Reciprocal stabilization of glycine receptors and gephyrin scaffold proteins at inhibitory synapses

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ABSTRACT Postsynaptic scaffold proteins immobilize neurotransmitter receptors in the synaptic membrane opposite to presynaptic vesicle release sites, thus ensuring efficient synaptic transmission. At inhibitory synapses in the spinal cord, the main scaffold protein gephyrin assembles in dense molecule clusters that provide binding sites for glycine receptors (GlyRs). Gephyrin and GlyRs can also interact outside of synapses, where they form receptor-scaffold complexes. Although several models for the formation of postsynaptic scaffold domains in the presence of receptor-scaffold interactions have been advanced, a clear picture of the coupled dynamics of receptors and scaffold proteins at synapses is lacking. To characterize the GlyR and gephyrin dynamics at inhibitory synapses, we performed fluorescence time-lapse imaging after photoconversion to directly visualize the exchange kinetics of recombinant Dendra2-gephyrin in cultured spinal cord neurons. Immuno-immobilization of endogenous GlyRs with specific antibodies abolished their lateral diffusion in the plasma membrane, as judged by the lack of fluorescence recovery after photobleaching. Moreover, the cross-linking of GlyRs significantly reduced the exchange of Dendra2-gephyrin compared with control conditions, suggesting that the kinetics of the synaptic gephyrin pool is strongly dependent on GlyR-gephyrin interactions. We did not observe any change in the total synaptic gephyrin levels after GlyR cross-linking, however, indicating that the number of gephyrin molecules at synapses is not primarily dependent on the exchange of GlyR-gephyrin complexes. We further show that our experimental data can be quantitatively accounted for by a model of receptor-scaffold dynamics that includes a tightly interacting receptor-scaffold domain, as well as more loosely bound receptor and scaffold populations that exchange with extrasynaptic pools. The model can make predictions for single-molecule data such as typical dwell times of synaptic proteins. Taken together, our data demonstrate the reciprocal stabilization of GlyRs and gephyrin at inhibitory synapses and provide a quantitative understanding of their dynamic organization.

INTRODUCTION

The postsynaptic scaffold at inhibitory synapses is characterized by the presence of dense clusters of gephyrin molecules that provide binding sites for inhibitory glycine receptors (GlyRs) and GABA type A receptors (GABA\textsubscript{A}Rs), as well as other synaptic components such as collybistin and neuroligin-2 (reviewed in (1)). Gephyrin has a particularly strong interaction with the intracellular domain of the \(\beta\)-subunit of the GlyR with a \(K_D\) in the nanomolar range (2–5). The presence of the \(\beta\)-subunit is therefore essential to anchor the pentameric GlyR complex in the postsynaptic membrane (6,7). Gephyrin is also involved in the forward trafficking of GlyRs toward the plasma membrane (8,9), where it remains associated with the receptor because of the high affinity of the GlyR\(\beta\)-gephyrin interaction (7,10).

In addition to receptor-scaffold interactions, the stability of inhibitory synapses is also dependent on scaffold-scaffold interactions. The basic unit of soluble gephyrin is a trimer, formed by homomeric interactions between the N-terminal
domains (G-domains) of gephyrin (11,12). Furthermore, the C-terminal domains of gephyrin (E-domains) can, under certain conditions, form dimers (2,13,14) that are thought to be required for synaptic clustering (15) (discussed in (1)).

The different molecular states of GlyRs and gephyrin are engaged in a dynamic equilibrium that can be largely accounted for by receptor-gephyrin and gephyrin-gephyrin interactions (10). In support of this view, expression of gephyrin and GlyRs in non-neuronal cells is sufficient to drive the spontaneous formation of membrane-associated gephyrin aggregates that resemble postsynaptic domains (e.g., (16,17)). Several models have been put forward to explain the formation of stable gephyrin domains arising from receptor-scaffold interactions (17–20).

Based on thermodynamic considerations, Sekimoto and Triller proposed a mechanism of phase separation between a condensed domain (phase) at synapses and a delocalized phase with lower receptor and gephyrin concentrations in the extrasynaptic space (18). Haselwandter and colleagues treated the GlyR and gephyrin populations as a reaction-diffusion system and proposed that postsynaptic domains are formed by a Turing-like instability (17,21). More recently, we hypothesized that gephyrin domains are in a nonequilibrium stationary state in which the desorption of synaptic gephyrin proteins into the cytoplasm is balanced by the capture of diffusing GlyR-gephyrin complexes (19,20). In this project, we set out to put these different ideas to the test by directly measuring the exchange dynamics of GlyRs and gephyrin at synapses using population measurements based on photoconversion and time-lapse imaging in cultured spinal cord neurons.

MATERIALS AND METHODS

Neuron culture and lentivirus infection

Primary spinal cord neurons of Sprague-Dawley rat embryos at embryonic stage E14 were cultured as described previously (22). Neurons were plated and grown on 18 mm diameter glass coverslips in Neurobasal Medium containing complement B27, 2 mM glutamine, 5 U/mL penicillin, and 5 μg/mL streptomycin at 37 °C and 5% CO2. Half of the culture medium was replaced twice a week with BrainPhys medium containing SM1 and antibiotics. Cultures were infected at day in vitro 3 or 4 (DIV 3 or 4) with lentivirus (15 μL per coverslip) driving the expression of Dendra2-gephyrin or Dendra2-GlyRα1 (see Supporting materials and methods) and used for experiments between DIV 13 and DIV 16.

Antibody cross-linking and live imaging

Before each experiment, the coverslips were rinsed in warm Tyrode solution (120 mM NaCl, 2.5 mM KCl, 2 mM CaCl2, 2 mM MgCl2, 25 mM glucose, 5 mM pyruvate, and 25 mM HEPES (pH 7.4)) and placed on a heating plate at 37 °C. GlyRs were immuno-immobilized by incubating the cultured spinal cord neurons with primary rabbit anti-GlyRα1 antibody (custom made, 1:100 dilution in Tyrode solution) for 10 min, rinsed twice, and incubated for another 10 min with Alexa Fluor 647- or Alexa Fluor 488-conjugated secondary antibodies (A647-coupled donkey anti-rabbit, A488 goat anti-rabbit, 1:100; Jackson ImmunoResearch, West Grove, PA). Coverslips were rinsed again, mounted in an imaging chamber on the microscope stage, and imaged for up to 1 h (typically 35–40 min) at 37 °C in Tyrode solution. Temperature and humidity were maintained using a H301-T-UNIT-BL-PLUS temperature control unit (Okolab, Ottaviano, Italy). Whenever direct control experiments were carried out (data in Figs. 3 and S5), all coverslips were treated in the same way, using Tyrode solution without antibodies in the control condition.

Photoconversion and time-lapse image acquisition

To determine the most suitable fluorophore for the photoconversion experiments, we compared the behavior of different photoconvertible fluorescent proteins (Dendra2, mEos2, and mEos4b) in COS-7 cells (Fig. S1). We noticed a strong increase of the fluorescence intensity of nonconverted mEos2 and mEos4b in response to low-intensity ultraviolet illumination. This photochromism of the Eos fluorophores introduces a nonlinearity in the intensity measurements that complicates data analysis. We therefore chose Dendra2 for our experiments because this fluorophore was the least affected by photochromic effects.

Fluorescence recovery after photobleaching (FRAP) and fluorescence decay after photoconversion (FDAP) were carried out in cultured spinal cord neurons expressing Dendra2-tagged gephyrin or GlyRαs. Images were acquired on an inverted Nikon Eclipse Ti microscope equipped with a perfect focus system (Nikon, Tokyo, Japan), a 100× oil-immersion objective (Apochromat, NA 1.49; Nikon), a 1.5× magnifying lens, a module for focusing the laser beam (Ti-FRAP, spot size ~1 μm2; Nikon), and an EMCCD camera (iXon Ultra, 512 × 512 pixels; Andor Technology, Belfast, Northern Ireland, UK). For widefield imaging, neurons were illuminated with a Solis-1C LED lamp (set at 1000 mA; Thorlabs, Newton, NJ) using specific excitation band-pass filters (485/20, 560/25, 650/13 nm; Semrock, Rochester, NY), a multiband dichroic mirror (410/504/582/669 nm), and the appropriate emission filters (440/40, 525/30, 607/36, and 684/24 nm). Photoconversion of Dendra2-gephyrin and Dendra2-GlyRα1 was done with a 405 nm laser (Obis, 120 mW; Coherent, Santa Clara, CA). Alternatively, GlyRs that were immuno-immobilized and labeled with A488 (data in Fig. 2) were photobleached using a 488 nm laser (Obis, 150 mW; Coherent). The intensity of the laser pulse was controlled by an acousto-optic tunable filter and introduced through an optical fiber via the upper filter turret of the microscope using a multiband dichroic mirror (405/488/543/635 nm) that was positioned in the light path only during laser illumination.

Photoconversion and acquisition parameters for time-lapse imaging were set in fixed neurons expressing Dendra2-gephyrin (Fig. S2). Images were acquired with NIS Elements software (Nikon) according to the following sequence: a single image was taken in the far-red channel, followed by three pairs of images every 10 s in the red and the green channels (baseline before FRAP or FDAP). Then, the 405 or 488 nm laser pulse was applied, after which a further 16 images were taken at regular intervals (10 s in fixed neurons) in the red and the green channels, followed by one final image in the far-red channel. In live-cell experiments, time-lapse images in the green and the red channels were acquired every 2 min or every 15 s with 200 ms exposure using a neutral density filter (ND 8) to obtain the best compromise between image quality, temporal resolution, and bleaching. Photobleaching in fixed samples was below 0.4% per acquired image in the green channel before FRAP or FDAP, and no further bleaching was detected after photoconversion in both channels throughout the recording (Fig. S2). The laser intensity was adjusted to maximize the rate of photoconversion. Application of a single 405 nm pulse (5% of the maximal laser output, 500 ms) reduced the average intensity of the green fluorescence in the targeted area (~1 μm2 spot) close to background levels (~50% of the baseline before FRAP or FDAP) while producing large gains in red fluorescence (~300 arbitrary units (au) above background in fixed samples). In the GlyR cross-linking experiments with A488-conjugated secondary antibodies, the dyes were bleached with a single pulse of a 488 nm laser (20% intensity, 1 s).
Image processing and data analysis

FRAP and FDAP image stacks were separated by channel. Seven areas of 9 × 9 pixels (106 nm pixel size, i.e., squares of ~1 μm²) were chosen in the green channel: one covering the synapse that was targeted by photoconversion (FRAP or FDAP), three synaptic puncta close to the center of the image (near controls), and three synaptic puncta far from the center (far controls). Two additional zones of variable dimensions were defined: one on the soma or on a segment of dendrite as a measure of the diffuse level of fluorescence of the neuron (background) and another outside the cell to determine the nonspecific fluorescence (offset). The selected points were tracked automatically using Openview software (Noam Ziv, Technion, Israel Institute of Technology), applying a manual correction if the position of the spot was lost after photoconversion. The average fluorescence intensity of the tracked spots (9 × 9 pixels) was measured in each channel (Fig. S2).

Data curation of the live Dendra2-gephyrin experiments

To homogenize the distribution of the initial fluorescence of the selected FRAP or FDAP spots between control and immuno-immobilized conditions, we excluded outliers in the control condition for which the average intensity in the green channel before FRAP or FDAP exceeded 3000 au (the maximal pre-FRAP or FDAP intensity observed in the immuno-immobilized condition was 2490 au). This is equivalent to requiring that intensities lie within 3.5 median absolute deviations of the median for the control condition, and 5 of 57 recordings were rejected based on this criterion. We furthermore excluded recordings in which the application of the FRAP or FDAP laser pulse did not produce a significant drop in fluorescence in the green channel. Specifically, we required that the intensity drop relative to the average pre-FRAP or FDAP intensity exceeded the baseline fluctuations of fluorescence intensity by a factor of four, with the size of the fluctuations being quantified by the standard deviation (SD) of the intensity measured in the three images taken before the pulse. In the control (immuno-immobilized) condition, this led to the exclusion of another four (two) recordings.

FRAP data analysis

In a pilot experiment with Dendra2-gephyrin in living neurons, we observed that low-intensity 405 nm light triggered a slight increase of the green fluorescence intensity (Fig. S3), a behavior that had not been seen in fixed samples (Figs. S1 and S2). This photochromism was factored out by normalizing the recovery data with the near control puncta. We applied the following normalization procedure: first, the intensity $I_{raw}(t)$ of each
FRAP spot was corrected by a multiplicative factor $n(t)$ that accounts for the time-dependent overactivation of the near control puncta, and was determined as the average intensity of all near control puncta at time $t$ divided by the average intensity of all near control puncta in the three images taken before FRAP. The corrected intensity $I_{\text{corr}}(t) = I_{\text{raw}}(t)/n(t)$ was then normalized and rescaled relative to its pre-FRAP average $I_{\text{pre}}$ and immediate post-FRAP value $I_0$ according to $I_{\text{norm}}(t) = (I(t) - I_{\text{pre}})/(I_0 - I_{\text{pre}})$. We then used a two-parameter exponential fit to characterize the observed FRAP dynamics (see Statistics and fitting).

**FDAP data analysis**

We normalized and rescaled the fluorescence intensity $I(t)$ in the red channel to its pre-FDAP average $I_{\text{pre}}$ and immediate post-FDAP value $I_0$ according to $I_{\text{norm}}(t) = (I(t) - I_{\text{pre}})/(I_0 - I_{\text{pre}})$. The intensity of the converted spot in the first image after the 405 nm pulse was $\sim$15% higher than in all subsequent images, both in fixed and live samples, a likely consequence of 560/24 nm excitation of newly converted Dendra2 fluorophores. We accounted for this overactivation by including an additional offset $f_{\text{off}}$ in the exponential fit of the FDAP dynamics (see below).

**Combination of FRAP and FDAP data**

In the stationary state and in the absence of imaging artifacts, nonlinearities etc., the average, normalized FRAP signal should follow the same dynamics as the average, normalized FDAP signal, with $\text{FDAP}_{\text{theoretical}}(t) = 1 - \text{FRAP}_{\text{theoretical}}(t)$. To make use of both FRAP and FDAP data in our theoretical model, we therefore fitted our model (Fig. 5) to averages of the FRAP and FDAP traces, in which we corrected the FDAP data for the overactivation at $t = 0$ min using the offset that best fitted the experimental data (see below): $\text{FRAP}_{\text{data,combined}}(t) = [\text{FRAP}_{\text{data}}(t) + 1 - \text{FDAP}_{\text{data,corr}}(t)]/2$, with $\text{FDAP}_{\text{data,corr}}(t) = \text{FDAP}_{\text{data}}(t)(1 - f_{\text{off}})$.

**Statistics and fitting**

Data are given in mean ± SD or standard error of the mean (SEM) as indicated. Pairwise comparison of intensity data of synaptic puncta (Fig. 4) was done using a nonparametric Mann-Whitney U-test (two-tailed).

Fits were performed in Python using the curvefit routine from the scipy.optimize module, a standard implementation of the least-sum-of-squares fit routine. To extract characteristic timescales of fluorescence recovery and decay as well as the associated stable fractions, FRAP and FDAP curves, respectively, were fitted with the following functions: for normalized FRAP intensity, we used $\text{FRAP}(t) = (1 - f_{\text{stable}}) \left(1 - e^{-t/\tau}\right)$, with characteristic time constant $\tau$ and stable fraction $f_{\text{stable}}$, whereas for normalized FDAP intensity, we introduced an additional offset $f_{\text{off}}$ to account for the observed overactivation in the first time frame after photoconversion and used $\text{FDAP}(t) = (1 - f_{\text{off}}) [f_{\text{stable}} + (1 - f_{\text{stable}}) e^{-t/\tau}]$.

**RESULTS**

**Exchange kinetics of GlyRs at synapses in spinal cord neurons**

An experimental FRAP and FDAP protocol was established to simultaneously quantify the recruitment and the loss of GlyRs or gephyrin at inhibitory synapses (see Materials and methods; Figs. S2 and S3). Receptors and scaffold proteins were tagged with the photoconvertible fluorophore Dendra2 and expressed in cultured spinal cord neurons using lentivirus infection. Dendra2 was photoconverted with a 405 nm laser focused on a single synaptic punctum, followed by time-lapse imaging to record the FRAP of the green (nonconverted) fluorescence over 30 min. Concurrently, we measured the FDAP in the red channel (photoconverted Dendra2) as an additional readout of the protein dynamics.

In living neurons, the GlyR-associated fluorescence of the bleached puncta recovered from close to background levels at $t = 0$ to $\sim$60% of its baseline value after 30 min (Fig. 1). We noticed that the Dendra2-GlyRα1 signals in the area surrounding the bleached punctum increased after the 405 nm pulse because of the low-intensity halo of the laser. The increase was much less pronounced at remote areas (far controls). To compensate for this photochromatic effect of Dendra2, the intensity data were normalized using control puncta in the proximity of the bleached spot (near controls; see Materials and methods for FRAP data analysis).
As expected, the red fluorescence of photoconverted Dendra2-GlyR at synaptic puncta decreased in parallel to the recovery of the green fluorescence (Fig. 1). The loss of fluorescence was similar to the rate of recovery, falling to \(\frac{1}{34}\) of the initial value after 30 min. Control puncta that were near the site of photoconversion also showed a slight increase in fluorescence in response to the 405 nm laser pulse, confirming that stray light can affect the fluorophores despite its low intensity. FDAP intensity traces were normalized and rescaled as described in the Materials and methods.

Our pilot experiments had shown that the recovery and the loss of Dendra2-gephyrin was roughly on the order of 50% after 30 min (Fig. S3). In other words, GlyR and gephyrin populations exchange on a similar timescale, which we thought could be an indication that the two components enter and exit synapses jointly in the form of GlyR-gephyrin complexes, consistent with earlier hypotheses (10,19). We reasoned that if this were true, the immobilization of the GlyRs should reduce the exchange rate of gephyrin at synapses.

**GlyR immuno-immobilization**

To interfere with the mobility of the GlyRs, we decided to cross-link the cell surface receptors using specific antibodies against the \(\alpha_1\)-subunit of the GlyR. Antibody cross-linking has been previously shown to fully block the lateral diffusion of neurotransmitter receptors at excitatory synapses (23). Using the same approach, spinal cord neuron cultures were treated for 10 min with high concentrations of primary antibodies against GlyRo1, followed by a 10 min application of Alexa Fluor 488 (A488)-
conjugated secondary antibodies. Because the Dendra2 fluorophore can mask the GlyRα1 epitope (Supporting material in (7)), these experiments were performed on naive neurons expressing endogenous GlyRs. FRAP was then carried out on the A488 dyes attached to the cross-linked endogenous GlyRs using a 488 nm laser (Fig. 2).

Antibody binding blocked the fluorescence recovery at GlyR puncta almost entirely. In absolute terms, the fluorescence intensity after bleaching of the A488 dyes remained at background levels throughout the recordings (Fig. 2). After normalization of the data, a minor recovery could be discerned; however, this is likely the result of the more pronounced photobleaching of the A488 fluorophores during image acquisition (control puncta). Nonetheless, it can be concluded that cross-linking had a dramatic effect on the mobility of endogenous GlyRs when compared with the exchange rates of recombinant Dendra2-GlyRα1 containing complexes under control conditions (Fig. 1). We did not observe any obvious differences in the subcellular distribution of GlyRs after cross-linking, as judged by immunocytochemistry using the vesicular inhibitory amino acid transporter as presynaptic marker (Fig. S4). Both endogenous GlyRs and Dendra2-gephyrin showed extensive colocalization with vesicular inhibitory amino acid transporter in the control condition as well as after immuno-immobilization.

Effects of GlyR immuno-immobilization on the exchange kinetics of gephyrin

Having demonstrated the efficacy of GlyR cross-linking, we examined the consequences of receptor immuno-immobilization (IMMO) on the dynamics of the synaptic gephyrin scaffold. To do so, we performed FRAP and FDAP experiments in spinal cord neurons expressing Dendra2-gephyrin. Endogenous GlyRs were immuno-immobilized as before using primary GlyRα1 antibody and A647-conjugated secondary antibody. Control neurons were treated in the same way in these experiments, except that the antibodies were omitted during the incubation in Tyrode solution (Figs. 3 and S5).

The recovery of the green Dendra2-gephyrin fluorescence was substantially reduced after cross-linking of the receptors compared to the control condition (44% vs. 70% of the baseline after 30 min; Fig. 3). The normalized FRAP curves recorded over a period of 30 min were fitted with two free parameters, a time constant \( t \) and a weighing factor \( f \) that describes the fraction of fluorophores in the stable pool. Interestingly, fitting of the FRAP data showed that the time constant of the recovery was not significantly different between the two conditions (\( t_{\text{CTRL}} = 16.8 \pm 1.2 \, \text{min} \), \( t_{\text{IMMO}} = 16.1 \pm 1.8 \, \text{min} \); 95% confidence interval reported from fit routine), but that the stable fraction of Dendra2-gephyrin was increased by GlyR cross-linking from 0.15 to 0.5. These observations were confirmed by the decay of the red fluorescence (FDAP). Again, immuno-immobilization increased the fraction of stable fluorescence (\( f_{\text{CTRL}} = 0.41 \pm 0.01 \), \( f_{\text{IMMO}} = 0.56 \pm 0.02 \)) but had only a minor

FIGURE 4 Cluster intensities after GlyR immuno-immobilization. (A) Time-lapse images of Dendra2-gephyrin in the control condition taken at the beginning (before photoconversion) and the end of the 30 min FRAP recordings (lower panels, green channel) are shown. The upper panels show the empty far-red channel (control condition without GlyR cross-linking). (B) Time-lapse images of immuno-immobilized GlyRs (top panels, far-red channel) and Dendra2-gephyrin (bottom, green channel) taken at the beginning (before FRAP or FDAP) and at the end of the recording (30 min) are shown. (C) Quantification of the average fluorescence intensity of A647-GlyRα1 at identified synaptic puncta at the beginning and at the end of the FRAP recordings is given (IMMO condition, \( n_{\text{before}} = 336, n_{30 \, \text{min}} = 336 \) from 54 cells, mean ± SEM). (D) Quantification of all Dendra2-gephyrin puncta in control and immuno-immobilized conditions at the beginning and at the end of the FRAP recording is given (\( n_{\text{CTRL}, \text{before}} = 3031, n_{\text{IMMO}, \text{before}} = 3318, n_{\text{CTRL, 30 \, \text{min}}} = 3261 \) from 56 fields of view; \( n_{\text{IMMO, 30 \, \text{min}}} = 3640 \) from 57 fields of view, mean ± SEM).
effect on the decay rate ($\tau_{\text{CTRL}} = 10.3 \pm 0.9 \text{ min}, \tau_{\text{IMMO}} = 13.5 \pm 2.0 \text{ min}$). It should be noted that the intensity in the red channel at the first time point after photoconversion was systematically higher than in the subsequent images, which is why we fitted an additional offset correcting for the over-activation at $t = 0$ (see Materials and methods).

To exclude the existence of a faster component that was not captured with a 2 min acquisition frequency, we conducted another series of FRAP and FDAP experiments with a higher acquisition rate (15 s over a period of 4 min). The Dendra2-gephyrin intensity showed only a small recovery and decay on this timescale. Moreover, the data could be approximated with a linear fit, indicating that no sizeable fast component of exchange was present (Fig. S5). However, there was a trend that the gephyrin exchange was reduced by GlyR immuno-immobilization (recovery slope $a_{\text{CTRL}} = 0.067 \pm 0.004 \text{ min}^{-1}, a_{\text{IMMO}} = 0.055 \pm 0.003 \text{ min}^{-1}$).

The fit of the FRAP and FDAP data recorded over a period of 30 min with two parameters ($r, f_{\text{stable}}$) implies that there is a seemingly immobile fraction of gephyrin that does not exchange with the extrasynaptic pool on this timescale. The fact that immuno-immobilization increases this immobile fraction suggests that the stable gephyrin...
population is dependent on receptor-scaffold interactions at synapses. In other words, it is possible that immobile GlyRs provide stable high-affinity binding sites for gephyrin at inhibitory synapses.

Effects of GlyR immuno-immobilization on synaptic receptor and gephyrin levels

Given that the size of synaptic gephyrin domains is thought to depend on the balance between GlyR-mediated diffusion and capture of extrasynaptic GlyR-gephyrin complexes and the loss of synaptic GlyR into the cytoplasm by desorption (19), we asked whether GlyR and gephyrin levels would be affected by immuno-immobilization of the receptor. We therefore compared the intensities of synaptic puncta at the beginning (before FRAP or FDAP) and at the end of our recordings ($t = 30 \text{ min}$; Fig. 4). We first verified that immuno-immobilization did not change synaptic GlyR levels, indicating that a stationary state was reached at the end of the treatment protocol. Indeed, the intensity of cross-linked GlyR puncta remained stable throughout the recording ($I_{\text{pre}} = 1206 \pm 911$ and $I_{\text{postmin}} = 1131 \pm 961$ au, mean $\pm$ SD, $n = 336$ from 54 cells, MW test $p = 0.114$; Fig. 4B). We then measured the average intensities of all clearly identifiable gephyrin puncta in the CTRL and IMMO conditions at both time points. The average intensity of gephyrin puncta was not different between the two conditions, suggesting that synaptic size is not dependent on GlyR-mediated gephyrin dynamics on the timescale of the experiment. The average intensity of synaptic puncta at the start of the FRAP/FDAP acquisition (approximately 5–15 min after immuno-immobilization) was $I_{\text{CTRL}} = 1279 \pm 825$ and $I_{\text{IMMO}} = 1240 \pm 737$ au ($n_{\text{CTRL}} = 3031$, $n_{\text{IMMO}} = 3318$, MW $p = 0.034$). At the end of the recordings (approximately 40–55 min after treatment), the average intensities were $I_{\text{CTRL}} = 1322 \pm 933$ and $I_{\text{IMMO}} = 1331 \pm 1059$ au ($n_{\text{CTRL}} = 3261$, $n_{\text{IMMO}} = 3640$, MW $p = 0.48$).

Model of receptor and scaffold dynamics at synaptic complexes

To integrate the different experimental data and to gain further insight into the interdependent kinetics of synaptic GlyRs and gephyrin, we devised a simple model of receptor-scaffold dynamics at inhibitory synapses (Fig. 5; Supporting materials and methods). For simplicity, we consider only three species or molecular states at the synapse: receptors $r$ that are diffusing and/or transiently attached to loosely interacting scaffold proteins $s$ and a population of more tightly bound receptor-scaffold complexes $c$.

The dynamics of receptor and scaffold populations arise from transitions between the states $r$, $s$, and $c$ and incoming as well as outgoing protein fluxes (Fig. 5A). Loosely bound receptors $r$ and scaffolds $s$ exchange with extrasynaptic pools, where receptors enter the synapse with a flux $J_{\text{on}}$ and exit with a rate $J_{\text{off}}$ into the extrasynaptic membrane. Scaffolds are recruited from and exit into the cytoplasm with an influx $K_{\text{on}}$ and at a rate $K_{\text{off}}$, respectively. In addition, extrasynaptic receptor-scaffold complexes enter into the synapse with a flux $G_{\text{on}}$, and individual receptor-scaffold complexes are released into the extrasynaptic membrane with a rate $g_{\text{off}}$. Inside the synapse, loosely bound receptors and scaffolds can form a more tightly cross-linked state $c$ with an effective binding rate $k_b$; inversely, more stable receptor-scaffold complexes in state $c$ can give way to loosely bound receptors $r$ and scaffolds $s$ with an effective unbinding rate $k_u$. For this reaction, we assume a fixed stoichiometry $\alpha = 1.5$ between receptors and scaffolds in state $c$ based on the oligomerization properties of GlyRs (containing two $\beta$ subunits) and gephyrin trimers (7); see Supporting materials and methods. In principle, the model is then completely characterized by the eight parameters $J_{\text{on}}, J_{\text{off}}, K_{\text{on}}, K_{\text{off}}, G_{\text{on}}$, $g_{\text{off}}, k_b$, and $k_u$. At the stationary state, receptor and scaffold in- and effluxes balance each other, which allows to determine the stationary values of all considered populations as a function of the model parameters (Supporting materials and methods). (We denote stationary values by an asterisk below.) By normalizing receptor and scaffold amounts by the total amount of scaffolds at the synapse, we can express $K_{\text{on}}$ as a function of all other parameters.

We mimicked the immuno-immobilization protocol by setting all fluxes involving receptors to zero because they cannot enter or exit from the synapse in the immobilized condition. Because we cannot exclude that antibody-mediated cross-linking of diffusing or loosely bound receptors in state $r$ affects their synaptic organization, we furthermore admit that a fraction $f$ of these receptors eventually end up in the highly interacting receptor-scaffold complex $c$. We present here the results covering the complete range $0 \leq f \leq 1$ (Fig. 5, D–F). Based on the experimental observation that the amount of scaffold proteins does not change among conditions, we require that the total amount of scaffolds $S = s + c$ is constant, which imposes an additional constraint on the parameters and reduces the number of free parameters to six (see Supporting materials and methods).

Our model then allows us to predict the time course of FRAP and FDAP experiments for receptors in the CTRL and scaffold proteins in the CTRL and IMMO conditions as a function of the model parameters (see Supporting materials and methods). To determine the parameters of the model, we tried and fitted the predicted time courses to the experimentally obtained time courses (Fig. 5, B and C for $f = 0$). Based on these fits, we can quantify the total amount of synaptic receptors ($R$) as well as the different contributions of loosely bound ($r, s$) or more strongly interacting ($c$) receptor and scaffold protein populations (Fig. 5, D and E; Fig. II in Supporting materials and methods). Our model predicts the ratio of receptors/scaffold proteins to be 0.8–1.1 (Fig. 5 D), which corresponds to a ratio of 0.3–0.4 pentameric GlyRs per gephyrin monomer because we take
gephyrin trimers to be the basic unit of scaffold proteins in the model. Furthermore, the model suggests that in the control condition, only a small fraction (~30%) of receptors are loosely bound and exchange with the extrasynaptic membrane, whereas the majority of receptors exist in the stable state (Fig. 5 D). According to the model, scaffold proteins are more equally distributed between the loosely bound and the stable states (Fig. 5 E).

We can furthermore ask what the respective fluxes of receptors and scaffolds exiting and entering the synapse are, as well as the fluxes related to binding and unbinding of receptors \( r \) and scaffolds \( s \) into the more stable complex \( c \) (Fig. 5 F). Our model suggests that the lateral influx \( G_{on} \) of receptor-scaffold complexes is similar to the influx \( J_{on} \) of receptors that enter the synapse without a scaffold protein attached, and the same holds for the exiting fluxes \( j_{off} \) and \( g_{off} \), respectively. The cytoplasmic recruitment \( K_{on} \) of scaffold proteins tends to outweigh the lateral receptor-mediated scaffold influx \( G_{on} \); the loss \( k_{off} \) of scaffolds to the cytoplasm is equal to \( K_{off} \), if not somewhat larger. The exchange between the loosely bound states \( r \) and \( s \) and the highly interconnected state \( c \) is considerably slower than all receptor and scaffold exchanges with extrasynaptic pools.

Whereas for \( f = 0 \), all fluxes are individually balanced (i.e., \( J_{on} = j_{off} \), \( G_{on} = g_{off} \), etc.; see Supporting materials and methods), we cannot exclude that there is a net influx of scaffold proteins arriving in the form of GlyR-gephyrin complexes by lateral membrane diffusion, as we find \( G_{on} > g_{off} \) for \( f > 0 \) (Fig. 5 F; Fig. III in Supporting materials and methods). A net influx would violate detailed balance and thus imply a departure from thermodynamic equilibrium; the synapse would be in a nonequilibrium stationary state. Our model shows, however, that the cytoplasmic recruitment of scaffold proteins at the synapse contributes significantly in all cases to the renewal of synaptic scaffolds in the control condition, and any departure from thermodynamic equilibrium would supposedly be minor.

**DISCUSSION**

Our data disclose the reciprocity of receptor and scaffold protein dynamics and clustering at glycinergic spinal cord synapses, mediated by strong interactions between the GlyR \( \beta \)-subunit and the synaptic scaffold protein gephyrin. A biophysical model of our data identified different degrees of receptor stability at synapses: on the one hand, a more loosely interacting population of receptors and scaffold proteins, and on the other hand, a more a tightly complexed receptor-scaffold network.

**FRAP and FDAP of gephyrin and GlyRs**

To determine the dynamic behavior of gephyrin and GlyRs in cultured spinal cord neurons, we established an analytical protocol based on the photoconversion and time-lapse imaging of fluorescently tagged recombinant proteins at synapses. The photoconvertible fluorophore Dendra2 was chosen for our experiments because it displays less photochromism in the green channel in response to near-ultraviolet illumination as opposed to mEos4b (24). Local reference puncta (near control points) were used to correct photochromic effects and the photobleaching during image acquisition.

There is some evidence that the overexpression of recombinant GlyRs and gephyrin does not substantially alter the copy numbers at synapses (7,25). In the case of the receptor, synaptic targeting is strictly dependent on the assembly of Dendra2-GlyRz1 with the endogenous \( \beta \)-subunit. This suggests that the overexpression of recombinant GlyRz1 replaces the majority of the endogenous \( \alpha \)-subunits without changing the absolute numbers at synapses. Even though it cannot be entirely ruled out that the overexpression and fluorescent tagging may have some impact on the synaptic structure, the synapses have most likely reached a steady state by the time the FRAP or FDAP experiments are carried out, given that lentiviral infection was done several days before synaptogenesis.

Our experimental results are largely consistent with earlier studies of the population dynamics of gephyrin and GlyRs. For instance, synaptic puncta of transfected Venus-gephyrin and mRFP-gephyrin, as well as endogenous (knock-in) mRFP-gephyrin in cultured spinal cord neurons, recover to ~40% of their initial fluorescence within 30 min (15). A relatively broad distribution of time constants on the order of hours was determined for synaptic Dendra2-GlyRz1 in motoneurons of transgenic zebrafish larvae (26). It is noteworthy that the FRAP and FDAP traces in our experiments were fitted with a single exponential recovery and decay, alongside a much slower component that we considered as stable within the duration of our recordings. It is therefore expected that the characteristic timescales obtained from our fits are faster than the respective timescales of a complete recovery.

In organotypic hippocampal slices, the rate of recovery of endogenous EGFP-gephyrin clusters was shown to be highly variable (27). In addition to a subtle size dependence of the exchange rate, the authors observed a strong developmental stabilization of gephyrin. However, these data are not directly comparable with our situation because inhibitory synapses in the hippocampus are overwhelmingly GABAergic and likely follow different clustering mechanisms (1).

The effect of receptor immuno-immobilization on scaffold protein dynamics

On a purely qualitative level, our data reveal that GlyR cross-linking reduces the exchange of Dendra2-gephyrin at synapses. A possible explanation could be that the dynamics of gephyrin depends to a certain extent on the entry and exit of
GlyR-gephyrin complexes at inhibitory synapses. This is due to the fact that the GlyRβ-gephyrin interaction is remarkably stable, allowing extrasynaptic GlyR-gephyrin complexes to integrate into the synaptic scaffold via multiple interaction sites (GlyR-gephyrin and gephyrin-gephyrin) (1). At excitatory synapses, immuno-immobilization of AMPA receptors did not produce a slowdown of the exchange rate of the scaffold protein βSAP97 (23), which is consistent with a role of βSAP97 in the forward trafficking of AMPA receptors to the plasma membrane but not their integration into the synaptic membrane (28).

Another possible explanation would link the reduction of gephyrin exchange to the cross-linking and immobilization of the synaptic GlyR population. In this scenario, immobile GlyRs form stable interactions with synaptic gephyrin molecules that are thus prevented from exchanging with extrasynaptic pools. In line with this interpretation, GlyR cross-linking did not change the steady state level of gephyrin at synapses, pointing to a mutual stabilization between receptors and scaffold proteins at the synapse.

A model of reciprocal GlyR-gephyrin stabilization

Based on our experimental observations, we aimed to develop a biophysical model that would provide quantitative insight into the synaptic organization and dynamics of GlyRs and gephyrin molecules beyond the apparent stable fractions and characteristic timescales. We propose a model in which the extrasynaptic receptor and scaffold pools exchange with loosely bound populations at synapses, which are in turn in a dynamic equilibrium with a tightly interacting receptor-scaffold complex. This simple model is sufficient to account for all experimental FRAP and FDAP curves, in which the cross-linking of GlyRs is mimicked by a suppression of all GlyR-associated fluxes.

Interestingly, our model does not require the existence of a fully stable component of synaptic GlyR or gephyrin, as one could infer from the heuristic fits of a single exponential decay to our experimental data. The model instead suggests that the observed dynamics arise from the interplay between the fast exchange of loosely bound synaptic populations and extrasynaptic pools on the one hand and their slow exchange with the tightly interacting synaptic receptor-scaffold complex on the other hand. The existence of a synaptic component with a higher degree of stabilization had been previously proposed based on a large fraction of GlyRs that do not appear to swap between synaptic and extrasynaptic locations on a timescale of minutes (10). To what extent the stable synaptic population relies solely on receptor-scaffold interactions or depends on additional factors such as gephyrin palmitoylation or binding to adhesion proteins cannot be decided at this stage (1).

Although in the model all receptor and scaffold quantities are expressed in terms of the total amount of scaffold proteins, we can obtain an estimate of the copy numbers of synaptic GlyRs from the typical number of synaptic gephyrin trimers. If we assume the latter to be ~100 (corresponding to ~300 gephyrin monomers/synapse on average), the model predicts the number of synaptic GlyRs to be of the order of 80–110, closely matching earlier experimental results (7). A majority of these (~70%) interact tightly with synaptic gephyrin, whereas the remainder are more loosely bound and exchange with extrasynaptic pools. We can furthermore obtain estimates of the number of extrasynaptic GlyRs and gephyrin proteins that enter the synapse per unit of time: for GlyRs, the flux amounts to ~5 GlyRs/min, and we obtain a slightly larger value for gephyrin with ~6 gephyrin trimers/min.

Single-molecule dynamics predicted by our model

Although our model relies on a coarse-grained description of receptor-scaffold dynamics at synapses, it allows predictions about single-molecule dynamics that go beyond the scope of this study. The existence of a small receptor population that exchanges with the extrasynaptic pool on a fast timescale implies that individual receptor dwell times can be considerably shorter than the typical time constant of GlyRs observed in our FRAP experiments (Fig. 6). Our model predicts that a considerable fraction (10–30%) of GlyRs leave the synapse in less than a minute, as opposed to a fluorescence recovery of comparable size in up to 10 min. The prediction of short receptor dwell times is consistent with the literature, in which very short timescales (<40 s), a distinction between swapping (exchanging) and stable receptors has been made and for which typical dwell times for the swapping population have been determined (e.g., (29)). However, a more detailed quantitative comparison with single-molecule data is hampered by experimental limitations such as the size of quantum dots (30) or the

FIGURE 6 Predicted dwell times of receptors and scaffolds after entry into the synapse. Although the characteristic FRAP and FDAP timescales of both receptors (blue) and scaffold proteins (orange) are similar and on the order of tens of minutes (Figs. 1 and 3), a sizable proportion of receptors have dwell times below a minute, as opposed to those of scaffold proteins (inset). Shaded bands indicate the range of values obtained for 0 ≤ f ≤ 1; solid and dashed lines represent the predicted dwell times for f = 0 and f = 1, respectively.
insufficient localization precision of single-molecule diffusion data (31).

Relation to previous models of receptor-scaffold organization at inhibitory synapses

Earlier modeling studies of receptor-scaffold organization at inhibitory synapses did not explicitly address the exchange kinetics of synaptic GlyRs and gephyrin, but we can try to assess the consequences of GlyR immobilization for each of them. In a recent study, we hypothesized that the size of the postsynaptic domain is maintained by the recruitment of extrasynaptic GlyR-gephyrin complexes (here denoted by $G_{on}$) that replace gephyrin molecules that are lost because of desorption ($k_{off} s^*$) (19). In our model, we considered that these fluxes dominated the lateral outward flux of scaffolding proteins bound to receptors ($g_{off} r^s$) as well as the incoming flux from the cytoplasm ($K_{on}$). In this limit, the resulting nonequilibrium stationary state depends on the diffusion of GlyR-gephyrin complexes in the extrasynaptic membrane; the immobilization of GlyRs should therefore lead to the depletion of synaptic gephyrin. This size decrease is not seen on the timescale of our experiments (Fig. 4). The incoming lateral flux $G_{on}$ and the outgoing flux $k_{off} s^*$ determined in this work are comparable with what we estimated earlier (19). However, the receptor-mediated lateral efflux $g_{off} r^s$ is found to be comparable with the corresponding influx $G_{on}$, and similarly, the scaffold influx from the cytoplasm ($K_{on}$) is found to compensate the losses due to scaffold desorption into the cytoplasm ($k_{off} s^*$), as would be expected if the postsynaptic domain was a structure at or close to thermodynamic equilibrium.

Our data, therefore, strongly suggest that the size of the synaptic gephyrin domain is determined by processes other than the simple recruitment of GlyR-gephyrin complexes, although it cannot be ruled out that more GlyR-gephyrin complexes enter the synapse ($G_{on}$ in the model) than leave the synapse ($g_{off} r^s$) (Fig. 5). However, our findings support the conclusion that all lateral fluxes contribute to the synaptic dynamics because a model in which $G_{on}$ and $g_{off} r$ are not considered does not fit the experimental data satisfactorily (Appendix B in the Supporting materials and methods). It remains to be mechanistically understood how the interaction between receptors and scaffold proteins with different degrees of stabilization, as well as their interactions with other synaptic proteins, contribute to synaptic size regulation.

It appears less straightforward to interpret our results in light of the reaction-diffusion model proposed by Haselwandter and colleagues (17,21). The authors identified several key reactions necessary for the spontaneous formation of scaffold domains, notably the recruitment of cytoplasmic scaffold proteins and cytoplasmic receptors (exocytosis) by synaptic scaffold proteins. These reactions are limited by steric repulsion of proteins, and incoming cytoplasmic fluxes are balanced by diffusive fluxes of receptors and scaffolds into the extrasynaptic membrane. The effect of cross-linking of GlyRs crucially depends on how the various reactions are modified in this setting and cannot be a priori estimated from the model equations. Because the size of the domains is entirely dependent on the interplay of receptor and scaffold spatiotemporal dynamics, however, receptor immobilization should significantly affect scaffold domain size in this model, which is not supported by our data. The Turing instability proposed to be at the root of postsynaptic domain size determination is intrinsically a nonequilibrium phenomenon and also appears at odds with the (at least approximate) balance of individual fluxes, a hallmark of thermodynamic equilibrium.

In contrast, our experimental results and the proposed model are broadly consistent with the quasiequilibrium model of Sekimoto and Triller (18), who predicted that condensed phases of scaffolds and receptors would arise spontaneously when the interaction between the two is sufficiently strong (see also (32)). In this model, the size of the domains is externally controlled and the nucleation of the condensed phase constrained to the synaptic area by additional molecular interactions at the synapse, in line with the kinetic model presented here. Although this earlier study convincingly argued that the reciprocal stabilization of scaffolds and receptors may support the formation of stable postsynaptic domains, we present here a detailed, quantitative account of the underlying reaction kinetics, fluxes, and synaptic organization.

Other models of synaptic scaffold protein dynamics have been proposed that did not specifically address inhibitory synapses or the role of receptors. Shomar et al. (33) argued that both scaffold recruitment from and desorption of scaffolds into the cytoplasm are cooperative processes, which would allow one to explain the observed skewed distributions of synapse sizes (34). However, this model cannot account for the observed changes in the FRAP and FDAP traces after receptor immobilization, and it is not clear from our data that scaffold binding and unbinding have to be cooperative processes.

CONCLUSIONS

The quantitative analysis of excitatory and inhibitory synaptic size dynamics has received much attention in recent years (reviewed in (35)), and concomitant theoretical modeling ranged from very generic statistical (34) to more biophysical models of synaptic scaffold dynamics (19,33). However, an integrated account of synaptic size dynamics that takes into account both receptors and scaffold proteins has so far been lacking. Although the large number of different molecular players at synapses (e.g., (36–38)) precludes a microscopically detailed biophysical model involving all relevant species in the foreseeable future, our model with three distinct receptor and scaffold populations
is a first step toward a more comprehensive picture of glycinergic synapse dynamics. Although we did not explicitly address synaptic size fluctuations in this work because we restricted our analysis to the average dynamics with a stationary synaptic size, it would be straightforward to extend our model to account for molecular fluctuations.

In this work, we identified loosely bound GlyR and gephyrin scaffold populations that coexist with a stable receptor-scaffold complex at inhibitory synapses. It is tempting to speculate that these mobile and stable populations differentially contribute to the plasticity and the stability of glycinergic synapses. More generally, it will be interesting to explore to what extent similar descriptions may apply to excitatory synapses that are also stabilized by scaffold proteins interacting with mobile receptors.

**SUPPORTING MATERIAL**

Supporting Material can be found online at https://doi.org/10.1016/j.bpj.2021.01.024.

**AUTHOR CONTRIBUTIONS**

V.H., A.T., J.R., and C.G.S. designed the research. T.C. and C.G.S. planned and performed the experiments. T.C., J.R., and C.G.S. analyzed the data. J.R. developed and analyzed the model with input from all authors. T.C., J.R., and C.G.S. wrote the manuscript. All authors reviewed and edited the final manuscript.

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