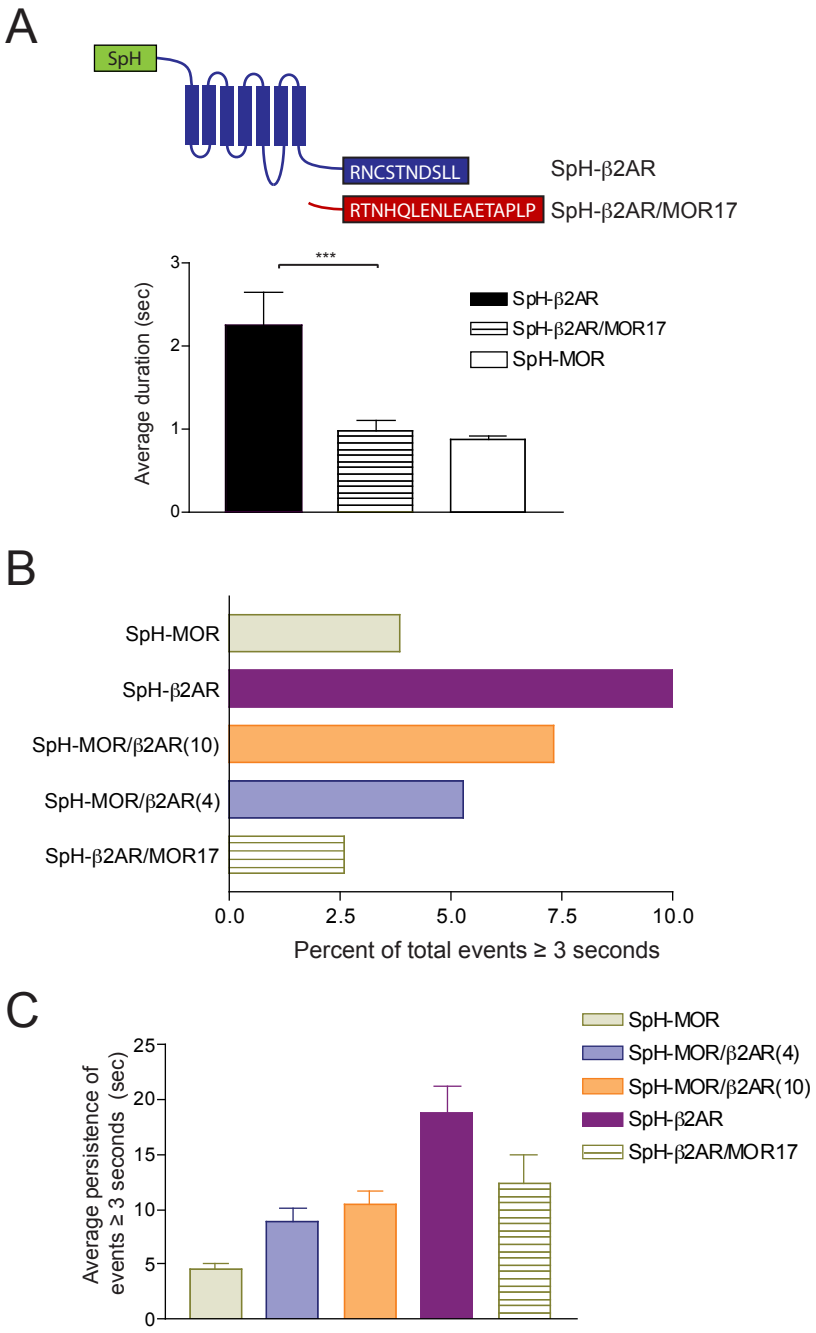

Figure 6. The cytoplasmic tail of the β2AR is necessary for persistent insertion events. (A) Schematic of wild-type SpH-β2AR and SpH-β2AR/MOR17 chimeric receptor, where the last 17 amino acids of the MOR cytoplasmic tail were substituted for the β2AR cytoplasmic recycling sequence. Bar graphs represent mean duration of all SpH-b2AR (n= 300) and SpH-β2AR/MOR17 (n= 647) insertion events (p< 0.0002). (B) Bar graphs show proportion of total events imaged for each receptor type that persisted for 3 or more seconds. (D) Bar graphs represent mean duration of insertion events lasting 3 seconds or longer for each receptor type.

DISCUSSION

The present results are the first to define a specific cell biological mechanism for regulating post-endocytic delivery of GPCRs into the somatodendritic membrane of CNS neurons. Our results demonstrate that both endogenous and recombinant MORs recycle within minutes in multiple primary neuronal populations after agonist-mediated endocytosis. This rapid receptor delivery occurs via discrete exocytic events occurring both in the cell body and throughout dendrites. We further show that recycling of MORs and β2ARs was driven by different cytoplasmic sorting sequences and are functionally interchangeable with respect to their ability to drive rapid insertion of receptors into the somatodendritic membrane. Surprisingly, these sequences produced markedly different effects on the lateral movement of receptors after their local exocytic insertion. We found a significant fraction of recycling events mediated by the β2AR sorting sequence were retained at the site of membrane insertion for a prolonged period of time before lateral dispsersion. In contrast, all exocytic recycling events driven by the MOR sorting sequence resulted in uniformly rapid lateral dispersion after insertion into the somatodendritic membrane. These results identify a novel function for GPCR recycling sequences and reveal a cell biological mechanism regulating the rapid dynamic properties of post-endocytic receptor delivery and mobility in CNS neurons.

Although previous studies have suggested that MORs are capable of recycling in physiologically relevant CNS neurons (Arrtamangkul et al., 2008), our study is the first to define a specific cellular mechanism driving this trafficking pathway. In addition to

affecting longer-term regulation of opioid responsiveness as previously suggested by other groups (Koch et al., 2005; Martini and Whistler 2007), rapid turnover of MOR is likely to be functionally important for the regulation of acute opioid signaling in response to both native peptide ligands and abused opioid drugs. Thus, sequence-directed recycling of MORs may play a major role in controlling physiological opioid responsiveness. More generally, that the specific sorting sequence required for rapid and efficient MOR recycling in neurons is the same as the one mediating this process in non-neural cells (Tanowitz and von Zastrow 2003) suggests that sequence-directed recycling is conserved in diverse mammalian types.

Our results are the first to indicate that biochemically distinct receptor domains differ significantly in their effect on the exocytic diffusion behavior of receptors during recycling. While sequence-dependent recycling has been described for several other GPCRs, there is a remarkable lack of conservation among these receptors (Tsao and von Zastrow 2000; Tanowitz and von Zastrow 2003; Gage et al., 2001; Vargas and von Zastrow 2004). The striking divergence in these sequences, which all function to sort receptors into the recycling pathway, begs the question of how sequence specificity functions in differentiating post-endocytic recycling behavior among different GPCRs. By imaging discrete insertion events using time-lapse TIRF microscopy, we have revealed that distinct MOR and β2AR sequences couple local exocytic insertion to distinct kinetic modes of surface receptor trafficking. The ability of the cytoplasmic tail sequence to govern this spatiotemporal insertion of receptors has previously been shown with AMPA receptors, where distinct sequences of different subunits determined the

trafficking kinetics of entire receptor (Passafaro et al., 2001). Thus, that cytoplasmic sequences can drive distinct patterns of surface receptor accumulation in neurons may be a more general phenomenon for GPCRs.

Because previous analysis of both β2ARs and the AMPA-type glutamate receptors revealed the presence of persistent insertion events (Yudowski et al., 2006, Yudowski et al., 2007), it was initially anticipated that persistent events might be a common characteristic of receptor insertion in general. However, we were surprised to find that not only were MOR insertion events transient in nature, but they also represented a more uniform population of events, compared to β2ARs (see Fig 4D). Given that receptor mobility in the plasma membrane is variable and may depend on the presence of anchoring and/or scaffolding proteins, it is highly possible that temporal differences in lateral diffusion of receptors could be due to interaction of cytoplasmic receptor domains with cytoskeletal and membrane proteins (Choquet and Triller 2003). Although surprisingly little is known about binding partners of the MOR cytoplasmic tail, a number of groups have suggested that various regulatory proteins can bind to this region and affect MOR function (Onoprishvili et al., 2003; Charleton et al., 2008). For example, the dendritic spine-enriched protein spinophilin has been shown to interact directly with and modulate MOR responsiveness and signaling (Charleton et al., 2008). Therefore, not only can distinct cytoplasmic sequences mediate post-endocytic sorting of receptors but could also contribute to regulation of downstream signaling.

Rapid turnover of signaling receptors under agonist stimulation presents the fundamental problem of proper receptor redistribution over the somatodendritic plasma membrane. Both our immunocytochemistry and time-lapse imaging data revealed that recycling occurs throughout the somatodendritic membrane in multiple neuronal populations. Based on this observation, we hypothesize that this problem of receptor redistribution is most efficiently resolved through local recycling and re-insertion of receptors. Moreover, the intrinsic limits of lateral diffusion suggest that exocytic insertion could be occurring at a more local level.

The remarkable lateral mobility of signaling receptors after re-insertion into the cell surface challenges the traditional hypothesis that lateral organization of the somatodendritic plasma membrane is achieved essentially by static scaffolding. Previously, this view was based largely on classic studies of signaling membranes such as the motor endplate, where nicotinic acetylcholine receptors turn over on a time scale of hours to days, as oppose to minutes. It has become increasingly clear that vesicular trafficking processes regulating the recycling of post-synaptic receptors occur at a much faster time scale. As a result, the kinetics of receptor insertion and dispersion into the surface membrane can significantly affect steady-state distribution and surface localization of signaling receptors such as the MOR and β2AR. Although subcellular expression of both receptors are reported to be throughout the somatodendritic membrane, MOR expression is primarily extrasynaptic, while β2AR distribution was predominantly observed to be co-localized with post-synaptic densities (PSD) in dendritic spines (Wang et al., 1997; Aoki et al., 1987). Our present data suggests that differences in

spatiotemporal insertion of receptors, driven by distinct cytoplasmic sequences, may be one possible way to achieve these specific patterns of surface distribution.

Further studies will be required to more fully investigate this dynamic patterning hypothesis, assess specific signaling consequences of receptor-specific recycling and local surface trafficking, and define biochemical mechanism(s) mediating the distinct trafficking effects of divergent receptor-derived sorting sequences. By establishing non-redundant effects of sequence-directed recycling in relevant neurons and by revealing an unanticipated ability of divergent recycling sequences to couple individual vesicular insertion events to kinetically distinct modes of local surface trafficking, our results have opened new avenues of exploration and make a significant step toward understanding fundamental principles and consequences of endocytic receptor delivery to the postsynaptic surface.

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CHAPTER 5: GENERAL DISCUSSION AND IMPLICATIONS

The current body of work reveals several novel findings on the regulation of MORs in physiologically relevant CNS neurons. Discussion of these results will aim to address potential implications of these findings and suggest their relevance to both the opioid field and the general study of GPCR trafficking in neurons.

Morphine-induced MOR internalization in striatal neurons

Contrary to a significant body of literature indicating morphine's inability to drive efficient internalization of MORs, we find that acute morphine treatment is capable of inducing rapid and pronounced MOR endocytosis with both endogenous and recombinant receptors in striatal neurons. This morphine-mediated internalization was also observed with endogenously expressed receptors both in cultured amygdala (Yu et al., 2009) and midbrain neurons (unpublished). Endocytosis was determined to be β-arrestin2 dependent, as expression of dominant-negative β-arrestin2 abolished both morphine- and DAMGO-mediated MOR endocytosis. This suggests that both morphine and DAMGO, at least in striatal neurons, are able to drive arrestin-dependent regulation of MOR. This contrasts with previous studies suggesting that morphine-activated receptors fail to undergo β-arrestin-dependent uncoupling from G-proteins and desensitization (Whistler and von Zastrow 1998; Johnson et al., 2006). However, in HEK 293 cells where these studies were conducted, morphine also fails to drive robust MOR internalization. Additionally, Johnson et al. (2006) examined β-arrestin1 (i.e., arrestin-2), but not β-arrestin2, translocation to the activated receptor. Thus, it is possible that either the

quantitative and/or qualitative differences in regulatory machinery (such as differences in GRK expression or β-arrestin isoforms) in the different cell types may account for the discrepancy between these studies and our observations in neurons.

That β-arrestin2 involvement is critical for morphine-induced MOR endocytosis is consistent with studies of β-arrestin2 knockout mice, where these animals showed a decrease in MOR desensitization, an increase in morphine-mediated analgesia, and a loss of tolerance (Bohn et al., 1999; Bohn et al., 2000). Although these studies were conducted under conditions of chronic morphine treatment, there was no difference in the number of MORs as assessed by [^{3}H]naloxone binding, indicating that the effects observed cannot be explained by downregulation of receptor expression. Thus, the potentiation and prolongation of morphine-induced analgesia suggests a lack of desensitization, presumably due to loss of MOR internalization in the absence of β-arrestin2. The specificity in β-arrestin2 involvement is further compelling in the light of data suggesting that β-arrestin1 (i.e., arrestin2) expression is actually higher in most brain regions, including the striatum, compared to β-arrestin2 (i.e, arrestin3) (Gurevich et al., 2002). It suggests that although other isoforms of β-arrestin may exist in brain regions implicated in opioid physiology, they are not sufficient to compensate for the lack of morphine-induced MOR desensitization observed in β-arrestin2 knockout mice.

Functional differences between the two isoforms may also play a role in their ability to drive MOR internalization. It has been suggested that some GPCRs, but not others, preferentially recruit β-arrestin2 over β-arrestin1 to clathrin-coated pits (Santini et al.,

2000). We assessed whether expression of this protein was different between neural versus non-neural cells but were unable to distinguish between isoforms among the cell types (see Fig. 7G in Chapter 2). Although yet to be demonstrated with MORs, it is possible that preferential recruitment of one β-arrestin isoform over another may be more important than the expression level differences of the two isoforms in different cellular backgrounds.

The direct relationship between MOR internalization, desensitization, and tolerance in neurons remains unclear. Data suggesting how these processes relate to morphine tolerance is difficult to reconcile, as there remains much discrepancy between studies of neural and non-neural cell models (Bohn et al., 2000; Williams et al., 2001; He et al, 2002; Alvarez et al., 2002; Bailey et al., 2005; Arrtamangkul et al., 2008). To date, there is no direct evidence showing that internalization is required for functional MOR recovery in neurons. It is possible that morphine's poor ability to produce desensitization, rather than its ability to drive internalization per se, is an important determinant of its effect on tolerance. Additionally, differences in MOR-induced signaling cascades in various brain regions may also play a role in MOR desensitization reported after chronic morphine treatment (Sim-Selley et al., 2000). It would be interesting in the future to investigate whether morphine can produce rapid desensitization in the same striatal neurons where we observed robust internalization. Nevertheless, our studies revealing morphine's ability to drive robust internalization in several brain regions suggests that perhaps this drug is not as different from other opioids as previous literature claims with

regards to how it produces long-term adaptive changes in select brain regions implicated in opioid physiology.

Why morphine drives rapid internalization of MOR in some cells but not others also remains unclear. Divergent endocytic behavior observed in various cellular environments raises the possibility that the processes driving morphine-mediated MOR desensitization are distinct depending on the cell type. In the very least, enhancement of MOR desensitization seems to be important for development of morphine tolerance in physiological relevant neurons. To complicate things even further, however, both internalization and desensitization of MORs can also be regulated by other GPCRs that interact with MORs. This will be further discussed in the following section describing a specific interaction between MORs and NK1Rs.

Cross-regulation of MOR and NK1R: Implications for trafficking and signaling

We found that co-activation of NK1Rs and MORs expressed on the same neuron resulted in a significant inhibition of MOR endocytosis in response to both DAMGO and morphine. This cross-regulation of trafficking was observed in multiple populations of neurons and also in a neurosecretory cell line. NK1R activation also diminished MOR desensitization in response to DAMGO, suggesting that inhibition of MOR endocytosis prolonged signaling as measured by cAMP accumulation. Additionally, NK1R-mediated inhibition of MOR was also observed with endogenously expressed receptors in both the amygdala and LC, suggesting that this may be a plausible mechanism for modulating MOR function in the native tissue. That this phenomenon was observed with

endogenously expressed receptors in the amygdala is especially compelling in light of a previous study indicating that selective ablation of NK1R-expressing neurons in the amygdala, but not other brain regions, of intact animals was responsible for a reduction in morphine reward behaviors (Gadd et al., 2003). Together, these studies indicate that the amygdala is a critical brain region for NK1R-mediated opioid reward.

Although MORs and NK1Rs were previously described to form heterodimers in HEK 293 cells (Pfeiffer et al., 2003), we did not see any evidence indicating the same was true in neurons. In fact, that activation of NK1Rs selectively and non-reciprocally inhibited MOR endocytosis reveals a novel mechanism by which these two receptors can interact. It is possible that the heterodimerization observed in HEK 293 cells, but not primary neurons, is an indication that cell-specific proteins are required for receptor oligomerization, or that over-expression of receptors facilitated an artificial interaction. The latter has been argued as a precaution against interpreting co-internalization of receptors as an indication of receptor oligomerization (Gurevich et al., 2008). This hypothesis proposes that massive phosphorylation of over-expressed receptors followed by arrestin recruitment and mobilization of clathrin to the activated GPCR could in effect "drag" another receptor that happened to be residing in the same membrane microdomain. Any one of these scenarios would explain the discrepancy observed between cross-regulation differences of NK1R and MOR activation in the different cell types.

The heterologous inhibition of MOR endocytosis by NK1R activation appeared to be β-arrestin2-dependent. High affinity endomembrane recruitment of the NK1R-β-arrestin complex is presumed to result in a net depletion of available arrestin in the cytoplasm for mediating MOR endocytosis. This was further confirmed by over-expression of β-arrestin2-EGFP, which enabled MOR endocytosis even in the presence of activated NK1Rs. Many other GPCRs also exhibit high affinity arrestin binding, where activation of the receptor results in internalization of recruited β-arrestins along with the receptor into endosomes (Oakley et al., 2001). Endomembrane recruitment of β-arrestin is also associated with a slower recycling and resensitization profile (Oakley et al., 2001). This particular mechanism is reported to be responsible for a number of cross-regulation between different GPCRs, including β2AR and vasopressin2 (V2) receptor, NK1 and NK3 receptors, vasopressin 1a (V1a) and V2 receptors, and the currently reported MOR and NK1R (Klein et al., 2001; Schmidlin et al., 2002; Terrillon et al., 2004; Yu et al., 2009). That this β–arrestin sequestration mechanism also mediates cross-regulation of receptors in CNS neurons suggests it may be a fundamental means by which GPCRs co-expressed on the same neuron can modulate trafficking and signaling behavior in physiologically relevant cells.

The stoichiometry of β-arrestins to actual receptors is unknown in the native tissue and would be important to establish in the future in order to better understand how expression levels of this protein might affect arrestin-mediated cross-regulation. We were able to observe β-arrestin2-dependent heterologous inhibition of MOR internalization in cells expressing native levels of both receptors and β-arrestin. This suggests that the actual

limiting factor is the functional activity of the protein itself. It is possible for there to be a sub-population of readily available β-arrestin distinct from the total amount of the protein in the cytoplasm. For instance, different phosphorylation states of β-arrestin have been reported to be important for mediating clathrin-mediated receptor endocytosis (Lin et al., 1997; Lin et al., 1999). Thus, post-translational modifications of β-arrestin could be an important factor in amount of functional β-arrestins available to mediate receptor endocytosis.

Not only can cross-regulation alter trafficking of receptors but it can also lead to changes in downstream signaling. Inhibition of MOR endocytosis via activation of NK1Rs resulted in a decrease in acute DAMGO-mediated MOR desensitization as measured by inhibition of cAMP accumulation. This is consistent with the idea that internalization may play a critical role in enhancing both receptor desensitization and presumably resensitization of receptor function. The latter mechanism was not specifically examined in the current study and it would be interesting to investigate whether NK1R-mediated inhibition of MOR endocytosis would result in an attenuation of MOR function after prolonged morphine and substance P treatment. In the intact animal, chronic morphine treatment did not result in either downregulation or loss of MOR function in the striatum of $NK1R^{-/-}$ mice (Murtra et al., 2000). Most opioid withdrawal symptoms were still exhibited by $NK1R^{-/-}$ mice, which is consistent with previous results indicating that chronic morphine in vivo can cause superactivation of adenylyl cyclase activity even in the absence of internalization and receptor downregulation (Stafford et al., 2001).

Interactions among different opioid receptor types, especially oligomerization, have been described extensively (Gomes et al., 2002), but there are few studies examining heterologous interactions between opioid receptors and other GPCRs. Recently, a novel relationship between MORs and the serotonin (5HT) 2A receptors was reported, where both expression and co-activation of 5HT2A receptors altered MOR function (Lopez-Gimenez et al., 2008). Specifically, co-activation of 5HT2A receptors resulted in morphine-mediated desensitization, internalization, and downregulation in HEK 293 cells that was previously lacking in the absence of activated 5HT2A receptors. Although this is somewhat contradictory to our observation, the difference in cellular model and agonist used (i.e., morphine vs. DAMGO) for measuring desensitization differs from our model. Additionally, a distinct cellular mechanism of action was not provided for this cross-regulation.

Our MOR and NK1R cross-regulation results provide a cell biological mechanism of how these two co-expressed receptors might interact in CNS neurons. We acknowledge that there remains a substantial gap between the described cell biology mechanisms and the complex neural circuitry involved in NK1R-mediated morphine reward, tolerance, and withdrawal physiology. However, our study provides a novel mechanism for MOR regulation by NK1Rs in CNS neurons. More generally, it suggests that trafficking and signaling of endogenous GPCRs in the native tissue can be significantly affected by other co-expressed receptors.

MOR recycling in neurons: new role in GPCR recycling sequences

Rapid recycling of MORs after agonist-induced endocytosis is an important regulatory mechanism for maintaining cellular responsiveness to opioids (Koch et al., 2005; Martini and Whistler 2007). In non-neural cells, several GPCRs possess distinct cytoplasmic tail sequences that are critical for the proper sorting of internalized receptors into either the recycling or degrading pathway (Tsao and von Zastrow 2000; Gage et al., 2001; Tanowitz et al., 2003; Vargas and von Zastrow 2004). We show that MOR recycling also occurs in a sequence-dependent manner in cultured striatal neurons and truncation of this sequence resulted in a mutant MOR that recycled poorly. This was the first demonstration that the same sorting sequence that drives rapid MOR recycling in non-neural cells is necessary and functional in its native receptor environment.

Using time-lapse TIRF imaging, we were able to observe discrete exocytic recycling events at the plasma membrane. Recycling events were initiated within minutes after agonist treatment and occurred throughout both the cell body and dendrites of striatal neurons. The lack of compartment specificity suggests that rapid recycling may be occurring at a local level throughout the neuron, which increases efficiency by eliminating the need for long-distance transport of recycled receptors (e.g., delivery of MORs to distal dendrites). Furthermore, endogenously expressed MORs in striatal neurons also showed rapid recycling throughout the somatodendritic plasma membrane, indicating that local recycling may be a general mechanism for rapid re-insertion and distribution of internalized receptors.

Individual exocytic events exhibited dramatically different lateral diffusion kinetics after re-insertion into the plasma membrane. Whereas a proportion of β2AR insertion events remained at the plasma membrane before dispersion (i.e., "persistent"), all MOR insertion events exhibited rapid and uniform lateral dispersion at the site of insertion (i.e., "transient"). This difference in insertion kinetics was dependent on the specific recycling sequences present at the carboxyl terminus of each receptor: substitution of β2AR cytoplasmic tail sequence resulted in the appearance of persistent MOR insertion events, and substitution of the MOR recycling sequence resulted in a significant decrease in the proportion of persistent β2AR insertion events. An interesting future question to address is where in the intracellular recycling pathway does the divergence between receptors that differ in their insertion kinetics occur. Do MORs and β2ARs traverse completely different endocytic routes on their way back to the cell surface? Or are they present in the same vesicles, and only upon membrane insertion does some unknown mechanism drive them to exhibit distinct kinetic modes of dispersion? These are all issues yet to be explored that would add to our understanding of how the recycling process and insertion kinetics might be related.

The single kinetic mode of MOR insertion was unanticipated due to the presence of persistent events observed with other signaling receptors in neurons (Yudowski et al., 2006; Yudowski et al., 2007). Although how exactly persistent or transient insertion events are distinguished mechanistically is uncertain, several possibilities exist. The cytoplasmic sequence of both β2ARs and AMPA receptors, where persistent events have been observed, contain PDZ (postsynaptic density 95/disc large/zonula occludens-1)-

binding domains that are known to interact with a number of different cytoskeletal scaffold proteins to regulate receptor trafficking (Dong et al., 1997; Leonard et al., 1998; Cao et al., 1999; Gage et al., 2001; Cong et al., 2001). MORs, on the other hand, lack a PDZ-binding domain in its carboxyl terminus and little is known about proteins that might bind to its recycling sequence. It therefore possible that [1)] unidentified proteins interacting with the MOR tail regulate insertion kinetics differently at the plasma membrane compared to PDZ-binding proteins, [2)] PDZ scaffold proteins function to slow down receptor dispersion, and because MOR recycling occurs independently from these interactions, all insertion events are transient and fast, or [3)] an unknown mechanism.

The physiological function of distinct exocytic insertion kinetics has yet to be determined. One possibility is that specific receptor sequences may drive distinct patterns of spatiotemporal distribution of receptors throughout the somatodendritic membrane. Cytoplasmic tail sequences have been proposed to encode temporal and spatial distribution of AMPA receptors in hippocampal neurons (Passafaro et al., 2001). The rate of surface insertion of AMPA receptors was dictated by the individual GluR1 and GluR2 subunits that comprised the receptor as a whole. Furthermore, the kinetics of subunit-specific receptor insertion was found to be dependent on the subunits' cytoplasmic sequences. Therefore, it is possible that one function of sequence-mediated recycling in neurons may be to achieve spatiotemporal specificity in receptor distribution after agonist-induced endocytosis.

There is considerable diversity among GPCR recycling sequences (see Hanyaloglu and von Zastrow 2007 for the most current list). What is remarkable is that although these

receptor domains have more or less the same function (i.e., sorting of receptors to the recycling pathway) and is a conserved mechanism among mammalian cells, their primary amino acid sequences are surprisingly diverse. Numerous studies have shown that these sequences can be interchanged between different receptors and yet retain their function as a regulator of post-endocytic sorting (Tsao and von Zastrow 2000; Gage et al., 2001; Tanowitz and von Zastrow 2003). Yet further differentiation of function between these distinct sequences remained unknown. Our observation that interchangeable recycling sequences are not redundant is the first to provide an additional function for GPCR recycling sequences. It also suggests that these cytoplasmic domains may be more sophisticated than previously thought and additional functions may reveal themselves with further investigation.

Our investigation of the cellular mechanisms underlying MOR recycling in neurons has revealed a novel function of GPCR recycling sequences. The study also provides further insight into the how signaling receptors are recycled and distributed back into the neuronal plasma membrane after agonist-induced endocytosis. Beyond its role for maintaining physiological responsiveness, rapid and local receptor re-insertion after internalization may help efficiently maintain the membrane complement of various GPCRs in the presence of agonist. Rapid recycling of receptors would therefore allow for proper preservation of receptor distribution at the cell surface and play an active role in the maintenance of normal brain function.

Together, these studies of MOR endocytosis, cross-regulation, and recycling have furthered our understanding of how MORs function in CNS neurons where they drive a vast array of physiological consequences. Both the pervasive analgesic and addictive properties of opiate drugs highlight the importance of studying MOR regulation in both complex neural circuits and at the single cell level. It is our hope that by advancing our cell biological knowledge of how opioid receptor function in neurons, we can better understand the processes underlying both cellular and behavioral adaptations to chronic opioid drugs.

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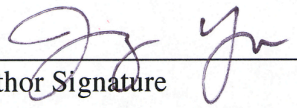

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