Formation and dissociation of M₁ muscarinic receptor dimers seen by total internal reflection fluorescence imaging of single molecules

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G-protein–coupled receptors (GPCRs) are the largest family of transmembrane signaling proteins in the human genome. Events in the GPCR signaling cascade have been well characterized, but the receptor composition and its membrane distribution are still generally unknown. Although there is evidence that some members of the GPCR superfamily exist as constitutive dimers or higher oligomers, interpretation of the results has been disputed, and recent studies indicate that monomeric GPCRs may also be functional. Because there is controversy within the field, to address the issue we have used total internal reflection fluorescence microscopy (TIRFM) in living cells to visualize thousands of individual molecules of a model GPCR, the M₁ muscarinic acetylcholine receptor. By tracking the position of individual receptors over time, their mobility, clustering, and dimerization kinetics could be directly determined with a resolution of ~30 ns and ~20 nm. In isolated CHO cells, receptors are randomly distributed over the plasma membrane. At any given time, ~30% of the receptor molecules exist as dimers, and we found no evidence for higher oligomers. Two-color TIRFM established the dynamic nature of dimer formation with M₁ receptors undergoing interconversion between monomers and dimers on the timescale of seconds.

acetylcholine receptor | dimerization | G-protein–coupled receptor | receptor clustering | receptor mobility

muscarinic acetylcholine receptors are members of the major subfamily A of G-protein–coupled receptors (GPCRs). The M₁ subtype is selectively distributed in the forebrain and is considered to play an important role in cognition and memory (1, 2). A critical question is how these receptors bind acetylcholine and produce downstream signaling via heterotrimeric G proteins. In particular, a major issue is whether muscarinic receptors exist as obligate dimers or higher oligomers. Initial events in the GPCR signaling cascade can be monitored by measuring radioligand binding to membrane-bound receptors and associated heterotrimeric G proteins and by measuring early biochemical or electrophysiological signals. However, the composition of receptor complexes is not generally known at the level of individual molecular species. Within the GPCR field there is considerable controversy regarding the molecular state of receptors at the surface of living cells. There is incontrovertible evidence that some members of the small subfamily C of GPCRs are constitutive dimers (reviewed in refs. 3 and 4) and substantial evidence that certain members of the largest subfamily A may exist as dimers (4–6) or even as higher oligomers (6, 7). There is also evidence that GPCR heterodimers may exist (reviewed in ref. 8). However, the principal experimental approach used for many in vivo studies has been resonance energy transfer, and interpretation of its data has led to controversy (9–12). Adding to the debate, some recent studies have indicated that the monomeric state of some GPCRs can act as a functional unit (13–16). Using bulk methods, it has been difficult to unambiguously determine the oligomeric state and mobility of molecules within living cells. However, single-molecule approaches can give direct information about how molecules move and interact within the cellular context. Total internal reflection fluorescence microscopy (TIRFM) is a wide-field imaging method that enables hundreds or thousands of individual molecules to be visualized within living cells at sufficiently high spatial and temporal resolution that they can be observed and tracked over a period of several seconds. Using this approach, we have been able to observe the transient formation and disruption of M₁ receptor dimers in real time.

Results

To study individual muscarinic receptors, it was necessary to label them specifically with a fluorescent species having suitable biophysical characteristics in terms of absorption wavelength and resistance to photobleaching. Two fluorescent muscarinic antagonists were synthesized (SI Text, Figs. S1–S3, and Tables S1 and S2), and one of these, Cy3B–telenzepine (17) (Fig. 1A), was found to have a very high affinity for M₁ receptors with a Kᵦ of 35 pM (~logKᵦ 10.46 ± 0.06) at 37 °C and very slow dissociation kinetics (t₀.₅ 7 ± 2 h at 37 °C and 23–110 h at 23 °C), determined by measuring the time-dependent binding of a fast-associating muscarinic radioligand, [³H]-N-methylscopolamine, in the presence and absence of Cy3B–telenzepine (SI Text, Fig. S4, and Table S3). A second ligand, Alexa488–telenzepine (Table S3 and SI Text), had a lower affinity for the M₁ receptor (Kᵦ 0.5 nM at 23 °C, ~logKᵦ 9.32 ± 0.07) but still had very slow dissociation kinetics (t₀.₅ 3.6 ± 0.6 h). The time dependence and equilibrium binding of both fluorescent antagonists were compatible with the simple model in which the receptor can be occupied by either antagonist or radioligand (SI Text) and not by models with more complex stoichiometries.

Visualization of Single M₁ Muscarinic Receptors. The high affinity of the Cy3B–telenzepine ligand allows stoichiometric labeling of M₁ receptors on CHO cells, with up to 97% being labeled by 0.1 nM concentrations (see SI Text). The slow off-rate means that any unbound or nonspecifically bound ligand can be washed away with no substantial loss of receptor labeling over a period of hours. The level of receptor expression used in the present study is in the


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range found for the expression of M₁ receptors in rat cerebral cortex [~1 pmol receptor per mg membrane protein (18)].

When the basal plasma membrane of live cells was viewed using laser-based TIRFM (SI Text), numerous moving fluorescent spots were observed, and their positions and intensities were recorded using an electron multiplying (EMCCD) video camera system at a rate of either 25 or 33 frames s⁻¹. To confirm specific labeling of M₁ receptors by the Cy3B–telenzepine ligand, cells were pretreated with 10⁻⁵ M atropine (a potent muscarinic antagonist). We found just a small number of stationary spots adhering to the glass coverslip surface (nonspecific binding), but cell labeling was completely blocked and no moving fluorescent spots were observed at the plasma membrane. Figure 1B shows a single video frame taken from a 48-s recording of the basolateral cell surface of a living CHO cell where the transfected M₁ receptors were labeled with 1 nM Cy3B–telenzepine. A large number of fluorescent spots with intensities comparable to those of single molecules adsorbed to a glass slide were observed. A region of interest in Fig. 1B is highlighted and enlarged to illustrate the pixelated appearance of the image of the individual receptors. The first 3 s of this recording is shown in Movie S1. At the laser excitation intensity used in this recording, fluorescent spots photobleached with a half time of ~5 s. If, after photobleaching, the laser was turned off for several minutes, unbleached labeled receptors diffused to the basal surface of the cell from regions that had not been exposed to the evanescent field produced by the TIRF illumination. This fluorescence recovery procedure could be repeated a number of times to obtain multiple recordings from the same cell.

Fluorescent molecules were identified and tracked using digital image analysis (19). An example of a recording is in Fig. 1C and Movie S2. The mean lateral diffusion coefficient of the Cy3B–telenzepine-labeled receptors at 23 °C was 0.089 ± 0.019 μm² s⁻¹ (mean ± SD for 35,208 tracks from 23 cells, range 0.06–0.15 μm² s⁻¹ for different cells). A similar diffusion coefficient was found for Alexa488-labeled M₁ receptors [0.079 ± 0.006 μm² s⁻¹ (mean ± SD for 15,944 tracks from nine cells)]. At 37 °C, the labeled receptors moved faster, with a mean lateral diffusion coefficient of 0.16 ± 0.04 μm² s⁻¹ (3,540 tracks from eight cells). The higher diffusion rate at 37 °C made it more difficult to track receptors at the expression levels found in most cells examined (range ~0.5–2 receptors μm⁻²).

**Distribution and Diffusion of M₁ Receptors on the Cell Surface.** To characterize the motion of receptors at the membrane, we calculated their mean squared displacement (MSD) over a range of time intervals (δt). The linear relationship between MSD and δt (Fig. 1D) shows that receptor diffusion is consistent with a
classical random walk. There was no evidence of restricted or anomalous diffusion over the timescales explored in this study (50 ms–5 s). To test whether receptors are clustered or corralled at the plasma membrane, we examined the distribution of tracks over the duration of the video records and of fluorescent spots observed at the very start of the recording. The distribution of molecules was examined either by counting within 3 × 3-μm² grids (or quadrants) overlaid on images of the cell or by performing a nearest-neighbor statistical analysis of the objects. We found no evidence for clustering of the receptors at any region of the plasma membrane of isolated cells. As expected, agonists showed no detectable effects when applied to cells where the M₁ receptors had been prelabeled to high occupancy by Cy3B-telenzepine.

**M₁ Receptors Exist as Monomers and Dimers.** A histogram (Fig. 1E) of the intensities of fluorescent spots observed at the beginning of the video record (Fig. 1B) shows an asymmetric distribution skewed to higher intensities than that expected for a single fluorophore. The origin of the asymmetry is evident when the intensities of individual tracks are plotted as a function of time (Fig. 2). Some tracks have intensities that can be categorized as “level 1” (corresponding to the intensity of a single fluorophore, as measured from the intensity of nonspecifically bound ligand molecules attached to the glass slide near the cell being observed (described in more detail in SI Text)), others as “level 2” (corresponding to the intensity of two fluorophores), and a third type of track switches from intensity 2 to 1 (termed 2 → 1) before a track terminates. As described earlier, the binding studies show that one molecule of Cy3B–telenzepine binds to only one M₁ receptor, so the first two types of track are compatible with the presence of monomers and dimers, respectively. The third type of track is characteristic of two-step photobleaching whereby two molecules of Cy3B–telenzepine are bound to a dimer, and they sequentially photobleach. However, tracks of type 2 → 1 are also compatible with dissociation of the dimer into two monomers because the tracking algorithm would follow only one of the two tracks that are generated upon dimer disruption. The distribution of fluorescence intensities for individual spots is compatible with the presence of monomers (mean ~60 counts/px) and dimers (mean ~120 counts/px) indicated by the black and red arrowheads in Fig. 1E.

Because the conditions used in these experiments were such that ~97% of the receptors were labeled, it is important to note that, if the M₁ receptors were constitutive dimers, the percentage of objects of intensity 2 at the start of the recording would be close to 100% but would progressively fall to zero as photobleaching occurred. We found that the starting percentage of dual-labeled receptors (level 2) never approached 100% (typically 10–15%) and showed no strong time-dependent change during the recording period (SI Text). These findings are incompatible with the idea that M₁ receptors form constitutive dimers. Furthermore, we did not observe objects at the plasma membrane with an intensity corresponding to three or more fluorophores or objects showing three or more photobleaching steps. Our data provide no evidence for the presence of receptor trimers or higher oligomers as suggested by atomic force microscopy observations of rhodopsin in retinal preparations (7). However, estimates of the steady-state fraction of M₁ dimers from these data are not precise because both photobleaching and the nature of the video tracking method lead to an underestimation.

**Evidence for Reversible Dimer Formation.** Visual inspection of individual track intensities from a single cell allowed assignment to three categories (1, 2, and 2 → 1), giving 82%, 7%, and 3%, respectively, of 1346 identified tracks. The remainder were more complex or could not be unambiguously assigned. To make this procedure more rigorous, an automated procedure was developed to assign intensity trajectories to specific categories, and this also allowed more complex intensity changes to be categorized for very large data sets (SI Text and Fig. S5). Using this procedure, the percentage of tracks in different categories for the same cell described above (i.e., 1, 2, and 2 → 1) was 81%,
9%, and 3%, respectively. The major population, that of level 1, showed no evidence of two-step photobleaching, which means that level 1 cannot correspond to a dually labeled receptor dimer nor level 2 to a receptor tetramer. We found that more complex intensity trajectories accounted for the remainder, and these categories were dominated by $1 \rightarrow 2$, $1 \rightarrow 2 \rightarrow 1$, and $2 \rightarrow 1 \rightarrow 2$ trajectories (3%, 3%, and 0.5%, respectively) (Fig. 2). These changes in intensity are compatible with reversible dimer formation, which can be tested specifically by inspecting the individual frames of original video data (Fig. 3). In many cases, as illustrated here, we found that the more complex intensity trajectories arose from two objects of intensity 1, coalescing to form a single spot of intensity 2, which then diffused for some time before the objects separated (i.e., the original spots returned to intensity 1). However, optical diffraction means that, if two point sources of light are separated by less than 300 nm (the Rayleigh limit), the images coalesce and appear as a single, bright spot. This can make interpretation of track intensity fluctuations ambiguous because it is not clear if molecules have associated or simply passed close by.

**Two-Color TIRFM Measurements of M₁ Receptor Dynamics.** To address the issue of whether M₁ receptors undergo reversible dimer formation, we devised a dual-color imaging technique in which receptors were labeled in a 1:1 ratio with Cy3B–telenzepine and Alexa488–telenzepine (*SI Text*). The known affinities of the two ligands, derived from bulk studies, enabled us to control the labeling ratio. We used alternating dual-color TIRFM (*SI Text*) to visualize the movements of receptors. Excitation was switched between two lasers (488 and 566 nm) so the specimen was illuminated with blue and then green light on alternate, sequential video frames. A dual bandpass emission filter allowed either the green or red fluorescence signals to be separated from the scattered laser excitation light so the monochrome video images represented the intensity of green and red fluorescence. Video recordings of up to 50 s were stored, and the image stack could later be displayed using appropriate color look-up tables (Fig. 4A–F, *SI Text, Movies S3 and S4, and Table S4*).

The initial densities of green and red fluorescently labeled receptors were each ~0.9 μm⁻², reflecting the 1:1 labeling stoichiometry. At this receptor density, individual receptors appear as diffraction-limited spots of light. When two adjacent frames representing green and red fluorescence intensities are overlaid (Fig. 4C), regions where both green and red channels are of high intensity give rise to a yellow color. [Yellow spots can indicate the presence of dimers or the chance presence of two monomers within the same diffraction-limited (~300 nm) distance in the membrane.] Furthermore, overlap between individual spots might be imperfect if the time delay (33 ms) between acquisition of the image pair had allowed a receptor dimer (labeled with one green and one red fluorophore) to diffuse by more than 300 nm. Although the root mean square displacement is $<x^2> = \sqrt{2D_{lat} \delta t}$, the random nature of diffusion means that larger and smaller excursions in position will occur between adjacent frames by chance.

By tracking green and red spots separately (12,485 red and 9,469 green tracks from 9 cells, giving $D_{lat} = 0.097 \mu m^2 \cdot s^{-1};$ Table S4), a
Given the diffusion coefficient of the receptors and assuming that they diffuse independently, the probability of the molecular trajectories overlapping (i.e., centroids of the spots remaining with 300 nm of each other) by chance for more than 10 consecutive frames is exceedingly low (<<1%). We used this as a criterion to distinguish overlap of the spots by chance from true association (dimerization) of the receptors. Analysis of coincidence of the two-color channels showed that 2089 tracks coincided (i.e., ~20% of the total observations) for part of their trajectory. As an independent check, we offset the green and red video frames of the cell shown in Fig. 4 (by shifting the coordinates of the red frames by 500 nm) and found that no tracks then overlapped. We found that dual-labeled tracks diffused more slowly (0.065 μm·s⁻¹ at 23 °C) than the estimate obtained for monomers, which is consistent with the increased radius of a dimer compared to a monomer (20). We found no evidence for anomalous diffusion of dimers, which might be expected if dimers were specifically anchored to a larger intracellular complex or localized to specific membrane domains or the cytoskeleton.

Closer inspection of the green and red coincident tracks revealed three types of behavior: (i) those that started as dimers and separated into monomers; (ii) those that started as monomers and formed dimers; and (iii) those that started as monomers, formed dimers, and then separated into monomers. The behavior of the last group is equivalent to that shown in Fig. 3 for the singly Cy3B–telenzepine-labeled receptors. Trajectories of examples of these three groups of tracks are shown in Fig. 4G. The x-coordinates of green and red tracks are shown in the upper sections of records 1–5 in Fig. 4G, and the y-coordinates in the lower sections. Coincidence of the green and red tracks, i.e., the presence of a dimer, is manifest by the overlap of green and red tracks in both the x- and y-coordinates. Although the tracks that show two-color coincidence represent ~20% of the total tracks (Table S4), these are dimers for only ~50% of the time (e.g., tracks in Fig. 4G), and thus the estimate of the percentage of two-color dimers at any one time, ts = ~10%.

From an analysis of the lifetimes of the 84 individual dimers in the third group (Fig. 4G: records 4 and 5 and inset), it was possible to estimate the dissociation rate of the dimers as ~1.3·s⁻¹ (or a lifetime of ~0.5 s at 23 °C). Analysis of the categories of tracks shown in Fig. 4G: records 1 and 2 gave estimated dimer lifetimes of ~0.5 s (1,243 tracks) and ~0.8 s (372 tracks), respectively.

For some tracks, we could also detect transient dissociation–dimerization events where the two-color dimers separated for short periods of time (Fig. 4G, record 3). Finally, we also observed that 5.7% and 4.6% of the green and red tracks did not overlap with the other color but instead exhibited two-step photobleaching compatible with the presence of homodimers of Alexa488–telenzepine- or Cy3B–telenzepine-labeled receptors (1,079 tracks, ~10% of the total observations; Table S4), which is equivalent to what is observed in the one-color data (Fig. 2C). Thus the total percentage of single-color and two-color dimers observed is ~20% (equivalent to ~50% of the total receptor molecules present as dimers).

**Discussion**

In this study, M₁ muscarinic receptors labeled with fluorescent telenzepine analogs were observed as individual, mobile, fluorescent spots that were evenly distributed on the cell surface. The high affinity and very slow off-rates of the telenzepine analogs enabled us to observe ~97% of all receptors present at the plasma membrane. Individual receptors were tracked with high spatial resolution using digital image analysis, and their lateral diffusion, on the timescale of seconds, was found to be consistent with the predictions of a random walk model and in good agreement with estimates from bulk studies of other GPCRs (21, 22). We found no evidence for restricted or anomalous diffusion of M₁ receptors, unlike reports for gold-tagged μ-opioid receptors (23, 24).

The simplest interpretation of our single-color imaging experiments is that each muscarinic receptor binds a single fluorescently tagged telenzepine molecule. This means that the intensities of the optical signals report the oligomeric state of receptor complexes. The pattern of intensity changes for individual spots of light tracked over time indicates that the receptors exist in a dynamic equilibrium between monomeric and dimeric states and that higher oligomeric states are completely absent. This finding was confirmed by dual-color labeling experiments in which transient formation and disruption of dual-labeled species were directly observed. Although this is the
simplest interpretation of the data, we cannot exclude the possibility that muscarinic receptors exhibit “half of the sites binding.” Each fluorophore would then report the position of a dimer, and instead the data would suggest dimer-to-tetramer transitions. It has been suggested from bulk studies of muscarinic receptor subtypes that they may be oligomers (25) (M2 receptors), covalently linked dimers (26) (M3 receptors), or predominantly constitutive dimers (27) (M1–M5 receptors). However, we know of no evidence that M1 receptors show “half of the sites binding” stoichiometry (see SI Text for further discussion) or reports showing that they form heterodimers with other endogenous GPCRs in CHO cells. So, our single- and dual-color imaging data are most easily explained by the presence of a steady-state mixture of M1 receptor monomers and dimers that associate and dissociate on the timescale of seconds.

In cell biology terms, M1 receptors are behaving as a poised system. The steady-state proportion of dimers in vivo will depend on the level of receptor expression, on any heterogeneity in their distribution within the membrane, and possibly on the cellular milieu. The pharmacological nature of any bound ligand, whether it be an agonist, neutral antagonist, or inverse agonist, could well be important if the dimers behave differently from monomers, for example, in their functional properties or in their ability to be delivered to the cell surface or internalized. If our findings of reversible dimer formation transfer to other members of family A of GPCRs, it is likely that the affinities of the receptors for each other (e.g., in homo- or heterodimers) and hence dimer lifetimes could well be different from values reported here. Transient receptor dimerization, as also suggested by recent fluorescence recovery after photobleaching studies (28), could have unanticipated physiological consequences as well as providing a molecular explanation for apparently conflicting data in the literature. Using the single-color and two-color imaging approaches described here, it is now practicable to follow and analyze quantitatively very large numbers of individual molecules of GPCRs (or other cell-surface proteins) in living cells with high spatial and temporal resolution.

Materials and Methods

Chemical Synthesis. The synthesis and characterization of Cy3b–telenzepine and Alexa488–telenzepine are described in the SI Text.

Determination of the Association and Dissociation Kinetics of the Fluorescent Ligands. The radioligand binding assay protocols used and the results are reported in the SI Text.

Labeling of the M1 Receptors on CHO Cells by the Fluorescent Ligands. The methods for labeling M1 receptors with the fluorescent ligands for single-color or dual-color TIRFM are described in the SI Text.

Detection of Single Molecules of M1 Receptors. The TIRFM imaging system used is described in detail in the SI Text.

Analysis of the TIRFM Data. The automatic single particle tracking algorithm used to identify and track individual muscarinic receptors has been described previously (19) (available at www.nimr.mrc.ac.uk/GMimPro). The algorithm used to identify and analyze pairs of moving “red” and “green” particle tracks in the two-color TIRFM experiments is described in the SI Text. The algorithm used to identify stepwise changes in intensity in the single-color imaging experiments also is described in the SI Text.

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The pharmacology and kinetics of given μ). Superimposed on this are the exist as stable (5 mg, 90% reactive dye, effective Sep-pak 527.7501. The material was dissolved in DMSO at 5 (25 cm; no S2 solution for the Alexa conjugate and were referenced and μ 5 was from Amersham Biosciences. Protons. Interconversion between the major ro-

Assignments and assignments are reported in the section NMR Spectra and Assignments (below) because of the spectral complexity. High-resolution mass spectra (HRMS) were recorded on a Waters Micromass Q-ToF Ultima instrument at the School of Pharmacy, University of London. Preparative TLC was carried out on Merck 1-mm Kieselgel 60 plates (20 x 20 cm; no fluorescence indicator) and reverse-phase purification was performed on C18 Sep-pak cartridges (Waters).

Telenzepine-Alexa488 conjugate (see Fig. S2). Aliquots (90 μL) of a solution of the telenzepine congener 1 (4 mg, 5.9 μmol) in dry dimethylformamide (DMF) (0.50 mL) that also contained diisopropylethylamine (DIPEA) (4.1 μL, 24 μmol) were added to each of three vials of Alexa488 TFP ester 3 (each vial contained 1 mg, 1.13 μmol). Final reactant quantities per vial were the following: congener 1, 1.07 μmol; congener 3, 1.13 μmol; DIPEA, 4.32 μmol. The vials were kept at room temperature for 24 h and then stored at ∼20 °C while purification methods were developed. Thin-layer chromatography (TLC) procedures showed strong streaking, and attempted partition between water and chloroform-methanol was unsuccessful in extracting the product from the organic layer. However, reverse-phase purification based on Sep-pak cartridges was more promising. Thus the contents of one vial were diluted with water (1.35 mL) and applied to a Sep-pak cartridge (previously washed with methanol and water) and eluted with water until free dye was removed (∼5 mL water). Stepwise elution with aliquots (2.5 mL) of 10% and 20% methanol-water (each 2.5 mL) and 30% methanol-water (1 mL) gave little material in the 10% and 20% washes, but some elution of fluorescent material was observed in the 30% wash. Elution with methanol-water (1:1, 6 mL) gave the conjugate 4 (0.70 μmol; measured from absorbance at 494 nm and based on an absorbance coefficient of 71,000 M⁻¹·cm⁻¹; Molecular Probes data). HRMS: Found, 527.7488 (M+2H)⁺. Calculated for (C₂₀H₁₇N₂O₅S₂ + 2H)⁺ 527.7501. The material was dissolved in DMSO at ∼200 μM concentration for storage at −80 °C.

NMR Spectra and Assignments. The NMR samples were ≈5-mM solutions in deuterated DMSO and/or deuterated methanol. All spectra were acquired at 27 °C on a Varian UNITY PLUS 500 MHz spectrometer. Spectra were processed by VNMR and NMRPipe (3) and analyzed using SPARKY (T. D. Goddard and D. F. Kneller, SPARKY 3, University of California, San Francisco). Assignments were extracted from the analysis of the 2D DQF-COSY spectra (4), TOCSY spectra (5) with an isotropic mixing time of 80 ms using the MLEV-17 sequence with a field strength of +2248.8 kHz and ROESY spectra (6) with a 400-ms mixing time. The simplified numbering systems used to report the assignments of conjugates 4 and 5 are shown in Figs. S2 and S3 and the assignments are in Tables S1 and S2. The spectra of conjugates 4 and 5 were complicated by restricted rotation about the exocyclic amide bond to the thieno[3,4-b][1,5]benzdiazepine-10-one ring, giving rise to duplication of the proton resonances on the ring and the 7-CH₃ protons. Interconversion between the major rotamer and minor rotamer (a) was detected in the ROESY spectra. Slowly interconverting conformations of a related compound,pirenzepine, have been observed previously (7). There was also restricted rotation about the amide bond between the linker and the Alexa488 and Cy3B ring systems. These rotamers were in an ∼1:1 ratio and resulted in duplication of the proximal methylene resonances (designated in Tables S1 and S2 as the main rotamer and rotamer b) as well as the nearby aromatic proton resonances of the carboxylated ring of Alexa488. Interconversion of these rotamers was detected in the ROESY spectra.

It should also be noted that it is likely that 4 and 5 exist as stable enantiomers due to nonplanarity of the tricyclic ring structure, as has been reported previously for telenzepine (8). These enan-

Radioligand Binding Assays. The pharmacology and kinetics of Cy3B-telenzepine and Alexa488–telenzepine binding to M₁ muscarinic receptors were determined by radioligand binding assays using [³H]-N-methylscopolamine ([³H]-NMS, obtained from Amersham International). For this assay, cell membranes prepared from CHO cells stably expressing M₁ muscarinic receptors as previously described (9). Briefly, membranes were prepared at 0 °C by homogenization with a Polytron followed by centrifugation (40,000 × g, 15 min). Washed once in 20 mM Heps + 0.1 mM EDTA, pH 7.4, and stored at −70 °C in the same buffer at protein concentrations of 2–5 mg/mL. Protein concentrations were measured with the BioRad reagent using BSA as the standard. Frozen membranes were thawed, re suspended in incubation buffer containing 20 mM Heps + 100

Supporting Information

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SI Materials and Methods

Chemistry. Synthesis of telenzepine–fluorophore conjugates. 4,9-Dihydro-3-methyl-4-(4-(10-aminodecyl)-1-piperazinyl)acetyl-10H-thieno [3,4-b][1,5]benzdiazepin-10-one dihydrobromide 1 (telenzepine amino congener) was obtained from the National Institute of Mental Health Chemical Synthesis and Drug Supply program and had been prepared as previously described (1). Cy3B N-hydroxysuccinimide (NHS) ester 2 was from Amersham Biosciences. Alexa488 tetrafluorophenyl (TFP) ester 3 was from Molecular Probes. The chemical structures are shown in Fig. S1. Other reagents were obtained from Sigma-Aldrich. NMR spectra were recorded in methanol-d₄ solutions for the Cy3B conjugates and in DMSO-d₆ solution for the Alexa conjugate and were referenced to the residual undeuterated solvent signal. The NMR methods and assignments are reported in the section NMR Spectra and Assignments (below) because of the spectral complexity. High-resolution mass spectra (HRMS) were recorded on a Waters Micromass Q-ToF Ultima instrument at the School of Pharmacy, University of London. Preparative TLC was carried out on Merck 1-mm Kieselgel 60 plates (20 x 20 cm; no fluorescence indicator) and reverse-phase purification was performed on C₁₈ Sep-pak cartridges (Waters).

The NMR measurements were carried out at the Medical Research Council Biomedical NMR Centre, National Institute of Medical Research (London).

Radioligand Binding Assays. The pharmacology and kinetics of Cy3B–telenzepine and Alexa488–telenzepine binding to M₁ muscarinic receptors were determined by radioligand binding assays using [³H]-N-methylscopolamine ([³H]-NMS, obtained from Amersham International). For this assay, cell membranes prepared from CHO cells stably expressing M₁ muscarinic receptors as previously described (9). Briefly, membranes were prepared at 0 °C by homogenization with a Polytron followed by centrifugation (40,000 × g, 15 min), washed once in 20 mM Heps + 0.1 mM EDTA, pH 7.4, and stored at −70 °C in the same buffer at protein concentrations of 2–5 mg/mL. Protein concentrations were measured with the BioRad reagent using BSA as the standard. Frozen membranes were thawed, re suspended in incubation buffer containing 20 mM Heps + 100
mM NaCl + 10 mM MgCl₂ (pH 7.4), and incubated with radioligand and unlabeled drugs at 20–23 °C or 37 °C in a volume of 1 mL. Membranes were collected by filtration over glass fiber filters (Whatman GF/B) presoaked in 0.1% polyethyleneimine, using a Brandel cell harvester (Semat), extracted overnight in scintillation fluid (ReadySafe, Beckman), and counted for radioactivity in Beckman LS6000 scintillation counters. Some binding studies were carried out in 1 mL 96-well polystyrene plates with binding stopped by filtration onto Wallac 96-Printed GF/B filter mats (PerkinElmer Wallac) using a Harvester 96, MACH III M (TOMTEC). The filter mats were washed twice with 3 mL cold deionized water and dried. A solid melt-on scintillator sheet (Meltlex B/HS, Wallac) was melted onto the filter mat containing the harvested membranes. Radioactivity was counted on a normalized, calibrated 1450 Microbeta Trilux counter (5 min/sample). Each time point was measured in duplicate or quadruplicate. Membrane protein concentrations were adjusted so that not more than about 15% of added radioligand was bound. Nonspecific [³H]-NMS binding was measured in the presence of 10⁻⁶ M QNB or 10⁻⁶ M scopoline (antagonists with picomolar potency) and accounted for 2–5% of total binding.

Initial experiments revealed that the inhibition of [³H]-NMS binding by Cy3B-telenzepine increased considerably when the incubation times were extended from 2 to 6 h, indicating that there was a slow component of Cy3B–telenzepine binding. The affinity and association and dissociation rate constants of Cy3B–telenzepine and Alexa488–telenzepine were determined by measuring the time dependence of up to three fixed concentrations of the fluorescent ligands to affect the binding of a fixed concentration of [³H]-NMS over a period of up to 6–18 h (for Cy3B–telenzepine) and 6–8 h (for Alexa488–telenzepine). In addition, the dissociation rate constant of the fast binding lidand [³H]-NMS and, in a number of experiments, its association rate constant (using one or two concentrations of [³H]-NMS) were measured using a “reverse time” strategy as described previously (10).

An example of the approach is shown in Fig. S4, where the dissociation rate of [³H]-NMS (blue symbols and curve) is well described by a simple exponential. The monoequivalent association rate of two concentrations of [³H]-NMS (0.16 and 0.36 nM) is shown by the light- and dark-gray symbols and curves. The progression curve for the binding of a higher concentration of [³H]-NMS (1.0 nM) in the presence of 3 nM Cy3B–telenzepine is shown by the magenta symbols. The binding rapidly rises to a maximum and then gradually decreases over the next 6 h but has still not reached equilibrium by that time.

The results were analyzed by simultaneous nonlinear least-squares analysis of the association and dissociation curves of the radioligand and the progression curve using Prism5 (GraphPad Software) and the equations derived by Motulsky and Mahan (11), which assume that the two ligands bind competitively (i.e., a 1:1 binding stoichiometry) with slope factors of 1 and monoequivalent association and dissociation kinetics. The curves represent an excellent fit to the data points. In some experiments, the [³H]-NMS kinetics in the absence of fluorescent ligand were not measured, but the well-defined [³H]-NMS dissociation rate constant and affinity constant were set as constants in the fitting procedure.

The kinetic and equilibrium parameters for [³H]-NMS and the fluorescent ligands at ∼22 °C and 37 °C are shown in Table S3. It can be seen that both fluorescent ligands have very slow association and dissociation rate constants (15- to 230-fold slower) relative to the radioligand [³H]-NMS. The slow kinetics may be attributed substantially to the presence of the telenzepine moiety because its association and dissociation kinetics are also 10- to 20-fold slower than NMS (8). We thank Angela Popham and Marlet Martinez Archundia for their skill in performing some of the complex radioligand binding assays.

Cell Labeling and Preparation for TIRFM Imaging. CHO cells stably expressing human muscarinic M₁ receptors were grown and maintained in DMEM/F12 medium containing 10% FBS at 37 °C and 5% CO₂.

Single labeling of M₁ receptors. CHO cells expressing the human M₁ receptors were seeded on 25 mm no. 1 coverslips. In some experiments, 1 mM carbacol was present to reduce the receptor number. Twenty-four hours later, the cells were washed several times with Hanks buffered salt solution (HBSS) and were labeled with 1 nM Cy3B-telenzepine or 3 nM Alexa488–telenzepine and incubated at 37 °C, 5% CO₂ for a further 16 h to give ~90–97% receptor occupancy. Cells were washed at least three times with HBSS before chamber assembly.

Dual labeling of M₁ receptors. The slow dissociation rate of Cy3B–telenzepine from M₁ receptors allowed a sequential labeling strategy. CHO cells expressing M₁ receptors were first labeled to ~50% occupancy with 0.03 nM Cy3B–telenzepine using the method described above. After 16 h incubation at 37 °C, unbound Cy3B–telenzepine was washed away, and the cells were incubated with Alexa488–telenzepine (3 nM) for 2–5 h at 23 °C, which labeled ~90% of the remaining unoccupied receptors.

After labeling, the coverslip with the cells attached was assembled into a circular, stainless steel imaging chamber that held two round coverslips separated by a silicone O-ring (Ø = 2.5 mm, 25-mm outer diameter). The imaging chamber was filled with HBSS containing 20 mM Hepes (pH 7.4) and 10% of FCS (all chemicals from Sigma-Aldrich). The chamber was mounted on an Axiovert 135 microscope (Carl Zeiss) enclosed in a thermostatically controlled incubator (Solen Scientific) set to 37 °C or 23 °C.

Imaging System. For single- and dual-color alternating imaging, we used a custom-built TIRFM system described previously (12). Briefly, the beam from a blue laser (488 nm, 20 mW, Protera 488, Novalux) was expanded using a Galilean beam expander (x10) to give the correct numerical aperture (far-field beam diameter of about 8 mm). Another beam expander (x10) was used to expand the beam from a green laser (556 nm, 100 mW, MGL-556-100, Suwtech). The expanded laser beams were then combined to follow the same light path using a dichroic mirror (FF495-Di02, Semrock). Laser light was focused, using a 160-mm focal length achromatic lens at the back focal plane of a high numerical aperture objective lens (AlphaPlan, 100×, NA1.45, Carl Zeiss). A front-surface silvered mirror (Ø = 3 mm) was used to direct the laser beams into the objective lens by positioning it immediately below and at the extreme edge of the back aperture (12). An identical mirror, placed on the opposite side of the back aperture, was used to direct the laser beams out of the microscope as they were reflected back. The average laser intensities at the specimen plane were ~10 μW·μm⁻² for 488-nm illumination and ~40 μW·μm⁻² for 556-nm illumination. A dual-band emission filter (59004m, Chroma Technology) was used for two-color imaging. One laser was activated during an image frame acquisition period, and the other was activated during the next image acquisition period. Alternating frames were therefore exposed to the two different excitation wavelengths. Because the fluorescence (green or red) emitted by the specimen goes through the same light path, the green and red images collected by the camera are in perfect geometrical register. Images were collected at a rate of 30 frames·s⁻¹ (15 frames·s⁻¹/channel) using an EMCCD camera (iXon-897BV, Andor). Sequences of images (records) were stored directly to a computer hard drive for subsequent analysis. Sequences of “green” and “red” images were stored in separate files for future analysis. The image acquisition and image analysis software was written using C++ (Borland CBuilder6).

In the present study, the intensity of a single fluorophore was estimated by measuring nonspecifically bound ligand molecules attached to the glass slide near the cell being observed. Because these molecules do not move, it is easy to measure their mean
intensity and to show that they exhibit only single-step photo-
bleaching.

The relatively broad distribution of spot intensities is due to a
combination of factors: (i) uneven illumination of the cell being
observed, (ii) variability of the contact distance between the cell
membrane and the glass coverslip and hence exposure of the
membrane to different evanescent field strengths, and (iii) the
image shown in Fig. 1B is just a single (30 ms) frame. So Fig. 1B is
a snapshot of the range of intensities, and there is no smoothing of
shot noise or camera noise. The temporal variation in intensity
shown in Fig. 2 enables the reader to estimate the noise and signal
levels for a given spot. The noise level for an individual spot is
dominated by photon noise, but the variation in average intensity
between spots is due to points (i) and (ii) above.

**Automatic Single-Particle Tracking and Subsequent Data Analysis.** An
automatic single-particle tracking algorithm was used to identify
and track individual muscarinic receptors labeled with Alexa-488–
telenzepine or Cy3B–telenzepine. A detailed description of the
algorithm has been reported previously (13) (also see www.nimr.
mrc.ac.uk/GMimPro). Briefly, single fluorescent molecules were
identified as Gaussian-shaped spots of diffraction-limited size
whose amplitude was similar to the fluorescence of a single
Alexa-488 or Cy3B molecule under identical imaging conditions
(Eq. 8, ref. 13). Objects identified in the sequence of images were
sorted using a nearest-neighbor algorithm to yield individual
particle tracks. Information about time, x–y coordinates, and flu-
orescence intensity was then stored for further statistical analysis.

The algorithm used to identify pairs of moving “green” and “red”
molecules (bound to each other) worked by measuring the dis-
cance from (with ∼20-nm resolution) between the closest “green” and
“red” particle tracks. If the tracks remained within a certain dis-
cance for a certain period, the tracks were identified as being co-
incident (i.e., the objects moved as one). The rationale for the time
and distance thresholding values could be set, a priori, knowing
that the root mean squared deviation (RMSD) in position of one
object relative to another over a given time interval = (2(D_{moff}−δt)^{1/2},
(D_{moff} = lateral diffusion coefficient; δt = time interval between
measurements). For an object with D_{moff} = 0.095 μm^2 s^{-1}, RMSD =
80 nm for a 33-ms time interval (i.e., one video frame). Therefore,
95% of movements should fall within a 160-nm radius. We used
this distance as a limit to determine how much the two images (red
and green) could move relative to one another between video
frames. Validity of the thresholding criteria (separation distance
and duration) could be tested by deliberately offsetting the red and
green images either by inverting the y-coordinates or by shifting
one color set by a distance of 500 nm so the registration of the
images was corrupted. Under these conditions, the density of
moving spots remains the same, so chance overlap between red
and green channels would occur at the same rate. At our chosen
threshold values we obtained zero coincident spots (i.e., we scored
no false events).

To obtain an estimate of the lifetime of the dimeric state, we
selected tracks in which pairs of receptors (red and green) bound
and were then seen to separate again during the observation
period. This allowed us to measure the duration of the dimeric
state and hence to estimate the rate of unbinding from the
exponentially distributed lifetimes (see main text).

**Automated Track Analysis.** To identify stepwise changes in intensity
in our single-color imaging experiments, the intensity variation
during each single molecule trajectory was analyzed either by
visual inspection or by an automated procedure. Intensity tra-
jectories for each molecule were inspected for sudden changes in
level and assigned a unique coding (Fig. S5A). Individual tra-
jectories were encoded as follows: An initial code value was set
at unity and then each rise or fall (→ve edge or →ve edge, re-
spectively) was coded by multiplying by 2 or multiplying by 2 and
adding 1. For example, a single fall gave a coding of 1 × 2 = 2, a
single rise would yield a value of 1 × 2 + 1 = 3, and a fall fol-
lowed by a rise would give a value of 1 × 2 + 1 × 2 + 1 = 5, etc.
Changes in fluorescence intensity can arise for several reasons,
including photobleaching, object loss during tracking, and co-
incidence of two intensity tracks either by chance overlap of the
images or by transient dimerization or dissociation (see cartoon
in Fig. S5B).

The automated procedure for coding the intensity changes of
each molecular trajectory consisted of several stages: First, we
computed the square of the first derivative of intensity versus time.
Peaks in this data series were then thresholded to yield transition
times identifying the start and the end of each level or “phase” of
the intensity plot versus time (Fig. S5C). The average fluorescence
intensity level during each phase was then computed (from
the mean of the intervening data) so a histogram of intensity levels
could be plotted (Fig. S5D). The distribution of intensity types (1,
2) →(1 →2), (2 →1), and (1 →2 →1)—was encoded as described
above and then plotted as a histogram (see Fig. S5E). Note that
nearly all intensity trajectories fitted to the rather simple behavior
of alternating between levels 1 and 2 (e.g., trajectory types 4 and 7
are absent in the data). There was an obvious lack of data showing
trajectories that increased or decreased more than once in suc-
cession (a behavior that would require three discrete levels).

The fraction of tracks showing the presence of dimers, was
measured in six sequential sets of 190 tracks from a single
recording. The ratios of dimers to monomers were 0.14, 0.06, 0.12,
0.05, 0.06, and 0.08 (in sequential order). Therefore, there is no
evidence at the shortest times of recording that there is a high
ratio of dimers to monomers or that the fraction of dimers
progressively decreases from 100% during the recording, as would
be predicted if M<sub>1</sub> receptors were constitutive dimers. The data
are compatible with reversible formation of dimers.

**Table S4** presents a summary of the analysis of recordings at 23°C
from nine CHO cells with the M<sub>1</sub> receptors labeled in a 1:1 ratio
to high occupancy with Alexa488–telenzepine (green) and Cy3B–
telenzepine (red). Table S4 shows that all of the cells exhibit a
consistent 1:1 red:green labeling stoichiometry at the beginning of
the recording. The two- to threefold intercell variation in the tracks
recorded for a given cell is linearly related to the area of the cell
being recorded, indicating a consistent surface expression of the
receptors. More red tracks were recorded because of the slightly
faster rate of photobleaching of the green objects under the illu-
mination conditions used in these experiments. Approximately 20% of
the tracks scored in the Table result from different colored ob-
jects that coincide for only part of their trajectory. This leads to an
overestimation of the percentage of two-color dimers at any given
instant because, for a considerable fraction of time (~50%), the
objects are not coincident but instead are moving independently
as monomers. When a correction is made for this time of nonoverlap,
the estimate of two-color dimers (RG and GR) becomes about
10%. The number of one-colored receptor dimers, e.g., green +
green (GG) and red + red (RR), should also be 10% on a statistical
basis, and the observed value of two-step bleaching from GG and
RR spots (from Table S4) is 5.4 + 4.7% = 10.1%, which agrees with
this prediction. Therefore, the dual-color approach gives a total of
∼20% receptor dimers (or the equivalent statement that ∼30% of
the receptor molecules are present as dimers at any one time).

Our estimate of the dimer:monomer ratio from the single-color
experiments (~10–15%), deduced from the proportion of two-step
photobleaching events, requires that both fluorophores photo-
bleach during the observation period or that intensity levels are
correctly assigned to level 2 and level 1. We believe that the single
color data set is less reliable because most of the errors associated
with the technique tend to lead to underestimation of the per-
centage of dimers. Both the single-color and the dual-color ex-
periments give comparable levels of the percentage of dimers, with
the higher value obtained with the dual-color method being more

Hern et al. www.pnas.org/cgi/content/short/0907915107
reliable because it allows unequivocal receptor:receptor association to be detected.

**Evidence for a 1:1 Stoichiometry of Ligand: M2 Receptor Monomer.**

The possibility that the fluorescent antagonists label only one binding site in a constitutive dimer in living cells has to be considered; i.e., these ligands exhibit “half of the sites binding.” If that were true, then our TIRFM observations would indicate that dimers were reversibly forming tetramers.

**Stoichiometry in pure receptor preparations and in crystal structures.** In pure receptor preparations, there is a 1:1 binding stoichiometry between orthosteric ligands and a molecule of muscarinic receptor (14, 15) even when the radioligand is used to label the receptor in membranes (16) and other class A G-protein-coupled receptors (GPCRs), for example, β-adrenergic receptors (17, 18). The same stoichiometry is found in the reported crystal structures of other GPCRs containing bound ligands (19–23) and in rhodopsin (24–26), reconstituted β-adrenergic (27), and leukotriene B4 receptors (28).

Only for the GABAB receptor, a class C GPCR constitutive heterodimer with one binding subunit not capable of binding orthosteric ligands (and possibly taste receptor heterodimers), does one molecule of agonist bind per dimer. In the case of metabotropic glutamate receptors (mGluRs, class C constitutive homodimers), the studies of Kniazeff et al. (29) indicate a 2:1 stoichiometry of orthosteric ligand per dimer (i.e., one ligand per receptor), and the same stoichiometry is also present in the x-ray structures of the binding domains of several liganded mGluRs (30–32).

**Stoichiometry in membrane preparations.** The evidence for a 1:1 stoichiometry of fluorescent ligand:radioligand is that the fluorescent antagonist inhibition curves, and their time dependence, are described by a model in which both the radioligand 3H-NMS and the fluorescent ligand bind with slope factors of 1 and with a 1:1 binding stoichiometry and monoenzyme kinetics (see Figure S5). The data are not compatible with a model for a binding stoichiometry of 1H-NMS to the fluorescent ligand of 2:1, which would give slope factors very different from 1. So there is a 1:1 stoichiometry between orthosteric antagonists and the muscarinic receptor monomer in pure preparations and a 1:1 stoichiometry between the orthosteric muscarinic antagonist radioligand and the fluorescent ligands in a membrane environment.

**Evidence for muscarinic receptor tetramers and higher oligomers.** Primarily on the basis of the different number of binding sites labeled by different radioligands and their antagonist inhibition curves, Wells and coworkers have suggested that, in some solubilized and purified membranes and reconstituted preparations, the antagonist binding properties of muscarinic receptors are compatible with the presence of tetramers or higher oligomers (33–35). The binding sites are modeled to interact in a negatively cooperative manner, but dissociation studies (36) appear not to quantitatively support the predictions of the model. “Half of the sites binding” was not assumed in the studies: if this were to occur, then the receptors would be present as octamers or higher oligomers.

**Implications of “half of the sites binding” in whole cells.** The homotropic negative cooperativity associated with “half of the sites binding” for antagonists within a dimer has to be very large and could possibly be reflected in the millimolar concentrations of antagonists required to begin to slow down the dissociation of the high-affinity radioligand binding from its site (one test for the presence of negative cooperativity). Even this slowing has been interpreted as a nonspecific effect (reviewed in ref. 37) or the effect of antagonists binding to an allosteric site (36). Furthermore, there has to be a strong bidirectional conformational change that is transmitted across the receptor dimer interface to generate the negative cooperativity. To rationalize “half of the sites binding” with the reported binding stoichiometry in isolated preparations, disruption of the dimer in living cells would have to occur at some point during the sequence: cells → membrane preparation → solubilization → purification. It would also be expected on thermodynamic grounds that such a disruption would give rise to a substantial change in antagonist affinity constants and/or an increase (doubling) in the number of binding sites. Neither of these effects has been reported. Muscarinic antagonist affinities in whole cells, membranes, and soluble and pure receptors are in general very similar to those observed in whole-tissue functional studies.

**Could antagonists bind at a muscarinic dimer interface?** Equally, there is no evidence of one antagonist molecule binding at a muscarinic receptor dimer interface. The residues important for antagonist (and agonist) binding face inward toward the core of the seven-helix transmembrane bundle (35–40) and not toward a putative dimer interface. On solubilization of M1 receptors there is no reported strong perturbation of the antagonist binding properties of the receptor, as would be expected to occur if ligand binding occurred at a dimer interface that was disrupted by solubilization.

**Conclusion.** In summary, we cannot exclude the possibility that muscarinic receptors exhibit “half of the sites binding” in these cells or in other preparations. Our interpretation of a binding stoichiometry of 1:1 receptor monomer: fluorescent antagonist (and other orthosteric antagonists) is the simplest way to explain the data on the M1 receptor reported in this paper.

Fig. S2. Simplified numbering system for the hydrogen-bearing carbon atoms of the substructural components of the telenzepine–Alexa488 conjugate 4. This numbering is used to report the $^1$H NMR spectral assignment of the conjugate.
Fig. S3. Simplified numbering system for hydrogen-bearing carbon atoms of the substructural components of the telenzepine–Cy3B conjugate 5. This numbering is used to report the $^1$H NMR spectral assignment of the conjugate.
Fig. S4. Experiment to determine the rate constants for the binding of $[^3H]$-NMS and Cy3B–telenzepine at 23 °C.
Fig. S5. (A) Coding system used to identify the series of intensity changes of an individual molecular trajectory (see text for details). The code numbers are shown alongside the idealized intensity trajectories (y axis = intensity, x axis = time). Red highlighted trajectories are readily explained by the presence of either one or two fluorophores within the detection region. The thickness of the red lines indicates the frequency of occurrence in our experimental observations: thick red lines were frequently observed, and black lines were extremely rare or absent in our data sets. (B) Complicated intensity trajectories (e.g., corresponding to code values 2, 6, 10, 26) (shown in red on the right) arise when molecules pass close to one another or undergo dimerization. The cartoons illustrate different ways in which paths of individual molecules might transiently overlap and how the intensity signals (right) might vary. Note that when two tracks merge or when a single track splits apart, this gives rise to “orphan” tracks, shown in gray). (C) Intensity versus time plot for 20 molecules (each molecule is shown as an alternate color along the x axis); the timescale is given in frames (here 30 ms/frame). Intensity-level changes were identified from changes in the first derivative of the data (shown as a solid line below). The intensity level between transitions (called “phases”) was averaged and plotted as a solid black line over the original data. The amplitudes were then plotted as a histogram. (D) Histogram of intensity levels derived from the analysis of several thousand individual molecular trajectories (a small subset of the data used to construct the histogram is shown in C). (E) The distribution of intensity track types indicates that most molecules remained at a single level throughout the record (code 1) or increased or decreased once (1 → 2 or 2 → 1, i.e., codes 2 or 3) or showed other “simple” behaviors described by the red highlighted tracks in the coding diagram (A).
Table S1. ¹H chemical shifts of Alexa488–telenzepine

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*Only chemical shifts of resonances of rotamers a and b that differ from those of the major rotamer are shown. See NMR Spectra and Assignments for explanation of the different rotamers.
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<tr>
<td>1 CH₂</td>
<td>3.239</td>
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<td>2 CH₂</td>
<td>1.557</td>
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</tr>
<tr>
<td>3 CH₂</td>
<td>1.367</td>
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<tr>
<td>4–7 (CH₂)₆</td>
<td>1.352</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 CH₂</td>
<td>1.359</td>
<td>1.378</td>
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<tr>
<td>9 CH₂</td>
<td>1.597</td>
<td>1.632</td>
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</tr>
<tr>
<td>10 CH₂</td>
<td>2.616</td>
<td>2.716</td>
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<td>Cy3B</td>
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<tr>
<td>1 CH₂</td>
<td>3.636</td>
<td></td>
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<tr>
<td>2</td>
<td>7.443</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>7.342</td>
<td></td>
<td></td>
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<tr>
<td>4 CH₂</td>
<td>3.976, 4.400</td>
<td></td>
<td></td>
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<tr>
<td>5 CH₂</td>
<td>2.085, 2.649</td>
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<tr>
<td>6</td>
<td>4.736</td>
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<td>8 CH₂</td>
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<td>9 CH₂</td>
<td>4.023, 4.426</td>
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<tr>
<td>10</td>
<td>7.345</td>
<td></td>
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<tr>
<td>11</td>
<td>7.961</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>7.983</td>
<td></td>
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</tr>
</tbody>
</table>

*Only chemical shifts of resonances of rotamers a and b that differ from those of the major rotamer are shown. See NMR Spectra and Assignments for explanation of the different rotamers.
<table>
<thead>
<tr>
<th>Table S3. Equilibrium and kinetic binding parameters for NMS, Cy3B–telenzepine, and Alexa488–telenzepine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binding kinetics of NMS</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>20–23 °C</td>
</tr>
<tr>
<td>37 °C</td>
</tr>
<tr>
<td>log $K$</td>
</tr>
<tr>
<td>$9.99 \pm 0.02$ (3)</td>
</tr>
<tr>
<td>$9.83 \pm 0.07$ (7)</td>
</tr>
<tr>
<td>$k_\text{off}$ min$^{-1}$</td>
</tr>
<tr>
<td>$0.054 \pm 0.004$ (3)</td>
</tr>
<tr>
<td>$0.13 \pm 0.01$ (7)</td>
</tr>
<tr>
<td>$t_\text{1/2}$ dissociation (min)</td>
</tr>
<tr>
<td>13</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>$k_\text{on}$ ($\times10^9$ M$^{-1}$min$^{-1}$)</td>
</tr>
<tr>
<td>$0.52 \pm 0.06$ (3)</td>
</tr>
<tr>
<td>$1.00 \pm 0.1$ (7)</td>
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<tr>
<td>Binding kinetics of Cy3B–telenzepine</td>
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<tr>
<td></td>
</tr>
<tr>
<td>20–23 °C</td>
</tr>
<tr>
<td>37 °C</td>
</tr>
<tr>
<td>log $K$</td>
</tr>
<tr>
<td>$10.73 \pm 0.20$ (3)</td>
</tr>
<tr>
<td>$10.46 \pm 0.06$ (7)</td>
</tr>
<tr>
<td>$k_\text{off}$ min$^{-1}$</td>
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<tr>
<td>$0.00023 \pm 0.00013$ (3)</td>
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<tr>
<td>$0.0023 \pm 0.004$ (7)</td>
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<tr>
<td>$t_\text{1/2}$ dissociation (h)</td>
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<tr>
<td>23–100</td>
</tr>
<tr>
<td>7 ± 2</td>
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<tr>
<td>$k_\text{on}$ ($\times10^9$ M$^{-1}$min$^{-1}$)</td>
</tr>
<tr>
<td>$(0.014 \pm 0.001)$ (3)</td>
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<tr>
<td>$0.072 \pm 0.022$ (7)</td>
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<td>Binding kinetics of Alexa488–telenzepine</td>
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<td>20–23 °C</td>
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<tr>
<td>log $K$</td>
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<tr>
<td>$9.32 \pm 0.07$ (5)</td>
</tr>
<tr>
<td>$k_\text{off}$ min$^{-1}$</td>
</tr>
<tr>
<td>$0.0030 \pm 0.0005$ (5)</td>
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<tr>
<td>$t_\text{1/2}$ dissociation (h)</td>
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<tr>
<td>4</td>
</tr>
<tr>
<td>$k_\text{on}$ ($\times10^9$ M$^{-1}$min$^{-1}$)</td>
</tr>
<tr>
<td>$0.0065 \pm 0.0015$ (5)</td>
</tr>
</tbody>
</table>
Table S4. Summary of the analysis of recordings at 23 °C from nine CHO cells with the M<sub>1</sub> receptors labeled in a 1:1 ratio to high occupancy with Alexa488–telenzepine (green) and Cy3B–telenzepine (red)

<table>
<thead>
<tr>
<th>Data set</th>
<th>Area (μm&lt;sup&gt;2&lt;/sup&gt;)</th>
<th>Density (object/μm&lt;sup&gt;2&lt;/sup&gt;)</th>
<th>No. of objects</th>
<th>Average $D_{lat}$ μm&lt;sup&gt;2&lt;/sup&gt;·s&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>Tracks showing two-step bleaching</th>
<th>Two-color coincident tracks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>N</td>
<td>%</td>
</tr>
<tr>
<td>1</td>
<td>320</td>
<td>1.09</td>
<td>1.14</td>
<td>554</td>
<td>680</td>
<td>0.814</td>
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<tr>
<td>2</td>
<td>660</td>
<td>0.93</td>
<td>1.13</td>
<td>1333</td>
<td>2256</td>
<td>0.59</td>
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<tr>
<td>3</td>
<td>550</td>
<td>1.04</td>
<td>1.15</td>
<td>1022</td>
<td>1171</td>
<td>0.873</td>
</tr>
<tr>
<td>4</td>
<td>780</td>
<td>0.96</td>
<td>1.04</td>
<td>1415</td>
<td>2003</td>
<td>0.706</td>
</tr>
<tr>
<td>5</td>
<td>370</td>
<td>1.21</td>
<td>1.23</td>
<td>639</td>
<td>798</td>
<td>0.801</td>
</tr>
<tr>
<td>6</td>
<td>690</td>
<td>1.07</td>
<td>0.95</td>
<td>1395</td>
<td>1691</td>
<td>0.825</td>
</tr>
<tr>
<td>7</td>
<td>650</td>
<td>1.12</td>
<td>0.97</td>
<td>1234</td>
<td>1522</td>
<td>0.811</td>
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<tr>
<td>8</td>
<td>820</td>
<td>0.87</td>
<td>1.19</td>
<td>1052</td>
<td>1394</td>
<td>0.755</td>
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<tr>
<td>9</td>
<td>450</td>
<td>1.07</td>
<td>1.13</td>
<td>925</td>
<td>970</td>
<td>0.851</td>
</tr>
<tr>
<td>Mean</td>
<td>588</td>
<td>1.02</td>
<td>1.09</td>
<td>1.01</td>
<td>0.78</td>
<td>0.098</td>
</tr>
</tbody>
</table>

“Area”: size of the basal cell membrane imaged. “Density”: number of objects identified at the beginning of the record divided by the cell area. “Ratio” (under “Density”): ratio of densities of green and red molecules. “No. of objects”: number of objects tracked during the whole record (slow-moving objects $D_{lat}$ < 0.05 μm<sup>2</sup>·s<sup>-1</sup> excluded). “Ratio” under “No. of objects”: ratio of green and red objects. “Average $D_{lat}$”: average lateral diffusion coefficient (slow-moving objects $D_{lat}$ < 0.05 μm<sup>2</sup>·s<sup>-1</sup> excluded). “Tracks showing two-step bleaching”: number of objects that exhibit two-step photobleaching (e.g., Fig. 2C, main text). Identification is based on a single fluorophore detection algorithm (Eq. 6, ref. 13). Threshold value S ≥ 1.5. “Two-color coincident tracks”: N = number of colocalized green + red objects (separation < 160 nm) moving for > 660 ms. These tracks were dimers for ~50% of the total track time. $D_{lat}$: Average lateral diffusion coefficient of green + red colocalized objects (i.e., dimers). Binding-splitting: number of green + red objects that initially move separately (distance > 200 nm) for > 200 ms before forming a two-color dimer (see above) and then separate again for > 200 ms. These data were used to calculate the dissociation rate of the M<sub>1</sub> receptor (see Fig. 4G, histogram, main text). Binding: number of green + red objects moving separately (distance > 200 nm) for > 200 ms before forming a dimer. Splitting: number of green + red objects moving together for > 660 ms, which then moved separately (distance > 200 nm) for > 200 ms.
Movie S1. First 3 s of a 48 s TIRF recording of the diffusion of individual M₁ receptors labeled with Cy3B–telenzepine on the plasma membrane of a single CHO cell. An image of this cell is shown in Fig. 1 (main text).

Movie S1

Movie S2. Tracking of the trajectories of individual Cy3B-Tz–labeled M₁ receptors on the plasma membrane of the cell shown in Fig. 1 (main text) and in Movie S1.

Movie S2

Movie S3. First 26 s of a two-color TIRF recording of individual labeled M₁ receptors on the plasma membrane of the cell illustrated in Fig. 4 A and B (main text).

Movie S3
Movie S4. Recording of 26 s of tracking of the trajectories of two-color M₁ receptor dimers on the plasma membrane of a small region of the cell shown in Movie S3 and in Fig. 4 A and B (main text).