Single Molecule Imaging Deciphers the Relation between Mobility and Signaling of a Prototypical G Protein-coupled Receptor in Living Cells*  
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Background: The mobility of G protein-coupled receptors in the plasma membrane is of central importance to regulate transmembrane signaling.

Results: In live cells, individual receptors show a broad mobility distribution with typical patterns for different phases of cellular signaling.

Conclusion: Heterogeneity of receptor mobility is critical in regulation of receptor activity.

Significance: These findings add further insights to the plasticity of receptor signaling.

Lateral diffusion enables efficient interactions between membrane proteins, leading to signal transmission across the plasma membrane. An open question is how the spatiotemporal distribution of cell surface receptors influences the transmembrane signaling network. Here we addressed this issue by studying the mobility of a prototypical G protein-coupled receptor, the neurokinin-1 receptor, during its different phases of cellular signaling. Attaching a single quantum dot to individual neurokinin-1 receptors enabled us to follow with high spatial and temporal resolution over long time regimes the fate of individual receptors at the plasma membrane. Single receptor trajectories revealed a very heterogeneous mobility distribution pattern with diffusion constants ranging from 0.0005 to 0.1 μm2/s comprising receptors freely diffusing and others confined in 100–600-nm-sized membrane domains as well as immobile receptors. A two-dimensional representation of mobility and confinement resolved two major, broadly distributed receptor populations, one showing high mobility and low lateral restriction and the other showing low mobility and high restriction. We found that about 40% of the receptors in the basal state are already confined in membrane domains and are associated with clathrin. After stimulation with an agonist, an additional 30% of receptors became further confined. Using inhibitors of clathrin-mediated endocytosis, we found that the fraction of confined receptors at the basal state depends on the quantity of membrane-associated clathrin and is correlated to a significant decrease of the canonical pathway activity of the receptors. This shows that the high plasticity of receptor mobility is of central importance for receptor homeostasis and fine regulation of receptor activity.

Membrane receptors are of utmost importance for cellular signaling, transferring the information of extracellular stimuli into intracellular responses. In this context, their lateral distribution and mobility in the plasma membrane play a critical role as random or directed movements in the membrane plane bring signaling partners efficiently into transient or stable contact (1–4). A fundamental issue of modern quantitative cell biology is to understand how the complex, highly dynamic spatial distribution of components of the plasma membrane influences central cellular signaling processes (5–7). Single molecule optical imaging, more specifically single particle tracking (SPT), is ideally suited to establish a tomogram of the distribution of individual plasma membrane components over time and space, revealing the full complexity of individual signaling reactions that would be hidden in ensemble measurements (8).

Here we concentrate on seven-transmembrane domain receptors, also known as G protein-coupled receptors (GPCRs). GPCRs establish the largest family of cell surface receptors converting extracellular signals into intracellular responses. As they are involved in many central physiological processes, they are also among the most important targets for drug development (9–12). After activation by extracellular stimuli, GPCRs are typically desensitized, internalized, and recycled. These processes occur from seconds (phosphorylation) over minutes (endocytosis) to hours (down-regulation) (13). In this context, the clathrin-mediated endocytosis (CME) machinery is essential for maintaining proper function of GPCRs on the cell surface (14). All this yields an amazingly diverse network of intra-

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4 The abbreviations used are: SPT, single particle tracking; GPCR, G protein-coupled receptor; CME, clathrin-mediated endocytosis; NK1R, neurokinin-1 receptor; SP, substance P; CCP, clathrin-coated pit; Qdot, quantum dot; ACP, acyl carrier protein; NCS, newborn calf serum; mβCD, methyl-β-cyclodextrin; ROCK, Rho-associated coiled coil kinase.
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FIGURE 1. Internalization and recycling of NK1R at the cell plasma membrane. The NK1R can be located in different regions of the plasma membrane and in intracellular endosomes: I, nanometer-sized membrane domains; 2, clathrin-related region; 3, clathrin pre-pits; 4, clathrin-coated pits closed by dynamin; 5, clathrin-coated vesicles; 6, early, late, or perinuclear endosomes; 7, lysosomes; 8, recycling vesicles. NK1R can be recruited in the different regions described above. I, receptor exchanges between clathrin-related region and free membrane. II, after agonist binding, receptor is phosphorylated, leading to the recruitment of β-arrestin. The receptor is targeted to clathrin pre-pits. III, receptor-clathrin complex is bound to dynamin-dependent invaginating regions. IV, receptor is internalized in clathrin-coated vesicles, which are then transformed to early endosomes. V, SP is removed from NK1R in intracellular endosomes and recycled to the plasma membrane of the cell. VI, NK1R is phosphorylated in clathrin-coated vesicles and in intracellular endosomes: VII, SP is removed from NK1R in late endosomes where either in VI SP and a fraction of the receptors are degraded in lysosomes or in VII a fraction of the receptors is recycled to the cell membrane.

Experimental Procedures

Cell Culture—Adherent 293T cells, stably expressing the NK1R genetically fused with an acyl carrier protein at the extracellular N terminus (ACP-NK1R) (33), were grown at 37 °C in DMEM/F-12 (Life Technologies) supplemented with 10% (v/v) newborn calf serum (NCS) (Sigma-Aldrich) and 200 µg/ml hygromycin B (Sigma-Aldrich) in a humidified atmosphere with 5% CO2. Cells were seeded 24–48 h before microscopy experiments in 8-well plates (Lab-Tek Nunc, Naperville, IL) in growth medium without antibiotics. Single receptors were imaged in colorless DMEM supplemented with 15 mM HEPES (Life Technologies) without antibiotics or serum.

Addition of Chemicals to Cells—NK1Rs were activated by adding freshly prepared SP to the extracellular medium (Tocris, Bristol, UK). Nocodazole (AppliChem, Darmstadt, Germany) was used at 1 µM, and cytochalasin B (Sigma-Aldrich) was used at 20 µM. Dynag-4a and PitStop 2 (Abcam, Cambridge, UK) were applied at 30 µM, and Dynasore (Sigma-Aldrich) was applied at 80 µM. Methyl-β-cyclodextrin (mβCD; Sigma-Aldrich) was used at 10 mM, and Y-27632 (Tocris) was used at 10 µM. All chemicals except SP were added to the extracellular medium 20–30 min before SPT measurements.

siRNA Knockdown of Clathrin—Clathrin depletion was achieved using ON-TARGETplus SMARTpool siRNAs (Dharmacon/GE Healthcare, Amersham Biosciences) against the human clathrin heavy chain 1 with the following target sequences: (i) 5'-GAG AAU GGC UGU ACG UAA U-3’, (ii) 5'-UGA GAA AUG UAA UGC GAA U-3’, (iii) 5'-GCA GAA UGA UCA ACG UUA U-3’, and (iv) 5'-CGU AAG AAG GCC CGA GAG U-3’. Transfection of siRNAs (15 pmol/well) was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. SPT measurements were performed 60 h after transfection.

Receptor Function Assay—The NK1R-mediated activation of the Gαq pathway was assayed by measuring the intracellular Ca2+ response after SP addition. 293T cells stably expressing ACP-NK1R were seeded into a 96-well plate (PerkinElmer Life Sciences) and grown in DMEM/F-12 supplemented with 10% NCS for 24 h. Cells were loaded with Fluo4-NW (Life Technol-
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Receptor Labeling—293T cells stably expressing ACP-NK1R were washed once with DMEM/F-12 containing 10% NCS and supplemented with 1% (w/v) bovine serum albumin (BSA). Labeling was performed by incubating the cells for 15 min at 37 °C in DMEM/F-12 containing 10% NCS and supplemented with 1% BSA, 10 mM MgCl₂, 1 μM 4′-phosphopentanehexyltransferase, and 5 μM CoA-biotin. After incubation and prior to the second step of labeling, cells were washed three times with DMEM/F-12 supplemented with 10% NCS and 1% BSA. Cells were then incubated for 5 min at room temperature with 10 μM streptavidin-coated CdSe-ZnS core-shell Qdots 655 (Invitrogen, Life Technologies) in Dulbecco’s PBS containing Ca²⁺ and Mg²⁺ and supplemented with 1% BSA. Prior to SPT measurements, cells were rinsed three times in colorless DMEM supplemented with 1% BSA to remove unbound Qdots. To account for cell variability and avoid NK1R-unrelated effects, experiments on the inactive NK1R state without addition of chemicals were performed systematically before each new experiment.

Microscopy—For single molecule microscopy, cells were mounted on a modified epifluorescence wide field microscope (Axiovert 200, Zeiss, Feldbach, Switzerland). To observe Qdots 655, light of a 488 nm Obis diode laser (Coherent Inc., Santa Clara, CA) was directed by a dichroic mirror (Q645LP, Chroma) into the microscope objective (C-Apochromat 63×/1.2 numerical aperture water corrected, Zeiss) to illuminate a 15-μm-diameter region of the sample. Fluorescence was collected by the same objective, passed through a filter (HQ710/100, Chroma) and a 1.6× Zeiss Optovar, and imaged on an electron-multiplying charge-coupled device camera (Ixon 887BV, Andor Technology, Belfast, UK). Single molecule trajectories were recorded at a frequency of 30 Hz with excitation intensities around 0.1 kilowatt/cm² for 33 s.

For confocal scanning microscopy, cells were mounted on a Zeiss LSM 510 microscope. Qdots were excited with the 488-nm line of an argon ion laser. Fluorescence signal was acquired with a 63×, 1.2 numerical aperture water immersion objective (Zeiss), filtered through an HFT 488/561 dichroic mirror and a 650-nm long pass filter (both Zeiss), and detected with an avalanche photodiode (PerkinElmer Life Sciences).

Single Molecule Tracking—Image sequences were treated using a home-written software in Igor Pro (Wavemetrics, Lake Oswego, OR). Each image of a single molecule time series was restored using a noise filter to remove (i) long range structures from inhomogeneous laser profile, detector fluctuation, and autofluorescence regions and (ii) short range discretization noise and charge-coupled device spikes (34). Local maxima were determined on the filtered image. Each local maximum was then fitted using a two-dimensional Gaussian fit. Size, shape, and fluorescence intensity time profile of a fitted fluorescent peak were verified to match single molecule features. Selected fluorescence peaks were then linked over an image series to obtain trajectories. To avoid bias in subsequent steps of analysis, only cells with more than 90% of approved visible labels were considered.

Analysis of Individual Trajectories—Diffusion coefficient, length of confinement region, moment scaling spectrum, and velocity were calculated from individual trajectories as described elsewhere (35). To extract a maximal amount of information, long trajectories were segmented using a sliding window of 100 frames. Each of the segmented trajectories was treated as an individual and analyzed to obtain five mobility-related features: 1) initial diffusion coefficient, $D_{1-10}$ (30, 36), computed by fitting the first 10 time intervals of a standard mean square displacement versus time lag ($t_{lag}$) curve and corresponding to a maximal time interval of 200 ms during which the diffusion is less affected by long range features; 2) Hurst parameter $S_{MSS}$ (37); 3) length ($L$), which is the diagonal of the smallest rectangle containing 95% of the point position; 4) asymmetry (38); and 5) efficiency (39). These features were then used to construct a vector, which described with high specificity the mobility state of the particular trajectory segment.

Population Analysis—$D_{1-10}$ and $S_{MSS}$ values characterize the mobility properties of individual receptors and, by extension, those of a receptor population. These two values were obtained with two independent calculations from the same data set. Median values of $D_{1-10}$ and $S_{MSS}$ were calculated for each experimental condition. Box plots were then computed to obtain population extent and 95% confidence interval (notches) using R (R Foundation for Statistical Computing). $D_{1-10}$ and $S_{MSS}$ Values were used to compare different experiments.

Two-dimensional probability functions of $D_{1-10}$ and $S_{MSS}$ were determined using the values obtained with the segment feature analysis. Two-dimensional mobility patterns were obtained using the kde2d function of R on $D_{1-10}$ and $S_{MSS}$. This two-dimensional kernel density estimator was then used to establish a mobility pattern for each condition. Bandwidths of 0.11 on the x axis, corresponding to $\log_{10}(D_{1-10})$, and 0.03 on the y axis, corresponding to $S_{MSS}$, were used unless otherwise stated.

Classification of Individual Trajectories—Trajectories were classified according to two methodologies: (i) ensemble analysis, calculating the proportion of trajectories exhibiting defined characteristics from the complete two-dimensional probability function described above, and (ii) a priori, or single trace, classification assigning a single trace to a category depending on the values of the mobility parameters described above.

For a priori classification, a linear support vector machine was used. Three homogenous sets of real trajectories (shown in Fig. 2, C, E, and G) served as a training set. The support vector machine was based on a four-dimensional vector comprising $D_{1-10}$, $S_{MSS}$, asymmetry, and efficiency. The training set was composed of 6969 type I, 5500 type II, and 7524 type III segments of trajectories.

The same classification machine was used to construct a time-dependent distribution. A sliding time window of 160 s was used to extract the evolution of the mobility parameters in...
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FIGURE 2. Heterogeneous diffusion of NK1R in the plasma membrane of 293T cells. Diffusion trajectories of individual NK1Rs labeled with Qdots 655 were measured by SPT in the plasma membranes of the cells. The mobility patterns of the receptor are presented as two-dimensional probability density functions calculated from measured trajectories, a selection of which are shown in C, E, and G. $S_{MSS}$ values of the trajectory segments are plotted versus $D_{1-10}$. $D_{1-10}$ and $S_{MSS}$ describe the diffusion coefficient and the type of motion of the receptor, respectively. A, single receptor trajectories from a single cell obtained at 30 Hz with an acquisition time of 29 ms. B, mobility pattern of 1342 individual receptors measured in the basal state. Boxes indicate the regions of the different mobility types depicted in D, F, and H. C, typical traces of type I diffusion with average $D_{1-10} > 0.008 \mu m^2/s$ and $S_{MSS} > 0.25$. The 12 trajectories depicted were selected from a collection of 633 individual receptors satisfying these conditions. D, mobility pattern of type I receptors of which selected trajectories are shown in C, E, typical traces of type II diffusion with average $D_{1-10} < 0.002 \mu m^2/s$ and $S_{MSS} < 0.1$. The 12 trajectories depicted were selected from a collection of 483 individual receptors satisfying these conditions. F, mobility pattern of type II receptors of which selected trajectories are shown in E, G, typical traces of type III diffusion with average $D_{1-10} < 0.02 \mu m^2/s$ and $S_{MSS} < 0.15$. The 12 trajectories depicted were selected from a collection of 131 individual receptors satisfying these conditions. H, mobility pattern of type III receptors of which selected trajectories are shown in G, I, closer view of a typical type III receptor trajectory. The color code scales with the indicated frequency (arbitrary units) of states.

time. The classification was performed for each time frame using the same support vector machine as above.

Dimension of Confined Regions—The dimension of a confined region ($L$) in the case of restricted receptor diffusion is not a straightforward value to obtain. It is generally estimated by fitting a mean square displacement versus $t_{lag}$ curve of either a single trajectory or an ensemble of trajectories using a complete (40) or a simplified (41) function of $t_{lag}$. Here we chose a simplified definition of the confinement length as the diagonal of the smallest rectangle enclosing 95% of the trajectory. This definition was retained to avoid artifacts due to noise and interdependence of $L$ and $D$.

Results

Heterogeneous Mobility of the NK1R in the Plasma Membrane of 293T Cells—To track a particular receptor equipped with one Qdot, we used a 293T cell line stably expressing ACP-NK1R (30, 32). The ACP-NK1R was labeled by the following sandwich method. We first covalently linked CoA-biotin to the ACP tag of the receptor and then bound to the biotin a streptavidin-coated Qdot (42). Labeling with Qdots did not prevent receptor endocytosis. The highly specific labeling of a few NK1Rs at the plasma membrane of a living cell allowed us to follow trajectories of individual NK1Rs over time periods between 30 s and 2 min at 33-ms temporal resolution (see supplemental Movie S1).

Trajectories of single receptors diffusing on the dorsal cell membrane were obtained from time lapse series of 1000 fluorescence images recorded at 30 frames per second using a wide field epifluorescence microscope. NK1R showed five distinguishable mobility regimes in the plasma membrane: (i) free Brownian diffusion, (ii) slow diffusion in confined regions, (iii) fast diffusion in confined domains, (iv) directed motion, and (v) immobile receptors (Fig. 2A).

Here, we determined the short term diffusion coefficient, $D_{1-10}$ and the Hurst parameter, $S_{MSS}$ (37). $D_{1-10}$ is related to the physical properties of the surrounding membrane and the interaction of the receptor with its close environment (31, 32, 40). $S_{MSS}$ describes the overall mode of motion; values below 0.5 are typical for confined diffusion, values equal or close to zero represent immobile molecules, Brownian movements are characterized by $S_{MSS}$ values around 0.5, and directed movements show $S_{MSS}$ values above 0.5 (37).

To augment the information content extracted from each single molecule trace, a window of 100 frames was moved along the trajectory, and each resulting segment was analyzed as an independent trajectory. The two central parameters, $D_{1-10}$ and $S_{MSS}$, were calculated from each segment and displayed as a two-dimensional probability density function named “mobility pattern.” This novel representation method allowed for resolving highly heterogeneous receptor populations as well as following their evolution over time.

Receptors in the basal state (Fig. 2, A and B) displayed distinct diffusion modes. Freely diffusing receptors had characteristic diffusion coefficients $D_{1-10}$ between 0.016 and 0.05 $\mu m^2/s$ and $S_{MSS}$ values >0.3. Importantly, receptors with $S_{MSS}$ values between 0.3 and 0.5 diffuse in the plasma membrane without
boundary even if they display anomalous behavior. Receptors with confined diffusion showed diffusion coefficients $D_{1-10}$ ranging from 0.002 to 0.02 $\mu$m$^2$/s and $S_{\text{MSS}}$ values spread between 0.1 and 0.3. Typically confined diffusion was restricted to domains of 120–550-nm size and in a few cases to 70–800-nm domains. Receptors were assigned as immobile when their $D_{1-10}$ values were lower than 0.002 $\mu$m$^2$/s and their $S_{\text{MSS}}$ values were lower than 0.1. Fast confined receptors had diffusion coefficients larger than 0.02 $\mu$m$^2$/s and $S_{\text{MSS}}$ values <0.15. Less than 2% of NK1Rs exhibited directed motion, and these comprised two distinct cases: receptors transported in the membrane plane outside of domains and receptors diffusing freely in moving nanodomains.

To simplify the analysis, receptors were classified into three categories: type I comprised the freely diffusing and directed receptors, type II comprised the confined and immobile receptors, and type III comprised the fast confined receptors as described above. Representative traces of each type and their corresponding mobility pattern are shown in Fig. 2, C–H. The interchange rate between these three different receptors was very low, less than 3%, during 30-s measurement windows corresponding to an interchange rate of $10^{-3}$ s$^{-1}$.

The mobility pattern of NK1Rs in the basal state exhibited two clear distinct peaks (Fig. 2B) corresponding to freely diffusing (type I) and to restricted (type II) receptors, respectively. For each population, both diffusion coefficients and Hurst parameters did not show a normal distribution; instead, the logarithm of $D$ had a bimodal distribution, and $S$ had a multimodal distribution (Fig. 3A). The median and the range of these distributions served as a basis for comparing receptor mobility between different experiments. Although diffusion trajectories of individual NK1Rs were highly heterogeneous in 293T cells (Fig. 2A), the mobility pattern of the ensemble population (Fig. 2B) was totally reproducible over days of measurement under identical experimental conditions.

In the following, the NK1R mobility was characterized either globally or as type I, II, or III. The overall parameters were obtained by including all the trajectories measured during an experiment, whereas the values for the particular mobility types were obtained considering only the trajectories of the corresponding type.

**Receptor Immobilization after Activation**—To probe the effect of an activating ligand on NK1R mobility, the cells were stimulated with various amounts of the natural agonist SP. After addition of SP, the portion of receptors confined in nanometer-sized domains significantly increased. This immobilization effect is clearly visible by comparing supplemental Movies S2 and S3, which were recorded in the absence or presence of SP, respectively. Fig. 3, A and B, show the two-dimensional distribution of $D_{1-10}$ and $S_{\text{MSS}}$ values for single receptor trajectories before and after receptor activation, respectively. Fig. 3, C and D, show the corresponding mobility patterns evaluated as described before. The mobile receptor population (type I; high values of $D_{1-10}$ and $S_{\text{MSS}}$) decreased in favor of the confined receptor population (type II; low values of $D_{1-10}$ and $S_{\text{MSS}}$) in which the immobile receptor subpopulation ($D_{1-10} < 2 \times 10^{-3}$ $\mu$m$^2$/s and $S_{\text{MSS}} < 0.1$) increased substantially. This transition occurred in the first minutes after addition of SP. Interestingly, this immobilization depended on the concentration of added SP with an EC$_{50}$ of about 100 pm for the average $S_{\text{MSS}}$ (Fig. 3E).

**Temporal Classification of Trajectory Segments**—To reach an accurate and reliable classification of trajectories, we applied a machine learning evaluation procedure. The support vector machine was based on the four mobility parameters $D_{1-10}$, $S_{\text{MSS}}$, efficiency, and asymmetry (for definitions see “Experimenical Procedures”), which were calculated from segments of archetypal trajectories. The support vector machine was run for both basal and activated states of the receptors. The data set of receptors in the activated state was limited to trajectories acquired during the first 1000 s after addition of SP. An increase of 27% of the type II receptor population correlated with a decrease of 23% of the type I receptor population rapidly fol-

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![Image](https://example.com/mobility.png)

**Figure 3. Effect of SP binding on NK1R diffusion in 293T cells.** A, $S_{\text{MSS}}$ versus $D_{1-10}$ plot of 1207 individual receptors in the absence of agonist. Probability density function (pdf) of $D_{1-10}$ (bandwidth = 0.11 in the logarithmic scale) (top) and of $S_{\text{MSS}}$ (bandwidth = 0.03) (right) is shown. B, same representation as in A of 433 NK1R in the presence of 83 nm SP. C, mobility pattern of NK1R in the basal state. D, mobility pattern of NK1R in the first 30 min after addition of 83 nm SP. The color code at the bottom of C and D scales with the indicated frequency (arbitrary units) of states. E, dependence of NK1R immobilization on SP concentration. The average value of the Hurst parameters, $S_{\text{MSS}}$, corresponds to the average receptor mobility for each concentration. An EC$_{50}$ value of ~100 nm SP was calculated for the mobility change by fitting the $S_{\text{MSS}}$ versus SP concentration data with a standard Hill function. F, support vector machine classification of trajectory segments according to the three defined types before and after addition of 83 nm SP. G, time evolution of the three classes of trajectory segments. A sliding time window of 160 s was used to obtain the support vector machine results in F and G. SP was added at $t = 0$. The evaluated time frames comprised between 21,747 and 68,834 trajectory segments. Error bars represent S.D.
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FIGURE 4. Long term evolution of NK1R mobility after SP exposition and Rho/ROCK pathway inhibition. A, mobility pattern of the NK1R in the basal state. Measurements started 1.5 h after receptor labeling. B, measurements started 1 h after addition of 83 nM SP and 1.5 h after receptor labeling. C, measurements started just after SP addition and 30 min after labeling in the presence of Y-26732, a Rho/ROCK pathway inhibitor. D, measurements started 1 h after SP addition and 1.5 h after receptor labeling in the presence of Y-26732, a ROCK inhibitor. The color code scales with the indicated frequency (arbitrary units) of states.

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lowed receptor activation, whereas the fraction of type III receptors remained stable (Fig. 3F).

To follow the intrinsic changes of receptor mobility over time, we measured the respective fractions of the mobility types for 1000 s at basal state (Fig. 3G). No significant changes were detected during this time interval. After addition of SP, the type II receptor population increased rapidly with a correlated decrease of the type I receptor population. 30 min after activation, type I receptors recovered to their initial level, whereas the type III receptor population started to increase significantly (Fig. 3G). This distinct fraction showed a high mobility (D > 0.02 \( \mu m^2/s \)) and a strong confinement (S_{MSS} < 0.2) (Fig. 2H) in circular 200–600-nm-sized domains (Fig. 2, G and I). After long term exposition to the agonist, the relative majority of receptors showed typical type III trajectories (Figs. 2G and 3G).

Contribution of the Cytoskeleton in NK1R Confinement—To determine the origin of the important fraction of confined receptors (type II) at the basal state, we first investigated the role of the cytoskeleton and cholesterol, two key players often associated with protein compartmentalization in the plasma membrane.

Role of the Cytoskeleton in NK1R Confinement—To determine the origin of the important fraction of confined receptors (type II) at the basal state, we first investigated the role of the cytoskeleton and cholesterol, two key players often associated with protein compartmentalization in the plasma membrane.

GPCRs can interact directly or indirectly with the cytoskeleton of the cell (14, 44, 45). Here we probed the influence of the cytoskeleton on NK1R diffusion using cytoskeleton-depolymerizing toxins. To investigate the interactions of the NK1R with microtubules, the cells were incubated with nocodazole, an antineoplastic agent interfering with microtubule polymerization. This resulted in a significant decrease of the overall mobility of the NK1R as seen by the median short term diffusion coefficient (Fig. 5E). This effect correlated with a significant increase of type II fraction, whereas the diffusion coefficient of the type I receptors remained unaffected (Fig. 5, A and B).

The hindered and heterogeneous mobility observed in SPT experiments can stem from contacts of the receptors with the actin-based membrane skeleton (46, 47). To probe the existence of such interactions, cells were treated with cytochalasin B, an inhibitor of actin filament polymerization. Cytochalasin B treatment did not significantly change the overall median diffusion coefficient (Fig. 5E). More specifically, \( D_{1-10} \) of the type I receptor population remained unaffected. However, the population of confined receptors increased substantially, resulting in significantly lower \( S_{MSS} \) values as revealed by the associated mobility pattern (Fig. 5C) in which the type III receptor population increased and the type I decreased. Importantly, neither the population nor the mobility features of type II receptors were affected by actin depolymerization. Theses results indicate that the restricted mobility of the NK1R is not due to direct interactions with the cytoskeleton of the cell.

Decrease of Receptor Mobility after Cholesterol Depletion—Cholesterol has been shown to play a role in the localization of NK1R in plasma membrane domains (32). To investigate the effect of cholesterol content in the plasma membrane on NK1R diffusion, the cells were treated with mβCD. We found that membrane cholesterol depletion by mβCD decreased the overall mobility of the receptor (Fig. 5E). In particular, we observed two independent effects: (i) a shift of the mobility pattern toward confined diffusion of type II (Fig. 5D) and (ii) a decrease of the median diffusion coefficient of the type I receptor population to 0.011 \( \mu m^2/s \), which differs significantly from the values observed for receptors in the basal state (0.016–0.025 \( \mu m^2/s \)).

NK1R Interaction with Clathrin—The NK1R is recycled through the CME pathway (27). Because the role of cytoskeleton and cholesterol appeared to be of minor importance in receptor confinement, we investigated the influence of clathrin on NK1R mobility. Three different CME inhibitors have been used to block formation and internalization of CCPs at different
Impairment of NK1R-mediated \( \text{Ca}^{2+} \) Signaling—The impact of the different chemical modulators used in the present study on NK1R activity was probed using a standard \( \text{Ca}^{2+} \) assay based on the fluorescent indicator FuraRed. Microtubule or actin filament disruption with nocodazole or cytochalasin B did not affect the cellular \( \text{Ca}^{2+} \) response induced by SP, whereas removal of cholesterol with \( \text{mBCD} \) almost abolished \( \text{Ca}^{2+} \) signaling (Fig. 8A). Addition of Dyngo-4a and PitStop 2, which down-regulate CME, also led to a significant decrease of the \( \text{Ca}^{2+} \) response (Fig. 8B). These results point out the importance of cholesterol but also clathrin in the regulation of NK1R activity.

To evaluate the ability of NK1R to bind SP in presence of the CME inhibitors, a fluorescent derivative of SP (SP-Cy5) was added to the cells, and binding of the fluorescent agonist was monitored using confocal microscopy. Dyngo-4a, PitStop 2, and Dynasore did not prevent SP-Cy5 binding (Fig. 8, C–E).

Discussion

Here we have investigated the mobility features of the neurokinin-1 receptor with an unprecedented level of mechanistic understanding. We used mobility patterns as a new, high content graphical representation of single molecule mobility. This representation is based on a two-dimensional density function of the short range diffusion coefficient \( D_{1-10} \) versus the mobility parameter \( S_{\text{MSS}} \), which is directly associated with the mode of motion of the receptor. This enabled us to visualize and analyze the complex information contained in a particular experiment within a single graph, substantially facilitating comparison of results obtained under different experimental conditions. Moreover, this method of analysis allowed us to easily define and classify the diffusing particles into different types according to their mobility regime.

Our study revealed that, despite the very broad distribution of the mobility and sizes of membrane confinement, the overall
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Different mobility patterns remain highly reproducible between different days of experiment. This suggests the presence of a very distinct and stable network of functional interactions between the receptor and other cellular components. As presented under “Results,” NK1R can be classified into three major classes. Receptors assigned to type I are free to diffuse in the cellular membrane. Their general diffusion properties are accessible by other measurement techniques such as fluorescence recovery after photobleaching or fluorescence correlation spectroscopy. The overall features of type I receptors are comparable with those observed by single molecule tracking of other GPCRs (31, 51–53). Low $S_{\text{RSS}}$ values measured here correlate with a restricted diffusion of the NK1R. A similar behavior was observed for other receptors in living cells and is often explained by multiple effects such as the rough, irregular shape of the plasma membrane; transient interactions with other membrane proteins; the heterogeneous composition of the plasma membrane; and the recruitment in caveolae (30, 54–57). The diffusion coefficients of GPCRs we and others have observed in the membranes of living cells are considerably lower than those of other membrane proteins of similar size or that of rhodopsin as an example of a class A GPCR in pure lipid bilayers (58, 59). The use of Qdots as a fluorescent label to track individual receptors allowed us to validate in an accurate and reliable manner previous single molecule diffusion measurements using organic dyes by Prummer et al. (30). Consequently, the use of Qdots does not interfere with the diffusion properties of the receptor.

Low diffusion coefficients are often explained by direct interactions of the membrane protein of interest with components of the cytoskeleton (60–62). According to the picket fence model (47), drug treatment causing actin depolymerization should result in increased receptor diffusion due to the reduction of actin filament barriers. The NK1R does not follow this behavior. In the present case, the type I mobility receptor population did not increase after actin fiber or microtubule depoly-
merization, strongly suggesting a lack of direct interaction of type I NK1Rs with the cytoskeleton. The relative low mobility of type I receptors could be explained by the high propensity of NK1R to form diffusing membrane domains a few tens of nanometers in size with high receptor density (32).

A high fraction of the NK1Rs exhibited a strictly restrained mobility and was therefore classified as type II. Indeed, more than one-third of the receptors in the basal state were found to be confined in submicrometer-sized domains. The low interchange rate between receptors of type II and other mobility regimes indicated that this restricted diffusion is not a consequence of the fast transient recruitment described in the picket fence model of the plasma membrane (59) but is more likely due to the existence of very stable membrane structures in which the NK1R is integrated. The low diffusion coefficients measured for type II receptors did not depend on intact actin filaments or microtubule structures as no differences were observed in type II diffusion features upon treatment of cells with cytochalasin B or nocodazole. The low $D_{1-10}$ values observed are very likely due to molecular crowding related to membrane regions of high protein content. The high frequency of our measurements (30 Hz) combined with the very high accuracy generated by the use of Qdots to localize individual receptors permitted us to exclude effects of domain size on the apparent diffusion coefficients observed elsewhere for other GPCRs with less photostable fluorophores (57, 63). A small part of the receptors in the basal state remained immobile; this population probably stems from constitutively internalized receptors.

Although our results do not yield any indication of direct or mediated interactions by a simple protein assembly of the NK1R with the cytoskeleton of the cell, the NK1R is nevertheless tightly related to its surrounding. Indeed, disruption of the cytoskeleton had an indirect influence on the mobility pattern of the receptor through structural modifications of the membrane.

**FIGURE 7. Mobility of NK1R in the plasma membrane of 293T cells after siRNA-mediated clathrin depletion.** $A$, mobility pattern of NK1R in nontransfected control cells. $B$, mobility pattern of NK1R after siRNA knockdown of the clathrin heavy chain. For both $A$ and $B$, bandwidths are $D_{1-10} = 0.21$ in the logarithmic scale and $S_{E,S}$ = 0.09. The color code scales with the indicated frequency of states (arbitrary units). Box plots of the NK1R diffusion coefficient $D_{1-10}$ (C) and the Hurst parameter $S_{E,S}$ (D) in the absence ($n = 61$) and presence ($n = 23$) of siRNA against clathrin. The median is drawn as a horizontal line. Notches indicate the 95% confidence interval of the median. Boxes represent the interquartile ranges of the distribution. The significance difference and ** indicating $p < 0.01$ with the null hypothesis (H0) that population A is not different from population B.

**FIGURE 8. Efficiency of the $G_{\alpha_q}$ activation pathway in 293T cells after cytoskeleton disruption, cholesterol depletion, and CME inhibition (A and B) and capacity of the NK1R to bind SP in presence of the CME inhibitors (C–E), A, intracellular Ca$^{2+}$ responses measured with Fluo-4 in the presence of an 83 nM concentration of the agonist SP before and after addition of (i) nocodazole (Noc), which disrupts microtubules, (ii) cytochalasin B (CytB), which prevents F-actin fiber formation, and (iii) mjICD, which depletes cholesterol from the plasma membrane. $B$, intracellular Ca$^{2+}$ responses before and after CME impairment by PitStop 2 and Dyngo-4a. $C–E$, fluorescence confocal micrographs showing 293T cells stably expressing ACP-NK1R labeled with CoA-Alexa Fluor 488 before (left) and after adding a 50 nM concentration of the agonist SP-Cy5 (right) in absence of CME inhibitor (C) or in presence of PitStop 2 (D) or Dyngo-4a (E). Scale bars, 20 µm.
Depolymerization of actin filaments is known to stimulate cell blebbing (64, 65). Under certain conditions, these blebs can be released as native microvesicles containing functional NK1R (66). The high fraction of type III receptors after cytochalasin B treatment is related to membrane blebbing. The protein content of native vesicles is different from that of the plasma membrane of the cell; in particular, they lack cytoskeletal structure (67). This explains the high $D_{1/2}$ observed for this population. Furthermore, the submicrometer size of the confinement region is in total agreement with the results obtained elsewhere (65, 66).

SP is a potent natural agonist of the NK1R. It triggers multiple signaling pathways and receptor recycling. After activation, the NK1R is recycled via two distinct pathways: in a fast process, receptors are recruited in plasma membrane domains or in early endosomes in close proximity to the plasma membrane (13, 29); in a slow process, the receptors are transported to low pH perinuclear late endosomes (68, 69). Both pathways are initiated by receptor phosphorylation and subsequent arrestin binding. Our SPT results in the presence of SP show a substantial decrease of NK1R mobility, consistent with an increased recruitment of the receptors in structures related to the recycling pathways. This effect correlates with a decrease of the overall diffusion coefficient mainly due to an increase of confinement as seen by an important shift from type I to type II receptors in the mobility patterns. Interestingly, only 30% of the receptors undergo a change of mobility after activation. It has been shown elsewhere that other cargo proteins associate transiently with CCPs during their formation and can dissociate before pit termination or internalization; the dwell times of this process display a very broad distribution from the second to hundred second regime (70). Taking this mechanism into consideration, our results can be explained by an increase of the receptor affinity for CCPs after agonist binding, thus increasing the dwell time and favoring internalization against release of the receptor in the plasma membrane.

It is remarkable that this immobilization effect associated with receptor recruitment in the CME pathway depends on agonist concentration. The dose-response curve resulting from the measurement of the average Hurst parameter with increasing SP concentrations shows an EC_{50} value of about 100 pm, which is comparable with the EC_{50} of the intracellular Ca^{2+} response, indicating that the ligand-coordinated receptor immobilization might regulate the intracellular response. The NK1R mobility change induced by the presence of low concentrations of agonist in the environment would quickly regulate the cell response and therefore limit the intracellular Ca^{2+} release in the case of long term agonist exposition.

An unexpected important result is that the decrease of CME functionality induced by the clathrin/dynamin inhibitors PitStop 2, Dyngo-4a, and Dynasore leads to a substantial increase of type II receptors confined in submicrometer membrane domains. Besides inhibition of endocytosis, these molecules induce an accumulation of clathrin at the plasma membrane, forming long lived clathrin structures (71), including membrane-attached vesicles (72). Clathrin can also exist in large patches on the plasma membrane without forming functional pits or invagination (73–75). The correlated decrease of both the diffusion coefficient and the Hurst parameter after inhibition of clathrin or dynamin indicates a stable interaction of NK1R with these clathrin-related structures. Importantly, this was observed with all three CME inhibitors, indicating a specific clathrin effect. It is thus possible to exclude domain recruitment due to clathrin-independent membrane processes, which would be affected by dynamin inhibitors (76). Receptors accumulate in a preinternalized state, that is in either clathrin lattices, prepits (77), coated pits (78, 79), or superficial early endosomes (28). The fast accumulation of NK1R after CME inhibition strongly suggests a high association rate with these structures. Further support for the specific interaction of clathrin with the NK1R comes from the observation that the distinct membrane organization of the receptor is strongly affected after clathrin depletion.

Interaction of NK1R with clathrin-dependent structures and immobilization of activated receptors are sequential events. In the absence of CME inhibitors, a large fraction of the receptors are localized in relatively stable domains in an intermediary mobility state between freely diffusing and internalized receptors. CME inhibitors promote this state by increasing the clathrin content at the membrane. In this state, receptors are diffusing in domains with lower diffusion coefficients and lower Hurst parameters. After activation with SP, the Hurst parameters remained unchanged, whereas the diffusion coefficients further decreased. The interactions involved in domain recruitment and in immobilization after activation are distinct. Non-activated receptors interact with clathrin-dependent structures, forming transient membrane domains, whereas activated receptors bind specifically to CCP through $\beta$-arrestin and AP2.

CME inhibitors also strongly impaired receptor-mediated intracellular calcium signaling. This decrease of the NK1R canonical activity can arise from several, non-exclusive reasons. (i) The agonist binding site is not accessible due to the shape of the invagination as depicted in Fig. 1. (ii) G proteins cannot bind the intracellular region of the receptor due to the densely packed clathrin structures (80). (iii) Receptor signaling is impaired by molecular crowding (81). Interestingly, disruption of microtubules, known to inhibit CME (82), does not alter NK1R activity in our case.

The correlation between the clathrin-dependent change of receptor mobility and the decrease of its activity, combined with the presence of a high fraction of type II receptors before activation, implies a clathrin-based mechanism for regulation of NK1R activity. Thereby, type II receptors could act as a non-activated receptor reservoir that is directly and quickly available at the cell membrane. This reservoir would have major implications in cell response to an agonist. In particular, it would allow responding to successive or long term agonist exposures. Indeed, for a short exposition time to an agonist, only a fraction of the receptors must respond. A gradual release of receptors from an inactive membrane reservoir could increase the response in the case of prolonged agonist exposition. Furthermore, such a mechanism would allow multiple intracellular Ca^{2+} responses to sequential agonist waves without the need of newly membrane-inserted receptors. This model is also compatible with the fast resensitization observed
elsewhere (29) albeit without the need of preliminary activation of NK1R.

Cholesterol removal with mβCD affects NK1R mobility and activity in a similar manner as CME inhibition. Indeed, cholesterol depletion provokes a substantial decrease of the overall diffusion of the receptor and practically abolished the intracellular Ca$^{2+}$ response. Thus, cholesterol, like clathrin, plays a major role in receptor mobility and is of critical importance for its activity, corroborating the close link between mobility and activity.

The fast diffusing type III receptor population confined in circular domains, which appears 30 min after NK1R activation with SP, results from the presence of receptors in membrane blebs or microvesicles. Membrane blebs are balloon-like structures of the plasma membrane in which the cytoskeleton elements are generally absent, leading to enhanced molecular diffusion. Tank et al. (83) found a considerable increase of the diffusion coefficients for both membrane proteins and lipids comparable with those found in liposomes (47). Besides the canonical signaling pathway leading to Ca$^{2+}$ release from the endoplasmic reticulum, SP induces cell membrane blebbing through the Rho/ROCK pathway by contraction of the actomyosin cell cortex (43).

Y-27632 is a highly specific and efficient cell-permeable ROCK inhibitor that prevents NK1R-induced blebbing without affecting the apoptotic state of the cells (43). The mobility pattern resulting from NK1R trajectories measured in cells treated with this inhibitor and stimulated with SP is characterized by the absence of type III receptors. It demonstrates that the type III receptor population is directly dependent on the activation of the Rho/ROCK pathway and thus on the presence of membrane blebs.

Membrane blebbing and excretion of microparticles are often associated with apoptosis. However, in our case, it has been shown that membrane blebbing is induced by activation of the NK1R by SP and is hence an apoptosis-independent phenomenon. This particular cellular mechanism may be of great importance for intercellular communication (84).

In summary, single particle tracking and multiparameter analysis allowed us to describe in detail the diffusional behavior of the neurokinin-1 receptor in the plasma membrane of living cells. The bimodal distribution of freely diffusing and confined receptors observed in the basal state is strongly shifted toward restricted mobility by receptor activation, whereas a new population of fast diffusing receptors in circular domains, corresponding to receptors in membrane blebs, resulted 30 min after activation of the Rho/ROCK pathway. Blocking of the CME pathway using different inhibitors leads to receptor confinement, which is correlated to a significant decrease of the receptor canonical pathway activity. Our results point to the central importance of clathrin, not only in receptor endocytosis and turnover but also in NK1R membrane homeostasis and fine regulation of its activity.

Author Contributions—H. V. initiated the project and was responsible for overall project management and strategy. L. V. and J. P. did the experiments and analyzed data. J. P. designed and implemented the computational methods. L. V., J. P., and H. V. designed the experiments, discussed the results, and contributed to the final manuscript.

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