Rapid Delivery of Internalized Signaling Receptors to the Somatodendritic Surface by Sequence-Specific Local Insertion

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The recycling pathway is a major route for delivering signaling receptors to the somatodendritic plasma membrane. We investigated the cell biological basis for the remarkable selectivity and speed of this process. We focused on the μ -opioid neuropeptide receptor and the β_2 -adrenergic catecholamine receptor, two seven-transmembrane signaling receptors that traverse the recycling pathway efficiently after ligand-induced endocytosis and localize at steady state throughout the postsynaptic surface. Rapid recycling of each receptor in dissociated neuronal cultures was mediated by a receptor-specific cytoplasmic sorting sequence. Total internal reflection fluorescence microscopy imaging revealed that both sequences drive recycling via discrete vesicular fusion events in the cell body and dendritic shaft. Both sequences promoted recycling via “transient”-type events characterized by nearly immediate lateral spread of receptors after vesicular insertion resembling receptor insertion events observed previously in non-neural cells. The sequences differed in their abilities to produce distinct “persistent”-type events at which inserted receptors lingered for a variable time period before lateral spread. Both types of insertion event generated a uniform distribution of receptors in the somatodendritic plasma membrane when imaged over a 1 min interval, but persistent events uniquely generated a punctate surface distribution over a 10 s interval. These results establish sequence-directed recycling of signaling receptors in CNS neurons and show that this mechanism has the ability to generate receptor-specific patterns of local surface distribution on a timescale overlapping that of rapid physiological signaling.

Introduction

The somatodendritic surface of CNS neurons is a highly dynamic membrane environment in which the number of specific signaling receptors accessible to extracellular ligands is tightly controlled by vesicular trafficking processes (Shepherd and Huganir, 2007; Newpher and Ehlers, 2008; Renner et al., 2008; Triller and Choquet, 2008). Endocytic removal of neural signaling receptors has been investigated in considerable detail (Carroll et al., 2001; Kennedy and Ehlers, 2006), but less is known about how signaling receptors are returned to the somatodendritic surface. Nevertheless, recycling from the endocytic pathway is thought to represent an important and highly versatile means for regulating signaling receptors in diverse cell types, including neurons (Sorkin and von Zastrow, 2009).

Endocytic recycling in neurons is remarkable both for its high throughput and biochemical selectivity. The ability of neurons to respond effectively to repetitive ligand-induced activation, and of the recycling pathway to support enhanced postsynaptic signaling on a timescale of minutes (Gainetdinov et al., 2004; Shepherd and Huganir, 2007), suggests that the recycling pathway is capable of delivering significant numbers of signaling receptors to appropriate regions of the somatodendritic surface very rapidly. The ability of neurons to sustain or potentiate responsiveness to some ligand-conveyed stimuli, while down-regulating responsiveness to others, suggests that only a selected subset of signaling receptors traverse the rapid recycling pathway after regulated endocytosis (Hanyaloglu and von Zastrow, 2008). These observations raise fundamental questions about the cell biological basis of receptor trafficking through the recycling pathway. First, given the high degree of selectivity evident in endocytic regulation, what determines whether a particular signaling receptor is capable of entering the rapid recycling pathway? Second, how are selected receptors delivered so rapidly and efficiently to the somatodendritic surface?

We investigated these questions in the present study by focusing on the μ -opioid neuropeptide receptor (MOR) and β_2 -adrenergic catecholamine receptor (β_2 AR). These receptors are so-called typical (family A) members of the seven-transmembrane superfamily of G protein-coupled receptors (GPCRs), the largest and most versatile group of signaling receptors expressed in the CNS (Gainetdinov et al., 2004). Both receptors undergo rapid endocytosis via clathrin-coated pits following ligand-induced activation but are natively dis-

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tributed in the cell body and throughout the elaborate dendritic arbor of medium spiny neurons at steady state (Aoki et al., 1989; Wang et al., 1997; Haberstock-Debic et al., 2003; Charlton et al., 2008). There is evidence for remarkable specificity in the endocytic trafficking of this receptor family, because the δ -opioid receptor (DOR), which is much more closely related to MOR than the β_2 AR, preferentially down-regulates following ligand-induced endocytosis in neuronal cells (Law et al., 2000; Scherrer et al., 2006; Pradhan et al., 2009). Thus, we reasoned that investigating these examples might provide useful insight to the cell biological basis for selective and efficient delivery of signaling receptors to the postsynaptic surface.

Materials and Methods

cDNA constructs. N-terminally Flag (DYKDDDD) epitope-tagged versions of the cloned murine MOR (MOR1 isoform), a mutant MOR construct lacking the C-terminal 17 residues (MOR Δ 17), and a murine DOR construct were described previously (Keith et al., 1996; Tanowitz and von Zastrow, 2003). SpH-MOR was generated by fusing supercliptic pHluorin (SpH) (Miesenböck et al., 1998; Sankaranarayanan et al., 2000), preceded by a cleavable signal sequence described previously (Yudowski et al., 2006), to the murine MOR1. SpH-MOR/ β_2 AR chimeric receptor constructs were generated using PCR to replace the C-terminal 17 residues of MOR1 with the terminal 4 or 10 residues derived from the human β_2 AR. The reciprocal SpH- β_2 AR/MOR17 chimeric receptor construct was generated from a previously described Flag-tagged version of this chimera (Tanowitz and von Zastrow, 2003) exchanged into SpH- β_2 AR using 5' *Hind*III and 3' *Eco*RV restriction sites. All constructs were verified by dideoxy sequencing (Elim Biopharmaceuticals) and subcloned into a pCAGG/SE expression vector (Niwa et al., 1991) using *Sac*I/*Xho*I restriction sites.

Cell culture and transfection. Dissociated primary cultures of striatal, cortical, and hippocampal neurons were dissected from embryonic day 17–18 Sprague Dawley rat embryos. The striatum (caudate–putamen and nucleus accumbens) was dissected based on the criteria of Ventimiglia and Lindsay (1998). The cortex and hippocampus were identified as described by Altman and Bayer (1995). Dissected tissue was dissociated in 1× trypsin/EDTA solution (Invitrogen) for 15 min before 1 ml of trypsin inhibitor was added for 5 min at room temperature. Cells were washed and triturated in DMEM plus 10% fetal calf serum (FCS; Invitrogen) using a glass pipette. Neuronal transfections were performed using electroporation (rat neuron nucleofector system; Amax Biosystems) immediately after dissociation and plated on poly-L-lysine-coated (1 mg/ml in 0.1 M sodium borate buffer, pH 8.5) glass coverslips at a density of 2×10^5 neurons per well. Electroporation was conducted using 5×10^6 dissociated cells, 3 μ g of plasmid DNA, and 100 μ l of rat neuron nucleofector solution. After transfection, neurons were kept in prewarmed RPMI media (University of California at San Francisco Cell Culture Facility) for 10 min for recovery before being transferred onto 24-well plates. Medium on the cells was replaced with Neurobasal medium supplemented with B27 and L-glutamine (Invitrogen) 24 h post-transfection.

Immunocytochemical localization of endogenous receptors. Cultured striatal neurons [7 days *in vitro* (DIV)] were pretreated with 10 μ g/ml cycloheximide for 1 h before incubations to assess regulated trafficking of receptors, and this protein synthesis inhibitor was left in the culture medium during subsequent manipulations. Cells were fixed immediately without exposure to added opioid and incubated for 30 min in the presence of 10 μ M [D-Ala², N-MePhe⁴, Gly⁵-ol]-enkephalin (DAMGO) or incubated for 30 min with DAMGO and incubated for an additional 45 min in the absence of DAMGO and the presence of 10 μ M naloxone to block possible residual agonist activity. Cells were then fixed in ice-cold methanol for 10 min and blocked in PBS containing 5% goat serum, 0.3% Tween-20, and 0.1% Triton X-100 in PBS for 45 min. Staining for endogenous MOR was performed using an affinity-purified rabbit antibody recognizing the C terminus of MOR1 (Keith et al., 1998) (gift from Dr. C. Evans, University of California at Los Angeles, Los Angeles, CA). Fixed, permeabilized specimens were incubated with primary antibody (1:1000) at room temperature for 2.5 h and then extensively washed in PBS. Redistribution of receptors was visualized after secondary labeling

with Alexa 488-conjugated goat anti-rabbit IgG (2 μ g/ml; Invitrogen) prepared in the same blocking solution as above and incubated for 30 min. 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 Diaphot microscope equipped with a 60×/numerical aperture (NA) 1.4 objective, mercury arc lamp illumination and standard dichroic filter sets (Omega Optical). Images were collected in the linear range with a 12 bit cooled CCD camera (Princeton Instruments).

Fluorescence ratio imaging and quantitative analysis of recombinant receptor recycling. Transfected neurons (14 DIV) plated on polylysine-coated coverslips were incubated with Alexa 594-conjugated M1 anti-Flag monoclonal antibody (5 μ g/ml; Invitrogen) for 30 min at 37°C to selectively label Flag-tagged receptors present in the plasma membrane. Surface-labeled live specimens were then subjected to one the following manipulations as indicated: (1) incubation in the absence of opioid for 30 min and fixed under nonpermeabilizing conditions using 4% paraformaldehyde freshly prepared in PBS for 15 min and then quenched in TBS (see Fig. 1, no agonist no strip); (2) incubation for 30 min in the presence of 10 μ M DAMGO (for MOR and MOR Δ 17) or 10 μ M [D-Ala²-D-Leu⁵]-enkephalin (DADLE; for DOR) for 30 min, selective “stripping” of labeled receptors remaining in the plasma membrane using multiple washes with cold PBS supplemented with 0.04% EDTA, and fixation/quenching (see Fig. 1, + agonist strip); (3) incubation with opioid peptide agonist followed by opioid washout/stripping as in manipulation #2, and then incubation at 37°C for an additional 45 min in the absence of agonist and presence of 10 μ M naloxone to prevent any confounding effects of possible residual agonist (i.e., re-endocytosis of receptors after recycling), then fixation/quenching (see Fig. 1, + agonist strip + naloxone). Following the indicated manipulations, fixed, nonpermeabilized specimens were blocked in 3% BSA in TBS for 1 h at room temperature and then incubated with fluorescein isothiocyanate-conjugated anti-mouse antibody (4 μ g/ml; Jackson ImmunoResearch Laboratories) for 1 h in blocking solution to selectively detect only those Flag-labeled receptors accessible at the cell surface. Epifluorescence microscopy was carried out using dichroic filter sets (Omega Optical) verified to selectively image each fluorochrome without significant bleedthrough. Twelve bit images, collected in a midfocal plane and in the linear range of the cooled CCD camera, were background subtracted, and integrated fluorescence intensity values measured in a region of interest including the soma were integrated and background subtracted using MetaMorph software (Molecular Devices) as described previously (Haberstock-Debic et al., 2005). Percentage of recycling was calculated from these values as described previously (Tanowitz and von Zastrow, 2003). For each experiment, 30–50 neuronal cell bodies, selected at random from at least five coverslips, were analyzed per condition. Results were compiled from five experiments for each cell type, and each experiment represented separate culture preparations derived from different animals. Statistical analysis of differences between experimental groups was performed using unpaired, two-tailed Student's *t* test calculated using GraphPad Prism software.

Biochemical analysis of receptor endocytosis and recycling using reversible surface biotinylation. Transfected cortical cultures (14 DIV) were analyzed using a particular variation of surface biotinylation and domain-specific cleavage (Tsao and von Zastrow, 2000). Briefly, cultures plated on plastic dishes were washed with cold Ca²⁺- and Mg²⁺-free PBS and incubated with 0.3 mg/ml sulfo-NHS-SS-biotin (Pierce) freshly prepared in PBS at 4°C for 30 min. Neurons were washed with TBS and placed in DMEM for 10 min at 37°C before incubation for 30 min in 10 μ M DAMGO. For the control stripping and internalization conditions, cells were washed on ice with TBS to remove and quench residual biotinylation reagent, and labeled receptors present at the neuronal surface were selectively “stripped” at 4°C by incubation of cultures three times for 15 min with ice-cold 100 mM 2-mercaptoethanesulfonic acid (MESNA, Sigma-Aldrich) freshly dissolved in 50 mM Tris, pH 8.8, 100 mM NaCl, 1 mM 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 min also at 4°C. For the “recycling” condition (see Fig. 2B, lanes 5 and 6), labeled/DAMGO-treated/stripped neurons were returned to reg-

ular culture media and incubated for an additional 45 min at 37°C in the absence of opioid peptide agonist (and the presence of naloxone to block possible residual agonist effects). Cells were then washed and, where indicated (see Fig. 2C, lane 6), subjected to a second strip at 4°C. At the end of the indicated manipulations, neurons maintained on ice were solubilized with lysis buffer containing 1 mg/ml iodoacetamide in immunoprecipitation (IP) buffer (0.2% Triton X-100, 150 mM NaCl, 25 mM KCl, 10 mM Tris, pH 7.4) supplemented with 0.1 mM EDTA and a protease inhibitor cocktail (Complete mini EDTA-free tablet, 1 tablet/10 ml; Roche). Cell debris was removed by centrifugation at $13,000 \times g$ for 15 min at 4°C, and 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 extensively with IP buffer, eluted, denatured under non-reducing conditions, and separated by SDS/PAGE. Equal aliquots of unfractionated cell extracts (see Fig. 2, lysate samples) were resolved similarly as a loading control in each experiment and for each dish. Gels were transferred to nitrocellulose membranes and probed for Flag-tagged receptors by immunoblotting with anti-Flag M1 mouse antibody (5 μ g/ml; Sigma) for 1 h followed by anti-mouse IgG horseradish peroxidase conjugate (1:5000; GE Healthcare) for 1 h. Immunoreactive receptors were detected using chemiluminescence (Pierce SuperSignal reagent) and analyzed by luminescence imaging in the linear range using a FluorChem instrument (Alpha Innotech). The major immunoreactive signal corresponding to the mature monomeric receptor protein was integrated in each lane and background corrected using FluorChem software for calculation of fractional internalization and recycling. Results were averaged over duplicate samples and mean determinations were averaged across three independent experiments. The significance of differences observed was tested using unpaired, two-tailed Student's *t* test.

Live imaging of surface receptor delivery using rapid total internal reflection fluorescence microscopy. Transfected striatal neurons expressing the indicated SpH-tagged receptors (7 DIV) were imaged by TIR-FM using a Nikon TE-2000 inverted microscope equipped with a 60 $\times$ /NA 1.49 objective. Evanescent illumination was generating by focusing a fiber-coupled 488 nm argon ion laser (Melles Griot) onto the outer back focal plane of the objective using a micrometer-guided illuminator (Nikon). Cultures were imaged in conditioned neurobasal medium supplemented with 10 mM D-glucose and 30 mM HEPES buffer, pH 7.4. Temperature was controlled at 37°C using a thermoelectric stage (Bioscience Tools) and an objective warmer (Biopetechs). Time-lapse sequences were collected under continuous illumination using a deep-cooled, electron-multiplying CCD camera (Andor) operated in frame transfer mode to achieve continuous acquisition at 10 Hz.

Statistical analysis and modeling of live imaging data. Discrete fusion events labeled with SpH-tagged receptors were identified and scored as described previously (Yudowski et al., 2006) using ImageJ software (Abramoff et al., 2004). To analyze the statistical significance of differences between selected receptor constructs in persistent-type insertion, we counted the number of events with duration ≥ 3 s in each independent experiment (i.e., separate imaging session and different dish of cultured neurons) and used either Mann–Whitney (see Fig. 4 for single comparison) or Kruskal–Wallis test with Dunn's post-test (see Fig. 5 for multiple comparisons) as conservative, nonparametric tests using the number of experiments as statistical *n*. To assess possible differences in total event numbers across constructs and experiments, we carried out the same analysis on the distributions of total insertion events (regardless of duration). As another metric for assessing general kinetic differences in receptor insertion properties, we computed the mean event duration measured for each receptor construct and in each imaging experiment and then compared this value across experiments. This value was near-normally distributed, so significance across experiments was analyzed using one-way ANOVA and Bonferroni's multiple comparison post-test, again with the number of experiments as statistical *n*. Statistical tests were calculated according to standard algorithms using GraphPad Prism software with a significance threshold of *p* < 0.05. The exact number of events and experiments included in each group is specified in the relevant sections of Results and figure legends. Maximum intensity projections and averaged surface receptor intensity distributions were computed

over the indicated time scales from 10 Hz image series using ImageJ. Analytical solutions of Fick's law for the indicated geometry and least-squares fitting of predicted curves to the averaged intensity data were calculated using Matlab software.

Results

Rapid recycling of μ -opioid receptors to both somata and dendrites of CNS-derived neurons

MOR has been shown previously to undergo reversible redistribution between the plasma membrane and endocytic vesicles when expressed endogenously or at near-endogenous levels in other neurons (Sternini et al., 1996; Arttamangkul et al., 2008). We verified similar behavior of endogenous MOR by indirect immunofluorescence microscopy applied to medium spiny neurons cultured from rat striatum (Fig. 1A). To visualize receptor localization with increased signal-to-background ratio and to unambiguously test the return of the internalized receptor pool to the surface of both somata and dendrites, we expressed a recombinant MOR construct possessing a Flag epitope tag fused to its N-terminal ectodomain and applied a previously established fluorescence imaging method based on surface antibody accessibility to follow the trafficking fate of receptors after specific labeling in the plasma membrane (Tanowitz and von Zastrow, 2003). In the absence of opioid, Flag-MOR was localized throughout the plasma membrane. This was indicated by uniform labeling of the somatodendritic surface by Alexa 594-conjugated M1 anti-Flag antibody and by accessibility of labeled receptors to a fluorescein-conjugated anti-mouse secondary antibody added to nonpermeabilized specimens (Fig. 1B, top). Incubation of neurons with 10 μ M DAMGO for 30 min resulted in redistribution of M1-labeled (red) receptors from a diffuse to punctate localization pattern (Fig. 1B). Redistributed receptors were resistant to EDTA-mediated stripping of bound anti-Flag antibody from the extracellular surface and were inaccessible to fluorescein-conjugated secondary antibody in nonpermeabilized cells (indicated by their failure to stain green). Both of these properties indicate that the redistributed pool represents receptors internalized from the cell surface. Within 45 min after DAMGO washout, the internalized pool of M1-labeled MOR regained a diffuse, peripheral localization and again became accessible to surface-applied secondary antibody, resulting in bright surface labeling with both fluorochromes (producing a yellow merge image) throughout the cell body and dendrites (Fig. 1B, bottom). Pronounced return of the internalized pool of Flag-MOR was also observed in somata and dendrites of mixed cortical cultures (Fig. 1B, right; a representative region of a dendrite for each condition is shown at higher magnification in the inset at far right) as well as dissociated hippocampal cultures, which are composed largely of excitatory neurons (data not shown). Together, these results indicate that MOR is capable of efficient trafficking through the rapid recycling pathway of multiple neuronal populations, and they establish the ability of these receptors to rapidly repopulate the surface of both somata and dendrites within minutes after endocytosis.

Efficient recycling into both surface domains requires a specific cytoplasmic sorting sequence

Efficient recycling of MOR in non-neural cells requires a specific structural determinant that is modular and contained within the distal 17 residues of the receptor's C-terminal cytoplasmic domain. This "MOR-derived recycling sequence" (MRS) is not conserved in DOR, accounting for the failure of DOR to recycle efficiently in such cell models (Tanowitz and von Zastrow, 2003; Hislop et al., 2004). To investigate whether this sequence func-

Figure 1. MOR recycling is sequence dependent and occurs in both the cell body and dendrites of neurons cultured from multiple brain regions. **A**, Representative epifluorescence micrographs showing localization of endogenous MOR in dissociated striatal cultures. Incubation with 10 μ M DAMGO for 30 min (+ DAMGO) caused a redistribution of receptors to intracellular puncta. Subsequent washout of agonist and recovery in the presence of 10 μ M naloxone produced a redistribution of internalized receptors back to a diffuse distribution (+ DAMGO + naloxone) similar to that observed in neurons not exposed to opioid (no agonist). Insets show the cell body at higher magnification to make it easier to appreciate the observed differences in subcellular distribution. **B**, Analysis of recombinant Flag-MOR trafficking by dual label fluorescence imaging. In neurons not exposed to opioids (top panels), Alexa 594-labeled (red) Flag-MOR remained accessible to fluorescein-conjugated secondary antibody (green), resulting in yellow surface distribution in the merged fluorescence image. Incubation of neurons with 10 μ M DAMGO for 30 min (middle panels) produced a redistribution of Alexa 594-labeled Flag-MOR to intracellular vesicles, as verified both by the failure of chelating extracellular calcium to dissociate or strip the red label from receptors and the inaccessibility of labeled receptors to green secondary antibody added to nonpermeabilized cells (producing exclusively red labeling). After selective labeling of the internalized receptor pool with red antibody, removal of DAMGO (and subsequent incubation with the opioid antagonist naloxone to prevent the confounding effects of re-endocytosis if residual agonist is present) resulted in redistribution of the internalized receptor pool to a surface distribution that was readily accessible to labeling with extracellular secondary antibody. This was indicated by recovery of green labeling producing a yellow merged image (bottom panels). **C**, Use of the dual-label fluorescence method to compare endocytic trafficking of Flag-MOR, Flag-MOR Δ 17, and Flag-DOR expressed individually in dissociated cortical cultures. Representative examples are shown of neurons fixed after manipulations identical to those described in **B**, except that 10 μ M DADLE was used instead of 10 μ M DAMGO to drive endocytosis of Flag-DOR. **D**, Quantification of recombinant receptor recycling in three populations of cultured neurons. Fluorescence ratio imaging was used to measure fractional recycling of receptors in the agonist washout condition (**B**, **C**, corresponding to bottom). Bar graphs represent mean percentage of receptor recycled as determined from analysis of 30–50 cell bodies selected at random in neuronal cultures and averaged over five independent cultures (and on different days). Error bars represent the SEM across the independent cultures. Student's *t* test (two-tailed) indicated significantly reduced recycling of Flag-MOR Δ 17 versus Flag-MOR, as well as Flag-DOR versus Flag-MOR, in all of the neuronal populations examined ($p < 0.001$ throughout).

tions in a relevant cellular background, we applied the dual label fluorescence method to compare in cultured neurons Flag-tagged MOR, DOR, and a truncated mutant MOR construct (Flag-MOR Δ 17) specifically lacking the MRS. All constructs were detected in the plasma membrane of transfected cortical neurons in the absence of opioid, suggesting that the MRS is not required for surface receptor delivery from the biosynthetic pathway. All constructs internalized rapidly following activation by the appropriate peptide agonist (Fig. 1C, top and middle, respectively), verifying that the MRS is also not required for receptor endocytosis. Full-length Flag-MOR recycled almost completely within 45 min after agonist washout, but Flag-MOR Δ 17 and Flag-DOR, in marked contrast, remained predominantly localized in endocytic vesicles (Fig. 1C, bottom). We quantified these effects across multiple, randomly selected neurons in independent culture preparations by fluorescence ratio imaging (Tanowitz and von

Zastrow, 2003) and then carried out the same analysis on trafficking of Flag-tagged receptors expressed in dissociated striatal and hippocampal neurons. While Flag-MOR recycled almost completely, Flag-MOR Δ 17 and Flag-DOR recycled to a significantly smaller degree in all of the neuronal populations examined (Fig. 1D) ($p < 0.001$ by Student's *t* test when compared to Flag-MOR in all three populations).

As an independent analytical approach and to obviate any possible effect of sampling bias in the microscopic analysis, we applied a biochemical assay of receptor internalization based on cell surface biotinylation (Schmid and Smythe, 1991; Cao et al., 1998). Flag-MOR or Flag-MOR Δ 17 lacking the MRS was biotinylated in the neuronal plasma membrane at the beginning of the experiment, revealing a major immunoreactive signal resolving in a molecular mass range corresponding to the complex-glycosylated receptor monomer (Fig. 2A). We then applied a sequential incubation

Figure 2. Biochemical analysis of sequence-dependent recycling of MOR in dissociated cortical cultures. **A**, Surface biotinylation followed by streptavidin pull-down and immunoblotting (IB) of Flag-MOR and Flag-MORΔ17. The strong immunoreactive signal resolving between the 49 and 62 kDa markers is as expected for the mature, complex-glycosylated receptor. Control lanes in each blot correspond to an equal protein load of untransfected neurons, verifying the specificity of recombinant receptor detection. **B**, Schematic of the biochemical trafficking assay. Receptors present in the plasma membrane were labeled by surface biotinylation and then exposed to a membrane-impermeant reducing agent at the indicated points in the experimental protocol to strip biotin from surface-accessible receptors and thereby assess internalization and recycling of the labeled receptor pool in sequential stages after agonist exposure and washout, respectively. **C**, Anti-Flag immunoblots of the biotinylated (streptavidin pull-down fraction, top row of each panel) and total (unfractionated lysate, bottom row of each panel) from a representative experiment. Lanes 1 and 2 verify the ability of MESNA to completely strip surface-localized receptors. Lanes 3 and 4 show that both Flag-MOR and Flag-MORΔ17 undergo DAMGO-induced endocytosis, as a significant fraction of surface-biotinylated receptors was rendered inaccessible to a first strip with MESNA (Strip I in **B**). Lanes 5 and 6 show nearly complete recycling of Flag-MOR and greatly reduced recycling of Flag-MORΔ17 after DAMGO washout and subsequent incubation for 45 min, as indicated by the selective inaccessibility of biotinylated Flag-MORΔ17 to a second exposure to MESNA (strip II in **B**). **D**, Quantification of surface biotinylation results across multiple experiments using scanning luminometry. Bars represent mean DAMGO-induced internalization (left) and recycling measured after DAMGO washout (right), averaged over three independent experiments. Error bars represent standard error of the mean determinations across the three experiments (** $p = 0.002$ by Student's t test).

protocol, based on the ability of a membrane-impermeant reducing agent to efficiently “strip” biotin specifically from surface-accessible receptors, as an independent means to assess internalization and recycling of the surface-biotinylated receptor pool (Fig. 2*B*). Both Flag-MOR and Flag-MORΔ17 were fully accessible to surface stripping following incubation for 30 min in the absence of added opioid ligand (Fig. 2*C*, compare lanes 1 and 2 in the streptavidin pull-down blots; the corresponding lysate blots show total receptors detected in the unfractionated extract as a loading control). Incubation of neurons for the same time interval in the presence of 10 μ M DAMGO rendered a significant fraction of surface-biotinylated receptors inaccessible to the membrane-impermeant reducing agent, confirming rapid opioid-induced internalization of both Flag-MOR or Flag-MORΔ17 (Fig. 2*C*, lanes 3 and 4, streptavidin pull-down blots). When DAMGO-treated neurons were incubated for 45 min in the absence of agonist after the first strip (resulting in selective labeling of the internalized receptor pool), a second reducing step (Fig. 2*B*, strip II) was used to assess the ability of the internalized receptor pool to return to the plasma membrane. Under these conditions the immunoreactive signal corresponding to biotinylated Flag-MOR was almost completely lost, consistent with efficient recycling of receptors, whereas the signal corresponding to biotinylated Flag-MORΔ17 was largely retained (Fig. 2*C*, lanes 5 and 6, streptavidin pull-down blots). Nevertheless, both receptors were clearly detectable in similar amount by immunoblotting of cell extracts representing the input fraction used in streptavidin affinity isolation (Fig. 2*C*, corresponding lysate lanes). Quantification of these results across multiple experiments and culture preparations verified that Flag-MOR and Flag-MORΔ17 were closely similar in their ability to undergo DAMGO-induced internalization (Fig. 2*D*, left graph) but differed significantly in ability to subsequently recycle to the plasma membrane (Fig. 2*D*, right graph) ($p = 0.002$ by Student's t test). Together, these data provide several lines of evidence establishing functional recycling activity of the MRS in CNS-derived neurons.

Real-time imaging of discrete vesicular fusion events mediating MOR recycling in neurons

While the data described so far clearly established the occurrence of sequence-directed recycling of MOR in neurons, they provided no insight into the location or nature of specific membrane trafficking events mediating this process. To address this question, we tagged the N-terminal receptor ectodomain with SpH (also abbreviated as SEP in some studies) and applied TIR-FM to resolve individual vesicular trafficking events mediating receptor removal from and delivery to the plasma membrane of neurons (Miesenbock et al., 1998; Sankaranarayanan et al., 2000; Steyer and Almers, 2001; Yudowski et al., 2006). Dissociated striatal neurons expressing SpH-MOR were preincubated with 10 μ M DAMGO for 15 min at 37°C, a time that is sufficient to drive MOR internalization essentially to steady state in striatal neurons (Haberstock-Debic et al., 2005), and cultures were transferred to a microscope stage heated to 37°C in the continued presence of DAMGO. Neurons were incubated for an additional 10–15 min on the heated stage to establish stable focus and reach a time point comparable to that examined in the microscopic and biochemical studies described above. Evanescent field illumination was then applied to the specimen and imaging was carried out continuously for a 1 min interval, which has been shown previously to be sufficiently short to avoid detectable phototoxicity in dissociated cultures (Yudowski et al., 2006, 2007).

Figure 3. TIR-FM imaging reveals vesicular insertion of SpH-MOR into somata and dendrites. Dissociated rat striatal neurons expressing SpH-MOR were incubated in the presence of 10 μ M DAMGO for 15 min, mounted, and equilibrated in a 37°C imaging chamber in the continued presence of DAMGO and then imaged by rapid TIR-FM. **A**, Sequential 100 ms exposures imaged by TIR-FM illumination 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. Insets within images show a higher magnification view of individual insertion events appearing in the boxed area. Time stamp (in seconds after starting the sequential TIR-FM imaging episode) is displayed in upper right corner of each image. **B**, Detailed view of sequential 100 ms frames showing similarly rapid lateral spread of SpH-MOR fluorescence after insertion into both the soma and dendritic shaft (top and bottom series, respectively). Time stamp (in seconds, adjusted so that surface insertion in both examples occurs at 0.2 s to facilitate direct comparison) is displayed in lower right corner of each panel. **C**, Line scan analysis of SpH-MOR surface fluorescence intensity detected at the indicated imaging intervals after initial appearance of the fluorescent spot relative to the center of the fluorescent spot initially detected (distance = 0 on the abscissa). sec, Seconds. **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 (on a linear scale) relative to this baseline value. **E**, Expanded view of the time course plotted in **C** (left) showing the method used to measure event duration (Δt). **F**, Histogram display of individual event durations ($n = 307$ derived from imaging 19 independent culture dishes).

A punctate distribution of SpH-tagged receptor fluorescence was observed in TIR-FM images of DAMGO-exposed neurons, both in somata and regions of dendritic shaft domain illuminated by the ~ 100 nm thick evanescent field (Fig. 3A). This distribu-

tion differed from receptor-containing endosomes discussed above (most of which are not illuminated by the evanescent field) and was reminiscent of endocytic “clusters” shown previously to represent receptor-containing clathrin-coated pits in the neuro-

nal plasma membrane (Yudowski et al., 2006). These punctate structures were not highly dynamic during the 1 min imaging episode, as expected because individual receptor-containing coated pits have a surface lifetime of ~ 1 min (Puthenveedu and von Zastrow, 2006). Rapid serial acquisition (10 Hz) of TIR-FM images revealed localized bursts of increased SpH-MOR fluorescence intensity on a much faster time scale (Fig. 3A). These bursts were easily distinguished from endocytic clusters by their substantially higher brightness and abrupt appearance within a single 100 ms frame, suggesting that they represent vesicular fusion events (Yudowski et al., 2006). Confirming this, SpH-MOR events were not observed when the membrane-impermeant acidic buffer (0.1M MES, pH 5.5) was added to the extracellular solution (data not shown), a manipulation that selectively quenches fluorescence from surface-exposed receptors (Sankaranarayanan et al., 2000). Together, these observations indicate that the localized bursts of SpH-MOR fluorescence observed using this method represent discrete vesicular fusion events mediating surface insertion of receptors.

Multiple SpH-MOR-containing insertion events were observed (16 ± 4.1 events/neuron/min; $n = 19$ neurons) and occurred with approximately equal frequency in the soma and in dendritic shaft domains when quantified across multiple neurons. Surface insertion events observed in both plasma membrane domains were often followed by lateral spread of SpH-MOR fluorescence (Fig. 3) (this is visibly evident in the time series shown), suggesting lateral movement of receptors away from the site of vesicular insertion. The MRS-deleted SpH-MOR $\Delta 17$ mutant receptor exhibited similar rapid DAMGO-induced concentration in coated pits, but the frequency of visible receptor insertion events was greatly reduced (< 2 events/neuron/min; $n = 12$ neurons). This observation is consistent with the previous conclusion that truncating the MRS produces a selective inhibition of MOR recycling without preventing receptor delivery from the biosynthetic pathway or regulated endocytosis of receptors. This observation also verified that most SpH-MOR-containing insertion events visualized by this method represent surface delivery of receptors from the recycling pathway.

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

We estimated the rate of dissipation of SpH-MOR from sites of vesicular insertion by measuring fluorescence intensity as a function of time in a small (3×3 pixel) region including the diffraction-limited spot representing the site of abrupt initial appearance (Fig. 3C). We then determined the time interval between initial appearance and decay of the spot's background-subtracted surface fluorescence intensity to $< 20\%$ of this peak value (Fig. 3D). Using this approach, the vast majority of SpH-MOR vesicular insertion events were found to dissipate within 1–2 s after appearance, with an average duration of 0.86 ± 0.05 s (Fig. 3E) ($n = 307$ events in 19 neurons).

This uniformly rapid dissipation of inserted SpH-MORs contrasted with that observed previously for similarly tagged β_2 ARs in hippocampal neurons. In these cells, SpH- β_2 ARs were found to linger at or near the site of insertion for a variable and sometimes prolonged interval before lateral spread (Yudowski et al., 2006). To determine whether our failure to observe such longer-lasting or persistent surface insertion events for SpH-MOR in medium spiny neurons reflects a receptor-specific or neuron-specific difference, we compared the surface delivery properties of each receptor construct when expressed at similar levels (estimated by fluorescence intensity averaged across the somatic plasma membrane) in striatal cultures. Using an identical incubation and im-

aging protocol (except for substituting the adrenergic agonist isoproterenol for DAMGO when imaging SpH- β_2 AR), discrete vesicular fusion events mediating surface insertion of SpH- β_2 AR were visualized in the soma and dendrites of striatal neurons. Surface-inserted SpH- β_2 ARs dissipated rapidly from $\sim 85\%$ of the events visualized (Fig. 4A shows a representative example and intensity time course), but surface dissipation was obviously delayed from the remaining $\sim 15\%$ of events (Fig. 4B). This was visually apparent by plotting event durations compiled across imaging experiments (Fig. 4C). Cumulative plots of these measurements resembled a monophasic distribution for SpH-MOR, whereas SpH- β_2 AR kinetics were considerably more heterogeneous (Fig. 4D). To determine whether this difference was spurious or systematic across experiments, we defined persistent events such as those with surface duration ≥ 3 s and compared the distributions of this number across multiple experiments using the non-parametric Mann–Whitney test. This verified a significant difference in the number of persistent-type insertion events for SpH-MOR compared to SpH- β_2 AR, which appeared to be systematic across experiments when using independent experiments as statistical n ($p = 0.006$, $n = 19$ and 21 experiments, respectively). As a control, we found no significant difference across the same series of experiments when this analysis was applied to the total number of events (i.e., scored regardless of their duration).

A role of receptor-specific cytoplasmic sorting sequences in differentiating the kinetic properties of discrete surface insertion events

Considering that recycling of each receptor depends on a distinct cytoplasmic sorting sequence, we next asked whether these determinants contribute to generating receptor-specific differences in the kinetic properties of discrete recycling events. To begin to address this question, we examined a chimeric mutant MOR in which the MRS was removed and replaced by the terminal four residues derived from the distal β_2 AR tail [SpH-MOR/ β_2 AR(4)] (Fig. 5A). This relatively short sequence has been shown previously to be sufficient to promote rapid recycling of receptors in non-neural cells (Gage et al., 2001). Striatal neurons expressing SpH-MOR/ β_2 AR(4) were exposed to $10 \mu\text{M}$ DAMGO, and the standardized TIR-FM imaging protocol was used to identify individual vesicular fusion events mediating surface insertion of SpH-tagged mutant receptors.

Sequential 100 ms exposures revealed frequent SpH-MOR/ β_2 AR(4)-containing vesicular insertion events in both somata and dendrites similar in frequency and peak fluorescence intensity to those observed in neurons expressing wild-type SpH-MOR, confirming the ability of this minimal β_2 AR-derived sequence to function effectively as a modular endocytic sorting determinant driving rapid surface delivery of a distinct GPCR in neurons. Visual inspection of scatter plots, which summarize individual event durations compiled across all experiments, revealed an increased number of persistent-type insertion events for constructs containing this minimal β_2 AR-derived sorting sequence (Fig. 5B, compare left and middle columns). Fusing a longer (10 residue) sequence derived from the β_2 AR tail, which includes this minimal determinant and six flanking residues [SpH-MOR/ β_2 AR(10)] (Fig. 5A) that are known to enhance recycling activity in non-neural cells (Gage et al., 2001), also increased the number of persistent-type insertion events observed (Fig. 5B, right). Using the same approach of scoring persistent-type events by ≥ 3 s duration and a non-parametric test suitable to multiple comparisons (Kruskal–Wallis test with Dunn's multiple comparison test), both the four and 10 residue β_2 AR-

derived cytoplasmic tail sequences were found to produce a significant increase in persistent-type events compared to SpH-MOR when independent experiments were used as statistical n ($p < 0.05$ and 0.01 , $n = 15$ and 16 experiments, respectively). In contrast, we did not detect a significant difference across the same series of experiments when this analysis was applied to the total number of events.

We next examined the effect of the converse tail swap, replacing the sorting sequence in the wild-type β_2 AR with the MRS derived from MOR (SpH- β_2 AR/MOR17) (Fig. 5C). Scatter plots representing the duration of individual insertion events did not reveal any obvious loss of persistent-type insertion events for SpH- β_2 AR/MOR17 relative to SpH- β_2 AR and, consistent with this, comparison of the two distributions across independent experiments (using Mann–Whitney test) failed to detect a significant difference ($n = 13$ experiments). Inspection of the cumulative duration data suggested, however, a distinct and possibly more complex change in the overall distribution of event kinetics (supplemental Fig. 1A,B, available at www.jneurosci.org as supplemental material). Because this change obviously shifted the mean of the cumulative distributions for SpH- β_2 AR/MOR17 relative to SpH- β_2 AR, we used this value determined in individual experiments as a simple metric to ask whether there exists a systematic difference across experiments. Because mean event durations measured in individual experiments were near-normally distributed, we compared these distributions using one-way ANOVA as a relatively robust parametric test. This analysis supported a systematic difference in mean event duration between SpH- β_2 AR/MOR17 and SpH- β_2 AR, as well as between the parental SpH- β_2 AR and SpH-MOR constructs, using independent experiments as statistical n (supplemental Fig. 1C, available at www.jneurosci.org as supplemental material). We did not detect a significant difference in this metric for the initial tail swaps (substituting the β_2 AR-derived sorting sequence into SpH-MOR), however, despite the significant effect produced by these swaps on persistent-type insertion events as established and described above from the same experiments. Together, these analyses provide two lines of evidence indicating that the receptor-specific sorting sequences produce distinguishable effects on the kinetic properties of discrete receptor insertion events, further distinguish the β_2 AR-derived sorting sequence from the MRS, and verify that the distinct effects of these respective sequences are systematic when examined across independent experiments. They also indicate that kinetic differences observed between wild-type GPCRs are more complex than can be represented by a single metric and that the defined recycling sequences—while they clearly influence receptor insertion kinetics—are not the only structural determinants contributing to this distinction.

Figure 4. SpH- β_2 AR and SpH-MOR exhibit distinct local surface trafficking kinetics following vesicular insertion. Dissociated rat striatal neurons expressing SpH- β_2 AR were incubated as in Figure 3, except in the presence of $10 \mu\text{M}$ isoproterenol instead of DAMGO, and imaged for 1 min by continuous TIR-FM at 10 Hz. **A**, Time series shows sequential 100 ms frames of a membrane region containing a representative transient SpH- β_2 AR insertion event in which lateral spread of inserted receptors is visually apparent (top panels). The time course of fluorescence intensity change measured at the site of this transient insertion event is shown below. **B**, Time series showing sequential 100 ms frames of a membrane region including a representative persistent SpH- β_2 AR insertion event. Time stamp (in seconds) is shown in lower right corner. Arrows indicate an interval of 9.6 s during which the insertion event remained visibly unchanged as a discrete fluorescent spot. The time course of fluorescence intensity change of this persistent event is shown below. **C**, Scatter plot comparison of the individual event durations measured as in Figure 3D by parallel analysis of SpH-MOR ($n = 307$ events from 19 culture dishes; data are replotted from Fig. 3F) and SpH- β_2 AR ($n = 300$ events from 21 culture dishes). The significance of differences was verified across independent culture dishes using the Mann–Whitney test, as described in the text. **D**, Cumulative probability plot of the same data.

Effect of kinetically distinct surface delivery modes on the surface distribution of receptors observed over physiologically relevant timescales

Despite the obvious prolongation of persistent-type recycling events evident in live image series, the high acquisition rate (10 Hz) used in this imaging may actually exceed the rate at which the G protein-linked transduction cascade normally operates (Lohse et al., 2008). This raised the question of whether kinetic differences observed among discrete recycling events influence the surface distribution of receptors over a physiologically relevant timescale. To investigate this, we focused on the β_2 AR, for which transient and persistent recycling events were frequently observed in close proximity and in the same 1 min imaging episodes. We did so also because catecholamine receptors are well known to signal over a broad temporal range. This has been described most extensively for dopaminergic receptors, which mediate tonic signaling on the order of minutes and phasic signaling within seconds (Carelli and Wightman, 2004; Zhang et al., 2009), but physiological responses mediated by norepinephrine span a similar temporal range (Xiang and Kobilka, 2003; Aston-Jones and Cohen, 2005). Considering the typical kinetics of each type of receptor insertion event defined by the 10 Hz analysis, we anticipated from first principles that transient and persistent events would likely produce different surface distributions of receptors in these different time domains (Fig. 6A–C). To test this experimentally, we averaged the surface distribution of SpH- β_2 AR intensity over 1 min or 10 s intervals, which were chosen to

Figure 5. Divergent sorting sequences are sufficient to confer receptor-specific differences in local surface trafficking from individual vesicular insertion events. **A**, Schematic of wild-type SpH-MOR and SpH-MOR/ β_2 AR chimeric receptors. MOR-derived structures are indicated in red and β_2 AR-derived structures in blue. Sequence details of the MRS and regions of the β_2 AR-derived sorting sequence used to replace this sorting determinant are indicated. **B**, Scatter plot summarizing individual event durations measured by imaging SpH-MOR, SpH-MOR/ β_2 AR(4), and SpH-MOR/ β_2 AR(10). Event numbers and statistical analysis verifying the reliability of differences across independent culture dishes are described in Results. **C**, Schematic of wild-type SpH- β_2 AR and the SpH- β_2 AR/MOR17 chimeric mutant receptor. β_2 AR-derived structures are indicated in blue and MOR-derived structures in red. The β_2 AR-derived sorting sequence and MRS used to replace this sorting determinant are indicated in single-letter amino acid code. **D**, Scatter plot summarizing individual event durations measured by imaging SpH- β_2 AR/MOR17, displayed opposite the corresponding data for SpH- β_2 AR (replotted from Fig. 4C) for comparison.

approximate timescales appropriate to tonic and phasic signaling, respectively (Fig. 6D,E). When averaged over the “tonic” (1 min) interval, both transient and persistent-type insertion events were found to produce a diffuse distribution of surface receptors (examples of such events are circles in red and yellow, respectively) (Fig. 6E). When averaged over the “phasic” (10 s) interval, however, persistent events uniquely produced a punctate localization of surface receptors while transient events still produced a largely diffuse surface distribution (Fig. 6D,E). Of course, relatively static structures, which did not change significantly over the imaging episode, appeared identical on both timescales (an example is circled in white) (Fig. 6D,E). Critical to this comparison, we verified in individual time series that the events compared appeared almost concurrently and in the middle of the imaging interval analyzed (supplemental movie 1, available at www.jneurosci.org as supplemental material). Based on these simple considerations, one can begin to appreciate the ability of kinetically distinct insertion events to rapidly generate complex spatiotemporal patterns of specific signaling receptors in the somatodendritic plasma membrane, and to do so over a physiologically relevant timescale.

Discussion

The present study investigated the specificity of receptor delivery from the endocytic pathway to the somatodendritic plasma membrane and examined the location and nature of vesicular insertion events mediating this process. Our results established the occurrence of efficient recycling of MORs in several populations of CNS-derived neurons at a rapid rate that is sufficient to replace the entire surface complement of receptors in both the soma and dendritic arbor within minutes. Such robust recycling was not observed for DORs, a closely related neuropeptide recep-

tor that is also expressed endogenously in medium spiny cells, and is consistent with the tendency of DORs to traffic to lysosomes and undergo proteolytic down-regulation after endocytosis in neurons (Law et al., 2000; Pradhan et al., 2009). We further established that this receptor-specific difference in rapid recycling is determined by a modular cytoplasmic sorting sequence that is present exclusively in the MOR and was mapped previously based on its ability to function in fibroblastic cells (Tanowitz and von Zastrow, 2003).

These features of MOR trafficking were reminiscent of another seven-transmembrane receptor, the β_2 AR, which requires a distinct cytoplasmic sorting determinant for rapid recycling in hippocampal neurons (Yudowski et al., 2006). We established that both receptors indeed recycle via sequence-directed vesicular fusion events occurring throughout the soma and the dendritic shaft. Taken together with a previous study of GluR1-containing AMPARs using similar methods (Yudowski et al., 2007) and a study of GluR2-containing AMPARs using a different experimental approach (Jaskolski et al., 2009), these findings suggest that disseminated vesicular insertion represents a general strategy for mediating rapid delivery of various signaling receptors to the elaborate dendritic arbor.

We were surprised to find marked differences in the behavior of individual receptor types with respect to their behavior after vesicular insertion. SpH-MORs dispersed rapidly from essentially all insertion events visualized, while SpH- β_2 ARs were inserted both by “transient” insertion events but also by kinetically distinct “persistent” events characterized by a variable lag of inserted receptors before lateral spread. Mutational studies indicated that structurally divergent sorting sequences required for efficient recycling of the respective receptors play a significant albeit complex role in specifying kinetic differences between recycling events. To our knowledge, the present results are the first to establish such a functional difference between divergent sorting sequences in neurons or in any cell type. We also established that persistent-type recycling events characteristic of the SpH- β_2 AR, while indistinguishable from transient-type events in their effect on surface receptor localization averaged over 1 min, differed when averaged over 10 s. This suggests that receptor-specific differences in the properties of discrete recycling events can indeed influence the local surface distribution of signaling receptors over a physiologically relevant timescale.

These results, taken together, provide new insight to the neural cell biology of opioid receptors pertaining to the effects of clinically relevant drugs on regulation of the opioid system. Endocytosis of native opioid receptors can occur in response to endogenous opioid neuropeptides under some conditions (Sinchak and Micevych, 2003) and is induced to varying degrees by exogenous administration of nonpeptide opioid drugs (Keith et al., 1998; Haberstock-Debic et al., 2003; Charlton et al., 2008). MOR endocytosis has been associated with reduced development of antinociceptive tolerance to μ -opioid agonists *in vivo* (Charlton et al., 2008; Kim et al., 2008), consistent with the general

hypothesis that endocytic trafficking of MORs through the recycling pathway supports sustained functional signaling (Martini and Whistler, 2007; Koch and Holtt, 2008). The opposite relationship, increased antinociceptive tolerance, was correlated with regulated endocytosis of DORs (Pradhan et al., 2009). While one must bear in mind the existence of other differences between δ - and μ -opioid receptor-dependent antinociception and its behavioral assessment (Inturrisi, 2002), sequence-directed sorting specifically of μ -opioid receptors into the recycling pathway suggests a parsimonious explanation for such highly divergent physiological consequences of receptor endocytosis.

The present results also provide insight, more generally, to the nature of membrane protein delivery to the complex somatodendritic surface. We are not aware of any precedent for the ability of receptor-specific sorting sequences to affect the kinetics of vesicular insertion events mediating local surface delivery. Our mutational data begin to define a structural basis for this unanticipated specificity in neural endocytic membrane traffic, but the present work falls short of providing a precise biochemical or physical understanding. One could imagine that receptor-specific structural determinant(s), by binding to a submembrane scaffold or driving vesicular insertion into compositionally refined surface membrane domains of different effective viscosity, could produce distinct effects on the rate of receptor lateral movement after insertion (supplemental Fig. 2A,B, available at www.jneurosci.org as supplemental material). Both models predict monotonic dispersion of receptors from the site of vesicular insertion, however, and neither can explain the tendency of the majority of receptors inserted by individual persistent-type events to abruptly “switch” from a variable (and sometimes prolonged) period of lateral immobility to rapid lateral spread. This more complex behavior suggests that surface receptor delivery occurs by a multistep mechanism in which lateral spread is determined separately from initial vesicular insertion. One such model, as discussed extensively elsewhere (Fernandez-Alfonso and Ryan, 2004; Smith et al., 2008), is “kiss-and-run”-coupled exo-endocytosis (supplemental Fig. 2C, available at www.jneurosci.org as supplemental material). Arguing against this particular hypothesis as an explanation for the present data, MES quenching of persistent-type receptor insertion events was apparently complete. Previous data suggest that membrane proteins delivered to the presynaptic plasma membrane by this mechanism are largely inaccessible to MES (Gandhi and Stevens, 2003). We also note

Figure 6. Effect of distinct receptor insertion modes on the longer-term surface localization of receptors. **A**, Schematic of fluorescence intensity traces representing kinetically distinct types of receptor distribution present in the somatodendritic plasma membrane, as investigated in the present study. “Static localization” refers to punctate distributions of receptors that remain unchanged during the 1 min imaging episode, resulting in a stable fluorescence intensity measurement. These include receptor-containing clathrin-coated pits, which have a typical surface lifetime of ~ 1 min and are plentiful in the somatodendritic plasma membrane of neurons imaged in the presence of agonist. Transient insertion events (e.g., Fig. 4A) produce a brief increase in surface fluorescence intensity at the site of vesicular fusion, followed by rapid decrease. Persistent insertion events (e.g., Fig. 4B) produce a plateau of localized surface fluorescence followed by a rapid decrease. **B**, All of these events appear as a bright spot (red) when the map of surface fluorescence intensity is visualized as a maximum intensity projection, making this representation useful for identifying all events and estimating peak fluorescence intensities. **C**, These events are expected to produce different effects on the surface distribution of receptors over longer timescales. When averaged over the entire 1 min imaging interval (blue), a time period on the order of tonic catecholamine signaling, both types of insertion event would be expected to produce diffuse surface distribution. When averaged over a 10 s interval (green), a timescale in the range of phasic catecholamine signaling, transient insertions would be expected to produce a diffuse surface distribution whereas persistent events would appear more punctate. **D**, **E**, Experimental test of these predictions based on analysis of a representative $8 \times 15 \mu\text{m}$ region including a flat portion of somatic plasma membrane that was uniformly illuminated by the evanescent field. **D** shows the maximum projection map of SpH- β_2 AR fluorescence intensity. An example of a transient insertion event is circled in red, a persistent event in yellow, and a relatively static structure (likely a receptor-containing coated pit) in white. **E** shows the fluorescence intensity distributions averaged over 1 min and 10 s that are expected to estimate, respectively, the effective surface distribution of receptors over timescales pertinent to tonic versus phasic catecholamine signaling. As predicted, the persistent-type insertion event uniquely produced a diffuse distribution in the tonic view and a punctate distribution in the tonic view. The two dim structures visible in the tonic view near the periphery of the yellow circle are distinct from the insertion event itself (this can be verified by centroid analysis) and likely represent nearby coated pits. Sequential 100 ms frames covering the entire 10 s interval analyzed are shown in supplemental Movie 1, available at www.jneurosci.org as supplemental material, verifying that the transient and persistent events chosen for comparison appeared in the plasma membrane nearly coincidentally.

that, while kiss-and-run-type delivery has been shown from the biosynthetic pathway both in neurosecretory cells and non-neural cell types (Taraska et al., 2003; Jaiswal et al., 2009), we have to date not observed persistent-type recycling of signaling recep-

tors in non-neural cells (Yudowski et al., 2009). Thus, we presently favor the hypothesis that the persistent-type insertion events observed here reflect the operation of a distinct multistep mechanism of receptor delivery to the somatodendritic surface, which might be called “kiss-and-wait”-coupled exocytosis dispersion. According to this hypothesis, receptors are inserted into a surface-exposed domain that remains in contact with the extracellular milieu in a “corralled” state for a variable time period. Subsequently, in a second step corralled receptors are abruptly “released” and undergo rapid lateral diffusion (supplemental Fig. 2D,E, available at www.jneurosci.org as supplemental material). Supporting this latter hypothesis, the dispersion kinetics of receptors from persistent events, after the release step has apparently occurred, are consistent with simple diffusion at rate ($0.2\text{--}0.5\ \mu\text{m}^2\ \text{s}^{-1}$) similar to that from transient-type insertion events (supplemental Fig. 2F, available at www.jneurosci.org as supplemental material).

Because our analysis of discrete surface insertion events was limited to two particular receptors, we do not yet know how widespread the observed behaviors are. Both of the selected examples are members of the largest known family of signaling receptors, however, and we are not aware of any reason to suppose that the observed behaviors are unique. We also note that ionotropic glutamate receptors, for which scaffold-mediated immobilization is known to play a major role in determining surface receptor localization (Newpher and Ehlers, 2008; Renner et al., 2008; Triller and Choquet, 2008), undergo surface delivery via kinetically heterogeneous vesicular insertion events differing in ability to drive rapid extrasynaptic versus synaptic receptor localization (Yudowski et al., 2007; Makino and Malinow, 2009). Thus, we believe that the present observations may indeed reveal general cell biological principles underlying dynamic and specific delivery of neural signaling receptors to spatiotemporally distinct surface domains.

References

- Abramoff MD, Magelhaes PJ, Ram SJ (2004) Image processing with ImageJ. *Biophotonics Int* 11:36–42.
- Altman J, Bayer SA (1995) *Atlas of prenatal rat brain development*. Boca Raton, FL: CRC.
- Aoki K, Zemcik BA, Strader CD, Pickel VM (1989) Cytoplasmic loop of beta-adrenergic receptors: synaptic and intracellular localization and relation to catecholaminergic neurons in the nuclei of the solitary tracts. *Brain Res* 493:331–347.
- Arttamangkul S, Quillinan N, Low MJ, von Zastrow M, Pintar J, Williams JT (2008) Differential activation and trafficking of micro-opioid receptors in brain slices. *Mol Pharmacol* 74:972–979.
- Aston-Jones G, Cohen JD (2005) An integrative theory of locus coeruleus-norepinephrine function: adaptive gain and optimal performance. *Annu Rev Neurosci* 28:403–450.
- Cao TT, Mays RW, von Zastrow M (1998) Regulated endocytosis of G protein-coupled receptors by a biochemically and functionally distinct subpopulation of clathrin-coated pits. *J Biol Chem* 273:24592–24602.
- Carelli RM, Wightman RM (2004) Functional microcircuitry in the accumbens underlying drug addiction: insights from real-time signaling during behavior. *Curr Opin Neurobiol* 14:763–768.
- Carroll RC, Beattie EC, von Zastrow M, Malenka RC (2001) Role of AMPA receptor endocytosis in synaptic plasticity. *Nat Rev Neurosci* 2:315–324.
- Charlton JJ, Allen PB, Psifogeorgou K, Chakravarty S, Gomes I, Neve RL, Devi LA, Greengard P, Nestler EJ, Zachariou V (2008) Multiple actions of spinophilin regulate mu opioid receptor function. *Neuron* 58:238–247.
- Fernández-Alfonso T, Ryan TA (2004) The kinetics of synaptic vesicle pool depletion at CNS synaptic terminals. *Neuron* 41:943–953.
- Gage RM, Kim KA, Cao TT, von Zastrow M (2001) A transplantable sorting signal that is sufficient to mediate rapid recycling of G protein-coupled receptors. *J Biol Chem* 276:44712–44720.
- Gainetdinov RR, Premont RT, Bohn LM, Lefkowitz RJ, Caron MG (2004) Desensitization of G protein-coupled receptors and neuronal functions. *Annu Rev Neurosci* 27:107–144.
- Gandhi SP, Stevens CF (2003) Three modes of synaptic vesicular recycling revealed by single-vesicle imaging. *Nature* 423:607–613.
- Haberstock-Debic H, Wein M, Barrot M, Colago EE, Rahman Z, Neve RL, Pickel VM, Nestler EJ, von Zastrow M, Svingos AL (2003) Morphine acutely regulates opioid receptor trafficking selectively in dendrites of nucleus accumbens neurons. *J Neurosci* 23:4324–4332.
- Haberstock-Debic H, Kim KA, Yu YJ, von Zastrow M (2005) Morphine promotes rapid, arrestin-dependent endocytosis of mu-opioid receptors in striatal neurons. *J Neurosci* 25:7847–7857.
- Hanyaloglu AC, von Zastrow M (2008) Regulation of GPCRs by endocytic membrane trafficking and its potential implications. *Annu Rev Pharmacol Toxicol* 48:537–568.
- Hislop JN, Marley A, Von Zastrow M (2004) Role of mammalian vacuolar protein-sorting proteins in endocytic trafficking of a non-ubiquitinated G protein-coupled receptor to lysosomes. *J Biol Chem* 279:22522–22531.
- Inturrisi CE (2002) Clinical pharmacology of opioids for pain. *Clin J Pain* 18:S3–S13.
- Jaiswal JK, Rivera VM, Simon SM (2009) Exocytosis of post-Golgi vesicles is regulated by components of the endocytic machinery. *Cell* 137:1308–1319.
- Jaskolski F, Mayo-Martin B, Jane D, Henley JM (2009) Dynamin-dependent membrane drift recruits AMPA receptors to dendritic spines. *J Biol Chem* 284:12491–12503.
- Keith DE, Murray SR, Zaki PA, Chu PC, Lissin DV, Kang L, Evans CJ, von Zastrow M (1996) Morphine activates opioid receptors without causing their rapid internalization. *J Biol Chem* 271:19021–19024.
- Keith DE, Anton B, Murray SR, Zaki PA, Chu PC, Lissin DV, Monteillet-Agius G, Stewart PL, Evans CJ, von Zastrow M (1998) mu-Opioid receptor internalization: opiate drugs have differential effects on a conserved endocytic mechanism in vitro and in the mammalian brain. *Mol Pharmacol* 53:377–384.
- Kennedy MJ, Ehlers MD (2006) Organelles and trafficking machinery for postsynaptic plasticity. *Annu Rev Neurosci* 29:325–362.
- Kim JA, Bartlett S, He L, Nielsen CK, Chang AM, Kharazia V, Waldhoer M, Ou CJ, Taylor S, Ferwerda M, Cado D, Whistler JL (2008) Morphine-induced receptor endocytosis in a novel knockin mouse reduces tolerance and dependence. *Curr Biol* 18:129–135.
- Koch T, Höllt V (2008) Role of receptor internalization in opioid tolerance and dependence. *Pharmacol Ther* 117:199–206.
- Law PY, Wong YH, Loh HH (2000) Molecular mechanisms and regulation of opioid receptor signaling. *Annu Rev Pharmacol Toxicol* 40:389–430.
- Lohse MJ, Hein P, Hoffmann C, Nikolaev VO, Vilardaga JP, Bunemann M (2008) Kinetics of G-protein-coupled receptor signals in intact cells. *Br J Pharmacol* 153 [Suppl 1]:S125–S132.
- Makino H, Malinow R (2009) AMPA receptor incorporation into synapses during LTP: the role of lateral movement and exocytosis. *Neuron* 64:381–390.
- Martini L, Whistler JL (2007) The role of mu opioid receptor desensitization and endocytosis in morphine tolerance and dependence. *Curr Opin Neurobiol* 17:556–564.
- Miesenböck G, De Angelis DA, Rothman JE (1998) Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. *Nature* 394:192–195.
- Newpher TM, Ehlers MD (2008) Glutamate receptor dynamics in dendritic microdomains. *Neuron* 58:472–497.
- Niwa H, Yamamura K, Miyazaki J (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 108:193–199.
- Pradhan AA, Becker JA, Scherrer G, Tryoen-Toth P, Filliol D, Matifas A, Massotte D, Gaviériaux-Ruff C, Kieffer BL (2009) In vivo delta opioid receptor internalization controls behavioral effects of agonists. *PLoS One* 4:e5425.
- Puthenveedu MA, von Zastrow M (2006) Cargo regulates clathrin-coated pit dynamics. *Cell* 127:113–124.
- Renner M, Specht CG, Triller A (2008) Molecular dynamics of postsynaptic receptors and scaffold proteins. *Curr Opin Neurobiol* 18:532–540.
- Sankaranarayanan S, De Angelis D, Rothman JE, Ryan TA (2000) The use of pHluorins for optical measurements of presynaptic activity. *Biophys J* 79:2199–2208.
- Scherrer G, Tryoen-Tóth P, Filliol D, Matifas A, Laustriat D, Cao YQ,

- Basbaum AI, Dierich A, Vonesh JL, Gavériaux-Ruff C, Kieffer BL (2006) Knockin mice expressing fluorescent delta-opioid receptors uncover G protein-coupled receptor dynamics in vivo. *Proc Natl Acad Sci U S A* 103:9691–9696.
- Schmid SL, Smythe E (1991) Stage-specific assays for coated pit formation and coated vesicle budding in vitro. *J Cell Biol* 114:869–880.
- Shepherd JD, Huganir RL (2007) The cell biology of synaptic plasticity: AMPA receptor trafficking. *Annu Rev Cell Dev Biol* 23:613–643.
- Sinchak K, Micevych P (2003) Visualizing activation of opioid circuits by internalization of G protein-coupled receptors. *Mol Neurobiol* 27:197–222.
- Smith SM, Renden R, von Gersdorff H (2008) Synaptic vesicle endocytosis: fast and slow modes of membrane retrieval. *Trends Neurosci* 31:559–568.
- Sorkin A, von Zastrow M (2009) Endocytosis and signalling: intertwining molecular networks. *Nat Rev Mol Cell Biol* 10:609–622.
- Sternini C, Spann M, Anton B, Keith DJ Jr, Bunnett NW, von Zastrow M, Evans C, Brecha NC (1996) Agonist-selective endocytosis of mu opioid receptor by neurons in vivo. *Proc Natl Acad Sci U S A* 93:9241–9246.
- Steyer JA, Almers W (2001) A real-time view of life within 100 nm of the plasma membrane. *Nat Rev Mol Cell Biol* 2:268–275.
- Tanowitz M, von Zastrow M (2003) A novel endocytic recycling signal that distinguishes the membrane trafficking of naturally occurring opioid receptors. *J Biol Chem* 278:45978–45986.
- Taraska JW, Perrais D, Ohara-Imaizumi M, Nagamatsu S, Almers W (2003) Secretory granules are recaptured largely intact after stimulated exocytosis in cultured endocrine cells. *Proc Natl Acad Sci U S A* 100:2070–2075.
- Triller A, Choquet D (2008) New concepts in synaptic biology derived from single-molecule imaging. *Neuron* 59:359–374.
- Tsao PI, von Zastrow M (2000) Type-specific sorting of G protein-coupled receptors after endocytosis. *J Biol Chem* 275:11130–11140.
- Ventimiglia R, Lindsay RM (1998) Rat striatal neurons in low-density, serum-free culture. In: *Culturing nerve cells*, 2nd Ed (Banker G, Goslin K, eds), pp 370–393. Cambridge, MA: MIT.
- Wang H, Moriawaki A, Wang JB, Uhl GR, Pickel VM (1997) Ultrastructural immunocytochemical localization of mu-opioid receptors in dendritic targets of dopaminergic terminals in the rat caudate-putamen nucleus. *Neuroscience* 81:757–771.
- Xiang Y, Kobilka BK (2003) Myocyte adrenoceptor signaling pathways. *Science* 300:1530–1532.
- Yudowski GA, Puthenveedu MA, von Zastrow M (2006) Distinct modes of regulated receptor insertion to the somatodendritic plasma membrane. *Nat Neurosci* 9:622–627.
- Yudowski GA, Puthenveedu MA, Leonoudakis D, Panicker S, Thorn KS, Beattie EC, von Zastrow M (2007) Real-time imaging of discrete exocytic events mediating surface delivery of AMPA receptors. *J Neurosci* 27:11112–11121.
- Yudowski GA, Puthenveedu MA, Henry AG, von Zastrow M (2009) Cargo-mediated regulation of a rapid Rab4-dependent recycling pathway. *Mol Biol Cell* 20:2774–2784.
- Zhang L, Doyon WM, Clark JJ, Phillips PE, Dani JA (2009) Controls of tonic and phasic dopamine transmission in the dorsal and ventral striatum. *Mol Pharmacol* 76:396–404.