Deciphering the conformational dynamics of gephyrin-mediated collybistin activation

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ABSTRACT Efficient neuronal signaling depends on the proper assembly of the postsynaptic neurotransmitter machinery. The majority of inhibitory synapses feature γ-aminobutyric acid type A (GABA_A) receptors. The function of these GABAergic synapses is controlled by the scaffolding protein gephyrin and collybistin, a Dbl family guanine nucleotide exchange factor and neuronal adaptor protein. Specifically, collybistin interacts with small GTPases, cell adhesion proteins, and phosphoinositides to recruit gephyrin and GABA_A receptors to postsynaptic membrane specializations. Collybistin usually contains an N-terminal SH3 domain and exists in closed/inactive or open/active states. Here, we elucidate the molecular basis of the gephyrin-collybistin interaction with newly designed collybistin Förster resonance energy transfer (FRET) sensors. Using fluorescence lifetime-based FRET measurements, we deduce the affinity of the gephyrin-collybistin complex, thereby confirming that the C-terminal dimer-forming E domain binds collybistin, an interaction that does not require E domain dimerization. Simulations based on fluorescence lifetime and sensor distance distributions reveal at least a two-state equilibrium of the SH3 domain already in the free/unbound collybistin, thereby illustrating the accessible volume of the SH3 domain. Finally, our data provide strong evidence for a tightly regulated collybistin-gephyrin interplay, where, unexpectedly, switching of collybistin from closed/inactive to open/active states is efficiently triggered by gephyrin.

WHY IT MATTERS Information processing in the mammalian brain heavily depends on the presynaptic release of neurotransmitters and their postsynaptic reception. Inhibitory signaling is primarily mediated by the neurotransmitter γ-aminobutyric acid (GABA), which is precisely sensed by cognate GABA type A (GABA_A) receptors. Postsynaptic plasma membrane GABA_A receptor clustering in apposition to presynaptic neurotransmitter release sites ensures GABA-induced postsynaptic membrane hyperpolarization and reduced excitability with impaired GABAergic signaling triggering numerous brain disorders. The proper assembly of the postsynaptic neurotransmitter machinery is regulated by the scaffolding protein gephyrin and the adaptor protein collybistin. This study highlights the molecular basis of the gephyrin-collybistin interplay and demonstrates that gephyrin activates collybistin by inducing a molecular transition from a closed to an open state.

INTRODUCTION

Inhibitory synaptic signaling in the mammalian brain heavily depends on the neurotransmitters γ-aminobutyric acid (GABA) and glycine, which are recognized by postsynaptic GABA type A (GABA_A) and glycine receptors, respectively. Postsynaptic GABA_A receptor clustering in direct apposition to the presynaptic transmitter release sites ensures fast signal transduction, inducing postsynaptic membrane hyperpolarization and reduced excitability (1–4). GABA_A receptor assembly and maintenance is coordinated by various postsynaptic neuronal factors. These core neuronal components (Fig. 1) include cell adhesion proteins of the neuroligin family, specifically neuroligin 2 (NL2) and NL4, the scaffolding protein gephyrin, and the adaptor protein collybistin (CB) (5,6).

Gephyrin serves as a prime scaffolding protein at inhibitory GABAergic and glycinegic postsynaptic specializations and is principally responsible for GABA_A and glycine
Gephyrin recruitment from intracellular deposits to postsynaptic membranes mainly depends on the adaptor protein CB (also referred to as ARHGEF9). As a member of the Dbl family of guanine nucleotide exchange factors (GEFs) it features tandem Dbl homology (DH) and pleckstrin homology (PH) domains (26). In addition to the DH domain catalyzing the GEF activity and the phosphoinositide-binding PH domain, most CB mRNAs encode an additional N-terminal src-homology 3 (SH3) domain (27). In rat, CB genes are expressed in the splice variants CB1, CB2, and CB3, which differ in their N- and C-termini, and the presence or absence of the SH3 domain (6,27,28). Structural and biochemical studies suggested that the most abundantly expressed, full-length, SH3 domain-containing CB isoform 2 (CB2-SH3^+), adopts a closed conformation wherein the N-terminal SH3 domain interacts intramolecularly with residues in the DH and PH domains, a conformation that does not interact with the cell division control protein 42 homolog (Cdc42), a CB-specific small GTPase (29,30). In contrast, the SH3 domain lacking CB2 splice variant (CB2-SH3^-) constitutively regenerates the GTP-bound state of Cdc42 (30–32). However, a recent study demonstrated that only TC10, another Dbl family GEF that is closely related to Cdc42 but not Cdc42, can effectively mediate the conformational activation of full-length CB (33). Interestingly, earlier biochemical and cellular studies (29,34) indicated that CB interaction with the cytosolic region of NL2 led to a similar activation of CB (Fig. 1).

Previous studies revealed that CB-mediated gephryin recruitment and clustering at the plasma membrane depend on the binding of its PH domain to phosphatidylinositol 3-phosphate, whereas the GEF activity of its DH domain is dispensable (31,35). Mutations causing a disruption of the CB interdomain association lead to an open/active (with respect to receptor anchoring) CB conformation with enhanced phosphatidylinositol affinity (29). In vivo experiments with CB-deficient mice indicated a reduction in synaptic gephryin and GABA_A receptor γ2-subunit clustering, decreased GABAergic synaptic transmission and impaired spatial learning (19). Surprisingly, CB-deficient mice do not exhibit deficits in glycinegic synaptic transmission, suggesting that CB is dispensable for gephryin-mediated glycine receptor clustering at glycinegic synapses, but is required for the clustering of certain GABA_A receptors (36,37), further stressing the vital role of CB in the initial assembly and maintenance of gephryin-GABA_A receptor clusters. However, owing to technical limitations, clear molecular insights into the association of CB and gephryin and the overall clustering process have been lacking. Although the CB-gephryin interaction has already been described in previous studies (28,32), no quantification of the interaction strength has been reported and the reciprocal

![FIGURE 1 Schematic description of the inhibitory signal transmission and postsynaptic architecture. The synapse is stabilized by interactions between the cell-adhesion molecules, neurexin and neurelin, present at the pre- and postsynaptic site, respectively. GABA and glycine released from the presynaptic neuron bind to their cognate receptors embedded in the postsynaptic membrane. The GABAA and glycine receptors are stabilized by the scaffolding protein gephyrin, which forms an extended structure underneath the postsynaptic membrane. Collybistin (CB) operates as an adaptor protein for gephyrin, which forms an extended structure underneath the postsynaptic membrane. Collybistin (CB) operates as an adaptor protein for gephyrin, which forms an extended structure underneath the postsynaptic membrane.](image-url)
regulation of the activities of both proteins remains poorly understood.

This study aims at elucidating the molecular basis of CB autoinhibition, its binding to gephyrin and whether this interaction leads to CB activation. We constructed novel intramolecular CB Förster resonance energy transfer (FRET) sensors to understand the conformational dynamics of CB by making use of picosecond-scale time-resolved fluorescence lifetime measurements. These studies confirmed that CB in isolation remains in a closed conformation while gephyrin binding takes place via its C-terminal E domain leading to an open conformation of CB. We quantified the interaction strength of gephyrin to wild-type and constitutively active, open-state mutant CB FRET sensors. Based on the interfluorophore distance distributions from the FRET sensors in the absence and presence of gephyrin, we modeled the overall three-dimensional conformational space accessible to CB with respect to the SH3 domain. Our data combined with simulation studies provide clear molecular evidence of gephyrin-mediated CB opening, thus suggesting a synergy between concurrent gephyrin scaffold assembly and CB targeting to the plasma membrane.

METHODS

Cloning and expression

The wild-type CB FRET sensor (F1D0) was constructed by inserting a tetra-cysteine motif (tCM) (38) after the first amino acid residue of the rat CB2-SH3+ splice variant, while CFP (39) was C-terminally attached (Figs. 1 b and S1 a) using restriction-free cloning (40) in pETM14 vector (Table S2). Amino acid replacements for the open-state mutant sensors (F1smD0 and F1sD0) were generated by site directed mutagenesis. Additional sensors were created by inserting tCM at the specified position (Fig. 1 b, Table S1). GephFL and the domain variants (GephG, GephE, and GephLE) were described previously (16,41,42). For the GephE monomeric mutant (GephEmm), residues 318–750 were subcloned into the IMPACT system vector pTYB12 (New England Biolabs, Ipswich, MA, USA) and point mutations were introduced by site-directed mutagenesis. The SH3 domain of CB was generated by subcloning the cDNA coding for residues 10–79 of rat CB2-SH3+ into the pETM14 vector. The intracellular cytosolic domain of NL2 (NL2icd) encompassing residues 700–836 was subcloned into the pETM11 vector.

All CB FRET sensors were expressed in the E. coli strain BL21 (DE3). Cell lysates were subjected to affinity chromatography on Protin Ni-IDA Resin (Macherey Nagel, Düren, Germany) equilibrated in buffer A (50 mM Tris-HCl [pH 8], 250 mM NaCl, 5% glycerol, and 5 mM β-mercaptoethanol). Samples were eluted using buffer A containing 300 mM imidazole and subsequently applied to a MonoQ 10/100GL column (Cytiva, Marlborough, MA, USA) and eluted using a linear NaCl gradient from 50 mM to 1 M NaCl. Finally, sensors were subjected to size-exclusion chromatography on a Superdex 200 column (GE Healthcare) and concentrated by ultrafiltration. GephFL and its domain variants (GephG and GephE) were purified as described before (16) with small modifications. GephE and its dimerization-deficient mutant (GephEmm) were initially subjected to affinity chromatography on chitin agarose beads (New England Biolabs), followed by ion-exchange chromatography and subsequent size-exclusion chromatography.

Circular dichroism spectroscopy

Circular dichroism (CD) spectroscopy was performed using a Jasco J-810 spectropolarimeter. Before measurements, the buffer was exchanged to phosphate-buffered saline (pH 8.0) using ultrafiltration (Sartorius Vivaspin 500, Götttingen, Germany). Far-UV CD spectra from 190 to 260 nm were recorded at a scanning speed of 50 nm/min with a response time of 1 s and a bandwidth of 1 nm. CD spectra were recorded repeatedly (n = 15) for each sample and averaged to optimize the signal/noise ratio. The buffer spectrum was also recorded and subtracted from the protein spectra. All measurements were conducted at room temperature.

In vitro FlAsH labeling

For FlAsH labeling (38,44), purified sensors, designated with the subscript D0, were first incubated at room temperature in labeling buffer (50 mM Tris-HCl [pH 8], 250 mM NaCl, and 5 mM β-mercaptoethanol). Afterward, FlAsH reagent (Cayman Chemicals, Ann Arbor, MI, USA) was added in a 10-fold molar excess to the sensors and the mixture was further incubated for 30 min at room temperature. Later, the mixture was dialyzed thoroughly against labeling buffer to remove unbound FlAsH, resulting in the corresponding DA fluorophore pair. Labeled sensors were flash frozen and stored at −80°C for later use.

Time-resolved setup and data acquisition

Time-resolved fluorescent measurements were conducted with a custom-built confocal microscope setup (IX 71, Olympus, Hamburg, Germany) equipped with a time-correlated single photon counting (TCSPC) system (Hydraharp 400, PicoQuant, Berlin, Germany) with data acquisition by the fluorescence lifetime correlation software SymPhoTime 64 (PicoQuant). The excitation laser (440 nm pulsed laser LDH-D-C-440, PicoQuant) was fiber coupled (Laser Combining Unit with polarization maintaining single mode fiber, PicoQuant) and expanded to a diameter of 7 mm by a telescope to fill the back aperture of the objective (60× water immersion, NA 1.2, Olympus), thus creating a diffraction-limited focal spot. Before entering the objective lens, the laser polarization was adjusted by an achromatic half-wave plate (AHWP05M-600, Thorlabs, Bergkirchen, Germany). A beam splitter (HC458 rp phase τ uf1, AHF) guided the laser through the objective, epi-illuminating the sample. In the detection path, a 100 μm pinhole (PNH-100, Newport, Darmstadt, Germany) rejected out of focus light before being projected on photon counting detectors (2× PMA Hybrid-40, PicoQuant) by a telescope in a 4f configuration (focal length of lenses: 60 mm; G06312600, Qioptiq, Rhy, UK). The beam was split via a polarization beam splitter cube (10FC16PB.3, Newport) into parallel (VV, detector 1) and perpendicular emission (VH, detector 2) after the first lens of the telescope. Emission filters (band pass filter Brightline HC 480/40 AHF, Tübingen, Germany) rejected unspecific light in each detection path. The laser was operated in pulsed mode at 20 MHz with a laser power at the sample of ~11 μW and the temporal resolution was set to 4 ps. Measurements with the CB FRET sensors (1 μM concentration) were performed on standard glass coverslips (Menzel-Gläser, Braunschweig, Germany; 24 × 40 mm, 1.5).

CB FRET sensors (F1D0/F1D1) were titrated with varying concentrations of gephyrin (or other ligands) in binding buffer (50 mM Tris-HCl [pH 8], 250 mM NaCl, 10 mM EDTA, and 5 mM β-mercaptoethanol) in
a final sample volume of 20 μL. Before data acquisition, samples were incubated overnight at 4 °C under dark light conditions. Samples were excited at 440 nm and the donor emission between 460 and 500 nm was recorded. Donor only (D0) and buffer solutions were measured as control samples and for background corrections. The data were acquired at room temperature for 5–10 min depending on photon counts. To determine the instrument response function, a K1-saturated solution of 3 μM fluorescein in double-distilled water was measured for 10–15 min. To determine the relative detection efficiency in the parallel to perpendicular channel, i.e., the g-factor, a 1 μM solution of Coumarin 343 was measured. Samples were measured in technical triplicates to calculate average and standard deviations for each condition.

**Time-resolved fluorescence decay analysis**

To accurately determine the interfluorophore distance distribution from the fluorescence intensity decays the magic angle intensity decay was determined based on the obtained g-factor from the VV and VH signals. Data were exported from the ptu Symphotime format into text files using the Jordi-tool of the Seidel-Software package (https://www.mpc.hhu.de/software/3-software-package-for-mfd-fcs-and-mfs). Here, in the text file VV and VH data are stacked as a single column. All data were exported in 16 ps bins, i.e., 4096 channels for each detector. Thus, the single-column text file contains 8192 channels in total with the first 4096 channels corresponding to the VV and the next 4096 channels to the VH decay. With a given g-factor, the analysis was done in the Chisurf software (45) (https://github.com/Fluorescence-Tools/chisurf) as described elsewhere (46). We have calculated the g-factor to 0.9 from the tail fitting of the calibration dye Coumarin 343. The decay curves were fitted with a multiexponential model function using an iterative reconvolution approach (46,47) as follows

\[ F(t) = \sum_{i} x_i e^{-t/\tau_i}, \]  

where \( x_i \) represents the species fraction, i.e., the fractional amplitude of this component to the total intensity amplitude with \( x_i = A_i / A_{total} \), and \( \tau_i \) the lifetime of the corresponding component. The species fractions are normalized such that \( \sum x_i = 1 \). Ideally, D0 should show a single component but, due to local quenching in donor-only samples, multiexponential decays were expected. The quality of the curve fitting was evaluated by the reduced \( \chi^2 \) values and the weighted residuals. Time-resolved fluorescence intensities for FLAsH-labeled (F1DA) and F1DA-ligand complexes (all gephrin variants, NL2_cdc and SH3) were also analyzed by Eq. 1 to obtain the species-weighted average fluorescence lifetime.

\[ \langle \tau \rangle_x = \sum_i x_i \tau_i, \]  

where

\[ \sum_i x_i = 1. \]  

**KD determination**

We titrated the F1DA sensor with different concentrations of GephFL (or other ligands) and measured the resulting time-resolved fluorescence intensities. The fractional saturation (%) at concentration \( i \) was determined based on the average fluorescence lifetime:

\[ \text{fractional saturation} (\%) = \frac{\langle \tau_{DA,i,M} \rangle - \langle \tau_{DA,0,M} \rangle}{\langle \tau_{DA,max} \rangle} \times 100, \]  

where \( \langle \tau_{DA,i,M} \rangle \) is the average fluorescence lifetime at concentration \( i \), \( \langle \tau_{DA,0,M} \rangle \) is the mean fluorescence lifetime of the FLAsH-labeled CB FRET sensor without addition of ligand, and \( \langle \tau_{DA,\max} \rangle \) is the longest mean fluorescence lifetime of the titration, usually obtained at the highest ligand concentration. The resulting data points were plotted against the concentration of ligand and fitted as follows (Origin9, OriginLab):

\[ f(x) = b + (a - b) \left( \frac{(c_x + K_D * x)}{2 * c_x} \right)^{1/6} \]

where \( x \) is the concentration, \( b \) the offset, \( a \), the final intensity, \( c_x \) the protein concentration, and \( K_D \) the dissociation constant.

**Förster distance calculation**

The Förster distance \( R_0 (\bar{A}) \) was calculated from the overlap integral of the emission spectrum of the donor and absorption spectrum of the acceptor from

\[ R_0 = \left[ \frac{9 \ln(10)}{128 \pi^2 \cdot N_A} \cdot \left( \frac{J(\lambda)}{\kappa^2} \cdot \phi_D \right)^{3/2} \right]^{1/6} \]

where \( \kappa^2 \) is a factor describing the relative orientation in space of the transition dipoles of the donor and the acceptor. The magnitude of \( \kappa^2 \) is assumed to be 0.66 for a random orientation of donor and acceptor. The refractive index (n) of the aqueous buffer is assumed to be 1.33. \( J(\lambda) \) is the overlap integral of emission of donor (CFP), and absorption (Fig. S1 c) of the acceptor (FLAsH) and calculated by

\[ J(\lambda) = \int_0^\infty I_D(\lambda) \epsilon(\lambda) \lambda^2 d\lambda / \int_0^\infty I_D(\lambda) d\lambda, \]

where \( I_D(\lambda) \) is the fluorescence emission of the donor in the wavelength region \( \lambda \) and \( \epsilon(\lambda) \) the extinction coefficient (M\(^{-1}\) cm\(^{-1}\)) of the acceptor FLAsH (41,000 M\(^{-1}\) cm\(^{-1}\) at 508 nm).

**Average FRET efficiency calculation**

The fluorescence lifetime values obtained from the TCSPC decays were used to calculate an average FRET efficiency (EFRET) using the following equation:

\[ E_{FRET} = 1 - \frac{\langle \tau_{DA} \rangle}{\langle \tau_{DA} \rangle}, \]  

where \( \langle \tau_{DA} \rangle \) and \( \langle \tau_{DA} \rangle \) are the species-weighted average fluorescence lifetimes in the absence (D0) and presence (DA) of FLAsH as calculated based on Eq. 2.
FRET distance distribution analysis

For distance distribution analysis for FlAsH-labeled (F1da) and F1da-ligand complexes we followed a method described earlier (46,47). The time-resolved fluorescence intensities of the FRET-sample and the donor-only reference sample are presented as:

\[ F_{\text{FRET}}(t) = N_0 \left[ (1 - x_{\text{FlAsH}}) F_{\text{DA}}(t) + x_{\text{FlAsH}} F_{\text{DD}}(t) \right] \]

\[ \otimes \text{IRF} + sc \cdot \text{IRF} + c \]

Here, \( sc \) is the scattered light from the sample, \( c \) is the constant offset of the fluorescence intensity, and \( N_0 \) is the total photon number. \( x_{\text{FlAsH}} \) is the no-FRET contribution from the unquenched donor. As stated earlier, we obtained multieponential fitting for the donor-only sample due to local quenching; however, the local quenching of the donor is not affected by FRET (48). Hence, the FRET-rate \( k_{\text{FRET}} \) depends on the relative orientation and donor-acceptor distance and the FRET samples can be fitted globally with the donor-only reference sample. In the presence of FRET, the donor fluorescence decay can be expressed with a Gaussian distance distribution \( (\rho) \) of donor-acceptor as

\[ F_{\text{DA}}(t) = F_{\text{DD}}(t) \cdot \int \rho_{\text{gauss}}(\sigma, \langle R(i) \rangle) \cdot \exp(-k_{\text{FRET}}(\langle R(i) \rangle) \cdot t) \text{d}R, \]

where \( \langle R(i) \rangle \) is the mean distance between donor and acceptor and \( \sigma \) the width of the interf luorophore distance distribution \( R(i) \). In the analysis, \( \sigma \) was fixed to a physically meaningful value of 5 Å (45). The Förster radius for CFP and FlAsH was 39 Å, as calculated following the method described in supporting material.

Uncertainty estimation of distance distribution

The experimental uncertainty in the TCSPC-based interf luorophore distance analysis mainly stems from three sources: 1) the uncertainty of the orientation factor \( (\kappa^2) \), \( \delta_{\text{DA},i=2} \), 2) the uncertainty in the D\text{copy} reference \( \delta_{\text{DA,reference}} \) (based on sample preparation, etc.), and 3) the statistical uncertainty based on the fitting, \( \delta_{\text{DA,in}} \) (45). Here, we estimated the uncertainty \( \delta_{\text{DA,in}} \) in the obtained distances (Gaussian distance distribution, Eq. 11) by sampling the \( \chi^2 \) surface in 50 steps in the range from 20 to 120% of the respective distance using the “Parameter Scan” option in ChiSurf (45). The value of the scattered distance, \( R_1 \) or \( R_2 \), respectively, is fixed, all other parameters are fitted, and the resulting \( \chi^2 \) is reported. The resulting \( \chi^2 \) surface (49) was plotted against the scattered distance (Fig. S11 b) and the limits were determined using a 3σ criterion based on an F test (1700 TCSPC channels, 9 parameters) to a relative \( \chi^2_{\text{ outliers}} = \chi^2_{\text{obs}} / \chi^2_{\text{ref}} \) of 1.012. To incorporate \( \delta_{\text{DA,reference}} \), we extended the limits for \( R_{\text{min}} \) and \( R_{\text{max}} \) in such a way that the overall \( R_{\text{max}} \) and \( R_{\text{max}} \) for the experimental triplicates were used (Fig S11 b). The uncertainty of the orientation factor \( (\kappa^2) \), \( \delta_{\text{DA},i=2} \), which is usually the largest source of uncertainty, was not considered.

Model-free distance distribution analysis

For a model-free description, we calculated the FRET-induced donor decay as described previously (45). In brief, in a first step, the fluorescence decay of the FRET sample \( I_{\text{DA}}(t) \) is divided by the (modeled) decay of the single-labeled sample \( I_{\text{DD}}(t) \). Next, the donor-only fraction, \( x_{\text{FlAsH}} \), i.e., the offset, is subtracted, and, finally, this ratio is multiplied with the time axis \( t \) to yield the FRET-induced donor decay \( \epsilon(t) \):

\[ \epsilon(t) = \left( \frac{l_{\text{DA}}(t)}{l_{\text{DD}}(t)} - x_{\text{FlAsH}} \right) \cdot t. \]

For an intuitive display, we converted the x axis from time \( t \) to critical distance \( R_{\text{DA,c}} \) by the following relation:

\[ R_{\text{DA,c}} = R_0 \cdot \left( \frac{t}{\tau_0} \right)^{1/6}, \]

where \( R_0 \) is the Förster radius of the respective FRET dye pair (here 39 Å) and \( \tau_0 \) the reference fluorescence lifetime of the donor fluorophore (here, 3 ns). Plotting \( \epsilon(t) \) against \( R_{\text{DA,c}} \) results in a peaking distribution, which reflects the probability density function of the underlying distance distribution of the original decay \( I_{\text{DA}}(t) \).

FRET-restrained Markov-chain Monte-Carlo sampling

FRET-restrained Markov-chain Monte-Carlo (MCMC) sampling was performed using the FRET Positioning Software (FPS) (50) based on the x-ray crystal structures of CB-SH3 (PDB: 4mt6) and CB-SH3 (PDB: 4mt6) (29). In addition, the NMR structure of the ReAsh-tag (BioMagResBank ID code 16041) (51) was used as a model for the FlAsH-tag, and the x-ray crystal structure of eGFP served as a template for CFP (PDBID 4eul). Two types of restraints were defined: 1) connectivity restraints and 2) FRET-based restraints (Fig. S11 a).

Connectivity restraints are based on the linear connectivity between the protein domains and labels, i.e., the SH3 domain is separated from the DH domain by 35 amino acids based on the structural models and 2B residues from the FlAsH-label incorporated at position 99 (Table S14). These flexible connections are modeled as worm-like chain polymers, where the uncertainty (or width of distribution) for FPS was determined as the 1σ region. One exception is the connection between the PH domain and the CFP. Here, we assumed that residues 439–456 stay in their α-helical conformation as found in PDB entry 2dfk (30), while residue 439 serves as a flexible hinge around which the helix and the ensuing CFP move about. Here, the uncertainty was set to the length of one amino acid residue (3.6 Å).

FRET-based restraints were based on the experimental distances obtained by the Gaussian distance fitting. The mean distance was set as the average from the experimental triplicates and the uncertainties were determined as described above (Table S15).

Next, the five entities (FlAsH-1, SH3 domain [from 4mt6], FlAsH-99, CB [4mt6 or 4mt7] and CFP) were loaded into FPS and the respective CB structure (4mt6 or 4mt7) was fixed in place. The structural models were docked for 100 times, followed by 100× sampling using MCMC of each generated structure. In this step, the reciprocal kT was lowered to a value of 2. The resulting 10,000 models were exported as .pdb files, translated into PDB format, and the docking and sampling results were verified by comparing the obtained restrained mean value with our input values. Next, the trajectories were generated using mdtraj (52) and the density based on the occupancy of the FlAsH-1, FlAsH-99, CFP, and SH3 domain was exported from VMD (53). The data were visualized using PyMol (The PyMOL Molecular Graphics System, Schrödinger, New York, NV, USA).

Statistical analysis

All quantitative data are expressed as mean values ± standard deviation (SD) unless stated otherwise. Origin 9 (OriginLab) was used for
RESULTS

Sensor engineering and characterization

To generate suitable FRET sensors we incorporated an Aequorea victoria-derived cyan variant (CFP) of the green fluorescent protein (39) and the biarsenical dye, fluorescent arsenical hairpin binder-ethanedithiol (FlAsH) (44), as suitable donor and acceptor, respectively (54) into CB (Fig. 2, a and b). The nonfluorescent molecule FlAsH forms a fluorescent complex with any protein to which a short tCM is genetically fused (Fig. 2 c, Table S1), with the amino acid sequence CCPCGCC possessing the highest specificity for FlAsH (38). We optimized the position for CFP attachment and the tCM insertion site so that the intramolecular CB-FRET sensor can be used to study ligand-induced conformational changes. Initial screening for suitable intramolecular CB-FRET sensors was done by attaching the CFP moiety at various C-terminal positions by utilizing different truncated forms of CB, while the tCM insertion site was kept constant at the N-terminus of CB. Insertion of tCM after the first amino acid residue and CFP after residue 456 of the CB2-SH3 splice variant (Fig. 2 b, Table S1), denoted as F1, yielded the best working wild-type CB FRET sensor in terms of FRET efficiency (E_{FRET}) along with sensor purity, while still maintaining proper folding (Fig. S1, a and b). To better understand CB conformational dynamics, we generated three additional CB-FRET sensors (Fig. 2 b, Table S1), where we inserted the tCM for the FlAsH labeling after residues 28 (F28), 73 (F73), and 99 (F99) of CB, while keeping the CFP position fixed after residue 456. In addition, we also constructed two open-state mutant CB sensors (Fig. 2, a and b). In the single-mutant sensor (sm), Glu262 was replaced with alanine, whereas in the double-mutant sensor (dm), an additional Trp24Ala variant was engineered while keeping the FlAsH and CFP at the same positions as in the F1 construct. The sensors in the absence of acceptor are referred to with the subscript D0 and in the presence of the acceptor as DA. Full-length CB is rather unstable and prone to degradation, while GephFL is susceptible to aggregation and degradation during purification (16,29). After optimization, we recombinantly purified stable constructs of full-length CB and GephFL (see Methods for details). We ensured that all sensors (F1D0, F1smD0, and F1dmD0) retained proper folding (Fig. S1 b).

Previous crystallographic studies (29) suggested that CB exists in a closed conformation in its inactive state, which would allow optimum resonance energy transfer between CFP and FlAsH (Fig. 2 c). For our initial studies we used the F1 construct which is expected to closely mimic wild-type CB2-SH3⁺. Time-resolved fluorescence intensities of CFP in the absence (F1D0) and presence of FlAsH (F1DA) revealed a significant reduction in the average fluorescence lifetime (\langle \tau_f \rangle) from 2.16 (± 0.06) ns to 1.20 (± 0.04) ns (Fig. 2, d and e, Table S3). The decrease in \langle \tau_f \rangle in F1DA is attributed to FRET from the C-terminally attached CFP to the FlAsH moiety bound to tCM. F1DA displayed a FRET efficiency (E_{FRET}) of ~44% (Eq. 6) and a Förster radius (R_0) of 39 Å (Eq. 6 and Fig. S1 c). These properties support the use of F1DA as FRET sensor to study ligand-induced CB conformational dynamics.

![FIGURE 2 CB FRET sensor design and characterization. (a) CB domain architecture with the SH3, DH, and PH domains in light gray, black, and dark gray, respectively. Amino acid positions Trp24 (W24) and Glu262 (E262) are highlighted. (b) Domain architecture of the ensemble of CB FRET sensors constructed in this study, highlighting the position of the tetra-cysteine motif (tCM) used for labeling with the fluorescent arsenical hairpin binder-ethanedithiol (FlAsH-EDT2) (green spheres) and C-terminal attachment site of CFP (teal). Individual sensors contained a single tCM inserted after residues 1 (F1), 28 (F28), 73 (F73), and 99 (F99), whereas the CFP position (after residue 456) was kept constant. F1D0, F28D0, F73D0, and F99D0 represent the individual FRET sensors in the absence of FlAsH and F1DA, F28DA, F73DA, and F99DA after FlAsH labeling. In the single-mutant FRET sensor (F1smD0) E262 was replaced with Ala (E262A) and in the double-mutant FRET sensor (F1smD0), W24 and E262 were replaced with Ala (W24A/E262A). (c) Cartoon representing the CB FRET sensor (F1D0) in the closed conformation highlighting its labeling with FlAsH-EDT2 reagent, resulting in F1DA. FlAsH-EDT2 (nonfluorescent) turns fluorescent (green) after forming covalent bonds with the cysteine residues present in tCM (green circle). (d) Time-resolved fluorescence intensities of CFP of the CB FRET sensor (F1D0, teal) and the FlAsH-labeled CB FRET sensor (F1DA, cyan). The instrument response function (IRF) is shown in gray. F1D0 and F1DA were excited (λ_{ex}) at 440 nm and emission (λ_{em}) data were collected between 460 and 500 nm. Data were scaled to a maximum of 100 for easier comparison. (e) Species-weighted (\tau_f) of CFP in F1DA (1.20 ± 0.04 ns) is reduced compared with F1D0 (2.16 ± 0.06 ns), corresponding to a FRET efficiency of 44% (Eq. 6). Data from three different batches of experiments are presented as mean values ± SD.](Image 54x368 to 292x544)
Gephyrin, NL2, and free SH3 mediate CB activation

Using the CB FRET sensor as a novel tool, we sought to delineate the molecular basis of the CB and GephFL interaction. For interaction studies, we measured the \(\langle \tau \rangle_x\) of CFP in F1D\(_{0}\) as well as F1D\(_{A}\) alone and in the presence of a 100-fold molar excess (100 \(\mu\)M) of GephFL. A significant increase in average fluorescence lifetime to 1.56 ± 0.04 ns (Table S3) of the F1D\(_{A}\)-GephFL complex compared with free F1D\(_{A}\) was observed (Fig. 3 a). In this case an E\(_{\text{FRET}}\) of ~27% was calculated for the F1D\(_{A}\)-GephFL complex (Table S3), while no substantial change in \(\tau_x\) of CFP was observed for F1D\(_{0}\) in the presence of GephFL (Table S4), indicating that GephFL binding does not alter the fluorescent properties of CFP. The decrease in E\(_{\text{FRET}}\) upon GephFL binding reflects changes in the CB conformation, leading to an increased distance between the fluorophores attached to the SH3 and PH domain.

Earlier studies hypothesized that the intracellular cytosolic domain of NL2 (NL2\(_{\text{icd}}\)) binds to the SH3 domain of CB, resulting in an open/active state capable of interacting with plasma membrane phosphoinositides (29,34,55). In the presence of 100 \(\mu\)M NL2\(_{\text{icd}}\) the \(\tau_x\) of CFP in the F1D\(_{A}\)-NL2\(_{\text{icd}}\) complex was 1.37 ns (Fig. 3 a) with an E\(_{\text{FRET}}\) of ~36% (Table S3), a slightly higher E\(_{\text{FRET}}\) than in the F1D\(_{A}\)-GephFL complex.

A previous biochemical analysis (29) suggested that the SH3 domain engages in a weak intramolecular interaction with the DH-PH domain tandem. To investigate whether this interaction is dynamic so that the SH3 domain also exists in a partially detached state, we incubated a 100-fold stoichiometric excess (100 \(\mu\)M) of recombinantly purified SH3 domain with F1D\(_{A}\). The \(\tau_x\) and E\(_{\text{FRET}}\) for the SH3-F1D\(_{A}\) complex were measured as 1.44 ± 0.06 ns and 33%, respectively (Fig. 3 a, Table S3), indicating that the free SH3 domain can displace the covalently linked SH3 domain, which hence must exist not only in a state where it interacts with the DH-PH tandem.

Encouraged by these initial results, we performed titration experiments to quantify the binding affinity between CB and GephFL (Fig. 3 b). With increasing (0.05 to 400 \(\mu\)M) GephFL concentrations (Table S5), a concomitant increase in F1D\(_{A}\) \(\tau_x\) was observed, reaching saturation at a GephFL:F1D\(_{A}\) molar ratio of 100:1 (Fig. 3, b and c). In contrast, when titrating with the free SH3 domain (Table S6), saturation was only obtained at a SH3:F1D\(_{A}\) 1200:1 molar ratio (Figs. 3 c and S3 a). By plotting the fractional saturation determined from the corresponding \(\tau_x\) (Eq. 4) against the GephFL and SH3 concentrations, the dissociation constants \(K_D\) of the F1D\(_{A}\)-GephFL and F1D\(_{A}\)-SH3 complexes (high- and low-FRET states) against the concentration of GephFL obtained when analyzing the time-resolved fluorescence intensities with the Gaussian distribution model (Eq. 9). Curves showing the fraction of F1D\(_{A}\) molecules in the closed/high-FRET state (filled squares, black) with a FRET pair distance \(R_1\) of 25 ± 1.1 Å and their gradual transition into the open/low-FRET state (open squares) exhibiting a FRET pair distance \(R_2\) of 46 ± 1.5 Å upon addition of GephFL. The transition of F1D\(_{A}\) from the high- to the low-FRET state is illustrated in cartoon representation.

FIGURE 3 Full-length gephyrin-mediated CB activation. (a) Average fluorescence lifetime of CFP in F1D\(_{0}\) (teal), F1D\(_{A}\) alone (cyan), and in the presence of GephFL (black), SH3 domain (orange), and NL2\(_{\text{icd}}\) (dark red). ***p < 0.005. Data from three different batches of experiments are presented as mean values ± SD. (b) Fluorescence lifetime of CFP in F1D\(_{A}\) and F1D\(_{A}\)-GephFL complexes with increasing concentrations of full-length gephyrin (GephFL, black). Data were scaled to a maximum of 100 for easier comparison. The decay histograms are fitted in two ways: 1) with a multexponential fitting model (Eq. 1) to determine \(K_D\) and 2) with two FRET species \(R(0)\) with a Gaussian distance distribution (half-widths 5 Å) and a single NoFRET species (see Methods). (c) GephFL binding affinity was determined based on the average fluorescence lifetime converted into the fractional saturation using Eq. 4. The data were fitted with Eq. 5. For the F1D\(_{A}\)-GephFL complex (black) a dissociation constant \(K_D\) of 4.5 ± 1.7 \(\mu\)M and for the F1D\(_{A}\)-SH3 complex (orange) a \(K_D\) of 373 ± 116 \(\mu\)M (data ± SD) were obtained. Data from three different batches of experiments are presented as mean values ± SD. (d) Plot of the contribution of the two FRET species (high- and low-FRET states) against the concentration of GephFL obtained when analyzing the time-resolved fluorescence intensities with the Gaussian distribution model (Eq. 9). Curves showing the fraction of F1D\(_{A}\) molecules in the closed/high-FRET state (filled squares, black) with a FRET pair distance \(R_1\) of 25 ± 1.1 Å and their gradual transition into the open/low-FRET state (open squares) exhibiting a FRET pair distance \(R_2\) of 46 ± 1.5 Å upon addition of GephFL. The transition of F1D\(_{A}\) from the high- to the low-FRET state is illustrated in cartoon representation.
complexes were assessed as 4.5 ± 1.7 and 373 ± 116 μM, respectively (Eq. 5) (Figs. 3 c and S3 a). These results indicate that GephFL and CB interaction is moderately tight, while the SH3 domain has a low affinity toward CB. Previous microscale thermophoresis data (29) with the free SH3 and the tandem DH-AF domain yielded a $K_D$ of 273 ± 34 μM, similar to the value obtained here. The interaction strength between F1DA and NL2icd could not be quantified as no systematic increase in $\langle \tau_x \rangle$ of CFP was observed when further incubated with an excess (more than 100 μM) of NL2icd.

Two-state dynamics during CB activation

To understand the mode of CB activation, we analyzed the time-resolved fluorescence intensities of F1DA and F1DA-GephFL complexes at various concentrations by Gaussian distance distribution models (Eq. 9). From Fig. S2 a it is evident that fitting with two FRET species with a Gaussian distance (R(i)) and a NoFRET ($\chi_{\text{NoFRET}}$) state is significantly better than assuming only a single FRET species, while three FRET species led to no further improvement. The half-widths of the Gaussian distributions were kept fixed to 5 Å. The results suggested that the F1DA molecules exist in two conformational states, a high-FRET and a low-FRET state (Fig. S2 b). The high-FRET state exhibited an average interfluorophore distance ($R_1$) of 25.5 ± 0.5 Å, while the low-FRET state showed an average interfluorophore distance ($R_2$) of 45.5 ± 0.9 Å (Table S3). Considering the size of CFP (diameter ~20 Å), the distance of the high-FRET state indicates that the FLAsH and CFP fluorophores are in very close proximity, in line with a compact/closed conformation, while F1DA adopts an open state in the low-FRET state. While gradual addition of GephFL did not induce any significant changes in the interfluorophore distances, it shifted the equilibrium toward the low-FRET state (Figs. 3 d and S2 b). It must be noted that increasing GephFL concentrations led to a stronger population of the $\chi_{\text{NoFRET}}$ state (Fig. S2 c), possibly indicating another state beyond the measurable FRET distance limit (>49 Å) for this FRET pair (56). The fluorescence lifetime-based FRET study along with distance distribution analysis of F1DA provides concrete evidence of GephFL-mediated CB opening and its transition from the closed to an open state. Binding of the free SH3 domain also resulted in a concentration-dependent increase in the low-FRET F1DA state and a simultaneous decline in the high-FRET F1DA population (Fig. S3 b and c), suggesting a displacement of the SH3 domain present in F1DA by the isolated SH3 domain. Again, we observed that rising SH3 concentrations resulted in an $\chi_{\text{NoFRET}}$ increase (Fig. S3 d).

The E domain of gephyrin mediates CB binding and activation

Next, we investigated which region of gephyrin mediates the interaction with CB by employing constructs containing only the G domain (GephG), the linker followed by the E domain (GephLE), and the isolated E domain (GephE) of gephyrin. The purified variants (GephG, GephLE, and GephE) were incubated with F1DA and F1DA and fluorescence lifetime measurements were performed. Fig. 4 a shows the $\langle \tau_x \rangle$ changes in F1DA when incubated with the domain variants. At comparable concentrations of 100 μM, GephLE showed the highest increase in $\langle \tau_x \rangle$ at 1.54 ± 0.04 ns, followed by GephE (1.36 ± 0.02 ns) (Fig. S4 a, Table S3). In contrast, GephG (1.26 ± 0.01 ns) did not display a fluorescence lifetime change, thus confirming previous studies reporting that the G domain is not involved in the interaction with CB (10,32). Note, the incubation of F1DA with the domain variants, as observed before for GephFL, did not alter $\langle \tau_x \rangle$ of CFP (Table S4). Taken together, these data suggest that GephE, with a possible minor contribution from the linker, mediates interaction and opening of CB.

To further map the binding site, we individually titrated F1DA with GephLE and GephE. F1DA displayed a concentration-dependent increase in $\langle \tau_x \rangle$ for both domain variants (Fig. S4, b and c, Tables S7 and S8). We quantified the binding strengths by plotting the fractional saturation based on the change in F1DA $\langle \tau_x \rangle$ (Eq. 4) upon increasing GephLE and GephE concentrations, respectively. Both, GephLE and GephE displayed identical affinities with $K_D$ values of 6.4 ± 1.9 and 6.3 ± 1.8 μM, respectively (Fig. 4 b, Eq. 5). Subsequently, we examined how GephLE and GephE mediate the high-FRET to low-FRET transition in F1DA. In line with their identical affinities, GephLE and GephE displayed comparable concentration-dependent effects in transitioning from the high-FRET/closed state to the low-FRET/open F1DA state (Figs. 4 c and S4, d and e). Like GephFL, both GephLE and GephE exhibited an increase of $\chi_{\text{NoFRET}}$ with higher concentrations of both ligands (Fig. S4, f and g). Hence, the fluorescence lifetime-based affinity interaction study with F1DA demonstrated that the E domain solely mediates the interaction with CB.

A monomeric E domain induces a stronger CB conformational change

As GephE is capable of forming a dimer, we next checked whether GephE dimerization plays a role in its recognition by CB. To investigate this aspect we recombinantly purified a dimerization-deficient, monomeric mutant of GephE (GephEmm) described earlier (42) (Fig. S5 a). We then measured the $\langle \tau_x \rangle$ change
observed for F1DA in the presence of GephEmm and compared it with that of GephE (Fig. 4a). Surprisingly, at comparable concentrations, GephEmm displayed a longer lifetime (1.78 ± 0.02 ns) compared with GephE (1.36 ± 0.02 ns) (Table S3), implying that GephEmm possesses a higher potential for changing the conformation of CB. To investigate the binding strength of GephEmm we titrated F1DA with increasing concentrations of CB. To investigate the binding strength of GephEmm (Table S9, Fig. S5) compared with GephE (Table S3) and the monomeric E domain variant (GephLE, blue). A previous study reported that residues Trp24 and Glu262 in the DH domain, play crucial roles in modu-

Design and characterization of active-state mutant sensors

A previous study reported that residues Trp24 and Arg70, which are located in the SH3 domain, and Glu262 in the DH domain, play crucial roles in modulating the equilibrium between the inactive and active conformations in full-length CB (29). The Trp24Ala and Glu262Ala variants promote the formation of the open state, which, with respect to its role in inhibitory synapse formation, is considered to be the active state (29). Small-angle x-ray scattering (SAXS) and atomic force microscopy data indicated a more extended conformation for the Glu262Ala single mutant, in contrast to the compact state of wild-type CB (29). The Trp24Ala/Glu262Ala double mutant could not be analyzed by these biophysical techniques due to enhanced instability of the protein (29). In this study, however, where only low protein concentrations are required, we could carry out experiments with the single (F1smd) as well as the double-mutant (F1dmd) CB FRET sensor.

The (r) of CFP in F1smd (2.14 ± 0.02 ns) and F1dmd (2.10 ± 0.01 ns) in the absence of the FlAsH, were identical to that observed for F1dd (2.16 ± 0.06 ns) (Fig. 5a, Tables S3 and S10). In contrast, the FlAsH-labeled sensors F1smd (0.32 ± 0.01 ns) and F1dmd (0.35 ± 0.01 ns) displayed a substantial decrease in the average CFP fluorescence lifetime compared with the wild-type sensor at 1.2 ± 0.04 ns (Fig. 5a, Tables S3 and S10). At the same time, F1smd and F1dmd exhibited comparable E (44%). Next, we checked the effect of GephFL upon interaction with the mutant sensors. As expected, no (r) change was observed in F1smd and F1dmd upon GephFL interaction (Table S10). However, to our surprise, an interaction of GephFL with F1smd and
F1dmDA resulted in a drastic increase in their average fluorescence lifetimes to 0.62 ± 0.01 and 0.64 ± 0.02 ns, respectively (Figs. 5 a and S6, a and b), indicating a substantial increase in interfluorophore distance upon GephFL binding.

We also investigated the GephFL affinity for the open-state mutant sensors and hence separately titrated F1smDA and F1dmDA with increasing concentrations of GephFL. GephFL titration with F1smDA and F1dmDA led to a gradual increase in their fluorescence intensities (Eqs. 12 and 13). Normalized distance distribution curves shown for F1smDA and F1dmDA were measured as 47 ± 14 and 43 ± 11 μM, respectively. Data from three different batches of experiments are presented as mean values ± SD. (b) GephFL binding affinity plot of F1smDA (orange) and F1dmDA (wine). Binding affinities were determined by first converting (r) into the fractional saturation using Eq. 4 and the data were further fitted with Eq. 5. The GephFL binding affinity constant (Kd) for F1smDA and F1dmDA were measured as 47 ± 14 and 43 ± 11 μM, respectively. Data from three different batches of experiments are presented as mean values ± SD. (c) Model-free description of the interfluorophore distance distribution underlying the time-resolved fluorescence intensities (Eqs. 12 and 13). Normalized distance distribution curves shown for F1smDA and F1dmDA in the absence and presence of GephFL. F1smDA and F1dmDA show a major peak at 28 Å and a shoulder at 36 Å, which is significantly different from F1DA, particularly for the major peak located in this case at ~43 Å (Fig. S9 d). Upon complexation with GephFL the major peak shifts to 36 Å with a weak shoulder at ~28 Å.

F1dmDA was significantly reduced compared to the wild-type sensor (F1DA). Our studies with the mutant sensors corroborate previous data (29), which suggest that the disruption of the intramolecular interaction leads to a conformational switch within CB.

We also tried to analyze the fluorescent lifetimes with a Gaussian distance distribution model (Eq. 11); however, the fast exponential decay of the fluorescent intensities in the beginning for both F1smDA and F1dmDA sensors made the fitting with the Gaussian distance distribution model challenging. Thus, we followed a model-free approach reported earlier (45) to visualize the distance distribution underlying the time-resolved fluorescence intensities of F1smDA and F1dmDA (Figs. 5 c, S7, and S8). For comparison, we analyzed the F1DA and F1DA-GephFL time-resolved fluorescent intensities in the same way (Fig. S9). The distance distributions of F1smDA and F1dmDA revealed a main peak around 28 Å and a shoulder around 36 Å, indicating a disruption in the intramolecular interaction between the SH3 and DH domains, which concomitantly became more flexible, in both the single- and double-mutant sensors (Fig. 5 c). Thus, the FIAH present at the N-terminus and CFP at the C-terminus move closer to each other, as reflected in the main peak at 28 Å (high-FRET state), while a second peak around 36 Å is observed (low-FRET state). Upon ligand interaction the F1smDA Sensor showed a shift of the main peak from 28 to 36 Å with a minor shoulder at 28 Å, further indicating that the distance between the donor and acceptor increases upon ligand interaction. A similar type of distance shift was also observed for F1dmDA upon interaction with GephFL. In contrast, in the case of F1DA and the F1DA-GephFL complex (Fig. S9), the main peak is at ~43 Å (low-FRET) with a small shoulder at ~25 Å (high-FRET). Thus, the high-FRET population is strongly increased in case of F1smDA and F1dmDA compared with F1DA and the F1DA-GephFL complex, indicating that the opening of the structure due to mutations is different from the opening caused by ligand interaction of the wild-type CBFRET sensor.

Gephyrin binding elicits differential responses in a series of FRET sensors

To investigate the orientation of the SH3 domain with respect to the DH-PH tandem during activation, we performed interaction studies of GephFL with CB
constructs displaying the tCM at three additional positions: after residues 28 and 73, i.e., at the start and end of the SH3 domain, assuming that these positions should be sensitive to SH3 domain reorientations during activation, and after residue 99, close to the DH domain, for understanding DH-PH domain reorientation. Initially, CFP ($\tau_x$) measurements were carried out for the FIAsh-labeled sensors denoted as F28DA, F73DA, and F99DA (Table S13). An identical ($\tau_x$) decrease was observed for F73DA (0.79 ± 0.01 ns) and F99DA (0.8 ± 0.01 ns), whereas F28DA (1.2 ± 0.06 ns) showed a similar value as F1DA at 1.2 ± 0.04 ns (Figs. 6 a and S10 a). Upon interaction with GephFL, F73DA, and F99DA displayed a significant increase in ($\tau_x$) with 1.03 ± 0.02 and 1.2 ± 0.01 ns (Fig. 6 a and S10 b), respectively, whereas F28DA (1.4 ± 0.01) displayed a comparable change in ($\tau_x$) as observed for F1DA (1.56 ± 0.04 ns) (Tables S3 and S13). GephFL addition to F28DA, F73DA, and F99DA did not cause any substantial change in their ($\tau_x$), further suggesting that GephFL binding does not alter the fluorescent properties of the attached CFP.

Distance distribution studies for all sensors suggested that the absence or presence of GephFL does not alter the high-FRET and low-FRET states, indicating that all sensors, including F1DA, displayed comparable interferfluorophore distances (Fig. 6 b, Table S13). The equilibrium between the high-FRET ($x_1$) and low-FRET ($x_2$) states upon GephFL interaction is comparable to F1DA in the case of F73DA and F99DA (Table S13). In contrast, F28DA showed a smaller equilibrium shift following GephFL binding, as is evident from the fractions of the $x_1$ and $x_2$ species (Table S13). It must be noted that $x_{nFRET}$ is very low for F73DA and F99DA, but increases significantly for all sensors upon interaction with GephFL (Table S13). In summary, the results clearly indicated that GephFL induces an overall spatial shift/translocation of the SH3 and DH domain in CB during the transition from the closed to the open conformation.

MCMC sampling studies reveal CB conformations

To comprehend the overall conformational changes between the open and closed states of CB, especially with respect to SH3 domain, which can be described as “attached” and “detached” states, we performed MCMC sampling studies based on the experimental distance distributions derived from our four FRET sensors (F1DA, F28DA, F73DA, and F99DA), their respective uncertainties (Table S14), and the molecular architecture of CB. As input for the MCMC sampling (50), the structures of CB without the SH3 domain (PDB: 4mt7) and with the SH3 domain in the closed state (PDB: 4mt6) were used and dissected into their domains connected by flexible hinges (see Methods, Table S15). For the sampling of the open, GephFL-bound state, the respective low-FRET distances...
were used, while for the closed, GephFL-free state, the respective high-FRET distances were used (Table S14). We evaluated the accessible volume of CFP, F99DA (Figs. 6 c and S12) and the SH3 domain in the open and closed state. As Fig. 6 c shows, the opening and the PH domain rotation/tilt with respect to the DH domain generates an “empty” space between these two domains and thus gives the C-terminally attached CFP and F99DA access to a larger space, while in the two domains and thus gives the C-terminally attached SH3 domain with respect to DH-PH domain is in agreement with previous SAXS data (29).

**DISCUSSION**

Despite the fundamental importance of CB as an adaptor protein ensuring the proper function of inhibitory GABAergic synapses, its interaction with the neuronal scaffolding protein gephyrin remained poorly understood. In this study, we address this long-standing conundrum through the aid of custom-designed CB FRET sensors. Here, we provide fluorescence lifetime-based FRET data, which, along with FRET-restrained modeling studies, elucidates the molecular mechanism of autoinhibition of CB and its activation by gephyrin.

Previous studies demonstrated that activation of CB upon interacting with NL2icd or TC10 leads to an open structure of CB, which allows CB to interact with phosphoinositides located in the postsynaptic membrane (29,33,34,55). The two-state Gaussian distributed distance fit of the CB F1DA sensor showed an increase in the population of the low-FRET state upon interaction with NL2icd, indicating NL2icd-mediated CB opening. However, at comparable concentrations, NL2icd displayed a smaller increase in average fluorescence lifetime compared with GephFL, thus suggesting that GephFL not only interacts with but also efficiently activates CB. Compared with NL2icd, GephFL even possesses a higher capability for CB activation. Contrary to a previously hypothesized notion (18,29), our data suggest that initial CB relief by NL2 binding is not essential for gephyrin-CB complex formation.

With the aid of the F1DA FRET sensor we could successfully quantify the previously unknown binding strength of the GephFL-CB complex, yielding a reasonably tight interaction ($K_D = 4.5 \pm 1.7 \mu M$) between the two proteins (Fig. 3 c). Furthermore, using a two-state Gaussian distributed distance fit model for F1DA, we demonstrate that CB can be described as a two-state system, encompassing a compact or high-FRET state and a relaxed or low-FRET state (Fig. S2 b). Quantification of CB molecules in the high and low-FRET state indicated that GephFL binding shifts the equilibrium from the closed state of CB (high-FRET F1DA) toward the open (low-FRET F1DA) state (Fig. 3 d). In addition, the gradual increase of $X_{\text{NOFRET}}$ upon addition of GephFL indicates that there might be another state beyond the measurable FRET distance of 49 Å for this FRET pair.

Our FRET study therefore provides concrete evidence of GephFL-mediated CB opening, further strengthening the role of gephyrin as a CB activator. Identical affinities for GephLE (6.4 ± 1.9 μM) and GephE (6.3 ± 1.8 μM), along with their highly similar behavior in mediating the transition from closed to open states of CB (Fig. 4, b and c), indicate that the E domain is responsible for CB activation. In addition, a monomeric (dimerization-deficient) variant of the E domain (GephEmm) was also able to facilitate CB activation, which demonstrates that GephE dimerization is not a prerequisite for its interaction with CB. However, the lower binding strength (44.1 ± 7.8 μM, Fig. 4 b) of GephEmm for F1DA indicates that GephE dimerization is required to enhance its affinity for CB, potentially by stabilizing the E domain.

The constitutively active mutant CB FRET F1sdMDA and F1dmMDA sensors, somewhat counterintuitively, exhibited an increased average FRET efficiency compared with the wild-type F1DA sensor. This might be because the disruption of the intramolecular SH3-DH/PH interactions rearranges the SH3 and PH domains and brings the FIASH and CFP moieties into closer proximity. A comparable $\langle \tau_x \rangle$ decrease (Fig. 5 a, Table S10) in both mutant sensors, along with their similar affinity for GephFL (Fig. 5 b), suggests that the single Glu262Ala variant is already capable of eliminating the intramolecular interactions between the SH3 domain and the DH-PH tandem, and both mutant sensors potentially attain similar tertiary structures.

Structural insights into full-length CB is limited to a low resolution apo-CB-SH3 crystal structure (29) and hence information about the SH3 domain orientation in active CB state is limited. With our series of CB FRET sensors, we could visualize the accessible space of the SH3 domain in the active state of CB (Figs. 6 c and S12). In all sensors, GephFL addition led to an increase in $\langle \tau_x \rangle$, thus suggesting an increase in the average interfluorophore distance in the respective sensors (Fig. 6, a and b). Overall, the studies with the F28DA, F73DA, and F99DA sensors provide strong evidence for a displacement of the SH3 domain following the interaction of CB with gephyrin. Furthermore, MCMC sampling (57,58) based on the experimentally obtained interfluorophore distances
of the four sensors and protein domain connectivity clearly identified distinct closed and open states of CB. In the closed conformation of F99DA, the two fluorophores were found to be in close spatial proximity. In contrast, GephFL addition led to a clearly distinguishable open state of CB, in which the probability densities of the two fluorophores were clearly separated (Figs. 6c and S12a), with enhanced CFP density being present on the opposite side of the connecting helix between the DH and PH domains. This further suggests that GephFL-mediated CB opening causes a disruption of the intramolecular interaction between the SH3 domain and DH-PH tandem, thereby also rendering the PH domain flexible and generating space between the DH-PH domains, which, in turn, can be occupied by the fluorophores. Please note that gephyrin was not present in the MCMC modeling studies; however, it would significantly restrict the space accessible to the fluorophores. As is evident from the extended density of the SH3 domain in the closed state and the presence of both low- and high-FRET states in the gephyrin-free measurement, our lifetime-based FRET experiments and modeling studies suggest an equilibrium between an SH3-attached and SH3-detached state already in the inactive, unbound state (Fig. S12d). This was not apparent in the available structure of the closed state where crystal packing forces presumably selected for a single closed state. However, only a flexibly attached SH3 domain, in equilibrium between an attached and detached state, would allow other proteins to bind in the region usually occupied by the SH3 domain.

Our in vitro and modeling data with CB FRET sensors led us to formulate a model (Fig. 7) summarizing the gephyrin-mediated CB activation. Taken together, our results reveal a clear interaction between GephFL (and its domain variants) and CB, an interaction that was first identified more than 20 years ago (28); however, owing to technical limitations, this could not be comprehensively characterized. Taken together, our data provide a framework to understand how CB acts as a dynamic molecular switch cycling between closed/inactive and open/active states in response to gephyrin binding.

DATA AVAILABILITY
All data needed to evaluate the conclusions in the paper are present in the paper or the supporting material. Raw data are available from the corresponding authors upon request.

SUPPORTING MATERIAL
Supporting material can be found online at https://doi.org/10.1016/j.bpr.2022.100079.

AUTHOR CONTRIBUTIONS
N.I. generated the constructs, purified, labeled the proteins, and performed the in vitro experiments. N.I. performed the time-resolved measurements. N.I. and S.C. analyzed the results. Uncertainty analysis and MCMC modeling were done by K.H. N.I., H.S., S.C., K.H., and K.G.H. prepared the manuscript. The project was supervised by H.S. and K.G.H.

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DECLARATION OF INTERESTS
The authors declare no competing interests.

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