Diffusion Dynamics of Glycine Receptors Revealed by Single-Quantum Dot Tracking
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In living cells, the ability to selectively detect one molecule (or a small number of molecules) is a powerful way to understand the dynamics of cellular organization (1). So far, access to single-molecule properties in living cells has been restricted by either the size of the probe (40-nm gold nanoparticles or 500-nm latex spheres) (2) or the photobleaching of the small (1- to 4-nm) fluorescent labels (3). QDs, which are suitable for advanced biological imaging. We used QDs to track individual glycine receptors (GlyRs) and analyze their lateral dynamics in the neuronal membrane of living cells for periods ranging from milliseconds to minutes. We characterized multiple diffusion domains in relation to the synaptic, perisynaptic, or extrasynaptic GlyR localization. The entry of GlyRs into the synapse by diffusion was observed and further confirmed by electron microscopy imaging of QD-tagged receptors.

Semiconductor quantum dots (QDs) are nanometer-sized fluorescent probes by the scaffolding protein gephyrin (11, 12). The issue of lateral mobility of receptors for neurotransmitters has become central to understanding the development and plasticity of synapses (13). The membrane dynamics of GlyRs has been studied previously in transfected neurons using latex beads (14). GlyRs diffuse rapidly in the neuronal plasma membrane and transient interaction with gephyrin decreases their diffusion. Comparable results were obtained for the metabotropic- and AMPA-type glutamate receptors and their corresponding scaffolding molecules (15, 16). These measurements, however, preclude analysis of receptor dynamics in the synaptic cleft because of the use of 500-nm beads. We aimed to develop a new approach that could both access the synapse and be tracked for long periods of time. The specific detection of endogenous GlyR α1 subunits at the surface of spinal cultured neurons was achieved by the use of a primary antibody (mAb2b), biotinilated anti-mouse Fab fragments, and streptavidin-coated QDs (Fig. 1) (17). QD-GlyR formed numerous clusters around the soma and dendrites (Fig. 1A), similar to observations from previous immunocytochemical studies using conventional fluorophores (18). GlyRs were detected within synaptic and extrasynaptic domains (Fig. 1, B and C).

QDs were then used to study the lateral movement of individual GlyRs in living neurons. Single QDs were identified by their blinking property, i.e., the random intermittency of their fluorescence emission (5, 19). The results of these experiments were compared with those that used Cy3-coupled antibodies. Trajectories of single QD-GlyRs in the membrane could be visualized easily for at least 20 min, whereas the duration was ~5 s for Cy3. The spots were detected with a signal-to-noise ratio of about 50 (integration time 75 ms), almost an order of magnitude higher than the signal obtained with fluorophores. Thus, the lateral resolution reached 5 to 10 nm, well below the 40 nm achieved with Cy3 dyes (20).

First, we used single-QD tracking (SDQT) to study the rapid lateral dynamics of GlyRs. Continuous sequences of 75-ms images were acquired for durations of ~60 s. Individual QD-GlyRs diffusing in the neuronal membrane were either detected in extrasynaptic regions or associated with boutons identified with the amphiphilic FM4-64 dye. SDQT enabled the observation of multiple exchanges between extrasynaptic and synaptic domains, in which a GlyR alternated between free and confined diffusion states, respectively (Fig. 2A and movie S1). A GlyR, initially located at a synapse, started to diffuse rapidly (Fig. 2, A1 to A5) and, after about 30 s, stabilized close to another synaptic site (Fig. 2, A6 to A8), 4 to 5 μm away from the starting point. To quantify this observation, the instantaneous diffusion coefficients (D) were determined along the trajectory (Fig. 2B). For the 0- to 30-s period, D was ~0.1 μm²/s, and the mean-square displacement (MSD) function varied linearly (Fig. 2C), as is expected for free Brownian diffusion (2). In the later part of the trajectory (30 to 63 s), D decreased to ~0.02 μm²/s, and the MSD exhibited a negative curvature, characteristic of a space-confined movement (Fig. 2D). The high photostability of QDs also allowed for the tracking of individual GlyRs in the same neuritic region for long durations. To avoid toxic continuous illumination of the cells, data were acquired in a
time-lapse recording of one 75-ms image per second for 20 min (movie S2), a duration inaccessible when using fluorophores or even beads, which tend to stick to the cell membrane after a couple of minutes. An extrasynaptic receptor diffused freely and covered a large surface of the membrane (Fig. 3A). In contrast, some GlyRs were stable at synapses, whereas others moved but in a confined region around the bouton. These patterns, observed repeatedly, led us to classify receptors as synaptic for FM4-64 overlapping spots, perisynaptic for spots with centers that were localized within two pixels of the border of the FM4-64 spot, or extrasynaptic for spots farther away from the synapse (Fig. 3B). At any given time, about 80% of the GlyRs were perisynaptic or synaptic (Fig. 3D), a proportion consistent with the results obtained with fixed neurons (18). We focused here on the properties of these receptors and plotted their location every 5 min for 40 min (Fig. 3C). Several observations deserve to be highlighted: (i) Some receptors remained synaptic or switched between perisynaptic and synaptic localization. (ii) GlyR moving from an extrasynaptic to a synaptic state and vice versa always transited by a perisynaptic state lasting for up to a few minutes. (iii) No receptor remained perisynaptic over the entire recording. The fractions of receptors in a given state did not vary much over time (Fig. 3D). Thus, the neuronal somatodendritic membrane is organized in three domains (extrasynaptic, perisynaptic, and synaptic) with distinct diffusion properties. The existence of a perisynaptic domain may be explained by the adhesion molecules present at the periphery of synapses [references in (13)] and/or by the gephyrin scaffolding molecules that overextend slightly past the limit of the synaptic complex (27).

The diffusion coefficients of QD-GlyRs were determined as a function of their membrane location in about 230 trajectories (Fig. 3E). For extrasynaptic receptors, the mean \( D \pm \text{SEM} \) was 0.10 \( \pm 0.02 \) \( \mu \text{m}^2/\text{s} \) (\( n = 83 \)), which was about four times as large as those measured with the beads \( (0.029 \pm 0.005 \mu \text{m}^2/\text{s}) \) (14), indicating that the use of the beads significantly slowed down the receptor motion. For perisynaptic and synaptic receptors, the average diffusion coefficients were lower, at 0.023 \( \pm 0.005 \mu \text{m}^2/\text{s} \) (\( n = 70 \)) and 0.015 \( \pm 0.004 \mu \text{m}^2/\text{s} \) (\( n = 82 \)), respectively (22, 23). However, the mean coefficients did not reflect the wide range of movements that were observed. Therefore, we compared each receptor \( D \) to \( D_1 \), where \( D_1 = 0.01 \mu \text{m}^2/\text{s} \). This value was selected because \( \approx 80\% \) of the extrasynaptic movements that we measured had faster diffusion. Receptors with \( D > D_1 \) were classified as rapid; those with \( D < D_1 \) were classified as slow. The mean diffusion coefficients in the perisynaptic and synaptic regions were, respectively, \( 0.053 \pm 0.009 \mu \text{m}^2/\text{s} \) (\( n = 29 \)) and \( 0.073 \pm 0.013 \mu \text{m}^2/\text{s} \) (\( n = 16 \)) for rapid receptors and \( 0.0020 \pm 0.0004 \mu \text{m}^2/\text{s} \) (\( n = 41 \)) and \( 0.0010 \pm 0.0002 \mu \text{m}^2/\text{s} \) (\( n = 66 \)) for slow receptors. However, the relative fraction of rapid GlyR was significantly higher in the perisynaptic domains (41.4%) than it was in the synaptic domains (19.5%) (Fig. 3F). At synapses, rapid GlyRs are likely to be those that do not interact with gephyrin (14, 24).

Estimates of diffusion coefficient values may be altered by the size of QDs (10 to 15 \( \text{nm} \)) for particles conjugated to streptavidin and/or...
by their coupling to a divalent antibody that may crosslink receptors. As a control, we performed experiments with a Cy3-labeled Fab fragment of the primary antibody, a smaller (~3 nm) and monovalent molecule. The use of Fab fragments did not significantly modify the results (25). Indeed, the antigenic determinant for mAb2b is localized in the vestibule of the GlyR channel (26, 27). Given the size of this type of receptor channel (28), the distance between the epitopes of adjacent receptors would be at least 10 nm, which does not allow for cross-linking by divalent antibodies. Our experiments could not exclude an influence of the steric hindrance of QDs on the diffusion of tagged receptors in the synaptic cleft. However, we were able to record comparable proportions of rapidly diffusing receptors with Cy3 and QDs.

The precise localization of diffusing GlyRs in the neuronal membrane was definitively established by electron microscop-
Hematopoietic Cell Regulation by Rac1 and Rac2 Guanosine Triphosphatases

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The Rho guanosine triphosphatases (GTPases) Rac1 and Rac2 are critical signaling regulators in mammalian cells. The deletion of both Rac1 and Rac2 murine alleles leads to a massive egress of hematopoietic stem/progenitor cells (HSC/Ps) into the blood from the marrow, whereas Rac1−/− but not Rac2−/− HSC/Ps fail to engrant in the bone marrow of irradiated recipient mice. In contrast, Rac2, but not Rac1, regulates superoxide production and directed migration in neutrophils, and in each cell type, the two GTPases play distinct roles in actin organization, cell survival, and proliferation. Thus, Rac1 and Rac2 regulate unique aspects of hematopoietic development and function.

Rho GTPases, members of the Ras superfamily, are critical regulators of cellular function and signal transduction pathways in eukaryotic cells. In mammalian cells, the best-studied members—Rho, Rac, and Cdc42—play distinct roles in regulating actin assembly and motility (1). However, the roles of Rho GTPases in hematopoietic cell development and function have only recently begun to be elucidated. There are three Rac GTPases—Rac1, Rac2, and Rac3—and their high degree of homology suggests potential overlapping functions (2, 3). Rac1 is ubiquitously expressed, whereas the expression of Rac2 is restricted to cells of hematopoietic origin (2, 3) and Rac3 is expressed primarily in the brain (4).

We have previously reported the critical roles of Rac2 in a wide variety of primary hematopoietic cells, including the regulation of adhesion, migration, oxidative activity, and gene expression (5–10). These same functions have been attributed to Rac1 in nonhematopoietic cells in which Rac2 is not expressed (11–14). However, the roles of Rac1 compared with the roles of Rac2 in blood cells, which express both GTPases, remain to be elucidated.

Because homozygous Rac1-deficient mice die at ∼E8 (embryonic day 8) in utero (15), we generated mice with a conditional Rac1 (flox) allele (Rac1fl/fl) (16) (fig. S1). These were compared with mice that were homozygous for both the Rac1 flox allele and a Rac2-null allele (Rac1fl/fl/−Rac2−/−). Floxed Rac1 sequences were deleted by means of two methods, and the deletion of Rac1 sequences was confirmed with polymerase chain reactions (PCRs) and immunoblots (figs. S2 and S3) (17).

Most prominently, the absence of Rac1 led to a significant reduction compared with that of the wild type in the ability of hematopoietic stem/progenitor cells (HSC/Ps) to reconstitute hematopoiesis in a non-obese diabetic/severe combined-immunodeficiency (NOD/SCID) engraftment model (18) (Fig. 1A). Engraftment is a multistep process requiring proliferation and differentiation of stem cells after the movement of these cells into the bone marrow from the blood and adhesion in the hematopoietic microenvironment (19). Rac2−/− cells demonstrated normal short-term engraftment, indicating that defective engraftment was specific for Rac1 deficiency. Although modest movement (mobilation) of HSC/Ps out of the marrow cavity into the circulating blood was observed in Rac2−/− mice, as previously reported (6), the absence of both Rac1 and Rac2 resulted in a massive mobilization of progenitor colony-forming unit cells (CFU-C) into the peripheral circulation (Fig. 1B). These phenotypes occurred despite the normal expression of β1 integrin adhesion molecules (20) and the normal expression of CXCR4, the receptor for the stromal-derived factor-1 (SDF-1), both of which have previously been implicated in the engraftment and mobilization of stem cells (21) (Fig. 1C). However, whereas Rac1−/− HSC/Ps showed normal adhesion to fibronectin, Rac2−/−/− HSC/Ps and, more prominently, Rac1−/−:Rac2−/− cells displayed significantly decreased adhesion to fibronectin (Fig. 1D), strongly suggesting that Rac2 has a predominant but overlapping role with Rac1 in integrin-mediated stem cell adhesion. Moreover, mobilization of Rac1−/−:Rac2−/− HSC/Ps was associated with significantly increased expression of CXCR4 (Fig. 1C).

Rac1−/− HSC/Ps also displayed impaired growth factor–stimulated in vitro growth, as determined by progenitor colony formation (Fig. 2A and fig. S4) and expansion in liquid culture (fig. S5). Reduced Rac1−/− HSC/P growth was associated with significantly decreased thymidine incorporation (Fig. 2B). Rac1−/−:Rac2−/− cells had a more severe reduction in proliferation compared with that of the wild-type and Rac2−/− cells, and they formed profoundly abnormal colonies with no cellular halo, suggesting combined effects of impaired growth and migration. Indeed, this severe phenotype in Rac1−/−:Rac2−/− cells was associated with reduced proliferation (Fig. 2B), increased apoptosis associated with Rac2 deficiency (Fig. 2C and fig. S6), and profoundly decreased migration in response to SDF-1, as compared with that of wild-type cells (Fig. 2D).

To determine the mechanism of reduced HSC/P growth, we undertook additional analysis. Significantly fewer Rac1−/− HSC/Ps entered S and G2/M over 24 to 48 hours in response to stem cell factor (SCF), a growth factor for primitive hematopoietic cells, as compared with those in wild-type or Rac2−/− cells (Fig. 3A and fig. S7). We found that levels of cyclin D1, which is required for slow receptors. For synaptic receptors, the corresponding values were 0.016 ± 0.0002 μm²/s (n = 12) and 0.0037 ± 0.0002 μm²/s (n = 90). The fraction of rapid receptors was 27 and 12% for perisynaptic and synaptic GlyRs, respectively (Fig. 3F).

23. The Kolmogorov-Smirnov test indicated significant differences between the distributions of D in the different domains (P < 0.0001).

24. At glutamatergic synapses, rapid and slow diffusion of AMPA receptors have been recently described with comparable diffusion coefficient values [C. Tardin, L. Cognet, C. Bats, B. Louzins, D. Choquet, EMBO J. 22, 4656 (2003)].