



Inhibitory Postsynapse Regulation by Gephyrin Phase Separation

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List of abbreviations

1.6-Hex.	1.6-Hexandiol
Å	Angstrom
ADP, AMP, ATP	adenosine diphosphate, - monophosphate, - triphosphate
AP	action potential
AU	arbitrary unit
CaMKII	Ca ²⁺ /calmodulin-dependent protein kinase II
CB	collybistin
CDK	Cyclin dependent kinase
CNS	central nervous system
DDM	Dodecyl-β-D-maltosid
DH-domain	Dbl homology domain
DLC1/2	dynein light chain 1/2
DTT	Dithiothreitol
ECD	extracellular domain
GABA	gamma-amino butyric acid
GABA _A Rs	gamma-amino butyric acid type A receptor
GABARAP	GABA _A R-associated protein
GDP, GTP	guanosine diphosphate, - triphosphate
GEF	guanine nucleotide exchange factor
Geph	gephyrin
GephG, -C, -E	gephyrin G-, C- and E-domain
GlyRs	glycine receptor
HEK cells	human embryonic kidney cells
i/ePSD	inhibitory/ excitatory postsynaptic density
ICD	intracellular cytosolic domain
ITC	isothermal titration calorimetry
KD	dissociation constan
LGICs	ligand gated ion channels
LLPS	liquid-liquid phase separation
LS	lumazine synthase
LS-αICD	lumazine synthase with included GlyR-α1ICD
LS-αβICD	co-expressed Lsα1 and Lsβ1

LS-βICD	lumazine synthase with included GlyR-βICD
MoCD	Moco deficiency
Moco	molybdenum cofactor
NL	Neurologin
NMDA, NMDAR	N-methyl-D-aspartate, NMDA receptor
nNOS	neuronal nitric oxide synthase
palmitCoA	palmitoyl-Coenzyme A
PDB	Protein data base
PH-domain	pleckstrin homology domain
Pin1	peptidyl-prolyl isomerase NIMA-interacting protein 1
PIP	phosphoinositide
PKC	protein kinase C
PNS	peripheral nervous system
PSD	post-synaptic density
PSD95	post-synaptic density protein 95
PTM	post-translational modification
ROS, mROS	reactive oxygen species, mitochondria-derived ROS
SD	subdomain
SEC	size exclusion chromatography
SH3	src homology 3
SNARE	soluble N-ethylmaleimide-sensitive-factor attachment receptor
SSD	sub-synaptic domains
TM	transmembrane helices
vGAT	vesicular GABA transporter
w/o	with out
WT	wildtype

Abstract

Inhibitory synaptic transmission depends on the precise postsynaptic clustering of glycine and GABA type A receptors, a process orchestrated by the multifunctional scaffolding protein gephyrin. Gephyrin self-assembles into higher-order structures that undergo liquid-liquid phase separation (LLPS) to form the inhibitory postsynaptic density, a process driven by a complex interplay between its conserved G- and E-domains. Yet the molecular mechanisms governing this self-assembly and its dysregulation in neurological disease remain incompletely understood. This thesis provides new structural, biophysical, and cell biological insights into gephyrin self-assembly, identifying novel regulatory mechanisms operating across all of its domains and at the level of post-translational redox modification.

A *de novo* pathogenic missense variant in the gephyrin G-domain (G134R), identified in a patient presenting with epilepsy, developmental delay, microcephaly, and short stature, is characterized in the first part of this thesis. Biochemical and cellular analyses reveal that G134R severely impairs higher-order oligomerization, LLPS and prevents synaptic cluster formation in neurons. G134R further exerts a dominant-negative effect on wild-type gephyrin, disrupting inhibitory synapse formation. These findings highlight an indispensable role for the G-domain in gephyrin self-assembly, shifting focus beyond the previously E-domain-centric view of gephyrin function and revealing a novel molecular mechanism underlying epilepsy.

Building on the observation that gephyrin clustering is dynamically regulated, the second part of this thesis demonstrates that surface-exposed cysteine residues act as redox switches governing gephyrin multimerization and synaptic localization. Oxidation of these residues triggers reversible synaptic multimerization, enhancing receptor binding capacity and conferring proteolytic protection. Cys419, located at a critical interface within the E-domain important for LLPS, emerges as a key residue modulating phase separation in a redox-dependent manner. Surface-exposed cysteines are further shown to be required for maintaining fast miniature inhibitory postsynaptic current kinetics, establishing a direct link between neuronal redox state and inhibitory synapse function.

Using single-particle cryo-EM, this work demonstrates that E-domain dimerization drives the formation of gephyrin filaments in which adjacent dimers are connected through Z-shaped interfaces between their subdomains II (SDII). Deletion of SDII, charge neutralization at the interface, or introduction of epilepsy-associated

pathogenic variants each abolish filament formation, *in vitro* phase separation, and synaptic receptor clustering in hippocampal neurons, identifying E-domain filaments as the core structural scaffold underlying gephyrin phase separation and receptor clustering.

Together, this thesis advances the mechanistic understanding of gephyrin self-assembly and phase separation, revealing how structural, genetic, and metabolic perturbations converge to regulate inhibitory synapse formation and, when disrupted, contribute to epilepsy and neurodevelopmental disorders.

Zusammenfassung

Die inhibitorische synaptische Übertragung hängt von der präzisen postsynaptischen Clusterbildung von Glycin- und GABA-Typ-A-Rezeptoren ab, ein Prozess, der durch das multifunktionale Gerüstprotein Gephyrin gesteuert wird. Gephyrin ordnet sich selbst zu quervernetzten Strukturen, die eine Phasentrennung durchlaufen, um die inhibitorische postsynaptische Dichte zu bilden, ein Prozess, der durch ein komplexes Zusammenspiel zwischen seinen konservierten G- und E-Domänen angetrieben wird. Dennoch sind die molekularen Mechanismen, die diese Selbstorganisation steuern, sowie deren Dysregulation bei neurologischen Erkrankungen noch nicht vollständig verstanden. Diese Dissertation liefert neue strukturelle, biophysikalische und zellbiologische Einblicke in die Gephyrin-Selbstorganisation und identifiziert neuartige Regulationsmechanismen, die über alle Domänen hinweg sowie auf der Ebene posttranslationaler Redoxmodifikationen wirken.

Im ersten Teil dieser Arbeit wird eine *de novo* auftretende pathogene Missense-Variante in der G-Domäne von Gephyrin (G134R) charakterisiert, die bei einer Patientin mit Epilepsie, Entwicklungsverzögerung, Mikrozephalie und Kleinwuchs identifiziert wurde. Biochemische und zelluläre Analysen zeigen, dass G134R die Oligomerisierung höherer Ordnung sowie die Phasentrennung stark beeinträchtigt und die Bildung synaptischer Cluster in Neuronen verhindert. G134R übt zudem einen dominant-negativen Effekt auf Wildtyp-Gephyrin aus und stört die Bildung inhibitorischer Synapsen. Diese Ergebnisse unterstreichen die unverzichtbare Rolle der G-Domäne bei der Selbstorganisation von Gephyrin, verlagern den Fokus weg von der bisher auf die E-Domäne ausgerichteten Sichtweise der Gephyrin-Funktion und

decken einen neuartigen molekularen Mechanismus auf, der der Epilepsie zugrunde liegt.

Ausgehend von der Beobachtung, dass die Clusterbildung von Gephyrin dynamisch reguliert wird, zeigt der zweite Teil dieser Arbeit, dass oberflächenexponierte Cysteinreste als Redoxschalter fungieren, die die Multimerisierung von Gephyrin und dessen synaptische Lokalisierung steuern. Die Oxidation dieser Reste löst eine reversible synaptische Quervernetzung aus, wodurch die Rezeptorbindungskapazität erhöht und ein Schutz vor proteolytischem Abbau gewährleistet wird. Cys419, das sich an einer für die Phasentrennung wichtigen kritischen Schnittstelle innerhalb der E-Domäne befindet, erweist sich als Schlüsselrest, der die Phasentrennung auf redoxabhängige Weise moduliert. Es wird ferner gezeigt, dass oberflächenexponierte Cysteine für die Aufrechterhaltung einer schnellen Kinetik des inhibitorischen postsynaptischen Miniaturstroms erforderlich sind, wodurch ein direkter Zusammenhang zwischen dem neuronalen Redoxzustand und der Funktion inhibitorischer Synapsen hergestellt wird.

Mithilfe der Einzelpartikel-Kryo-EM zeigt diese Arbeit, dass die Dimerisierung der E-Domäne die Bildung von Gephyrin-Filamenten antreibt, in denen benachbarte Dimere über Z-förmige Schnittstellen zwischen ihren Subdomänen II (SDII) miteinander verbunden sind. Die Deletion der SDII, die Ladungsneutralisierung an der Schnittstelle oder die Einführung von epilepsieassoziierten pathogenen Varianten unterbinden jeweils die Filamentbildung, die *in-vitro*-Phasentrennung und die synaptische Rezeptorclusterbildung in Hippocampusneuronen, wodurch E-Domänen-Filamente als das zentrale strukturelle Gerüst identifiziert werden, das der Gephyrin-Phasentrennung und der Rezeptorclusterbildung zugrunde liegt.

Insgesamt trägt diese Arbeit zum mechanistischen Verständnis der Gephyrin-Selbstorganisation und Phasentrennung bei und zeigt auf, wie strukturelle, genetische und metabolische Störungen zusammenwirken, um die Bildung inhibitorischer Synapsen zu regulieren, und wie Störungen dieser Prozesse zu Epilepsie und neurologischen Entwicklungsstörungen beitragen.

Chapter 1: General introduction

1.1 The central nervous system

Life on earth eventually gave rise to organisms whose survival depended not only on sensing the world, but on integrating vast amounts of information across space and time within their own bodies. To meet this demand, higher animals evolved nervous systems: distributed networks of specialized cells that transform fleeting physical and chemical signals into coherent internal coordinated actions. Within such a system, individual cells do not remain isolated; they exchange information rapidly, selectively, and repeatedly, so that local events in one part of the body can influence distant targets with exquisite precision. In vertebrates, these communication modes are organized within a hierarchical architecture that distinguishes the central nervous system (CNS; Fig. 1A), comprising brain and spinal cord, from the peripheral nervous system (PNS), which connects the CNS to sensory epithelia, muscles, and visceral organs, thereby embedding neural computation into the physical body (Swanson *et al*, 2025).

The human CNS stands out for the sheer scale and richness of its connectivity. It is now estimated to contain on the order of 86 billion neurons, accompanied by a roughly equal number of non-neuronal cells such as glia, challenging long-held dogmas about higher glia-neuron ratios and emphasizing that the human brain is a quantitatively, rather than qualitatively, exceptional primate brain (Azevedo *et al*, 2009; Lent *et al*, 2012; Hawkins & Ahmad, 2016). Each neuron can, in principle, establish thousands of contacts with other neurons, so that even under conservative assumptions the number of possible network configurations far exceeds any intuitive grasp (Hawkins & Ahmad, 2016). In a brain with tens of billions of neurons the heuristic or informal argument upholds to this day that these configurations can, hypothetically, exceed estimates for the number of atoms in the observable universe. Within this vast combinatorial space, the communication mechanism between neurons is provided by synapses.

Synapses are the specialized junctions at which the axon of one neuron interfaces with the dendritic compartment of another, representing the critical sites where individual cells exchange and integrate information. On the one hand there are electrical synapses, in which gap junctions establish direct cytoplasmic continuity between neighboring neurons and permit near-instantaneous, bidirectional ionic current flow (Bennett, 1997). In the mammalian nervous system, however, chemical

synapses predominate. At these synapses, electrical signals are converted into chemical signals through the regulated release of neurotransmitters from the presynapse and subsequently back into electrical responses in the postsynaptic cell (Fig. 1B). Although this mode of transmission introduces a short time delay, it enables amplification, nonlinear signal processing, and a remarkable diversity of short- and long-term plasticity mechanisms (Manolov & Ovtscharoff, 1982). At these synapses, a fundamental distinction is made between chemical synapses that employ ligand-gated ion channels, where neurotransmitter binding directly triggers ion flux across the membrane, and those that utilize so-called G protein–coupled receptors (GPCRs), in which ion channel opening is mediated indirectly via downstream intracellular signaling pathways.

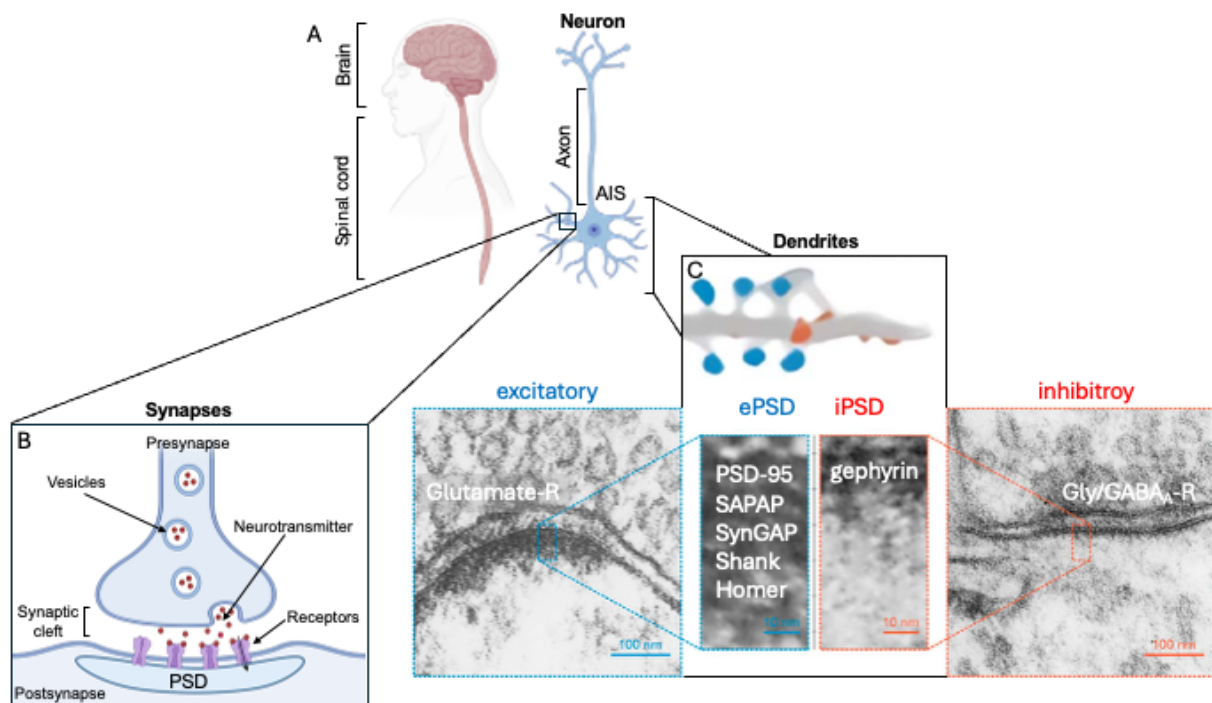


Figure 1: From the central nervous system to postsynaptic densities.

A) Cartoon breaking down the CNS, from brain and spinal cord over neuron with axon initial segment (AIS) and synapse to finally the post synaptic density (PSD). **B)** Schematic of a synapse, comprising presynapse with vesicles containing neurotransmitter that are released into the synaptic cleft. Neurotransmitters bind specific receptors in the postsynaptic membrane, which are located at the PSD **C)** EM images show excitatory postsynaptic density (ePSD) in blue and very thick, and inhibitory postsynaptic density (iPSD) in red and thin with indication for proteins present at the respective PSD. (Figure adapted from Bai and Zhang, 2022. Created with BioRender.com)

1.1.1 Chemical synaptic transmission

The electrical signals that initiate the process of synaptic transmission arise from changes in neuronal membrane potential. Neurons maintain a resting membrane potential through the unequal distribution of ions across the plasma membrane and the

selective permeability of ion channels. A central contributor to this ionic asymmetry is the Na^+/K^+ -ATPase, one of the most energy-consuming enzymes in the human brain, which continuously transports three Na^+ ions out of the cell and two K^+ ions into the cell at the expense of ATP hydrolysis (Peluffo & Hernández, 2023; Meyer *et al*, 2022). This active transport establishes transmembrane Na^+ and K^+ concentration gradients and generates a net negative charge inside the cell relative to the extracellular space of -40 mV to -90 mV. When synaptic input or intrinsic membrane conductance perturb this electrochemical equilibrium, local changes in membrane potential can propagate along the neuronal membrane. If sufficiently strong, these depolarizations activate voltage-gated ion channels, an influx of positively charged Na^+ ions and give rise to an action potential, a regenerative electrical signal that travels along the axon toward presynaptic terminals (Bean, 2007).

At the canonical chemical synapse, an invading action potential opens presynaptic voltage-gated Ca^{2+} channels and generates a steep Ca^{2+} gradient at so-called active zones, specialized regions of the presynaptic membrane where neurotransmitter-loaded vesicles are docked and primed for release. The Ca^{2+} in turn triggers the fusion of synaptic vesicles via SNARE (soluble N-ethylmaleimide-sensitive-factor attachment receptor) proteins with the presynaptic plasma membrane and the rapid release of neurotransmitter into a synaptic cleft of only about 20 nm width. Across this minuscule extracellular gap, neurotransmitter molecules diffuse within microseconds to bind to postsynaptic receptors, which themselves can open up for ion fluxes across the membrane or work as GPCRs (Südhof & Rothman, 2009; Südhof, 2013).

In neurons of the CNS, synaptic inputs impinge predominantly on dendritic spines and dendritic shafts, two structurally distinct postsynaptic compartments that already hint at a deeper functional dichotomy. Synapses located on spine heads are classically excitatory, whereas those more frequently targeting dendritic shafts are inhibitory, a morphological distinction that foreshadows the fundamentally different roles these two synapse types play in shaping postsynaptic integration (Fig. 1C) (Gray, 1959). At the biophysical level, these inputs generate excitatory and inhibitory postsynaptic potentials that are integrated across the dendritic arbor and soma. Excitatory inputs typically arise from the influx of Na^+ , K^+ or Ca^{2+} ions, resulting in a net inward flow of positive charge that depolarizes the membrane toward the threshold for generating another action potential. In contrast, inhibitory inputs are most commonly

mediated by Cl^- influx through ligand-gated ion channels, introducing negative charge or counteracting depolarizing currents and thereby hyperpolarizing the membrane. The net outcome of this integration is evaluated at the axon initial segment (AIS), where the exceptionally high density of voltage-gated ion channels confers sensitivity to even small changes in membrane potential. When the summed synaptic drive at the AIS crosses a defined threshold, another action potential is initiated and actively propagated along the axon (Bean, 2007; Xu *et al*, 2021).

The overall balance between excitatory transmission, primarily mediated by glutamatergic synapses, and inhibitory transmission, mediated largely by synapses that react to gamma-aminobutyric acid (GABA) and glycine, is essential for stable network operation. Glycine receptors (GlyR) and GABA type A receptors ($\text{GABA}_\text{A}\text{R}$), the principal neurotransmitter receptors concentrated at inhibitory synapses both belong to the cys-loop family of pentameric ligand-gated ion channels that become permeable for Cl^- ions upon neurotransmitter binding (Gamlin *et al*, 2018; Mihic & Harris, 1997). GABA type B receptors ($\text{GABA}_\text{B}\text{R}$) are members of the GPCRs that initiate inhibitory inputs via downstream intracellular signaling (Shaye *et al*, 2021).

GlyR mediates fast inhibitory glycinergic transmission and is typically composed of two types of subunits: a 48 kDa α subunit, which contains the glycine-binding site, and a 58 kDa β subunit, which interacts with postsynaptic scaffolds (Kim *et al*, 2006; Grenningloh *et al*, 1990; Grünewald *et al*, 2018). While α subunits can form functional homomeric channels, these receptors are predominantly extrasynaptic, and homomeric β subunits are unable to assemble into functional channels (Yu *et al*, 2021; Patrizio *et al*, 2017). GlyRs are mostly found in the brain stem and spinal cord and can be pharmacologically probed with antagonists such as strychnine (Grenningloh *et al*, 1987; Young & Chu, 1990).

$\text{GABA}_\text{A}\text{Rs}$ mediate the majority of fast inhibitory transmission in the mammalian CNS. They exhibit a highly diverse subunit composition, with 19 subunit-encoding genes identified in mammals, giving rise to a very high number of combinatorial variability of receptor isoforms with distinct pharmacological and kinetic properties (Sieghart *et al*, 1999). Among these, the $\gamma 2$ subunit is critical for synaptic targeting and clustering, making it a common postsynaptic marker in imaging studies (Essrich *et al*, 1998). The heterogeneity of $\text{GABA}_\text{A}\text{Rs}$ allows fine-tuning of inhibitory signaling across different neuronal populations and synaptic compartments (Wagner *et al*, 2021). The

molecular mechanisms that support inhibitory synaptic transmission will be examined in more detail in this thesis.

1.1.2 The postsynaptic density

Electron microscopy (EM) studies first revealed that the postsynaptic membrane is associated with an electron-dense thickening of variable size, which became known as the postsynaptic density (PSD) (Fig. 1C) (Gray, 1959). These specialized structures represent highly organized protein assemblies that concentrate neurotransmitter receptors, scaffold proteins, and signaling molecules directly opposite presynaptic release sites.

Excitatory postsynaptic densities (ePSD), display a disk-like morphology with lateral diameters of approximately 200–800 nm and a thickness of about 12–116 nm that contain glutamate receptors. (Gray, 1959; Tao-Cheng, 2019; Tao *et al*, 2018; Carlin *et al*, 1980).

Inhibitory postsynaptic densities (iPSD), by contrast and much later recognized as such, are more sheet-like and generally occupy smaller postsynaptic areas with a thickness of approximately 12 nm. They are specialized for the clustering and regulation of GABA_ARs and GlyRs at dendritic shafts (Carlin *et al*, 1980; Walikonis *et al*, 2000).

Proteomic analyses of isolated PSD fractions have underscored that, despite substantial differences in protein composition, ultrastructure, and subcellular morphology, excitatory and inhibitory synapses share a comparable functional organization in which presynaptic release sites are aligned with postsynaptic receptor–scaffold assemblies to ensure efficient signal transduction (Walikonis *et al*, 2000; Xu *et al*, 2021; Giesemann *et al*, 2003). This alignment is mediated in part by cell adhesion molecules such as neuroligins (NLs), which interact trans-synaptically with presynaptic neurexins, establishing physical and functional connectivity between pre- and postsynaptic elements (Ali *et al*, 2020; Pouloupoulos *et al*, 2009; Varoqueaux *et al*, 2004; Liu *et al*, 2022).

Scaffolding proteins form the core of these postsynaptic assemblies that organize receptor clustering: in ePSDs this organization is largely structured around the scaffold protein PSD-95, a member of the membrane-associated guanylate kinase (MAGUK) family whose name reflects both its approximate molecular weight of 95 kDa

and its enrichment in the PSD (Chen *et al*, 2015, 2008). PSD-95 acts together with additional scaffold proteins, including members of the SAPAP, SynGAP, Shank and Homer families, to form the structural backbone of the ePSD (Fig. 1) (Zhu *et al*, 2017; Li *et al*, 2019; Jia *et al*, 2025a). In contrast, the iPSD relies predominantly on a single central scaffold protein: gephyrin. The comparatively lower complexity of the inhibitory scaffold likely contributes to the thinner ultrastructural appearance of iPSDs observed in electron microscopy (Fig. 1C). Together, these observations highlight that, despite being structurally thinner and historically less studied than ePSDs, the iPSD is a highly organized molecular platform that ensures precise and reliable inhibitory neurotransmission. Therefore, the clustering mechanisms at inhibitory synapses will be further investigated in this thesis, with a major focus on gephyrin as the key regulator of inhibitory postsynaptic architecture.

1.2 Gephyrin as a moonlighting protein

The central iPSD scaffold protein, gephyrin, was originally identified in the late 20th century as a 81 kDa protein that co-purified with GlyR and was initially thought to represent a receptor subunit itself, before being recognized as a cytoplasmic anchoring protein that links GlyRs to subsynaptic actin filaments (Prior *et al*, 1992; Pfeiffer *et al*, 1982; Kirsch *et al*, 1991). Accordingly, it was named “gephyrin” from the Greek word γέφυρα (“*gephyra*”) for bridge. Gephyrin forms oligomeric assemblies beneath the postsynaptic membrane that bind GlyR and GABA_AR complexes, interact with neuroligins and other adhesion molecules that couple pre- and postsynaptic specializations, and associate with cytoskeletal elements that restrict the lateral diffusion of the receptors within the membrane (Hoon *et al*, 2009, 2011; Kim *et al*, 2006; Maric *et al*, 2014; Liu *et al*, 2022).

At the structural level, gephyrin harbors three major domains. The N-terminal G-domain that forms trimers, while the C-terminal E-domain mediates dimerization; these oligomerization interfaces are connected by a central linker region referred to as the C-domain (Fig. 2). The G- and E-domain nomenclature reflects the evolutionary origin of gephyrin related to two bacterial proteins, MogA and MoeA, which participate in the biosynthesis of the molybdenum cofactor (Moco) (Liu *et al*, 2000; Xiang *et al*, 2001). In eukaryotes, these ancestral enzymes became fused into single multidomain proteins such as Cnx1 in *Arabidopsis thaliana*, cinnamon in *Drosophila melanogaster*,

NIT-9 in *Neurospora crassa* or gephyrin in humans (Kamdar *et al*, 1994; Heck & Ninnemann, 1995; Schwarz *et al*, 2000).

Consistent with this evolutionary origin, gephyrin performs two distinct biological functions. First, it catalyzes the final steps of Moco biosynthesis, an essential metabolic pathway required for the activity of several molybdoenzymes, which will be described in detail in the following chapter (Stallmeyer *et al*, 1999; Sabatini *et al*, 1999). Second, in vertebrates, gephyrin has acquired the additional role as the principal scaffold protein of inhibitory synapses (Craig *et al*, 1996; Kirsch *et al*, 1993; Feng *et al*, 1998; Essrich *et al*, 1998).

This dual functionality places gephyrin among the growing class of so-called moonlighting proteins (Chen *et al*, 2018), whose evolutionarily conserved enzymatic functions coexist with newly acquired cellular roles in complex multicellular systems.

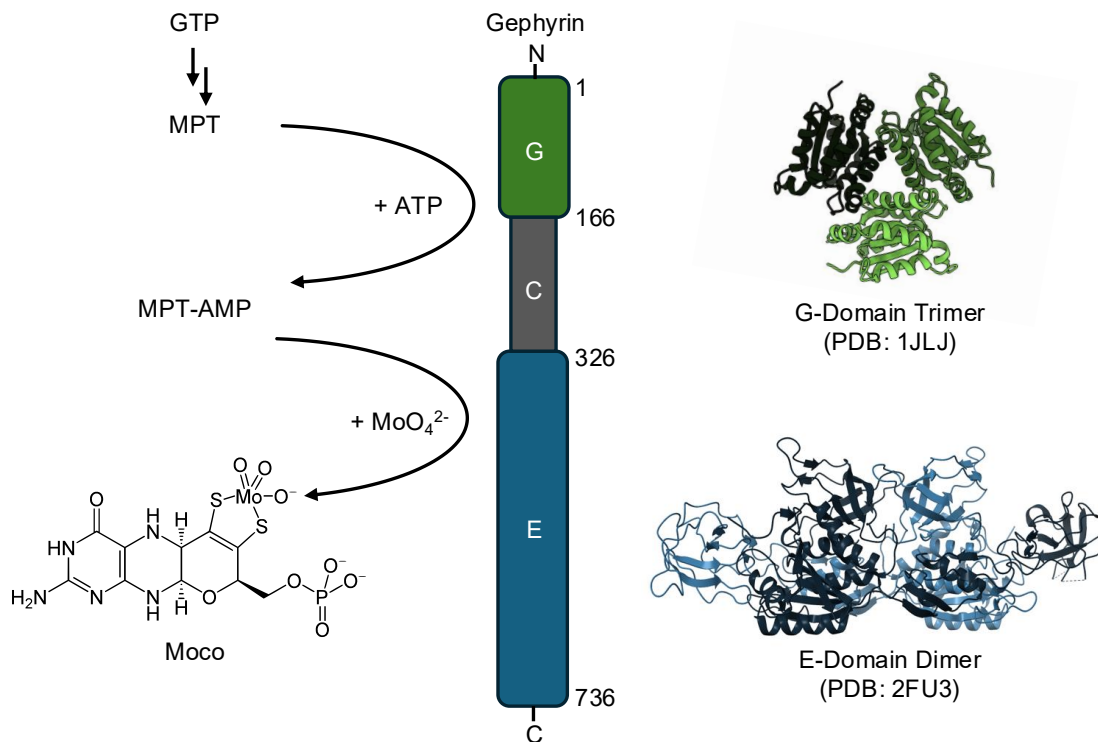


Figure 2: Gephyrin domain structure and steps in Moco biosynthesis.

The Moco biosynthesis starts with GTP, the subsequent steps until MPT are performed by proteins of the MOCS family. Gephyrin G-Domain performs adenylation to MPT-AMP and gephyrin E-Domain the final insertion of the molybdenum into the pterin backbone. Shown are the crystal structures of the isolated G-Domain trimer in green and E-Domain dimer in blue with PDB codes indicated.

1.2.1 Molybdenum cofactor synthesis

Moco is a highly conserved prosthetic group required for the catalytic activity of several enzymes that participate in redox reactions within nitrogen, carbon, and sulfur metabolism (Schwarz, 2005). The biosynthetic pathway of Moco is evolutionarily conserved across all kingdoms of life and involves a multistep enzymatic cascade that ultimately converts the nucleotide guanosine triphosphate (GTP) into the active molybdenum-containing cofactor (Fig. 2) (Stallmeyer *et al*, 1999).

In the first step in humans, GTP is converted into cyclic pyranopterin monophosphate (cPMP) within mitochondria by the enzymes MOCS1A and MOCS1B (Hover *et al*, 2015; Mayr *et al*, 2021). Subsequently, cPMP is transported to the cytosol, where it is converted into molybdopterin (MPT) by the concerted action of the proteins MOCS2A, MOCS2B, and MOCS3 (Leimkühler *et al*, 2003). The final two steps of the pathway are catalyzed by gephyrin (Fig. 2): the G-domain catalyzes the adenylation of MPT. During this reaction, ATP is utilized to generate the activated intermediate molybdopterin–adenylate (MPT–AMP) (Llamas *et al*, 2004). The adenylated MPT intermediate is thought to use the AMP moiety as a molecular “handle” or anchor that facilitates coordinated binding and transfer between the catalytic sites of the G- and E-domains (Kuper *et al*, 2004). Interestingly, one subdomain of the E-domain, namely subdomain III (SDIII; Fig. 3A), exhibits particularly strong structural similarity to the G-domain. Both regions contain conserved nucleotide-binding architectures, including Rossmann-like folds, which enable recognition of the nucleotide–pterin scaffold of MPT–AMP (Kasaragod & Schindelin, 2016).

At the E-domain of gephyrin, where the final maturation step of the cofactor takes place, molybdate (MoO_4^{2-}) is inserted into the dithiolene group of MPT to form the molybdenum–molybdopterin complex (Mo–MPT), which constitutes the functional Moco (Belaidi & Schwarz, 2013). Structural and biochemical studies in the plant orthologue Cnx1 have shown that this reaction is coupled to Mg^{2+} -assisted hydrolysis of the adenylate moiety (Hercher *et al*, 2020). Following molybdate insertion and hydrolysis of the adenylate group, the matured Moco can be released in its final phosphorylated form.

The newly synthesized Moco is then incorporated into various molybdoenzymes, where it is coordinated in slightly different structural environments depending on the enzyme. One of the best-characterized examples is sulfite oxidase (SUOX), in which the molybdenum atom of the cofactor is coordinated by the dithiolene

group of molybdopterins and a conserved cysteine residue within the enzyme's active site (Rudolph *et al*, 2003). In SUOX, through switches in the oxidation states of the molybdenum from its +6 state Mo(VI) via reduction to +4 Mo(IV), Moco enables catalytic redox reactions that are essential for cellular metabolism (Wang, 2012). Here the recycling back from Mo(IV) is achieved via single electron transfers to the heme-domain via Mo(V) back to Mo(VI) (Kisker *et al*, 1997).

During evolution, a gene fusion event between the bacterial enzymes MogA and MoeA occurred at least twice independently, giving rise to multidomain proteins with different domain arrangements in plants and in animals/fungi. In animals and fungi, the fused protein contains as described an N-terminal MogA-like domain (the G-domain), followed by a flexible linker or C-domain, and a C-terminal MoeA-like domain (the E-domain). In fungi, this protein is commonly referred to as NIT-9 (Rabenow *et al*, 2024). In contrast, plants exhibit the reverse domain architecture: the *Arabidopsis thaliana* protein Cnx1 contains an N-terminal MoeA-like domain, followed by a very short 12-residue linker and a C-terminal MogA-like domain. Despite these differences in domain order, the fundamental catalytic functions of the domains are conserved (Rabenow *et al*, 2024). The evolutionary fusion of MogA and MoeA is considered to increase the efficiency of the enzymatic cascade by facilitating substrate channeling between the two catalytic domains, thereby reducing the diffusion distance of intermediates and improving the overall kinetics of the pathway (Belaidi & Schwarz, 2013).

1.2.2 Scaffolding at the inhibitory post-synapse

In addition to Moco synthesis, gephyrin fulfills a second major function as principal scaffold protein at inhibitory postsynapses. Understanding this scaffolding function requires a detailed view of the oligomeric properties of gephyrin, the structural organization of its domains, and the mechanisms through which inhibitory receptors interact with the scaffold.

Several evolutionary rationales have been proposed to explain how an ancestral metabolic enzyme evolved into a neuronal scaffold protein. One possibility is that the multidomain architecture of the fused MogA–MoeA like proteins provided an inherent structural framework suitable for higher-order assembly, whereby the large C-domain, which links MogA and MoeA in the precise orientation required for catalysis, became a springboard for the evolution of novel functions. (Megrian *et al*, 2025).

At the structural level, gephyrin assembly relies on the distinct oligomerization properties of its terminal domains. The G-domain forms stable homotrimers (Sola *et al*, 2001; Schwarz *et al*, 2001) while the E-domain dimerizes (Fig. 2) (Sola *et al*, 2004; Schrader *et al*, 2004). Melting curve analyses revealed that the G-domain trimerization is significantly more stable than the corresponding dimeric arrangement of the E-domain (Sander *et al*, 2013). A key structural element in this trimerization process is α -helix 5, which forms the central trimerization interface and stabilizes the globular trimeric architecture of the domain.

The E-domain dimer resembles a “handshake”-like interaction in which two monomers interlock with each other (Sola *et al*, 2004; Schrader *et al*, 2004). In this analogy, each E-domain monomer represents one hand, while the intracellular loop of inhibitory receptors is positioned between the two domains like a thread that is held in place by the handshake (Kim *et al*, 2006; Maric *et al*, 2014). This structural arrangement forms the molecular basis for receptor binding. The E-domain itself is composed of four subdomains (SDI – SDIV; Fig. 3A). The dimer interface is formed primarily by interactions between SDI, SDIII, and SDIV of the two monomers. Within this assembly, the receptor-binding pocket is created by a hydrophobic cavity formed by subdomains III and IV of one monomer together with SDIV' of the opposing

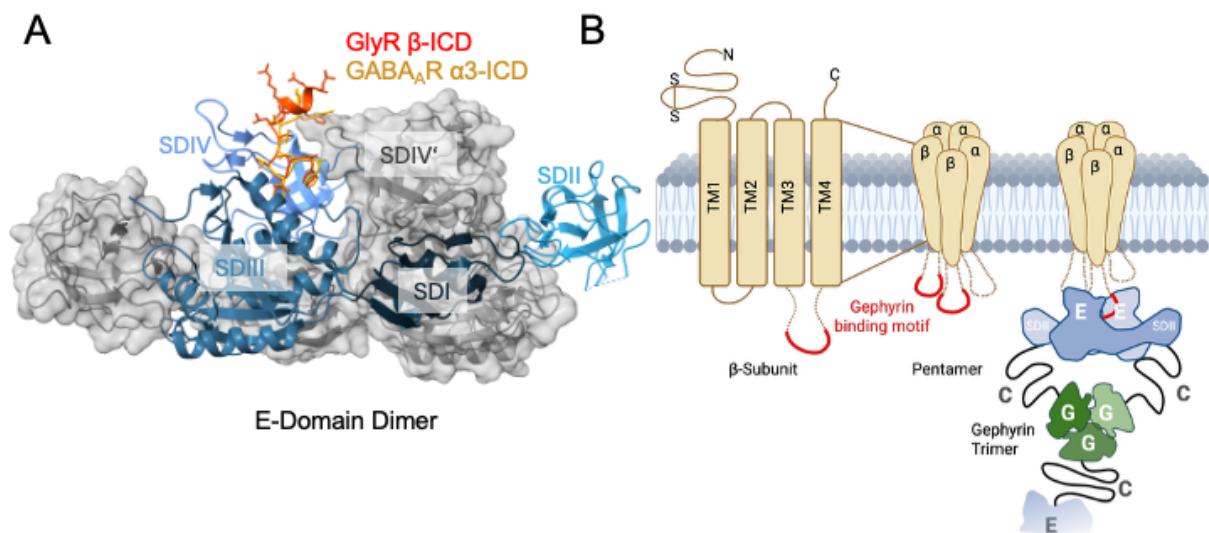


Figure 3: Receptor topology and structural basis of gephyrin-interaction

A) Overlay of the dimeric gephyrin E-domain (colored blue by its subdomain organization, PDB: 2FU3) bound to the GlyR β -ICD (red, PDB: 2FTS) and the GABA $_A$ α 3-ICD motif (orange, PDB: 4TK1) shows that both receptors engage an identical binding pocket formed by E-domain SDIII, SDIV, and SDIV'. Second monomer of the E-domain shown in grey. **B)** Cys-loop ligand gated ion channel is shown with the N-terminal name giving disulfide, four transmembrane domains (TM1-4), the gephyrin binding motif containing intracellular loop domain (ICD) in one subunit. Five subunits build one pentameric channel, here shown for GlyR with a 2 β :3 α stoichiometry. Dimeric gephyrin E-domain can bind the ICDs. (Figure Created with BioRender.com)

monomer. This pocket accommodates the intracellular peptides (formally called loops) of the respective inhibitory receptors (Fig. 3A) (Kim *et al*, 2006; Maric *et al*, 2014; Sola *et al*, 2004). At the same time, the active site for the enzymatic activity of the E-domain is formed by SDII of one monomer together with SDIII' of the opposing monomer (Krausze *et al*, 2017; Probst *et al*, 2021). Among the four subdomains, SDII is structurally most remote and functionally intriguing. In the handshake analogy, SDII can be imagined as the “thumb” of the hand. It protrudes somewhat away from the core of the E-domain monomer and displays a great degree of conformational flexibility within the structure (Nichols *et al*, 2007). This flexible positioning suggests that SDII may play an important role in conformational regulation of the E-domain. To which extent such a conformational diversity would impact receptor clustering, will be a subject of Chapter 4 of this thesis.

Additional insight into gephyrin oligomerization comes from *in vitro* studies of the full-length protein. In particular, the presence of the central and flexible C-domain appears to influence the oligomerization behavior while at the same time making it hard to determine the crystal structure of full-length gephyrin. When gephyrin is recombinantly expressed and purified from bacterial expression systems, the C-domain suppresses stable E-domain dimerization and favors the formation of G-domain-mediated trimers in which the E-domains remain largely monomeric (Bedet *et al*, 2006; Sander *et al*, 2013). These assemblies are often referred to as gephyrin trimers or low-order oligomers. Such species likely represent fundamental building blocks from which larger scaffold assemblies can form within the cellular environment.

The ability of gephyrin to anchor inhibitory receptors arises from direct interactions between the E-domain and the intracellular cytosolic domains (ICD) of the GlyR β -subunit and GABA_AR α - as well as GABA_AR β -subunits (Fig. 3A and B). Importantly, both receptor families bind to the same hydrophobic pocket within the E-domain dimer interface, resulting in direct competition for the same binding site (Maric *et al*, 2011). However, the two receptors exhibit markedly different binding affinities. The interaction between gephyrin and the GlyR β -subunit occurs with nanomolar affinity, which is approximately two orders of magnitude stronger than the corresponding interaction with GABA_AR α -subunits (Meyer *et al*, 1995; Grünewald *et al*, 2018; Schrader *et al*, 2004). This difference in affinity likely contributes to differences in receptor clustering efficiency and synaptic organization: quantitative super-resolution imaging has revealed that gephyrin copy numbers differ systematically

between inhibitory synapse subtypes. Glycinergic synapses in the spinal cord contain on the order of 300–600 clustered gephyrin molecules, whereas GABAergic synapses in the CNS typically harbor around 130–190 gephyrin molecules within their iPSDs (Nusser *et al*, 1998; Patrizio *et al*, 2017; Specht *et al*, 2013).

Beyond anchoring synaptic receptors, gephyrin also participates in receptor trafficking: it can transiently bind GlyRs *en route* to synaptic sites with the vesicular compartment and/or within the extrasynaptic space, whereas extrasynaptic GABA_ARs rely on distinct mechanisms and do not stably associate with gephyrin outside synapses (Maas *et al*, 2006; Christie & de Blas, 2002; Glykys & Mody, 2007; Brünig *et al*, 2002). In some regions, inhibitory synapses are mixed, containing both GlyRs and GABA_AR within a shared iPSD, where gephyrin-based scaffolds form nanodomains that differentially recruit and regulate these receptor populations (Dumoulin *et al*, 2000; Triller *et al*, 1987).

The fundamental receptor–gephyrin interaction can be investigated using a variety of well-established *in vitro* experimental systems. A commonly used approach involves peptides derived from the core gephyrin-binding motif within the receptor ICD of the GlyR- β ICD, typically spanning residues 378–426, which contain the core gephyrin-binding motif or of the GABA_AR- α ICDs (Grünwald *et al*, 2018; Meyer *et al*, 1995). The binding towards these peptides can be analyzed using techniques such as isothermal titration calorimetry (ITC) or surface plasmon resonance (SPR) spectroscopy to quantify binding affinity. Such experiments revealed that full-length gephyrin displays bimodal receptor binding behavior, with both high-affinity and low-affinity binding modes observed (Schrader *et al*, 2004). This contrasts with experiments using isolated E-domains, which typically display a single binding mode. To mimic the multivalent architecture of native receptors, the ICD peptides were fused to oligomerization domains creating chimera-proteins. For example, fusion to the dimeric coiled-coil domain of the yeast leucine zipper transcription factor GCN4 (Bai *et al*, 2021) generates a controlled dimeric receptor-binding module that has been widely used in biochemical studies, including in the work presented in this thesis. Alternatively, the Schwarz laboratory has developed a pentameric receptor model by fusing receptor loops to the pentameric yeast lumazine synthase scaffold, thereby mimicking the pentameric architecture of Cys-loop receptors (Macha *et al*, 2022a). Those novel multivalent systems, uncovered that multivalent receptor interactions promote gephyrin

liquid-liquid phase separation (LLPS), a proposed mechanism of synaptic gephyrin clustering addressed in the next chapters and throughout this thesis.

1.3 Liquid-liquid phase separation

The formation of intracellular compartments that are not enclosed by lipid membranes has been observed for more than a century. Early cell biological studies already described prominent structures such as the nucleolus, which is responsible for ribosomal ribonucleic acid (RNA) synthesis, as well as other nuclear structures including Cajal bodies (Fig. 4A) (Taliany *et al.*, 2023; Lafontaine *et al.*, 2021; Strom & Brangwynne, 2019). In the cytoplasm, additional examples such as P- or stress-granules have been identified (Protter & Parker, 2016; Brangwynne *et al.*, 2009; Banani *et al.*, 2017). All these structures were originally recognized primarily because of their striking appearance in microscopy. They often appear as highly condensed, protein-rich regions that can be readily detected with conventional staining approaches and are particularly prominent in EM. The dense appearance of such structures is reminiscent of other protein-rich cellular compartments, including the PSD, which likewise appears as an electron-dense layer at synapses in EM images (Feng *et al.*,

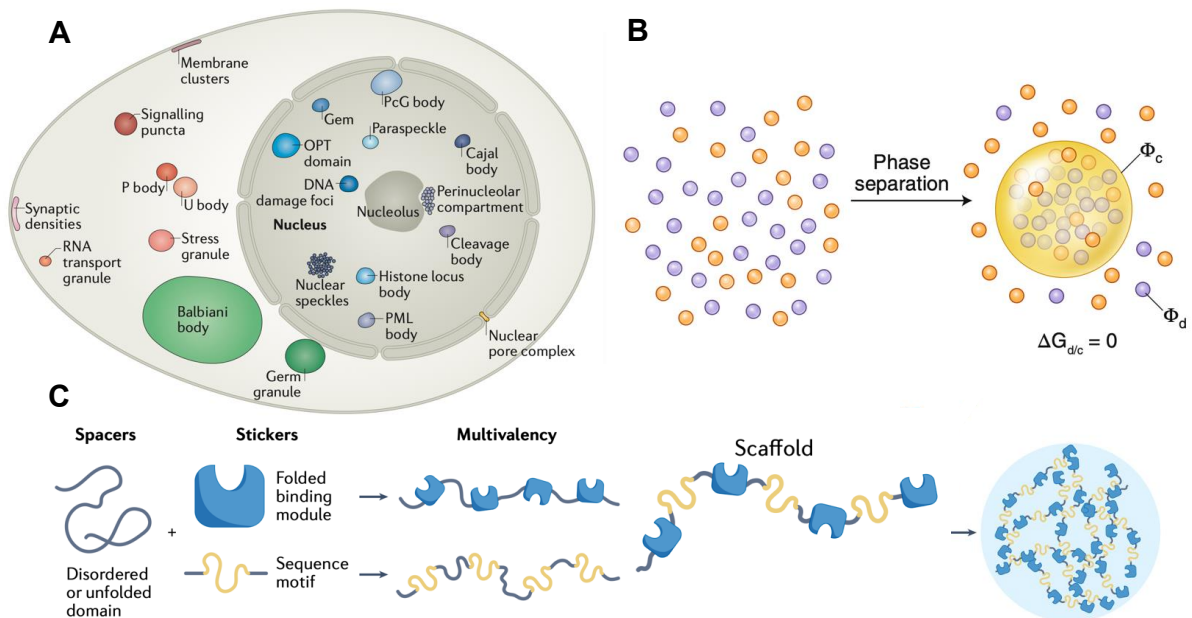


Figure 4: Membraneless organelles and mechanisms of phase separation

A) Schematic representation of membraneless organelles proposed to form through phase separation (Adapted from Banani *et al.*, 2017). **B)** Molecules in the dilute phase spontaneously assemble into a condensed (Φ_c) and dilute (Φ_d) phase, depicted as a rounded droplet where exchange between the phases is possible without energy costs ($\Delta G_{d/c} = 0$) (Adapted from Chen *et al.*, 2020). **C)** The spacer and sticker model serves to show how intrinsically disordered regions between folded binding modules that interact with one another can promote phase separation (Adapted from Mehta & Zhang, 2022).

2019). Membrane-less compartments serve important cellular functions by spatially organizing biochemical processes without physically separating them through lipid bilayers. Instead of enclosing reactions within a membrane-bound organelle, these assemblies create locally concentrated molecular environments that facilitate specific interactions while simultaneously excluding unrelated components. In this way, they act as dynamic molecular hubs that can compartmentalize and regulate cellular reactions while still remaining embedded within the surrounding cytoplasm or nucleoplasm (Feng *et al*, 2019).

In recent years, the concept of LLPS has emerged as a prominent framework to explain the formation of membrane-less assemblies. In physical terms, LLPS describes a process in which macromolecules preferentially interact with one another rather than with the surrounding solvent, driving the spontaneous de-mixing of a homogeneous solution into two coexisting phases: a dense macromolecule-rich phase (Φ_C) and a dilute phase (Φ_D ; Fig. 4B). Notably, these interactions occur in a multimodal fashion, engaging through a diverse repertoire of contacts rather than a single, defined interface as seen in crystalline states. This transition is strongly influenced by environmental conditions such as ionic strength and the presence of additional macromolecules, all of which modulate the balance between intermolecular interactions and solvent interactions (Alberti *et al*, 2019).

A large portion of LLPS research has focused on systems in which RNA acts as a central scaffold. In structures such as the nucleolus or stress granules, RNA provides a multivalent binding platform for numerous RNA-binding proteins, thereby promoting the formation of dense condensates (Roden & Gladfelter, 2021). However, LLPS can also occur in systems composed primarily or entirely of proteins (Alberti *et al*, 2025).

The driving forces for phase separation are typically associated with intrinsically disordered regions (IDRs) within proteins. IDRs lack a stable secondary or tertiary structure and instead exist as flexible, dynamic polypeptide segments. Because they do not adopt rigid conformations, these regions can engage in a wide variety of weak and transient interactions with multiple partners. These interactions may include hydrophobic contacts, electrostatic attractions, cation- π interactions, π - π stacking between aromatic residues, and other weak polar interactions (Choi *et al*, 2020). The cumulative effect of many of such interactions can generate a network of transient contacts that stabilizes a dense phase. Importantly, these interactions are highly sensitive to external factors. Changes in ion concentration, the presence of competing

macromolecules, or additional multivalent binding events can significantly modulate the propensity for condensate formation (Mittag & Pappu, 2022).

Despite the growing popularity of the LLPS framework, the concept remains the subject of ongoing scientific debate. It is argued that the interaction modes typically invoked to explain phase separation are extremely weak and highly sensitive to environmental fluctuations, raising questions about how such assemblies could be precisely regulated *in vivo*. Others have suggested that what is interpreted as phase separation may in some cases represent rather phase transitions, early steps toward irreversible protein aggregation, such as the formation of amyloid fibrils (Tang *et al*, 2025). According to this view, condensates might represent metastable intermediates along a pathway that can ultimately lead to pathological protein assemblies (Babinchak & Surewicz, 2020). These discussions highlight that the study of LLPS is an actively evolving field. New experimental approaches and theoretical frameworks continually refine the current understanding of how biomolecular condensates form and function. Rather than representing a weakness of the concept, this ongoing revision reflects a fundamental strength of the scientific process. Biological theories are not static; they are continuously questioned, amended, refined, or replaced as new data emerge. The investigation of phase separation follows this same principle, with hypotheses being rigorously tested and revised through experimental observation (Alberti *et al*, 2025).

Consequently, the theoretical description of LLPS has undergone substantial development over time. Early models, such as the spacer and sticker model (Fig. 4C), focused primarily on the macroscopic description of droplet formation, including distinctions between microphase and macrophase separation, the energetic stability of condensates, and the influence of environmental parameters such as ion concentration (Choi & Pappu, 2020; Mehta & Zhang, 2022). More recently, these classical views have been complemented by biophysical models that emphasize the role of network formation within condensates. One such framework is percolation theory, which proposes that phase transitions mediated by IDRs can be further stabilized by specific interactions between the classical stickers: well-folded protein domains (Shen *et al*, 2023). In this model, folded interaction modules act as defined nodes within a larger multivalent network. When the connectivity of this network exceeds a critical threshold, a system-spanning interaction network forms, resulting in a percolated state. Within such a percolated network, interactions extend throughout the entire condensate, effectively linking one side of the assembly to the other. This network architecture

provides a physical basis for the characteristic viscoelastic properties observed in many biomolecular condensates, which combine aspects of both liquid-like and gel-like behavior. The implications of percolation for synaptic scaffold assembly will be discussed in greater detail throughout this thesis but specifically in Chapter 5.

The integration of multiple concepts, weak multivalent interactions, environmental modulation, and network connectivity, has significantly expanded the theoretical framework for understanding biomolecular phase separation. In the context of neuronal synapses, these principles are particularly relevant, as synaptic compartments such as the PSD display many features reminiscent of protein condensates (Fig 5A). The following section will therefore examine how the concept of phase separation applies to synaptic organization and how these ideas contribute to our current understanding of scaffold proteins such as gephyrin.

1.3.1 Liquid-liquid phase separation at the postsynaptic density

Phase separation at excitatory synapses has been studied extensively in recent years. The central scaffold network of the ePSD is organized around the multidomain scaffold protein PSD-95 and its interacting partners, including members of the synGAP, SAPAP, Shank, and Homer protein families (Fig. 5B) (M *et al*, 2016; Chen *et al*, 2023; Jia *et al*, 2025a; Chen *et al*, 2020). These proteins form an interconnected interaction network that organizes glutamatergic receptors and associated signaling complexes. Recent work has significantly advanced the understanding of this system by integrating the concept of percolation into models of PSD organization. In particular, it was shown that targeted disruption of specific network nodes within the PSD-95-centered network can fundamentally alter the structure of the resulting condensates (Jia *et al*, 2025b). For example, interference with the PSD-95–SAPAP1 (alias GKAP) interaction using a synthetic peptide (“DLS-PBM”) disrupted system-spanning LLPS and led to the emergence of two distinct subregions within the condensate. These consisted of a dense “core” containing PSD-95 together with the auxiliary glutamate receptor subunit stargazin, and a surrounding “pallium” enriched in SAPAP1, Shank3, and Homer2. Further perturbation of the network could be achieved by replacing tetrameric homer with the splice variant Homer1a, which lacks the ability to form homo-tetramers. Because homer oligomerization is required for the integrity of the pallium network, expression of Homer1a effectively disrupted this outer condensate layer. These

experiments illustrate how specific interaction nodes can determine the connectivity of the interaction network and thereby modulate the architecture of PSD condensates,

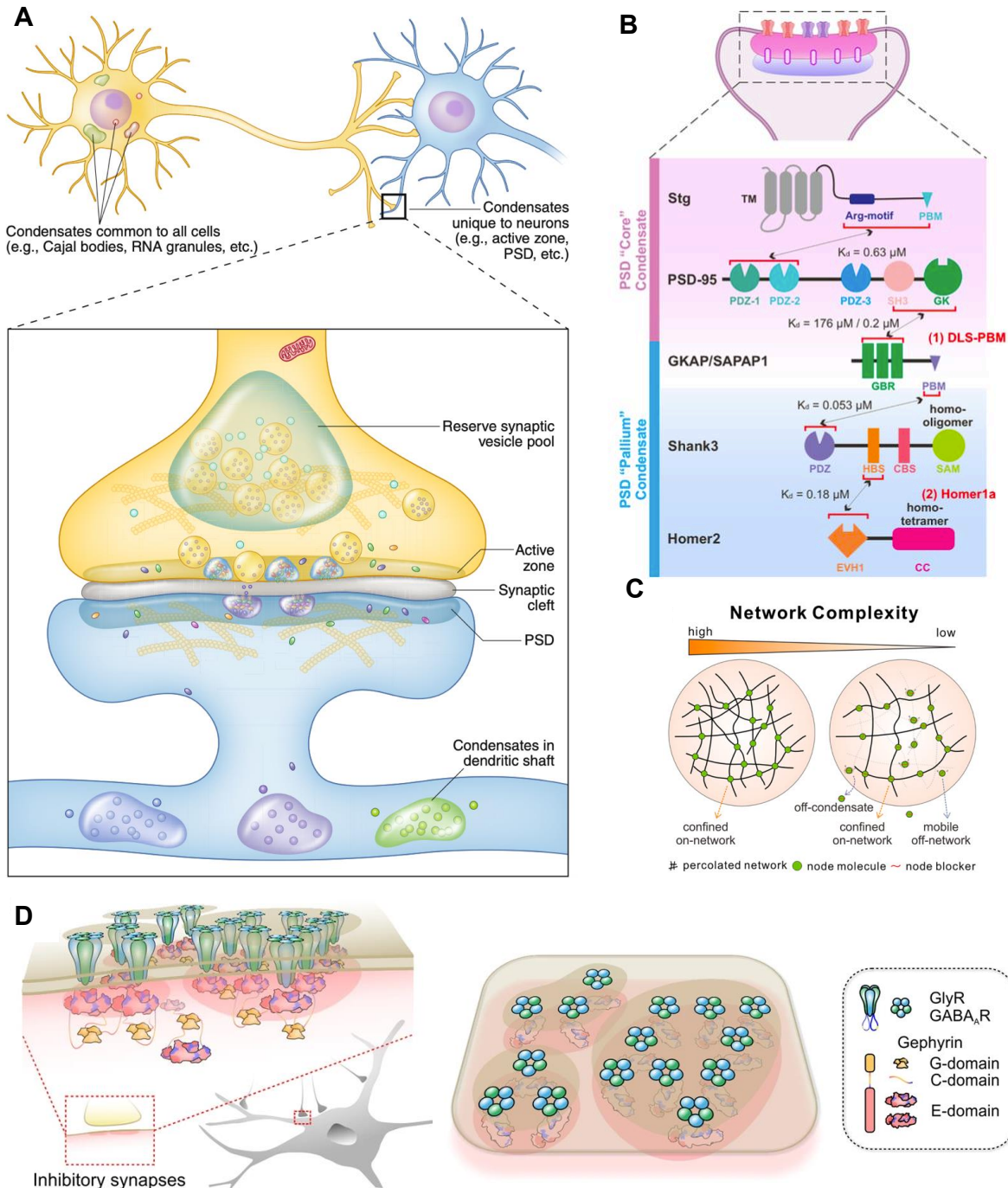


Figure 5: Phase separation at the synapse

A) Schematic representation of membraneless organelles in the nervous system. Common condensates found in many cells (e.g. Cajal bodies, RNA granules) are present in the soma, alongside synapse-specific assemblies (top). In presynaptic boutons, synaptic vesicle pools and active zones are organized via phase separation (bottom). On the postsynaptic side, both ePSD and iPSD proteins can form condensates that resemble native PSDs (Adapted from Chen *et al.*, 2020). **B)** Molecular organization of the ePSD in reconstituted condensates. Arrows indicate domains that were purified for interaction studies (Adapted from Jia *et al.*, 2025b). **C)** Schematic representation illustrating how inhibition of a central node within a percolated molecular network reduces overall network connectivity. As a result, more molecules disengage from the network, exhibit increased mobility, and show reduced enrichment within the condensate (Adapted from Jia *et al.*, 2025b). **D)** Conceptual depiction of phase separation between gephyrin and its associated receptors at inhibitory postsynaptic sites. (Adapted from Bai *et al.*, 2021)

providing strong experimental support for the emerging framework that combines LLPS with percolation theory (Fig. 5C) (Shen *et al*, 2023).

In contrast to the complex multi-protein ePSD scaffold with multiple interaction nodes that regulate phase separation, the iPSD is organized around one central scaffold protein: gephyrin. Historically, gephyrin condensation was first observed in heterologous expression systems, where expression of gephyrin in human embryonic kidney (HEK) cells led to the appearance of characteristic intracellular “blobs” or *puncta* without the link to biophysical processes such as LLPS (Kirsch *et al*, 1995). With the development of the LLPS framework hypothesis, these observations gained new significance. The first direct demonstration that gephyrin can undergo LLPS was provided only recently by *in vitro* studies using purified protein (Bai *et al*, 2021). In this study, several key criteria that define biomolecular phase separation were established. Fluorescence recovery after photobleaching (FRAP) experiments demonstrated the dynamic exchange of molecules within gephyrin condensates, consistent with liquid-like behavior. Sedimentation assays using the previously mentioned dimeric GlyR model system based on GCN4 revealed concentration-dependent condensate formation. Furthermore, experiments on giant uni-lamellar vesicles (GUVs) confirmed that gephyrin condensates could assemble at membrane surfaces (Bai *et al*, 2021). Together, these experiments provided compelling evidence that gephyrin can form phase-separated condensates that resemble the protein-dense structures observed at inhibitory synapses (Fig. 5D). The findings and their implications for inhibitory synapse organization have been summarized extensively (Bai & Zhang, 2022).

A central element of the ongoing discussion about gephyrin phase separation concerns the role of the central C-domain. This region of the protein is an IDR and has therefore been considered a potential driver of LLPS. However, different experimental approaches have produced partially divergent results regarding its precise role on phase separation. Cellular studies have shown that the gephyrin C-domain is indispensable for scaffold condensation and can even be functionally replaced by heterologous IDRs, in some cases enhancing gephyrin’s phase separation capacity (Lee *et al*, 2024). In contrast, *in vitro* experiments with purified proteins have shown that segments of the C-domain can inhibit LLPS driven by the E-domain. Under these conditions, interactions between the C-domain and the E-domain appear to restrain E-domain-mediated phase separation, thereby acting as an intrinsic regulatory

mechanism that limits spontaneous scaffold condensation (Bai *et al*, 2021). Despite these differences in interpretation, both experimental systems converge on the conclusion that multivalent interactions with receptor-binding partners boost robust phase separation. Without these multivalent receptor interactions, gephyrin does not readily form condensates *in vitro*, while in cellular systems these interactions further strengthen the previously described “blop”-formation.

1.4 Regulation of gephyrin clustering

Multiple regulatory mechanisms have evolved that modulate gephyrin structure, interactions, and oligomerization behavior, thereby influencing its capacity to interact with receptors, form higher-order assemblies, and participate in phase-separated synaptic condensates. One fundamental layer of this regulation is alternative splicing of the *GPHN* gene. Alternative splicing of gephyrin was first described for the rat *gphn* gene (Ramming *et al*, 2000). The gene architecture varies between species, with the

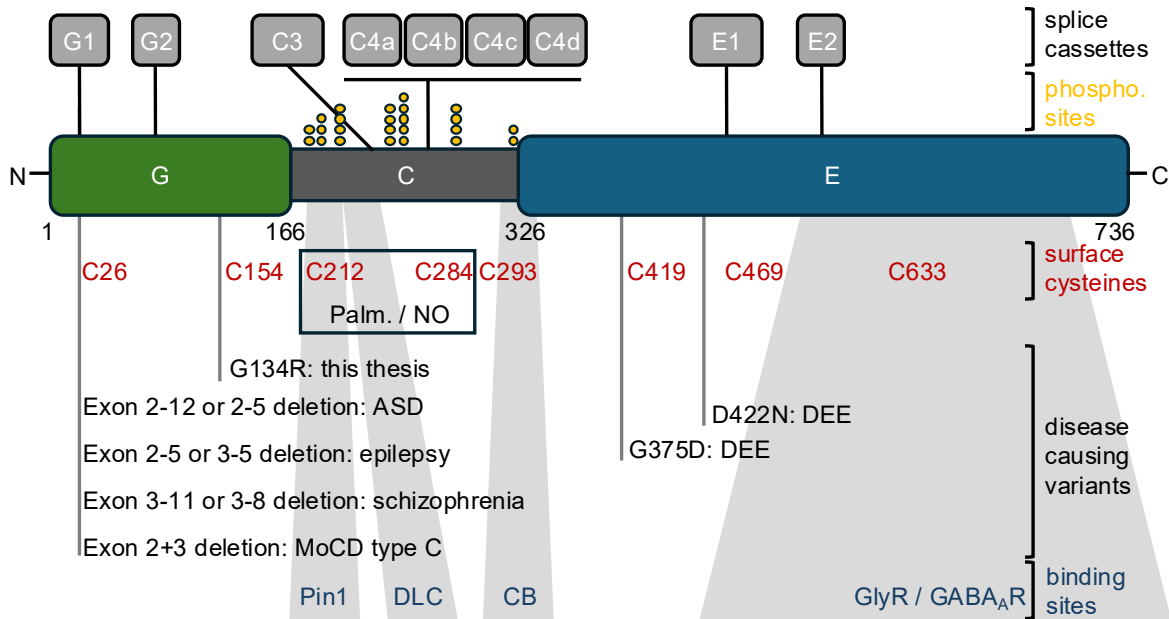


Figure 6: Gephyrin structural and regulatory landscape

Splice cassettes (named according to the affected domains) are indicated by grey boxes at their respective positions. Phosphorylation sites are shown in yellow, from left to right: 1[T198 + T199], 2[S188 + S194 + S200], 3[S205 + S222 + S226 + T227], 4[S262 + T266 + S268 + S270], 5[T276 + S277 + S280 + T281 + T286], 6[S295 + S303 + S305 + S319], 7[T324 + S325]. Surface cysteines are shown in red, with C212 and C284 being targets for palmitoylation and nitrosylation. Disease causing variants for Autism spectrum disease (ASD), epilepsy, schizophrenia, molybdenum cofactor deficiency disease (MoCD) or developmental and epileptic encephalopathy (DEE) are shown with location. Binding sites for Pin1 at phosphorylation site 2[S188 + S194 + S200], DLC at phosphorylation site 3[S205 + S222 + S226 + T227], collybistin (CB) at phosphorylation site 7[T324 + S325] and GlyR / GABA_AR are highlighted at the bottom.

murine *gphn* gene containing 40 exons (Dos Reis *et al*, 2022) and the human *GPHN* gene comprising 28 exons (Rees *et al*, 2003). Combinatorial exon usage gives rise to extensive transcript diversity, with more than 277 splice variants reported (Dos Reis *et al*, 2022).

The nomenclature of gephyrin isoforms has evolved alongside its increasing complexity. Early variants were named according to the domain in which alternative exons occurred (e.g., G1–G3, C3, C4a–C4d, E1, E2; Fig. 6). With the discovery of numerous transcripts, an alternative naming scheme based on exon composition and abundance was introduced (Dos Reis *et al*, 2022). Under this classification, the most abundant neuronal isoform, previously referred to as P1, is designated GPHN-1, which contains exons 1, 6, 8, 10, 12–14, 17, 25, 27–31, and 33–40.

Most alternative splicing events occur within the central C-domain (Fig. 6). Some splice variants show tissue-specific expression patterns, such as the C3-containing isoform, which is predominantly expressed in non-neuronal cells and is thought to primarily support gephyrin's enzymatic function in Moco biosynthesis (Smolinsky *et al*, 2008). In addition, splice variants can influence the subcellular localization of gephyrin within neurons. For example, the C4c-containing variant preferentially localizes to distal dendritic compartments and modulates inhibitory synaptic transmission (Liebsch *et al*, 2023). Together, these findings indicate that alternative splicing contributes to both the functional diversification and spatial regulation of gephyrin in neuronal and non-neuronal contexts. Beyond alternative splicing, gephyrin clustering and synaptic function are further regulated by posttranslational modifications (PTMs) and interactions with additional synaptic proteins.

1.4.1 Post translational modifications

A variety of PTMs have been identified on gephyrin, many of which occur within the C-domain, where they can influence scaffold dynamics and synaptic stability. They range from phosphorylation over acetylation and SUMOylation (small ubiquitin-like modifier proteins) to specific cysteine modifications such as S-nitrosylation and S-palmitoylation (Fig. 6).

Among these, phosphorylation represents one of the most extensively studied modifications. More than 18 phosphorylation sites have been identified, predominantly

on serine and threonine residues within the C-domain (Fig. 6) (Zacchi *et al*, 2014; Herweg & Schwarz, 2012). Several kinases are known to target gephyrin, including glycogen synthase kinase 3 β (GSK3 β) (Tyagarajan *et al*, 2011), cyclin-dependent kinase 5 (CDK5) (Kuhse *et al*, 2012), extracellular signal-regulated kinase 1 (ERK1) (Tyagarajan *et al*, 2013), calmodulin-dependent protein kinase II (CaMKII) (Flores *et al*, 2015), and cAMP-dependent protein kinase (PKA) (Battaglia *et al*, 2018). A direct mechanistic link between C-domain phosphorylation and gephyrin's capacity to form condensates comes from work showing that specific serine residues within two proposed autoinhibitory regions of the C-domain can bidirectionally regulate gephyrin-mediated LLPS (Bai *et al*, 2021). Phosphomimetic substitution of S268 or S270 (targets of ERK, GSK3 β , and CDK5), located within the first of these regions, further suppressed phase separation with GABA $_A$ R- α 3, indicating that phosphorylation at these sites strengthens autoinhibition and would be expected to reduce receptor clustering at the synapse. Conversely, phosphomimetic substitution of S305 (a CaMKII target) in the second region, immediately preceding the E-domain, enhanced phase separation, likely by introducing a negative charge that neutralizes the local positive charge of that segment and thereby relieves its inhibitory effect on the secondary receptor-binding surface of the E-domain. Together, these findings suggest that the C-domain acts as a regulatory switch, where the balance of kinase activity converging on distinct serine residues can bidirectionally tune the condensation of gephyrin/receptor complexes and, consequently, the density of inhibitory receptors at the postsynaptic membrane.

Gephyrin is also regulated by cysteine-based lipid and redox modifications, particularly S-palmitoylation and S-nitrosylation (Fig. 6). S-palmitoylation occurs on Cys212 and Cys284 within the C-domain and is catalyzed by zinc finger DHHC domain-containing protein 12 (DHHC-12) (Dejanovic *et al*, 2014), which attaches palmitoyl residues to these cysteine residues. This modification promotes membrane association of gephyrin, thereby contributing to its synaptic localization. While palmitoylation can be enzymatically reversed by palmitoyl protein thioesterases (PPTs), the specific enzyme responsible for removing palmitoyl groups from gephyrin has not yet been identified.

S-nitrosylation is linked to the neuronal nitric oxide synthase (nNOS) (Dejanovic & Schwarz, 2014). Gephyrin colocalizes with nNOS, and nitric oxide (NO) can promote nitrosylation either directly or indirectly via glutathione-mediated *trans*-S-nitrosylation.

Multiple cysteine residues in gephyrin have been reported to undergo S-nitrosylation, and differential modification patterns have been observed in patient cohorts with or without Alzheimer's disease, suggesting potential links between gephyrin redox regulation and neurodegenerative processes (Yang *et al*, 2022; Seneviratne *et al*, 2016).

More broadly, cysteine residues can adopt a wide range of oxidative states, which often confer unique chemical properties to proteins. Such modifications can function as redox switches (Talwar *et al*, 2023), participate in catalytic mechanisms (Ohno *et al*, 1984), or mediate metal binding (Shi *et al*, 2021), as seen in diverse proteins including cysteine proteases, thiol oxidoreductases such as thioredoxin, and metal-binding enzymes containing cofactors such as Fe–S clusters or Moco. Cysteine residues can theoretically transition between multiple states, including free thiol (S–H), persulfide (S–SH), disulfide (S–S), S-nitrosothiol (S–NO), S-palmitoyl (S-palm), sulfenic acid (S–OH), sulfinic acid (S–O₂H), and sulfonic acid (S–O₃H), each of which may influence protein-protein interaction behavior (Pace & Weerapana, 2013). Proteins that contain cysteine residues exposed on their surface are particularly susceptible to redox-dependent modifications. The accessibility of these cysteines has also been associated with their evolutionary conservation and functional relevance (Marino & Gladyshev, 2010). In this context, the presence of solvent-exposed cysteines in gephyrin suggests that their positioning could be functionally meaningful, potentially pointing toward a role in redox-mediated regulation.

Many of these oxidative modifications are influenced by reactive oxygen species (ROS), which function both as cellular signaling molecules and as potential sources of oxidative stress. In neurons, ROS can arise from mitochondrial electron transport chain activity (mROS) or from nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complexes such as NOX2 (Vignais, 2002; Turrens, 2003). The potential role of ROS-dependent redox regulation in modulating gephyrin function will be explored in more detail in Chapter 3 of this thesis.

1.4.2 Interacting Proteins

Several interacting proteins have been identified that influence gephyrin localization, oligomerization, or signaling at inhibitory synapses (Fig. 6). Those binding partners

belong to the family of cytoskeletal, membrane-interacting, motor and signal transduction proteins.

One such partner is collybistin, a guanine nucleotide exchange factor (GEF) whose name derives from the Greek *κόλλυβος* “*kóllymbos*” or “*colly*” (“to exchange”) and *βύζω* “*byzo*” or “*bystin*” (“to plug”). Collybistin functions as an adaptor linking gephyrin to membrane lipids. Structurally, it contains three main domains: an SRC homology 3 (SH3) domain, which mediates interactions with proteins such as neuroligin-2 and the $\alpha 2$ subunit of GABA_AR; a Dbl homology (DH) domain, which confers GEF activity; and a pleckstrin homology (PH) domain, which binds phosphoinositide lipids in the plasma membrane. Recent work demonstrated that gephyrin, collybistin, and specific plasma membrane phosphatidylinositol phosphate (PIP) lipids can assemble into large complexes exceeding 5 MDa, which remain enzymatically active and contribute to the regulation of gephyrin oligomerization and synaptic clustering (Burdina *et al*, 2025).

Another interacting protein is dynein light chain (DLC), originally described as the protein inhibitor of neuronal nitric oxide synthase (PIN) (Jaffrey & Snyder, 1996). In this context, DLC may act as a capping factor, potentially limiting the interaction between gephyrin and neuronal nitric oxide synthase (nNOS), thereby modulating gephyrin nitrosylation. DLC functions as a receptor transporter protein and binds within the gephyrin C-domain. As a motor protein it has been linked to gephyrin transport along the cytoskeleton but its interaction with gephyrin has also been linked to LLPS in the presence of dimeric receptor models (Bai *et al*, 2021).

Pin1 (peptidyl-prolyl *cis*–*trans* isomerase NIMA-interacting 1) (Zita *et al*, 2007) binds to a proline-rich region within the gephyrin C-domain and catalyzes *cis*–*trans* isomerization of peptide bonds preceding proline residues. Through this conformational regulation, Pin1 can influence the interaction between gephyrin and the β subunit of GlyR, thereby affecting receptor stabilization at inhibitory synapses.

Gephyrin can also be regulated through proteolytic processing by calpain, a widely expressed calcium-dependent protease that cleaves target proteins at different sites that are not fully described for gephyrin yet (González, 2019). Calcium signals that activate calpain frequently originate from excitatory synapses, suggesting that calpain-mediated cleavage of gephyrin may represent a mechanism of cross-talk between excitatory and inhibitory synaptic signaling (Kumar *et al*, 2017). This becomes especially important during progressed disease phenotypes, discussed in the next chapter.

1.5 Gephyrin in disease

Due to its dual functions in both neuronal scaffolding and Moco biosynthesis, dysfunction of gephyrin can lead to two major classes of disease phenotypes: metabolic disorders associated with impaired Moco synthesis and neurological disorders resulting from disrupted inhibitory synaptic transmission. In some cases the symptoms are overlapping due to neurological manifestation of both pathologies resulting in hyperexcitation by either metabolic activation of glutamate receptors or loss in inhibition.

Defects in the metabolic function of gephyrin contribute to Moco deficiency (MoCD) type C, a severe metabolic disorder affecting enzymes that depend on Moco (Johannes *et al*, 2022; Reiss *et al*, 2001). In case of type C MoCD, the last branch of the synthesis is affected while in type A (MOCS1A/B affected) the first reaction step and type B MoCD (MOCS2A/B or MOCS3 affected) the intermediate reactions are impaired. Biochemical diagnosis of MoCD relies on characteristic biomarkers including sulfite (SO_3^{2-}), S-sulfocysteine (SSC), and thiosulfate ($\text{S}_2\text{O}_3^{2-}$) (Johannes *et al*, 2022). While certain therapeutic approaches exist for forms of MoCD type A (Veldman *et al*, 2010; Schwahn *et al*, 2015; Schwarz *et al*, 2025), no effective treatment is currently available for deficiencies caused by the final step of the biosynthetic pathway involving gephyrin, i.e. MoCD type C.

Complete loss of gephyrin function is lethal in humans, with affected individuals typically surviving only a few days after birth and often remaining undiagnosed due to the severity and rapid progression of the phenotype. Similarly, gephyrin knockout mice die within the first hours of life (Feng *et al*, 1998; Essrich *et al*, 1998). Importantly, restoration of Moco synthesis alone is not sufficient to rescue this lethality, highlighting the essential neuronal role of gephyrin in addition to its metabolic function (Grosskreutz *et al*, 2003).

Gephyrin dysfunctions have been associated with several neurological phenotypes, including autism spectrum disorder (ASD) and epileptic encephalopathy. Missense mutations have been reported in patients, often accompanied by larger mono-allelic microdeletions spanning the G-, C-, or E-domains of the protein (Lionel *et al*, 2013; Dejanovic *et al*, 2015; Macha *et al*, 2022b). These alterations can impair gephyrin's ability to organize inhibitory receptor clusters, thereby disturbing the balance between excitatory and inhibitory signaling in neuronal circuits.

Disruption of gephyrin function can also occur through proteolytic cleavage by calpain. During periods of strong neuronal excitation, elevated intracellular Ca^{2+} levels can activate calpain, leading to cleavage of gephyrin and destabilization of gephyrin–receptor clusters. This process contributes to pathological excitation–inhibition imbalance, particularly under conditions of hyperexcitation. Notably, SSC, one biomarker of MoCD, acts as a glutamate analogue, further promoting excitatory signaling and Ca^{2+} influx, which can amplify calpain activation and thereby exacerbate the disruption of inhibitory scaffolds (Kumar *et al*, 2017).

Impairment of gephyrin interactions with inhibitory receptors can also produce distinct neurological phenotypes. Disrupted gephyrin–GlyR interactions interfere with glycinergic inhibition and have been linked to disorders characterized by muscle stiffness and exaggerated startle responses, such as stiff-person syndrome and hyperekplexia (Butler *et al*, 2000; Ryan *et al*, 1994). Similarly, disturbances in GABAergic inhibition can contribute to a broader range of neurological disorders, including epileptic encephalopathy.

Several single point mutations in gephyrin have previously been associated with epilepsy-related phenotypes, including the pathogenic variants G375D and D422N affecting the E-domain (Dejanovic *et al*, 2015; Macha *et al*, 2022b). Although both mutations exhibit reduced binding to model receptor peptides in ITC measurements, they are located within SDII of the E-domain, spatially separated from the primary receptor binding interface. This suggests that their pathogenic effects cannot be explained solely by impaired receptor interaction. In the case of D422N, the presence of nonsense-mediated decay (NMD) on the second *GPHN* allele results in exclusive expression of the mutant protein, leading to severe phenotypes consistent with strongly impaired glycinergic and GABAergic inhibition (Macha *et al*, 2022b).

In contrast, G375D occurs in a heterozygous context, resulting in milder but still pronounced symptoms, indicative of a dominant-negative effect (Dejanovic *et al*, 2015). Notably, G375D abolishes Moco synthesis, a defect that is at least partially compensated by the expression of active protein from the other wild-type allele. Beyond receptor binding, G375D shows multiple deficits, including impaired LLPS, reduced formation of higher-order oligomers, loss of NL-2 interaction, and diminished collybistin-dependent clustering (Kim *et al*, 2021; Bai *et al*, 2021). Together, these findings indicate that both G375D and D422N interfere with the structural and network-forming properties of gephyrin, supporting the notion that disruptions in condensate

organization and scaffold connectivity, rather than receptor binding alone, contribute substantially to disease pathology. In the context of this thesis, these earlier observations provide important background for the characterization of a newly identified patient mutation impacting gephyrin's G-domain. This variant will be examined in detail in Chapter 2.

1.6 Aims of this thesis

This thesis investigates the molecular principles underlying gephyrin-mediated inhibitory synapse organization, with LLPS serving as the central conceptual theme linking all chapters. With each chapter I aim to address a distinct aspect of gephyrin regulation and assembly, while together providing a coherent framework for an improved understanding of inhibitory clustering in health and disease.

Chapter 2 – G-domain regulation of gephyrin phase separation:

This chapter expands the analysis of gephyrin phase condensation behavior within the framework of percolation theory by investigating the role of the G-domain. While previous studies of gephyrin LLPS have largely focused on the receptor-binding E-domain, the G-domain has received comparatively little attention due to its distance from the receptor interaction site. In this chapter, I analyze the functional consequence of a newly identified *de novo* point mutation in the *GPHN* gene found in a 16-year-old patient suffering from intractable nocturnal epilepsy since birth. The mutation results in a glycine-to-arginine substitution at position 134 (G134R) within the highly conserved G-domain of gephyrin. Our study represents the first detailed investigation of how a patient-derived mutation in this domain affects gephyrin function in neurons.

Chapter 3 – Redox-dependent modulation of gephyrin clustering:

This chapter examines how posttranslational modification of surface cysteine residues influences gephyrin phase behavior and clustering with inhibitory receptors. We advance the previously known modifications on cysteines in the central C-domain of gephyrin via palmitoylation and nitrosylation to oxidative modifications *in vitro*, cellular experiments and also mouse experiments while also looking into detailed effects of single cysteine residues *in vitro*.

Chapter 4 – Structural basis of gephyrin filaments:

Here, we define the molecular architecture underlying inhibitory postsynaptic densities via combining multiple ideas of the fundamental structure at the iPSD with recent findings in its connection to LLPS. The patient derived single amino acid exchanges G375D and D422N help to pinpoint and connect previous models into a common structural basis while at the same time are being put into a whole new perspective in their structure giving role. With state-of-the-art cryo-EM imaging and *in vitro* biochemistry this chapter rounds off previous assumptions on gephyrin organization.

Collectively, the major aim of this thesis is to connect structural motifs, redox regulation, and phase separation into a unified model of gephyrin-mediated inhibitory synapse organization. By dissecting how these mechanisms intersect, the thesis aims to advance our mechanistic understanding of inhibitory clustering and its dysregulation in neurological disorders.

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Chapter 1 – General introduction

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Chapter 2: Impaired gephyrin G-domain trimerization and phase separation in a patient with developmental epileptic encephalopathy

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2.1 Author contributions

Emanuel H. W. Bruckisch, Günter Schwarz and Filip Liebsch designed the study. Emanuel H. W. Bruckisch, Günter Schwarz and Filip Liebsch wrote and corrected the manuscript. Emanuel H. W. Bruckisch performed protein purifications, SEC studies, ITC measurements, *in vitro* Moco analysis, *in vitro* phase separation and BN-PAGE experiments. Filip Liebsch and Ann-Kathrin Huber performed HEK cell experiments. Filip Liebsch performed murine hippocampal neuron culture experiments. Marcelo de Melo Aragão, Thais dos Santos Rhode and Mateus de Oliveira Torres performed medical examination of the patient as well as karyotyping, chromosomal microarray analysis and next-generation sequencing of a gene panel for epilepsy and metabolic disorders.

2.2 Abstract

Epilepsy, a common neurological disorder is frequently linked to genetic variants in synaptic proteins. Here, we describe a *de novo* pathogenic missense variant in the gephyrin G-domain (G134R) identified in an individual with developmental delay, epileptic seizures, microcephaly, dysmorphic features and short stature. Functional analyses reveal that G134R disrupts higher-order oligomerization, leading to impaired liquid-liquid phase separation (LLPS) and synaptic clustering. Recombinant G134R-gephyrin variant forms lower oligomers and retains only 60% of its molybdenum cofactor (Moco) synthesis activity while binding to glycine receptor models is unaffected. In non-neuronal cells, G134R fails to oligomerize beyond dimers with Moco synthesis activity reduced to 5%. In neurons, G134R is unable to form synaptic clusters and exerts a dominant-negative effect on WT-gephyrin, severely disrupting inhibitory synapse formation. Our findings highlight a critical role for the G-domain in gephyrin self-assembly and LLPS, shifting the focus from the E-domain-centric view of gephyrin function and providing a novel molecular mechanism for epilepsy linked to G-domain mutations.

2.3 Introduction

Epilepsy, affecting over 50 million people worldwide, is a common neurological disorder characterized by recurrent seizures caused by an imbalance between excitatory and inhibitory neuronal activity (Schwartzkroin 2012; Yardi 2025). Among the most severe forms are developmental and epileptic encephalopathies (DEE) (Scheffer et al. 2025), which often arise from *de novo* genetic mutations and manifest in early childhood with seizures and are regularly accompanied by developmental delay, and cognitive impairment (Cross and Guerrini 2013). Mutations in genes involved in synaptic transmission are frequent causes of DEE, with inhibitory synaptic proteins playing a prominent role (Guerrini et al. 2023; Uzay and Kavalali 2023; Reinthaler et al. 2015). Gephyrin is the essential postsynaptic scaffold protein at inhibitory synapses. It interacts with glycine receptors (GlyRs) (Meyer et al. 1995) and GABA type A receptors (GABA_ARs) (Maric et al. 2011), thereby anchoring and clustering these receptors to maintain inhibitory strength (Fritschy et al. 2008; Tyagarajan and Fritschy 2014). Gephyrin assembles into higher-order oligomeric complexes, mediated by trimerization of its N-terminal G-domain (Schwarz et al. 2001; Saiyed et al. 2007) and dimerization of the C-terminal E-domain (Kim et al. 2006). Recently, gephyrin oligomerization has been linked to liquid-liquid phase separation (LLPS), proposed as a central mechanism for the formation and plasticity of inhibitory synapses (Bai et al. 2021; Bai and Zhang 2022; Zhu et al. 2024; Lee et al. 2024).

Besides its synaptic role, gephyrin catalyzes the final steps of molybdenum cofactor (Moco) biosynthesis, a function conserved from bacteria to humans (Belaidi and Schwarz 2013). In this pathway, the G-domain catalyzes the adenylation of molybdopterin (MPT) to generate MPT-AMP, while the E-domain subsequently hydrolyzes AMP and incorporates molybdate to yield active Moco (Belaidi and Schwarz 2013). Mutations in gephyrin can thus impair metabolic functions, and, in the only reported case to date, a homozygous deletion of exons 2–3 in *GPHN* resulted in complete loss of gephyrin protein and caused molybdenum cofactor deficiency (MoCD) type C (Fig. 1A) (Schwarz et al. 2009; Johannes et al. 2022; Reiss et al. 2001).

Pathogenic variants and exonic microdeletions in the gephyrin gene (*GPHN*) have been identified in patients with a spectrum of neurodevelopmental and neuropsychiatric disorders, including autism spectrum disorder (ASD), schizophrenia, and epilepsy (Fig. 1A) (Lionel et al. 2013). Mechanistic studies on patients

demonstrated that missense variants within the E-domain, such as G375D and D422N reduce gephyrin receptor-binding and clustering, thereby disrupting inhibitory neurotransmission and causing DEE (Fig. 1A) (Macha et al. 2022; Dejanovic et al. 2015). Additionally, single particle cryogenic electron microscopy analyses revealed that these two variants fail to form filaments via subdomain II interactions of adjacent dimers, which likely explains their inability to form higher oligomers and undergo LLPS (Bai et al. 2021; S. Kim et al. 2021; Macha et al. 2025).

Here, we describe the first pathogenic gephyrin patient variant with a single amino acid exchange located in the G-domain, derived from an epileptic patient. We identified a heterozygous *de novo* missense mutation (c.400G>C, p.Gly134Arg “G134R”) in a patient with developmental seizures born to unrelated and unaffected parents. Functional analyses revealed that G134R disrupted the ability of gephyrin to form higher-order oligomers. We hypothesize that while the E-domain serves as the primary driver of phase separation and provides the interface for receptor binding (Macha et al. 2025), the G-domain plays a crucial role in interconnecting gephyrin conjugates into higher-order oligomers, thereby enhancing the capacity for phase separation and supporting synaptic clustering. Consequently, G134R-gephyrin failed to generate functional receptor-scaffold complexes at inhibitory synapses and similar to G375D-gephyrin decreased the density of synaptic WT clusters in a dominant-negative fashion.

2.4 Results

2.4.1 Clinical description of the patient with a *de novo* G134R-gephyrin variant

We report a 15-year-old female patient from Brazil, born late preterm after an uneventful pregnancy and delivery. No prenatal, perinatal, or postnatal complications were reported. Her parents are unrelated, and there is no family history of epilepsy or other neurological disorders. Developmental delay was noted within the first year of life, and at the age of nine months she was diagnosed with epilepsy. Seizures occurred predominantly during sleep and were characterized by apnea, generalized hypotonia, and episodes of tonic posturing of all four limbs followed by clonic movements lasting several minutes. During childhood, she was further diagnosed with intellectual disability and short stature.

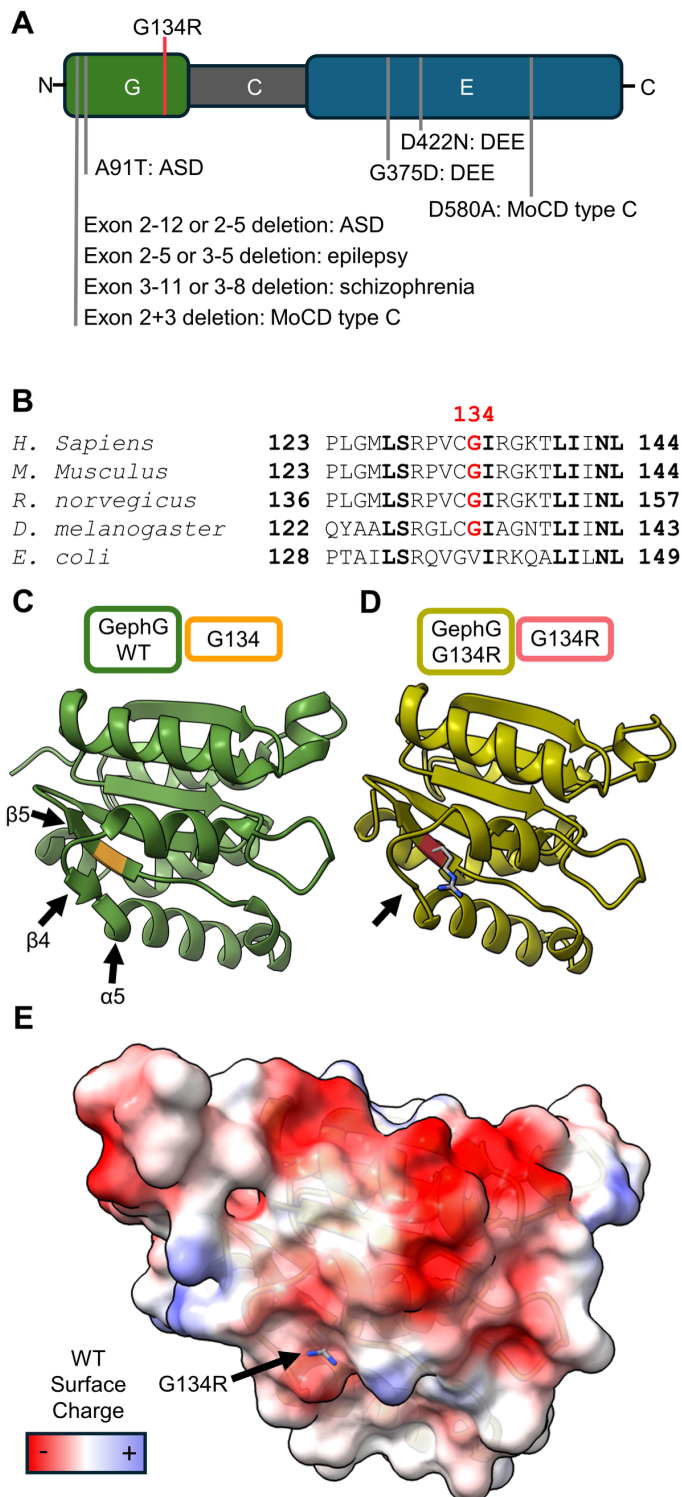


Fig. 1: Localization of the identified GPHN mutation G134R

(A) Gephyrin domain structure with localization of G134R. (B) Local sequence alignment of homologous G-domains in different species. Amino acids in bold are conserved. (C) X-ray crystal structure of human gephyrin G-domain monomer (PDB: 1JLJ). Yellow marks glycine 134. Arrows indicate alpha-helix 5 ($\alpha 5$) which is important for trimerization, beta-sheet 4 ($\beta 4$) and beta-sheet 5 ($\beta 5$) which neighbour alpha-helix 5. (D) AlphaFold3 prediction of G134R-gephyrin G-domain monomer. Magenta marks the position of G134R with arginine's carbon atoms depicted in grey and nitrogen in blue. Arrow marks the position of the lost beta-sheet 4 ($\beta 4$). (E) Electrostatic surface prediction taken from (C) overlayed onto (D) with arrow marking G134R. Red indicates negative charge, blue positive charge. The positive arginine side chain protrudes into a net negative surface charge.

Clinical follow-up began when the patient was 10 years old. Neurological examination at that time showed microcephaly and minor dysmorphic features, including bilateral epicanthus, upslanting almond-shaped palpebral fissures, and small, low-set ears with attached earlobes. Brain MRI performed at 5 years of age revealed no structural abnormalities. Electroencephalography (EEG) at 10 years demonstrated epileptiform discharges, predominantly in the left temporal region during drowsiness. It is likely that the patient's epileptic events began during infancy, at

approximately nine months of age. During this period, the semiology consisted of brief episodes of apnea and hypotonia lasting a few seconds, consistently occurring during sleep. Between the ages of 13 years and 7 months and 13 years and 11 months, prior to the initiation of treatment with the benzodiazepine clobazam, she exhibited a seizure frequency of approximately one episode per month. These events were characterized by tonic posturing of all four limbs accompanied by staring, occurred exclusively during sleep, and lasted several minutes. Before starting clobazam treatment, the patient had undergone therapeutic trials with topiramate, valproate, lamotrigine, and levetiracetam. However, adherence was consistently poor, as she did not tolerate or adequately accept these medications. Clobazam treatment was initiated at the age of 14 years and 3 months, at a dose of 5 mg, and she remained on this regimen thereafter. At the age of 15 years and 1 month, she experienced a single brief breakthrough seizure, prompting an increase in the dose to 10 mg at the age of 15 years and 3 months, approximately one year after treatment initiation. Since the dosage adjustment, she has maintained good seizure control, with no further reported events.

Genetic testing was performed to further investigate the cause of her condition. Both karyotyping and chromosomal microarray analysis yielded normal results. Subsequent next-generation sequencing (NGS by Invitae Corporation, San Francisco, USA) of a gene panel for epilepsy and metabolic disorders identified a heterozygous missense variant in the gephyrin gene (*GPHN*; NM_001024218.2: c.400G>A; p.Gly134Arg). Although an additional intronic variant in *SCN9A* (encoding the sodium voltage-gated channel alpha subunit 9) was identified, it was only classified as “likely benign”, already detected in the unaffected mother, and is primarily associated with altered pain perception rather than epilepsy (Ghanty et al. 2025; Furtleman et al. 2006); therefore, we focused our analyses on the gephyrin variant. This gephyrin variant was initially classified as a variant of uncertain significance (VUS). Segregation analysis demonstrated that the variant had arisen *de novo*. The affected residue Gly134 is located within the G-domain of gephyrin (Fig. 1A) and is conserved throughout higher eukaryotes (Fig. 1B) suggesting an important structural role. Looking at the published crystal structure it becomes obvious that Gly134 resides within the β -sheet 5 structure adjacent to α -helix 5, which forms the trimerization interface (Fig. 1C; PDB 1JLJ). *In silico* prediction tools uniformly support a deleterious effect of the Gly-to-Arg exchange: PolyPhen-2 predicts the substitution to be “probably damaging” (score 1.000), SIFT predicts it to “affect protein function” (score 0.01), MutationTaster25 classifies the

variant as “deleterious” and the combined annotation dependent depletion (CADD) score lists it in the top 0.05% most deleterious potential substitutions across the human genome (score 33.0). Furthermore, the human genome variant database “GnomAD” does not list any allele frequency for this variant, underlining the extreme rarity (variant ID: 14-66916013-G-A). AlphaFold3-based structural prediction suggests that the G134R substitution perturbs the β -sheet 4 element (Fig. 1D), with the bulky, positively charged arginine side chain protruding from and disrupting the otherwise electronegative surface defined by the WT crystal structure (Fig. 1E). Additionally, to complement the AlphaFold3 prediction, we used the web server DDMut-PPI (prediction of mutation-induced changes in protein–protein interaction) to calculate the predicted change in binding free energy of G-domain trimerization ($\Delta\Delta G_{\text{Binding}}$), which yielded a decreased affinity value (-2.53 ± 0.3 kcal/mol). Based on these findings, we decided to further study the molecular and functional consequences of G134R-gephyrin *in vitro* and in cells.

2.4.2 Impaired gephyrin self-assembly and structural destabilization by G134R

To investigate the biochemical properties of G134R-gephyrin, we expressed and purified recombinant isolated G-domain variants and full-length gephyrin from *E. coli*. Size-exclusion chromatography (SEC) of the purified G-domain confirmed the expected trimeric assembly for wild-type (WT) gephyrin, yielding a single, well-defined peak at 75 mL elution volume (Fig. 2A). In contrast, the G134R G-domain did not elute as a single trimer peak but produced partially overlapping peaks surrounding the elution of the WT peak. All fractions were positive for the G-domain as detected by Western blot analysis, indicating that the protein variant is present in multiple distinct oligomeric states (Fig. 2B). These findings suggest that the canonical trimerization of the G-domain is altered by the G134R substitution.

Analysis of full-length gephyrin revealed the characteristic oligomerization elution-profile previously described for WT protein, with specific populations corresponding to lower trimeric and higher oligomeric assemblies (50 mL and 60 mL respectively in Fig. 2C), in addition to aggregates eluting at 40 mL and degradation products eluting at 80 mL. Strikingly, full-length G134R-gephyrin completely lacked the high-oligomer peak at 50 mL, while only the low trimeric fraction was detectable. These trimers eluted at 60 mL, migrated at ~85 kDa on SDS–PAGE, and were

indistinguishable from WT gephyrin by Western blot analysis, indicating that these units remain intact but fail to engage in higher-order assembly (Fig. 2D).

Given these profound alterations in the oligomerization properties of G134R-gephyrin, we next asked whether the protein retains its biological functions. We therefore performed in-depth biochemical analyses of the two major activities of

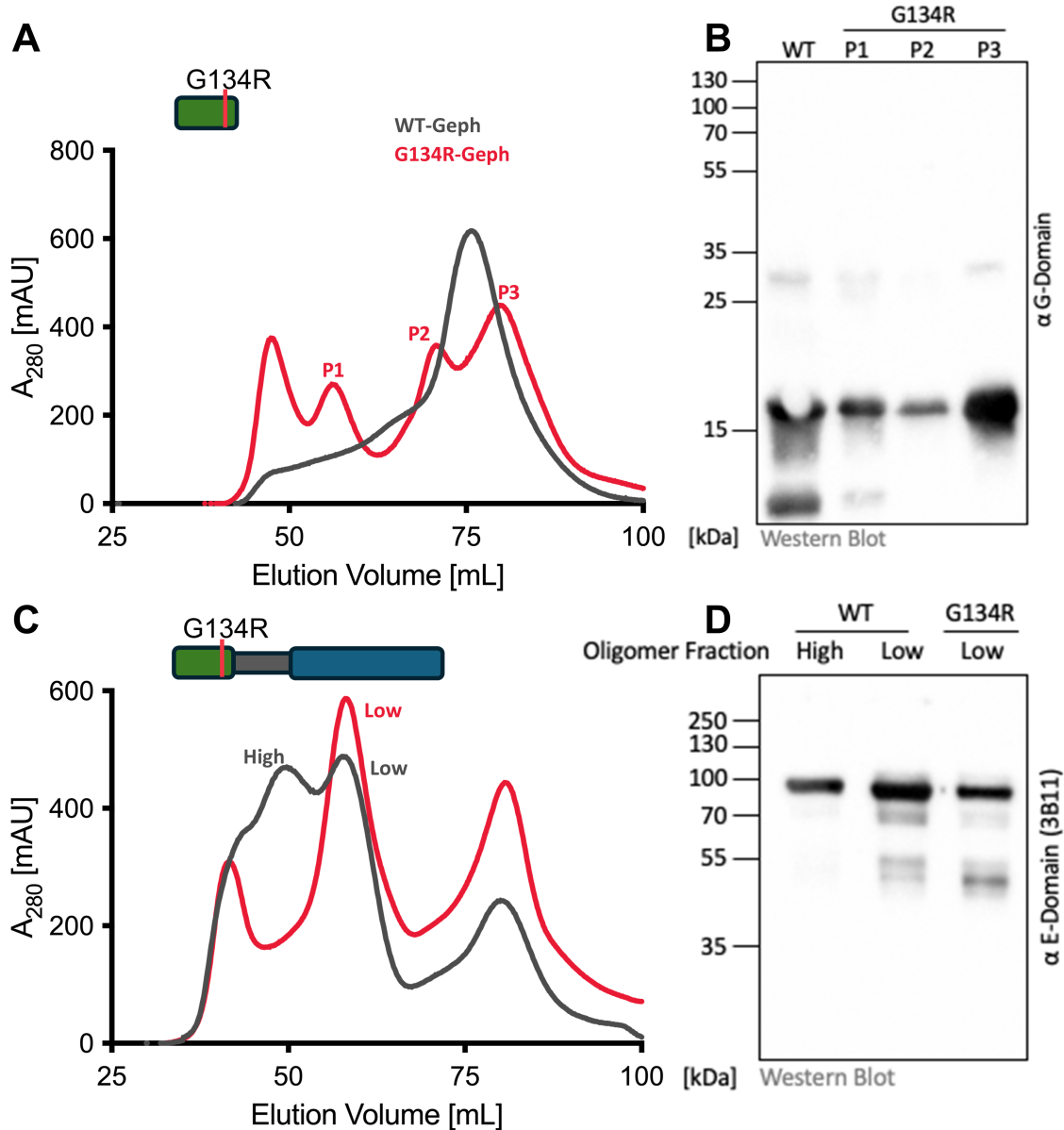


Fig. 2: G-domain and full-length G134R-gephyrin show distinct properties *in vitro*

(A) Size exclusion chromatography (SEC) elution profile of G-domain WT-gephyrin (grey) and G134R-gephyrin (magenta). While WT-gephyrin shows distinct trimeric elution peak, G134R-gephyrin splits up into 4 peaks. **(B)** Western blot analysis of eluted fractions from (A) identifies G134R-gephyrin peaks P1-3 via G-domain specific antibody. The peak before 50 mL depicts cut-off peak at void volume. **(C)** SEC elution profile of full-length WT-gephyrin (grey) and G134R-gephyrin (magenta). While WT-gephyrin shows elution peaks for trimeric low-oligomerization gephyrin and an additional peak for high-oligomerization, G134R-gephyrin is missing the latter. Peaks before 50 mL and after 75 mL depict cut-off peaks at void volumes. **(D)** Western blot analysis of eluted fractions from (C) identifies G134R-gephyrin via E-domain specific antibody (3B11). Only low-oligomer fractions show characteristic double band behavior.

gephyrin: clustering of inhibitory neurotransmitter receptors and catalysis of the molybdenum cofactor (Moco) biosynthesis.

2.4.3 G134R-gephyrin showed unaffected receptor binding and reduced but functional Moco synthesis activity

E-domain dimers of gephyrin mediate the postsynaptic anchoring of inhibitory receptors by binding to conserved intracellular loop domains of GlyR β - and GABA_AR α 3-subunits. Although Gly134 is located distantly in the N-terminal G-domain, the loss of higher-order oligomerization in G134R-gephyrin prompted us to test whether this variant affects receptor binding. To this end, we performed isothermal titration calorimetry (ITC) experiments using a synthetic peptide derived from the GlyR β intracellular loop domain (GlyR β -ICD). As previously reported (Macha et al. 2022), this peptide is a suitable model to study gephyrin–receptor interactions since GlyRs and GABA_ARs compete for the same binding site on gephyrin (Maric et al. 2014). In agreement with earlier studies, WT-gephyrin showed two distinct binding sites, a high-affinity interaction in the sub-micromolar range ($K_D = 0.27 \mu\text{M}$, $N = 0.33$) and a low-affinity interaction in the double-digit micromolar range ($K_D = 15.11 \mu\text{M}$, $N = 0.71$) (Fig. 3B). Strikingly, the G134R variant displayed binding curves and thermodynamic parameters highly similar to those of WT protein (Fig. 3A), with no statistically significant differences in affinity or stoichiometry (Fig. 3B). These results indicate that, despite its profound oligomerization defects, G134R-gephyrin retains intact receptor-binding capacity *in vitro*.

Gephyrin is a moonlighting protein with a second essential function in cellular metabolism: catalysis of the final two steps in Moco biosynthesis. We therefore assessed whether structural alterations in the G-domain of G134R-gephyrin impair its enzymatic activity. *In vitro* Moco synthesis assays revealed that G134R-gephyrin variant produced approx. 400 pmol Moco, corresponding to $64 \pm 9 \%$ of WT activity (Fig. 3C). Thus, although the G134R substitution markedly reduces catalytic efficiency, it does not completely abolish enzymatic activity. Importantly, when WT and G134R proteins were mixed in a 1:1 ratio mimicking the heterozygous expression condition in the patient, Moco production was restored to ~ 700 pmol, corresponding to $105 \pm 3\%$

of WT activity (Fig. 3C). These findings suggest that G134R-gephyrin does not exert a dominant-negative effect on the enzymatic function of WT-gephyrin.

Taken together, our results demonstrate that G134R-gephyrin preserves its two core functions *in vitro*: receptor binding and Moco synthesis. The partial Moco activity is likely mediated by the trimers that still form *in vitro* and are presumed to remain catalytically competent. Consistent with the absence of structural brain abnormalities on MRI and with the lack of biochemical or clinical signs of Moco deficiency this is not expected to be clinically relevant. Instead, our data point toward defective higher-order

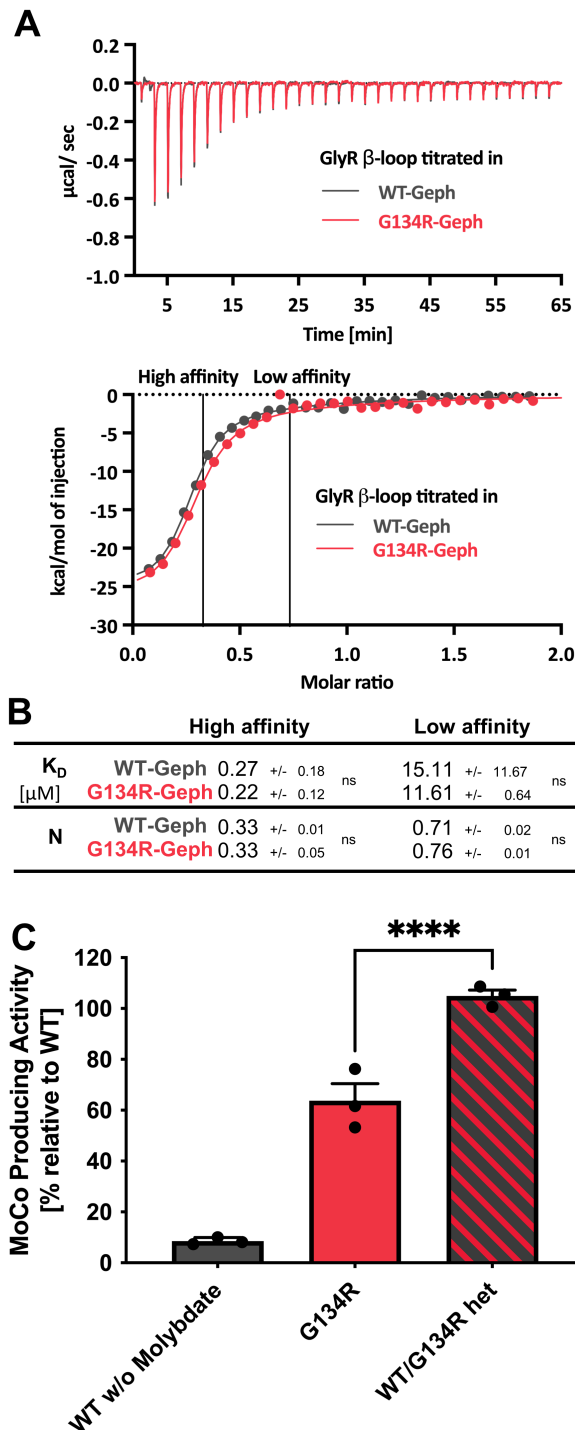


Fig. 3: Preserved GlyR β -Loop binding and Moco activity in G134R-gephyrin *in vitro*

(A) Representative isothermal titration calorimetry (ITC) binding curves illustrating the interaction of the GlyR β -loop with either WT-gephyrin or G134R-gephyrin are shown. Experimental data were fitted with a two-site binding model (solid lines), distinguishing high- and low-affinity interaction sites. **(B)** Quantitative binding parameters obtained from the ITC measurements, including dissociation constants (K_D , μM) and stoichiometry values (N), are presented. Data are reported as mean $\pm$ SD ($n = 3$, derived from three independent protein purifications) and statistical comparisons were performed using an unpaired, two-tailed Student's t -test. No significant differences were detected for any of the parameters (ns, $p \geq 0.05$). **(C)** *In vitro* molybdenum cofactor (Moco) biosynthesis assay was performed using 150 pmol of purified 6His-tagged gephyrin proteins as described earlier (Belaidi and Schwarz 2013). Presented as % relative to WT-gephyrin activity. Reaction lacking molybdenum (w/o molybdate) were included as internal negative control to validate assay performance. Data are reported as mean $\pm$ SD ($n = 3$, derived from three independent measurements) and statistical comparisons were performed using an one-way ANOVA ($F = 170.5$, $p < 0.0001$) with Tukey's multiple comparisons test ($p_{G134R \text{ vs } WT/G134R \text{ het}} < 0.0001$).

self-assembly as the critical impairment of this variant. Given the established link between gephyrin receptor clustering and LLPS at inhibitory synapses, we next investigated whether the inability of G134R-gephyrin to form higher-order assemblies translates into impaired phase separation capacity.

2.4.4 Loss of higher-order oligomers impairs gephyrin phase separation

Given that gephyrin synaptic clustering has been linked to LLPS at inhibitory synapses, we next tested whether the inability of G134R-gephyrin to form higher oligomers alters its phase separation behavior. We employed an established assay (Bai et al. 2021), in which the ICDs of inhibitory receptors are fused to the dimeric yeast zinc finger protein GCN4 and used them to probe receptor-induced gephyrin phase separation in sedimentation experiments (Fig. 4A). As described previously, the GlyR β -ICD represents the strongest known gephyrin interactor, the GABA_AR α 3-ICD shows intermediate affinity, and the GABA_AR α 1-ICD interacts only weakly.

When tested with the high oligomeric fraction of WT-gephyrin (isolated after SEC purification), we observed robust receptor-specific phase separation. Within the sedimented pellet fraction, $70 \pm 7\%$ of gephyrin were recovered when GlyR β -ICD fusion protein was present, while $50 \pm 5\%$ sedimented with GABA_AR α 3-ICD, and $19 \pm 5\%$ with GABA_AR α 1-ICD, consistent with the expected order of interaction strengths (Fig. 4B and C “High”). In contrast, the lower trimeric fraction of WT gephyrin showed significantly reduced phase separation with GlyR β -ICD ($p = 0.0001$) and α 3-ICD ($p < 0.0001$) pelleting decreased to $36 \pm 9\%$ and $15 \pm 5\%$, respectively, while α 1-ICD interactions remained unchanged (Fig. 4E and C). These results highlight the critical contribution of higher-order oligomers for phase separation. G134R-gephyrin, which lacks the ability to form higher oligomers, behaved similarly to WT trimers in LLPS (Fig. 4D and C “Low”). However, since the variant protein lacks higher-order assemblies, the majority of the phase separation-competent protein population was lost. We therefore conclude that G134R-gephyrin exhibited a strongly reduced propensity for LLPS, due to its inability to form higher oligomers.

Importantly, these findings suggest that although receptor binding to the E-domain is preserved, the structural instability of gephyrin assemblies lacking higher oligomers may compromise the integrity of the inhibitory postsynaptic density assuming that G134R-gephyrin fails to generate the protein-dense, phase-separated

scaffold required for stabilizing receptor clusters, thereby weakening inhibitory inputs. To directly test this hypothesis in a physiological setting, we next transitioned from *in vitro* assays to cellular models, first expressing G134R-gephyrin in HEK293T cells

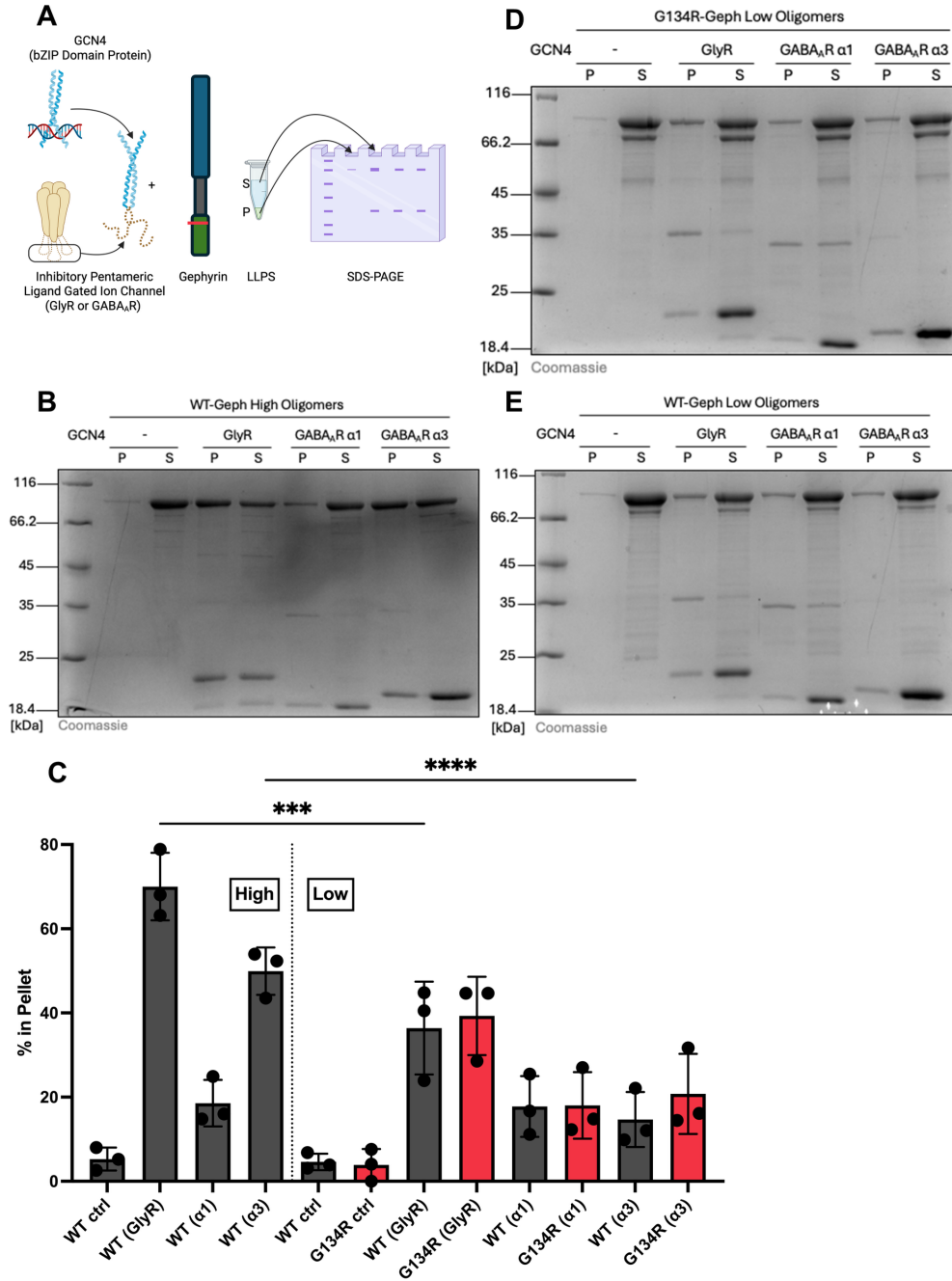


Fig. 4: A loss of higher oligomers reduces G134R gephyrin LLPS *in vitro*

(A) Cartoon depicting GCN4-based receptor models (Bai et al. 2021) and experimental design of liquid-liquid phase separation (LLPS) sedimentation assay. **(B, D, E)** Representative Coomassie stained SDS-PAGE of LLPS sedimentation assay from WT-gephyrin high oligomers (B), G134R-gephyrin low oligomers (D) and WT-gephyrin low oligomers (E) with GCN4-based receptor models for GlyR, GABA_AR-α1 and GABA_AR-α3 showing the amount of protein present in pellet (P) and supernatant (S) fractions. **(C)** The band intensities in the pellet fraction were measured and are shown as mean ± SD from three independent experiments (n = 3) and statistical comparisons were performed using a two-way ANOVA ($F(15,22) = 25.69$, $p < 0.000001$) with Tukey's multiple comparisons test ($p_{\text{WT oligomer vs WTtrimert (GlyR)}} = 0.000123$, $p_{\text{WT oligomer vs WTtrimert (α3)}} = 0.000061$). Panel C is based on the data in (B), while panel F summarizes the data from (D) and (E).

lacking endogenous gephyrin expression to assess both its oligomeric state, Moco forming activity and whether its altered LLPS propensity manifests as discrete fluorescent condensates in living cells. Building on these experiments, we then extended our analysis to primary hippocampal neurons to determine whether G134R-gephyrin can still support the formation of functional inhibitory synapses.

2.4.5 In HEK cells, G134R-gephyrin fails to oligomerize, resulting in disrupted Moco synthesis and absence of LLPS

Our HEK cell-based experiments reveal a severe defect in oligomerization of the G134R-gephyrin variant compared to both WT-gephyrin and previously characterized control variants. Using blue native PAGE, we demonstrate that G134R fails to form native trimers or higher oligomers, running at the same molecular weight as the dimeric control (G2-gephyrin splice variant; (Saiyed et al. 2007; Bedet et al. 2006)), while WT-gephyrin migrates above the trimeric control (S505F-gephyrin E-domain mutant; (Burdina et al. 2025)), confirming its ability to assemble into larger complexes (Fig. 5A). This defect is more pronounced in cells than *in vitro*, where trimers were still observed. To assess functional consequences, we co-expressed WT- or G134R-gephyrin with sulfite oxidase (SUOX) in HEK gephyrin knockout cells (KO). SUOX activity, which depends on Moco biosynthesis by gephyrin, was restored only by WT-gephyrin, indicating that G134R lacks sufficient catalytic activity for Moco synthesis in this context (Fig. 5B). Similar protein expression was validated via Western blot analysis (Fig. 5C).

Gephyrin forms discrete cytoplasmic condensates in a concentration dependent manner in cultured mammalian cells, consistent with LLPS (Lee et al. 2024). Similarly, widefield fluorescence microscopy of living HEK293T gephyrin KO cells revealed that overexpressed mScarlet-tagged WT-gephyrin forms cytosolic blobs (Fig. 5D). Blob formation emerged over a defined range of cytoplasmic fluorescence intensities, consistent with a threshold-like behavior. Using logistic regression, we estimated this onset as the intensity at which the probability of blob formation reached 50%. The estimated onset occurred at 79.6 arbitrary units (AU) of cytosolic gephyrin fluorescence intensity (95% confidence interval (CI): 67.5–93.5 AU) (Fig. 5E). Disrupting G-domain trimerization (G2-gephyrin) and E-domain dimerization (S505F-gephyrin) (Saiyed et al. 2007; Burdina et al. 2025) shifted this onset to higher intensities, with thresholds of 376 AU (95% CI: 334–424 AU) and 345 AU (95% CI: 301–391 AU), respectively

(Fig. 5E). Similarly, G134R-gephyrin also exhibited an elevated onset threshold of 406 AU (95% CI: 361–448 AU) (Fig. 5E). Co-expression with inhibitory postsynaptic receptor models lowered the saturation concentration of gephyrin (Lee et al. 2024). Consistent with this, co-expression of the mEGFP-tagged dimeric receptor model GCN4-GlyR promoted mScarlet-tagged WT-gephyrin condensation (Fig. 5F,G). Blob formation depended on the combined abundance of both proteins, as captured by a joint logistic model. To account for this, onset thresholds for gephyrin were estimated at the median GCN4-GlyR intensity within each condition. At median GCN4-GlyR

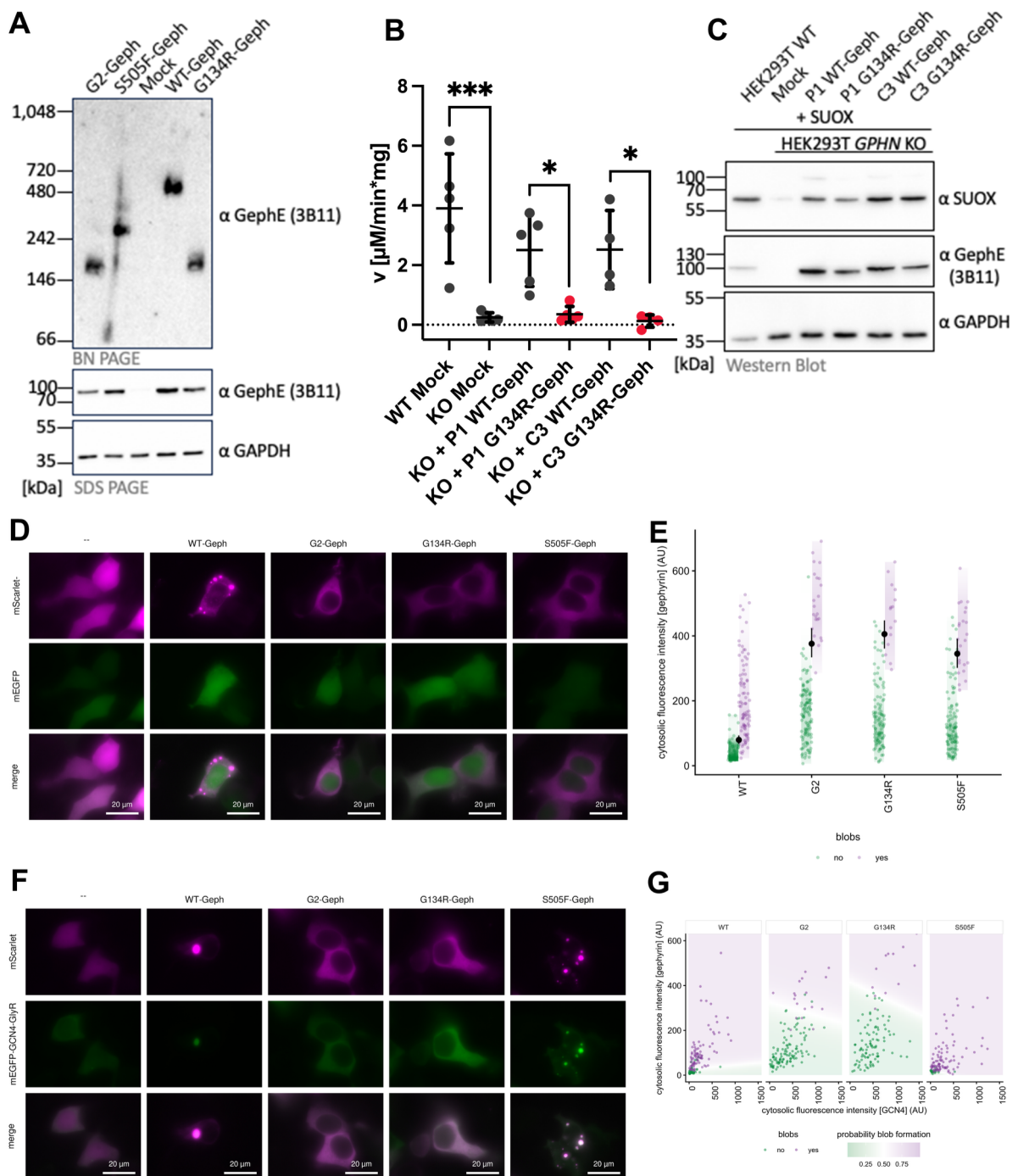


Fig. 5: Loss of oligomerization in G134R-gephyrin reduces Moco synthesis and LLPS in HEK cells

Fig. 5: Loss of oligomerization in G134R-gephyrin reduces Moco synthesis and LLPS in HEK cells
(A) Representative Western blot analysis after blue native PAGE of HEK293T *GPHN*KO cells transfected with indicated gephyrin variants. As control, Western blot analysis of SDS-PAGE was performed. **(B)** Sulfite oxidase-specific activity in whole cell lysates. Either HEK293T WT (WT) or *GPHN* KO (KO) were transfected with indicated gephyrin variants or empty plasmid (Mock) (n = 5 independent experiments; mean $\pm$ SD are indicated). Data were analyzed by one-way ANOVA ($F_{5,22} = 10.524$, $p = 2.94 \times 10^{-5}$), Tukey's post hoc test; $p_{WT \text{ Mock vs. KO Mock}} = 0.000241$; $p_{KO + P1 \text{ WT-Geph vs. KO + P1 G134R-Geph}} = 0.0416$; $p_{KO + C3 \text{ WT-Geph/KO vs. C3 G134R-Geph}} = 0.0435$ **(C)** Representative Western blot analysis of cell lysates from (B). **(D, F)** Representative widefield microscopy images of HEK *GPHN* KO cells expressing mScarlet, mScarlet tagged WT-gephyrin (isoform P1), G2-gephyrin, G134R-gephyrin or S505F gephyrin in the presence of mEGFP (D) or mEGFP tagged GCN4-GlyR (F). Scale bars represent 20 μm . **(E, G)** Cytosolic fluorescence intensity of individual mScarlet-gephyrin/mEGFP **(E)** and mScarlet-gephyrin/mEGFP-GCN4-GlyR **(G)** expressing cells. Data points are color-coded for the presence or absence of cytosolic blobs. **(E)** The shading density represents probability density, with higher color saturation indicating higher density. Black points and error bars indicate the estimated onset threshold (50% probability) and 95% confidence interval for each condition. **(G)** Probability background indicates the combinations of GCN4-GlyR and gephyrin intensities at which the predicted probability of blob formation reaches 50%. **(E)** WT 152 cells, G2 144 cells, G134R 127 cells, S505F 129 cells **(G)** WT 131 cells, G2 130 cells, G134R 109 cells, S505F 114 cells **(E, G)** from three independent transfections.

levels, the onset of blob formation with respect to WT-gephyrin intensity occurred at 29.1 AU (95% CI: 22.1–36.0 AU). In contrast, impaired G-domain trimerization markedly increased the onset threshold with values of 301 AU (95% CI: 258–357 AU) for G2-gephyrin and 369 AU (95% CI: 322–435 AU) for G134R-gephyrin (Fig. 5G). By comparison, impaired E-domain dimerization (S505F-gephyrin) showed an onset comparable to WT 15.3 AU (95% CI: 0.507–19.8 AU) (Fig. 5G). Together, these results support the conclusion that G-domain trimerization is critical for gephyrin condensation, whereas E-domain dimerization may be even dispensable in the presence of receptor scaffolding.

These findings indicate that the G134R variant disrupts both oligomerization and phase separation in cells comparative to G2-gephyrin, impairing gephyrin's metabolic and scaffolding functions. Building on these results, we next aimed to extend our analysis to neuronal cell culture to investigate whether G134R-gephyrin can still support the formation of functional inhibitory synaptic clusters.

2.4.6 G134R-gephyrin disrupts neuronal clustering and acts dominant negatively when co-expressed with WT-gephyrin

We finally tested the G134R variant in primary hippocampal neurons. To achieve expression in cells lacking endogenous gephyrin, we generated dissociated hippocampal cultures from floxed gephyrin mice (O'Sullivan et al. 2016). Knockout of endogenous gephyrin was achieved by Cre expression, which was combined with Cre-

dependent expression of mScarlet-tagged WT- or G134R-gephyrin (Fig. 6A). Under these conditions, WT-gephyrin formed robust somatic and dendritic clusters colocalizing with the presynaptic inhibitory marker vGAT and the $\gamma 2$ subunit of GABA_AR, confirming their synaptic identity (Fig. 6B and C). In contrast, neurons expressing only G134R-gephyrin displayed a dramatic loss in the number of synaptic clusters, with markedly fewer puncta colocalizing with vGAT and $\gamma 2$ (Fig. 6B and C).

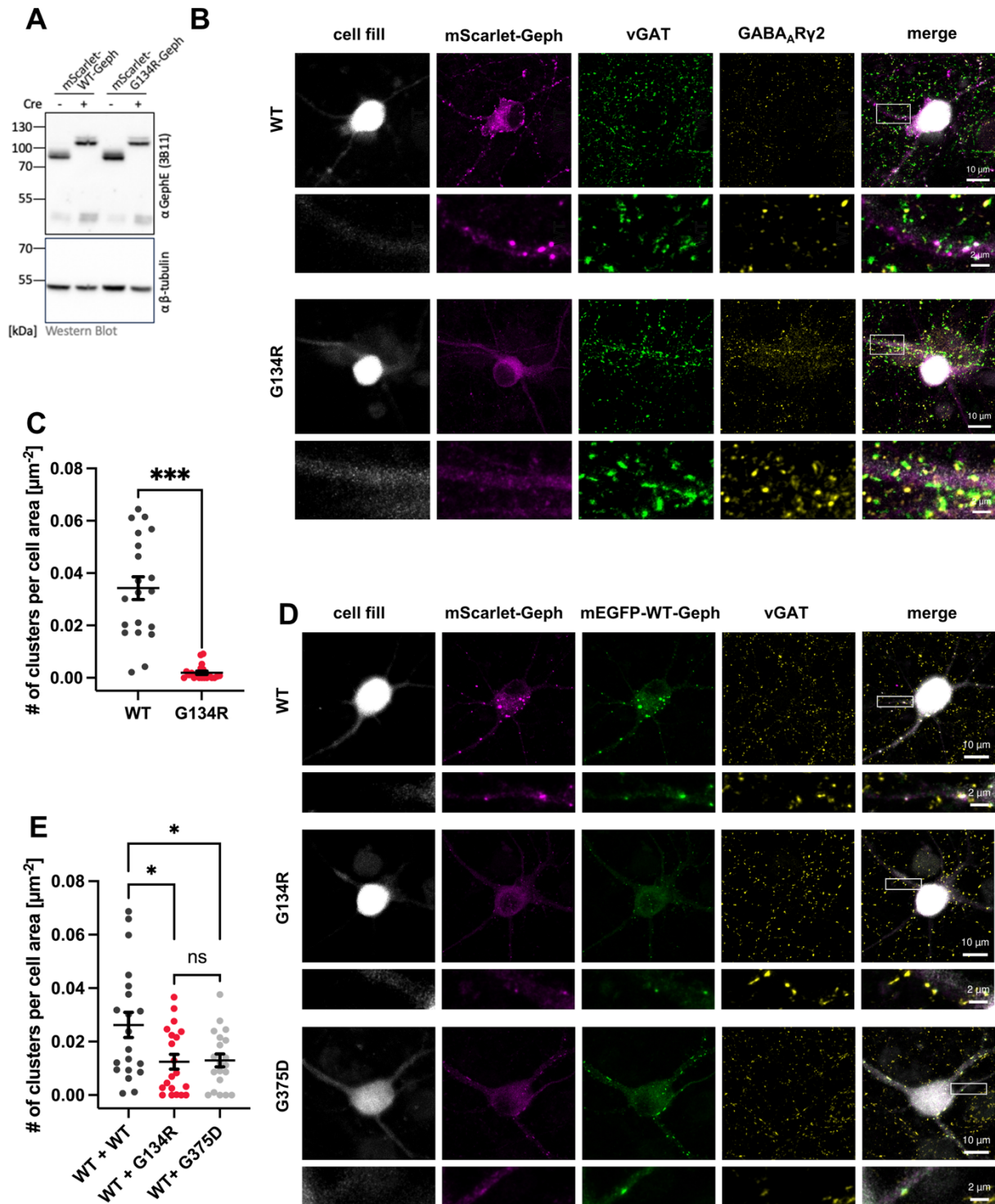


Fig. 6: Differential expression of WT- and G134R-gephyrin in primary hippocampal neuron culture

Fig. 6: Differential expression of WT- and G134R-gephyrin in primary hippocampal neuron culture
(A) Representative Western blot analysis of lysates from gephyrin floxed/floxed murine hippocampal neurons infected with moxBFP (-) or moxBFP-P2A-Cre (+) together with either mScarlet-WT-Geph or mScarlet-G134R-Geph. **(B, D)** Representative confocal images of gephyrin^{floxed/floxed} murine hippocampal neurons transfected with moxBFP-P2A-Cre and (B) infected with either mScarlet-WT-Geph or mScarlet-G134R-Geph and immunostained against vesicular GABA transporter (vGAT, green) and GABA_AR γ 2 subunit (yellow) or (D) infected with mEGFP-WT-Geph and either mScarlet-WT-Geph or mScarlet-G134R-Geph and immunostained against vesicular GABA transporter (vGAT, yellow). **(C, E)** Quantification of mScarlet-tagged gephyrin clusters was carried out using automated synapse analyses. Each data point represents synaptic cluster density (# per cell area μm^2) per acquired image; $n = 20$ cells per condition from five independent cultures, mean $\pm$ SD are indicated. Data were analyzed by (C) Mann-Whitney U test $U = 391$, $p = 0.00000023$, effect size $r = 0.820$ (E) one-way ANOVA ($F_{2,57} = 5.139$, $p = 0.009$), Tukey's post hoc test; $p_{WT + WT \text{ vs. } WT + G134R} = 0.0176$; $p_{WT + WT \text{ vs. } WT + G375D} = 0.0228$; $p_{WT + G134R \text{ vs. } WT + G375D} = 0.995$

These results demonstrate that G134R-gephyrin is unable to fully support the assembly of inhibitory postsynaptic scaffolds in neurons by itself.

To model the heterozygous state found in the patient, we tested whether the G134R variant exerts a dominant-negative effect on WT-gephyrin, potentially contributing to the observed epileptic phenotype. We co-expressed mEGFP-tagged WT-gephyrin and mScarlet-tagged G134R in neurons, enabling clear distinction between the two proteins based on their respective fluorescent labels. As a positive control, we included the previously described dominant-negative variant G375D (Fig. 6D). Heterozygous expression of G134R significantly reduced the density of WT inhibitory postsynaptic clusters to a similar extent as G375D, supporting a dominant-negative effect (Fig. 6E). These findings indicate that G134R-gephyrin interferes with WT-gephyrin function, providing a plausible molecular mechanism for the patient's epileptic phenotype.

2.5 Discussion

Our study identifies G134R-gephyrin as the first epilepsy patient-derived variant from a missense mutation in the *GPHN* gene affecting the G-domain of gephyrin, demonstrating that a single amino acid substitution disrupts gephyrin's oligomerization and LLPS. However, we acknowledge the inherent limitations of panel-based next-generation sequencing (NGS), as variants outside the captured regions cannot be formally excluded. Previously, two missense mutations affecting gephyrin's G-domain, namely V43L and A91T were identified in patients with autism spectrum disorder (Lionel et al. 2013). However, functional analyses revealed only moderate clustering deficiencies for A91T, while V43L behaved normally under the experimental conditions (S. Kim et al. 2021). Recombinant and purified G134R-gephyrin formed trimeric lower-

order oligomers, shows reduced Moco synthesis, while a dominant-negative effect on its enzymatic activity was not observed as expected from the patient's biomarker profile. While we could not resolve the structural consequences of this variant at atomic resolution, our aim was to provide an integrative functional characterization that advances the understanding of this disease-associated mutation, and could even go beyond by identifying key and novel principles of gephyrin dependent phase separation at the PSD. However, in a human cellular model, G134R-gephyrin failed to form higher-order oligomers beyond E-domain mediated dimerization and lost its Moco synthesis function entirely. While G134R-gephyrin retains the ability to interact with the intracellular domain of the glycine receptor, it is unable to form synaptic clusters in dissociated hippocampal neurons, both in a homozygous and heterozygous context, where it acts dominantly negative on WT-gephyrin; although these analyses are limited to neuronal cell culture and an entire knock-in mouse model would provide a more comprehensive *in vivo* assessment, they nevertheless allow us to evaluate synaptic targeting and clustering in a native neuronal context. This dominant-negative effect, which mirrors the behavior of the epileptogenic E-domain mutant G375D (Dejanovic et al. 2015), provides a direct molecular explanation for the patient's epileptic phenotype by effectively depleting functional inhibitory receptor-scaffold complexes at synapses.

Previous work from our laboratory identified the two E-domain gephyrin variants G375D and D422N in patients with developmental epilepsy. Both variants perturb LLPS, thus establishing the E-domain as a crucial structural hub for condensate formation and inhibitory synapse integrity (Dejanovic et al. 2015; Macha et al. 2022, 2025). Structural and functional analyses revealed that the subdomain two (SDII) within the E-domain is essential for the recently discovered filament formation via SDII–SDII' interactions, which in turn underlie LLPS and define the structural basis of postsynaptic gephyrin scaffolds (Macha et al. 2025). The G375D variant, located within SDII, causes unfolding of this subdomain, abolishing SDII–SDII' mediated filament formation and thereby eliminating LLPS and synaptic clustering capacity (Macha et al. 2025). In contrast, the D422N variant disrupts a critical salt bridge with R379, yet preserves the overall SDII fold, still resulting in loss of LLPS and impaired inhibitory synaptic transmission, highlighting that subtle perturbations of the SDII network suffice to destabilize condensate formation (Fig. 7 left).

Taken together with our current observations, we propose a refined model for establishing the postsynaptic architecture: while the E-domain is the primary driver of

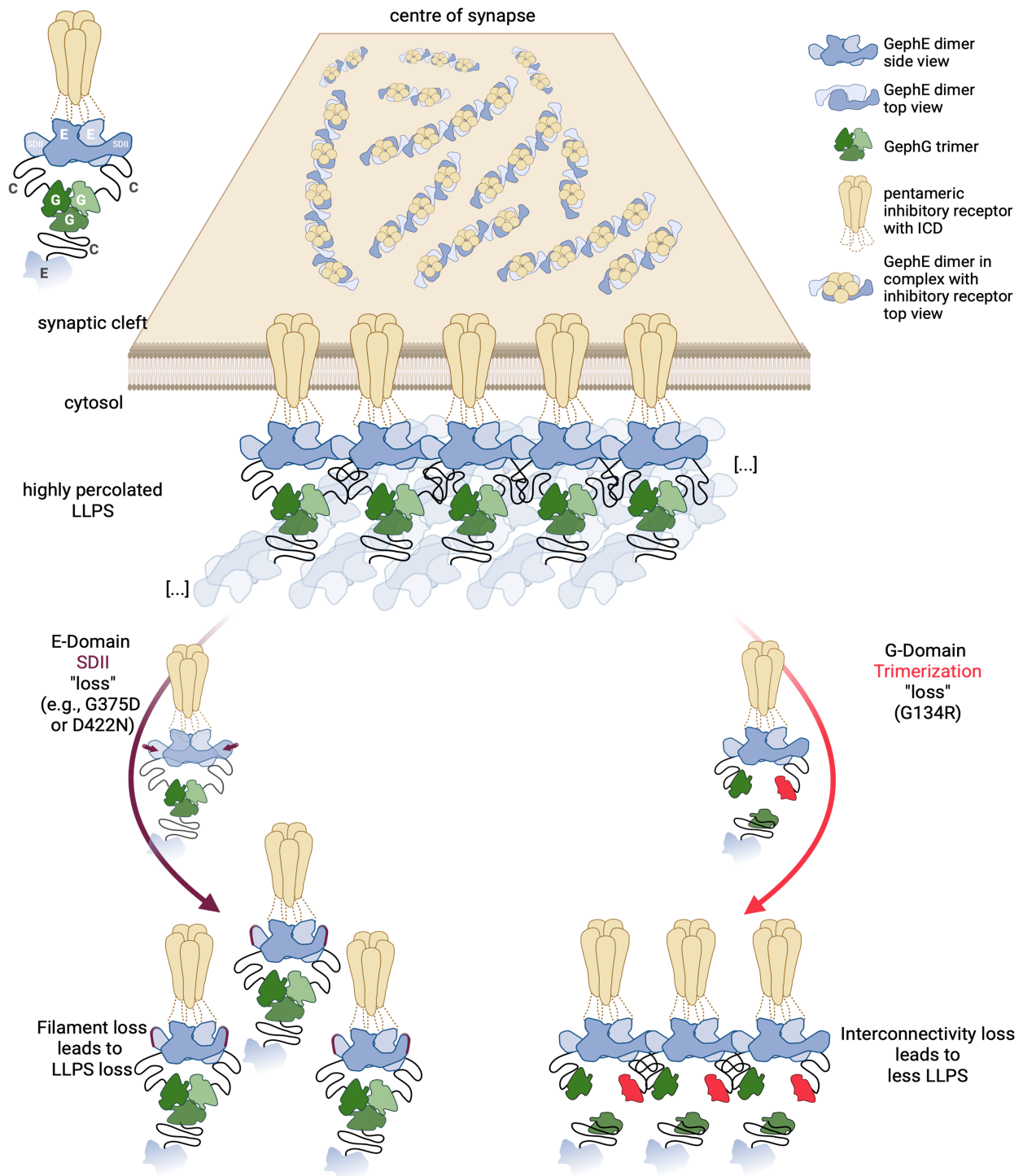


Fig. 7: Refined model for gephyrin LLPS

Schematic illustrating the proposed impact of disease-associated gephyrin variants on higher-order network assembly and LLPS. **(Center)** WT gephyrin forms a highly percolated network through coordinated G-domain trimerization and E-domain dimerization, which we hypothesize is required for efficient receptor immobilization and stable postsynaptic clustering. **(Left)** Previously characterized E-domain variants affecting subdomain II (G375D and D422N) disrupt filament formation and impair LLPS as reported in the literature. **(Right)** The newly identified G134R variant, located in the G-domain, is proposed to selectively disrupt G-domain-mediated intermolecular connectivity without affecting E-domain interactions, thereby reducing network percolation and impairing higher-order assembly and LLPS. Together, this model suggests that both G- and E-domain interactions contribute to the percolation threshold required for gephyrin condensate formation and functional synaptic clustering. (Figure created with BioRender).

phase separation and provides the receptor-binding interface, the G-domain is crucial for interconnecting E-domain gephyrin conjugates into higher-order oligomers, thereby amplifying LLPS capacity and stabilizing synaptic clustering (Fig. 7 right). The strong G-domain trimerization (Herweg and Schwarz 2012) may provide a hierarchical organizing principle that ensures E-domain dimers efficiently find and engage each other, thereby reaching the threshold concentrations required for LLPS and filament maturation under physiological conditions. Simultaneously, E-domain dimers nucleate filaments through SDII–SDII' interactions, and G-domain trimerization could interlink these E-domain-based filaments into a robust three-dimensional scaffold that can maintain the protein concentrations and geometric connectivity observed *in vitro* by cryo-EM and required *in vivo* at synapses. Within this framework, G134R-gephyrin is as functionally disruptive at the cellular and clinical levels as the E-domain SDII variant G375D, despite residing outside the critical receptor binding and filament forming domains of gephyrin, indicating that G-domain-mediated higher-order oligomerization is equally important as SDII-dependent filament assembly for LLPS and synapse formation.

Our convergent mechanistic insight suggests an additional evolutionary advantage for the fusion of G- and E-domains via the flexible C-domain in vertebrates within a single polypeptide chain: it serves both, product-substrate-channeling in Moco synthesis (Belaidi and Schwarz 2013) and precise control over condensate assembly for postsynaptic receptor clustering. The flexible C-domain likely permits the G-domain trimeric core to be spatially separated from the E-domain filament lattice, enabling dynamic regulation of scaffold connectivity without steric interference with filament formation.

The central C-domain of gephyrin, which behaves as an intrinsically disordered region (IDR), is indispensable for scaffold condensation and can be functionally replaced by heterologous IDRs, in some cases even enhancing gephyrin's LLPS capacity, indicating that the C-domain acts as a charged spacer that thermodynamically modulates phase separation rather than as a classical self-associating IDR (Lee et al. 2024). In apparent contrast, segments of the C-domain can inhibit LLPS driven by the E-domain *in vitro*, suggesting that under these conditions C-domain–E-domain contacts can restrain E-domain phase separation and thus provide a built-in brake on spontaneous condensation (Bai et al. 2021). Integrating these findings, the conformation and exposure of the C-domain operates as a dynamic hub

that senses local receptor density and postsynaptic architecture, defining the boundary of the inhibitory postsynaptic density so that gephyrin condensates remain confined to receptor-enriched nanodomains, while remaining permissive to rapid remodeling. In this scenario, the G-domain not only interconnects E-domain filaments but also allosterically controls C-domain compaction *versus* extension, thereby toggling between C-domain–shielded states that limit LLPS and C-domain–exposed states in which favorable electrostatic C–E interactions and increased effective valency promote percolation of a gephyrin network. This polydisperse nature of gephyrin conformation has been seen already in early structural studies using single angle X-ray spectroscopy (Sander et al. 2013). From the perspective of percolation-based phase separation hypothesis (Mittag and Pappu 2022; Shen et al. 2023), where the condensed phase emerges once a system-spanning, multivalent interaction network forms, the G-domain-mediated trimerization becomes a fundamental requirement: by providing strong, high-valency crosslinks between SDII-based E-domain filaments, G-domain connectivity helps to drive the system across the percolation threshold into a macroscopically connected, viscoelastic condensate, as described for other synaptic and PSD-like assemblies (Zhu et al. 2024) (Fig. 7 center). Consequently, pathogenic disruption of G-domain trimerization, as here discovered for G134R-gephyrin, is predicted not only to lower LLPS propensity but to prevent formation of a percolated scaffold network that is necessary to immobilize and concentrate inhibitory receptors within the postsynaptic density, thereby unifying G-domain and E-domain mutations within a common percolation framework for gephyrin-dependent inhibitory synapse failure.

The observation that both E-domain (G375D, D422N) and G-domain (G134R) epilepsy variants converge on a shared deficit in gephyrin LLPS implies that fine-tuning of G-domain trimerization and E-domain filament assembly constitutes a key regulatory axis to strengthen or weaken inhibitory synaptic inputs. Such multilayered regulation of filament formation, trimer crosslinking, and LLPS likely equips gephyrin with the ideal biophysical properties to function as a tunable postsynaptic scaffold in neurons, while at the same time making the system exquisitely sensitive to disease-causing missense perturbations in both the G- and E-domains.

2.6 Material and methods

Human subject

Parents/legal guardians of subject included in this study signed an informed consent form for participation. All experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

Expression constructs

6His-tagged gephyrin and GlyR β -loop-intein expression vectors have been described before (Macha et al. 2022; Dejanovic et al. 2015). GCN4 based GlyR-ICD and GABA_AR- α 1/3-ICD were kindly provided by the Zhang lab (Bai et al. 2021). 6His-tagged gephyrin G-domain and full-length gephyrin (isoforms P1 and C3 in pQE80) G134R variants were cloned by site-directed mutagenesis using forward (GACCAGTGTGTAGAATAAGAGGGGAAAA) and reverse (TTTTCCCTCTTATTCTACACACTGGTC) primers. 6His-tagged full-length gephyrin (isoforms P1 in pQE80) S505F variant was cloned by site-directed mutagenesis using forward (CTCATTCCCTGTGAACATAACGGCAAC) and reverse (GTTGCCGTTATGTTTACAGGGAATGAG) primers. 6His-tagged full-length gephyrin G2 in pQE80 was cloned by overlap extension PCR using G2 forward (CACGAGATGTCACTCCAGAGAAATTCCCAACATTCCCATTTTGTGGGC) and G2 reverse (CCCGTTCTATTACTTCTTTTGTGGCCCCTTTCTGGAGCCCACAAAATGGGAAT) with gephyrin flanking forward (GACCGAGGGAATGATCCT) and flanking reverse (AGGATCATTCCTCGGTC) primers. Full-length gephyrin and its variants were subcloned into pcDNATM3.1/Zeo (+) (Invitrogen) using NEBuilder HiFi DNA Assembly (NEB). Gephyrin was initially subcloned into pmEGFP-C2 (Liebsch et al. 2023), derived from pEGFP-C2 (Clontech) using XhoI and KpnI restriction sites. mScarlet-Gphn constructs in this plasmid were generated by replacing EGFP with mScarlet from pAAV-hSyn-mScarlet (a gift from Karl Deisseroth, Addgene plasmid #131001; <http://n2t.net/addgene:131001>; RRID:Addgene_131001) using NEBuilder HiFi DNA Assembly (NEB). mEGFP-GCN4-GlyR in pEGFP-C2 (Clontech) was generated by PCR amplification of GCN4-GlyR and subcloned into pmEGFP-C2 using XhoI and KpnI. Full-length gephyrin (isoform P1) G134R was amplified with forward

(GACTCAGATCTCGAGCATGGCGACCGAGGGAAT) and reverse (TAGCCGTCCGATGACC) primers and subcloned into pAAV-hSyn-FLEX-mScarlet-Gphn_P1 (Macha et al. 2025) using XhoI and BshTI restriction sites. mEGFP-tagged full-length gephyrin (isoforms P1) was subcloned into pAAV-hSyn-FLEX-mScarlet-Gphn_P1 using NcoI restriction site. The plasmids pAdDeltaF6 (Addgene plasmid # 112867; RRID:Addgene_112867) and pAAV2/1 (Addgene plasmid # 112862; RRID:Addgene_112862) were gifts from James M. Wilson. All DNA constructs were confirmed by Sanger sequencing (Eurofins), and the integrity of AAV2 ITR sites was confirmed by analytical SmaI restriction digest.

Protein expression, purification and size exclusion chromatography (SEC)

All gephyrin variants used in this study were recombinantly expressed in *E. coli* BL21 (DE3) rosetta cells in 1x LB medium containing 0.1 mg/ml ampicillin. Prior to expression, cells were grown until OD₆₀₀ of 0.3-0.4 was reached. His-tagged full length gephyrin was expressed for 16 h at 18°C. His-tagged GephG was expressed for 16 h at 30°C. Protein expression was induced addition of isopropyl-β-D-thiogalactopyranosid (IPTG) to an end-concentration 250 μM in the cell-culture. After protein expression, cells were harvested by centrifugation at 4000 g and resuspended in lysis buffer (100 mM Tris/HCl pH 8, 250 mM NaCl, 0.05 % (v/v) Tween20, 5 mM β-mercaptoethanol, 1x protease inhibitor (cOmplete, Roche, Basel, Switzerland), 6.5 mg lysozyme per L expression culture from chicken egg (Sigma-Aldrich) and 25 μg DNase per mL lysis Buffer) and stored at – 20°C for further use. For purification, the cells were lysed mechanically using EmulsiFlex (Avestin, Ottawa, Canada) and ultrasound for 3x 30 s at 45 % amplitude. Cytosolic extract was separated from cell debris by centrifugation at 50000 g for 45 min. Prior to Ni-NTA chromatography the cell extracts were supplemented with imidazole to 20 mM end concentration. The cell extract was applied to 10 ml Ni-NTA resin and proteins were washed by 10 column volumes (CV) of lysis buffer, 10 CV of lysis buffer containing 10 mM imidazole and 10 CV of lysis buffer containing 20 mM imidazole. Proteins were eluted with 3 CV lysis buffer containing 400 mM imidazole. All proteins were further purified and analyzed via size exclusion chromatography (SEC) (Superdex 16/600 prep grade, GE Healthcare) using protein buffer (20 mM Tris/HCl pH 7.5, 250 mM NaCl, 5 mM β-mercaptoethanol, 5 % glycerol) at a flow rate of 1 mL/min. All purification steps were performed at 4 °C. GlyR-βloop was expressed and purified as stated previously (Grünwald et al. 2018). GlyR-

β ICD, GABA_AR- α 1ICD and GABA_AR- α 3ICD were purified according to the protocol provided by Bai and colleagues (Bai et al. 2021).

Isothermal titration calorimetry (ITC)

All experiments were performed with a MicroCal Auto-ITC200 (Malvern, Malvern, United Kingdom) in ITC buffer (20 mM Tris/HCl pH 7.5, 250 mM NaCl, 5 mM β -mercaptoethanol, 5 % glycerol). The sample cell was filled with gephyrin variants at concentration of 20 - 30 μ M. GlyR- β loop was filled into the syringe at a concentration of 250-350 μ M. Experiments were conducted at 37 °C with an injection volume of 1.5 - 2 μ l, a set reference power of 5 μ cal/sec, spacing time of 180-300 s and a stirring speed of 750 rpm. Data was analyzed using Origin 7 (OriginLab Corporation) and binding parameters were derived by applying a two-site binding model.

Sedimentation assay of *in vitro* phase separation

Prior the assay proteins were pre-cleared at 17,000 $\times g$ for 10 min at 25 °C. For sedimentation assays, proteins were mixed at designated combinations and with 10 μ M gephyrin and 20 μ M receptor model in 50 μ L final volume in assay buffer (25 mM Tris pH 7.4, 150 mM NaCl). After 10 min incubation at 25°C, the mixtures were centrifuged at 17,000 $\times g$ for 10 min at 4 °C. Right after centrifugation, the supernatant and pellet were separated. The pellet was brought to the same volume as the supernatant. Proteins recovered in supernatant and pellet were analyzed by SDS-PAGE with Coomassie Blue staining. The band intensities in SDS-PAGE gels were quantified by Image Lab software. Relative and absolute amounts of proteins in supernatant and pellet were calculated based on the relative band intensities. Data of three repeats were presented as means $\pm$ SD.

***In vitro* MoCo synthesis assay**

The *in vitro* MoCo synthesis assay has previously been described in detail (Belaidi and Schwarz 2013). About 150 pmol *E. coli*-expressed and purified gephyrin was used per condition. Samples without gephyrin or without molybdenum were used as control and show MoCo produced either chemically or by trace amounts of molybdenum in the buffers. Proteins from two independent purifications were used for the assay.

SDS-PAGE, Coomassie, and Western Blot

Samples were supplemented with 1× sample buffer (5×: 250 mM Tris/HCl pH 6.8, 30% glycerol, 0.1% Bromophenol blue, 10% SDS, 5% β-mercaptoethanol) and incubated for 5 min at 95°C. Protein separation was performed with 12% SDS acrylamide gels, followed by Coomassie staining (30% EtOH, 10% acetic acid, 0.25% Coomassie brilliant blue R250) or immunoblotting using standard protocols with a chemiluminescence and an ECL system. The following antibodies were used and diluted in Tris-buffered saline/0.5% Tween containing 1% dry milk: anti-gephyrin E-domain (3B11, 1:20, self-made, RRID: AB_887719); anti-GAPDH (1:1000, G9545, Sigma), anti-mouse HRP-coupled (1:10 000, AP181P, Sigma); anti-rabbit HRP-coupled (1:10 000, AP187P, Sigma). Image acquisition of Coomassie stained gels and immunoblot detection were performed with a ChemiDoc™ Imaging System (BioRad). Band intensities were quantified using Image Lab 6.1 (BioRad).

Blue Native PAGE Analysis

Samples for blue native PAGE analysis were prepared by 1:3 dilution using H₂O and the addition of 10× blue native loading buffer (312 mM imidazole, 500 mM 6-aminohexanoic acid, 5% CBB G250, 6.25 mM EDTA, 40% glycerol). Samples were applied to 4%–16% native PAGE (TM Novex Bis-Tris Gel) and protein separation was performed according to the manufacturer's protocol. Afterward, gels were subjected to western blotting.

Structural predictions by AlphaFold3

Structural predictions were run in AlphaFold3 (Jumper et al. 2021). For structural comparison the GephG WT (PDB ID: 1JLJ) was used. For G134R substitution the gephyrin WT (UniProt ID: Q9NQX3) sequence was used. Predictions were run three times, with all five output predictions used for analysis. Sequence alignments were conducted on the UniProt website (The UniProt Consortium 2025).

Generation and transfection of HEK293T *GPHN* KO Cells

HEK293T (DSMZ no. ACC 635) and HEK293T *GPHN* KO cell were not recently authenticated but are tested annually for mycoplasma contamination. Generation of HEK293T *GPHN* KO cells was described by N. Burdina and colleagues (Burdina et al. 2025). 1×10⁶ HEK293T *GPHN* KO cells were plated per well into 6-well cell culture

plates or 2×10^5 HEK293T *GPHN* KO cells were plated on poly-D-lysine (Sigma) coated 35 mm glass-bottom dishes (ibidi) and incubated 24 h in Dulbecco's modified Eagle's medium supplemented with 10 % FCS and 2 mM L-glutamine at 37 °C and 5% CO₂. Transfection was performed using 200 ng (per well in 6-well) or 1000 ng (per 35 mm glass-bottom dish) of the respective plasmid DNA with polyethylenimine (PEI, Sigma) according to the manufacturer's protocol. 14 h after PEI transfection, cells were washed twice in ice cold PBS and lysed in native lysis buffer (20 mM HEPES pH 8.0, 150 mM NaCl, 5% Glycerol, 5 mM EDTA, 1% DDM, 2x protease inhibitor (cOmplete, Roche, Basel, Switzerland)). Lysates were cleared at 4 °C and 14,000 x g for 20 min and total protein concentration determined using Bicinchoninic Acid assay (Sigma). 16 - 18 h after PEI transfection live cells were imaged using a Nikon Ti2-E microscope (see below).

Sulfite oxidase activity in *GPHN* KO HEK293T cells

HEK293T cells and *GPHN* KO HEK293T cells were seeded in 6-well plates and co-transfected the next day with human sulfite oxidase in pcDNA5 FRT (Bender et al. 2019) and untagged gephyrin constructs (or empty plasmid, Mock) in pcDNA™3.1/Zeo (+) using Polyethylenimine. 24 h post-transfection cells were washed with PBS and lysed for 1 h at 4 °C (150 mM NaCl, 5 mM EDTA, 1 % (w/v) IGEPAL, 0.5 % (w/v) desoxycholate, 0.1 % (w/v) SDS, 50 mM Tris/HCl pH 7.5 with 1x protease inhibitor mix (cOmplete, Roche, Basel, Switzerland)). Lysates were cleared at 4 °C and 14,000 x g for 20 min and total protein concentration determined using Bicinchoninic Acid assay (Sigma). Sulfite oxidase-specific activity in 100 µg whole cell lysate was measured at room temperature (aerobic conditions) using a plate reader equipped with monochromators (Tecan Spark). In 200 µL, 50 µM cytochrome c, 20 µg/mL catalase (bovine, Sigma-Aldrich), 50 µM KCN, and 300 µM Na₂SO₃ were combined in 100 mM Tris/Ac pH 8.0 and reduced cytochrome c was measured at 550 nm. Initial cytochrome c reduction velocities were determined and specific activities calculated using Lambert-Beer Law with $\epsilon_{550 \text{ nm}} = 19\,630 \text{ M}^{-1} \text{ cm}^{-1}$ and $d = 0.857 \text{ cm}$.

Primary hippocampal cultures

Floxed gephyrin mice (O'Sullivan et al. 2016), a gift from Ralph A. Nawrothki, were back-crossed to C57BL/6NRj. Mice were housed on a 12 h light-dark cycle with free access to food and water at 22 ± 2 °C and a humidity of 55 ± 10 %. Dissociated primary

hippocampal cultures from homozygous gephyrin floxed embryos (E17.5) were prepared of either sex. Embryonal hippocampi obtained from one pregnant mouse were pooled prior to cell dissociation. After dissociation, 65,000 cells were seeded on Poly-L-lysine-coated 13-mm cover slips or 91,000 cells per well on Poly-L-lysine-coated 24-well plates. Cultures were grown in 0.5 mL per well neurobasal medium supplemented with B-27, N-2, GlutaMAX™ (Thermo Fisher Scientific), and Pen/Strep. Recombinant AAV1 particles were diluted in Neurobasal medium with supplements before they were added to the cultures. Cultures were infected (for Western blot analysis) with 1×10^8 viral genome copies (GC) of moxBFP or moxBFP-P2A-Cre or transfected (for immunocytochemistry) with 400 ng DNA using Lipofectamine 2000 after 6 days *in vitro* (DIV) and with 5×10^8 GC of AAV-hSyn-FLEX-mScarlet constructs after after 9 DIV.

rAAV2/1 preparation

Recombinant AAV2/1 particles were prepared in HEK293T cells (DSMZ no. ACC 635), precipitated with PEG/NaCl, and subsequently cleared via chloroform extraction (Kimura et al. 2019). Purity of preparations was assessed with SDS-PAGE, and titers were determined using Gel green® (Biotium) (Xu et al. 2020).

Immunocytochemistry

Cells were washed with PBS and fixed at DIV17 with 4 % formaldehyde in PBS for 15 min and quenched with NH₄Cl in PBS at room temperature. After blocking/permeabilization for 1 h with 10 % goat serum, 1 % BSA, 0.2 % Triton X-100 in PBS, the following primary antibodies (Synaptic Systems) were used: anti-vesicular GABA transporter (vGAT) (1:1000, #131003) for inhibitory presynaptic terminals; anti-GABA_AR γ 2 (1:500, #224004) for postsynaptic GABA_ARs. The following secondary antibodies were used: goat anti-rabbit AlexaFluor 488 (1:500, #A-11034, Thermo Fisher Scientific), goat anti-rabbit AlexaFluor 647 (1:500, #A-21245, Thermo Fisher Scientific), and goat anti-guinea pig AlexaFluor 647 (1:500, #ab150187, Abcam). Coverslips were mounted with Mowiol/Dabco.

Widefield microscopy and image analysis

Image stacks [0.3- μ m z-step size, 1600 $\times$ 1200 (281.90 $\times$ 211.43 μ m)] were acquired on a Nikon Ti2-E inverse microscope with an Apo 60x Oil λ S DIC N2 objective,

equipped with TxRed(Ex560/Em630) and EGFP(Ex480/Em535) filter cubes and a QImaging Retiga 2000R Mono 12-bit CCD camera. A random forest classifier was used for pixel classification (binarization) of blobs. Cytoplasmic fluorescence intensities were manually measured from background corrected average intensity Z-projections. Blob formation (binary outcome) was modeled as a function of cytoplasmic fluorescence intensity using logistic regression. The onset threshold was defined as the intensity corresponding to a predicted 50% probability of blob formation. Logistic regression models including both gephyrin and GCN4-GlyR cytoplasmic fluorescence intensities were used to estimate blob formation probability. The onset threshold for gephyrin was defined as the intensity corresponding to a 50% predicted probability of blob formation, evaluated at the median mEGFP intensity within each condition. Confidence intervals were obtained by bootstrap resampling (1,000 iterations), stratified by gephyrin condition.

Confocal microscopy and image analysis

Image stacks [0.3- μ m z-step size, 2520 $\times$ 2520 (144.77 $\times$ 144.77 μ m)] were acquired on a Leica TCS SP8 LIGHTNING upright confocal microscope with an HC PL APO CS2 63 $\times$ /1.30 glycerol objective, equipped with hybrid detectors (Leica HyD) and the following diode lasers: 405, 488, 552, and 638 nm. LIGHTNING adaptive deconvolution using “Mowiol” setting was applied. A random forest classifier was used for pixel classification (binarization) of clusters. Images were segmented and analyzed in an automated fashion using Python 3.10.8 (scikit-image 0.26.0, pyclesperanto-prototype 0.2.1, apoc 0.12.0, numpy 2.3.5, pandas 2.3.3, scipy 1.16.3, napari_simpleitk_image_processing, os, shutil 1.8.1), and the code is available online at GitHub (https://github.com/FilLieb/automated_synapse_analysis).

Computation

No randomization procedure was performed when allocating samples to treatment. No samples were excluded from the analysis. Investigators were not blinded to sample allocation. Entire images from primary neuron cultures were analyzed in an automated fashion to minimize the effects of subjective bias. Statistical analyses for image analyses (analysis of variance (ANOVA) with Tukey’s post hoc test, Mann Whitney U test) were performed using R version 4.5.2, rstatix v. 0.7.2, tidyverse v. 2.0.0, ggdist v. 3.3.3, distributional v. 0.7.0 in Rstudio environment (<https://www.rstudio.com/>),

Platform: x86_64-w64-mingw32/x64. Data were tested for normality, using violation of the Shapiro–Wilk test at $p < 0.01$ as the criterion. For all other statistical analyses GraphPad Prism 10 Version 10.2.3 was used for analysis of variance (ANOVA) with Tukey's multiple comparisons test. Perplexity was used for language style improvement. OMERO, BioRender and ChimeraX-1.8 were used for Fig. design.

Data availability

No data that requires deposition in a public database.

Ethics statement

We complied with all relevant ethical regulations for animal testing and research and experiments were approved by local ethics committees (Germany, Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, reference 2021.A450). The study is reported in accordance with the ARRIVE guidelines.

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The authors declare that they have no conflict of interest.

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Chapter 3: Redox-dependent synaptic clustering of gephyrin

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3.1 Author contributions

Maria-Theresa Gehling, Emanuel H. W. Bruckisch, Filip Liebsch and Günter Schwarz designed the study. Maria-Theresa Gehling, Filip Liebsch, Emanuel H. W. Bruckisch, Konrad Benting and Günter Schwarz wrote and corrected the manuscript. Emanuel H. W. Bruckisch and Konrad Benting performed protein purifications, SEC studies and *in vitro* phase separation experiments. Maria-Theresa Gehling performed all mouse and cell culture imaging experiments as well as protein purifications of gephyrin, CD spectrometry, redox shift assays, calpain digest assays, ITC, *in vitro* Moco assays and mRNA level determination. Filip Liebsch prepared and Fynn Eggersmann performed electrophysiological measurements in murine hippocampal neuron culture. Methodical input was given from Lianne Jacobs and Jan Riemer regarding the manipulation of redox environments in neurons with the use of antimycin A and cytosolic D-amino acid oxidases. Filip Liebsch gave methodical input regarding the use of macros for the automated image analysis through Fiji/ImageJ. Methodical input was given from Peter Kloppenburg regarding electrophysiological measurements.

3.2 Abstract

Reactive oxygen species (ROS) have been demonstrated to play central functions as signaling molecules thus extending their role beyond oxidative stress in disease and aging. At inhibitory synapses the scaffolding protein gephyrin clusters glycine and GABA type A receptors. Postsynaptic gephyrin clustering is regulated by post-translational modifications such as phosphorylation, S-palmitoylation, and S-nitrosylation, which all critically impact its scaffolding function. Here, we show that oxidation of surface-exposed cysteine residues in gephyrin triggered reversible, synaptic multimerization providing more receptor binding sites and leading to proteolytic protection. Cys419, located at the previously published subdomain II interface important for liquid-liquid phase separation, was identified as a critical residue further regulating liquid-liquid phase separation of gephyrin in a redox-dependent manner. We also observed that surface-exposed cysteines are required to maintain fast miniature inhibitory postsynaptic current rise times, supporting the functional relevance of gephyrin redox regulation. Collectively, our findings suggest that cysteines

in gephyrin regulate synaptic localization and clustering as redox-switches, thereby establishing a new link between neuronal and metabolic activity at inhibitory synapses.

3.3 Introduction

Neuronal activity is connected to oxygen consumption and the production of reactive oxygen species (ROS)¹, that were initially considered as neurotoxic². However recently, redox signaling has emerged as an important pathway for modulating synaptic plasticity. For instance, in molecular layer interneurons ROS can enhance neurotransmission at inhibitory synapses^{3,4}, which was attributed to the recruitment of $\alpha 3$ -containing GABA type A receptors (GABA_AR) to the synapse. This mechanism involves a complex cascade following Ca²⁺ entry through NMDA-type glutamate receptors, which increases neuronal nitric oxide synthase activity. Elevated NO levels trigger cGMP production and protein kinase G activation, which in turn stimulates NADPH oxidase. Topical NMDA application in the neocortex results in ROS production via the same pathway⁵. Finally, elevated ROS levels activate protein kinase C, which results in the recruitment of $\alpha 3$ -containing GABA_ARs⁴. Furthermore, in hippocampal pyramidal neurons H₂O₂ can increase GABA_AR-mediated tonic currents via an extracellular oxidative reaction, mediated by NADPH oxidase, while synaptic currents remained unaffected⁶.

Importantly, maintaining the proper relation of ROS production and clearance is crucial for healthy brain function, and disruptions in ROS homeostasis leading to oxidative stress have been linked to various neuronal diseases^{7,8}. Consequently, unraveling the underlying molecular link between ROS production and neuronal activity is of great interest for understanding synaptic plasticity and the onset of pathological processes.

One key component at inhibitory synapses is the bifunctional protein gephyrin. Gephyrin is crucial for the activity of molybdenum cofactor (Moco)-dependent enzymes involved in basic metabolism. The conserved N-terminal G-domain and C-terminal E-domain of gephyrin catalyze the last two steps of Moco biosynthesis⁹. In addition, in the CNS gephyrin serves as the main scaffolding protein at inhibitory synapses, where it clusters glycine receptors (GlyRs) and a subset of GABA_ARs to facilitate efficient neurotransmission^{10,11}. The synaptic clustering requires the self-assembly of gephyrin through E-domain dimerization and G-domain trimerization¹². The interaction between

receptors and gephyrin takes place between the intracellular domain (ICD) of one receptor subunit and the gephyrin E-domain¹². The gephyrin C-domain links the G- and E-domain, providing flexibility as well as sites for post-translational modifications (PTMs) that regulate receptor binding and clustering^{13,14}. The proteolytic cleavage within the gephyrin C-domain by the calcium-dependent protease calpain^{15,16} is an irreversible modification, which is important for gephyrin homeostasis and links excitatory calcium signaling to the shaping of inhibitory gephyrin scaffolds^{15,17}. Additionally, gephyrin phosphorylation¹⁸, acetylation, and SUMOylation¹⁹ regulate gephyrin clustering and can shape the structure and function of inhibitory synapses. Moreover, gephyrin participates in a complex network of protein interactions crucial for signal transmission and neuronal plasticity^{20,58}. Thus, dysfunction of gephyrin has detrimental consequences for both metabolic and neuronal functions, ranging from delayed development to seizures and early childhood death²¹⁻²³.

Recent studies revealed that gephyrin regulation by PTMs, including those targeting cysteines, is essential for synaptic plasticity^{24,25}. Palmitoylation at Cys212 and Cys284 was identified to increase synaptic gephyrin clusters²⁵, while S-nitrosylation resulted in smaller synaptic clusters²⁴.

Recently, liquid-liquid phase separation (LLPS) has emerged as a novel mechanism for creating subcellular microdomains and has been proposed as a possible mechanism for the formation of protein assemblies at presynaptic terminals and postsynaptic densities²⁶. Several proteins at excitatory postsynaptic densities can undergo LLPS^{27,28} and gephyrin together with model proteins of GlyR/GABA_ARs can be organized into concentrated, droplet-like compartments, without the need for membranes²⁹. Additionally, proteins at excitatory and inhibitory postsynaptic densities spontaneously segregate into distinct condensed molecular assemblies³⁰. Importantly, gephyrin condensation is critically regulated by its central intrinsically disordered region (IDR) linker through conformational collapse and electrostatic interdomain interactions, rather than IDR–IDR interactions, with other iPSD components modulating its stability and localization via a thermodynamically driven network of intermolecular interactions³¹. Recent structural work has begun to shed light on the molecular basis of gephyrin condensation. Using single-particle cryo-EM, our laboratory demonstrated that gephyrin E-domain dimerization drives the formation of filamentous assemblies in which adjacent dimers are linked through Z-shaped interfaces between their subdomain II (SDII) regions, identifying these SDII–SDII' interactions as the

fundamental structural unit underlying LLPS⁵⁷. Disruption of this interface, whether through SDII deletion, epilepsy-associated pathogenic variants, or charge neutralization, abolishes filament formation, *in vitro* phase separation, and synaptic receptor clustering, establishing gephyrin E-domain filaments as the structural foundation of inhibitory postsynaptic density organization. The importance of this finding is underlined by a second paper published recently, reproducing these structures⁵⁹. Moreover, while gephyrin's capacity for LLPS may be subject to redox regulation, the functional roles of its surface cysteines remain incompletely characterized. Here, we investigated the relationship between redox signaling and gephyrin surface cysteines, extending beyond previously described nitrosylation and palmitoylation events.

Our results demonstrate a dynamic redox-dependent regulatory mechanism for synaptic gephyrin clustering: Oxidation strengthened clustering and resulted in fast miniature inhibitory postsynaptic current (mIPSC) rise times. As a result of ROS-induced gephyrin multimerization, the local number of receptor binding sites increased, which is distinct from a previously reported redox-dependent recruitment of $\alpha 3$ -containing GABA_ARs to strengthen inhibitory neurotransmission^{3,4}. In conclusion, our study establishes a link between inhibitory synapses and ROS, and offers a feedforward pathway through redox-dependent synaptic clustering of gephyrin.

3.4 Results

3.4.1 Surface cysteines of gephyrin are required for synaptic function

A crucial aspect of inhibitory synaptic transmission is the ability of gephyrin to build a scaffold by multimerization. To test whether the oligomeric state of gephyrin is dependent on cysteines and if those are redox-sensitive, we tested whether gephyrin presents redox-sensitive behavior in native tissue. Therefore, we perfused mice with N-ethyl-maleimide (NEM)-containing PBS to stabilize free thiols and avoid unspecific oxidation. In subsequent Western blot analysis, we either used the reductant dithiothreitol (DTT) or omitted it, allowing to compare the presence of redox-dependent

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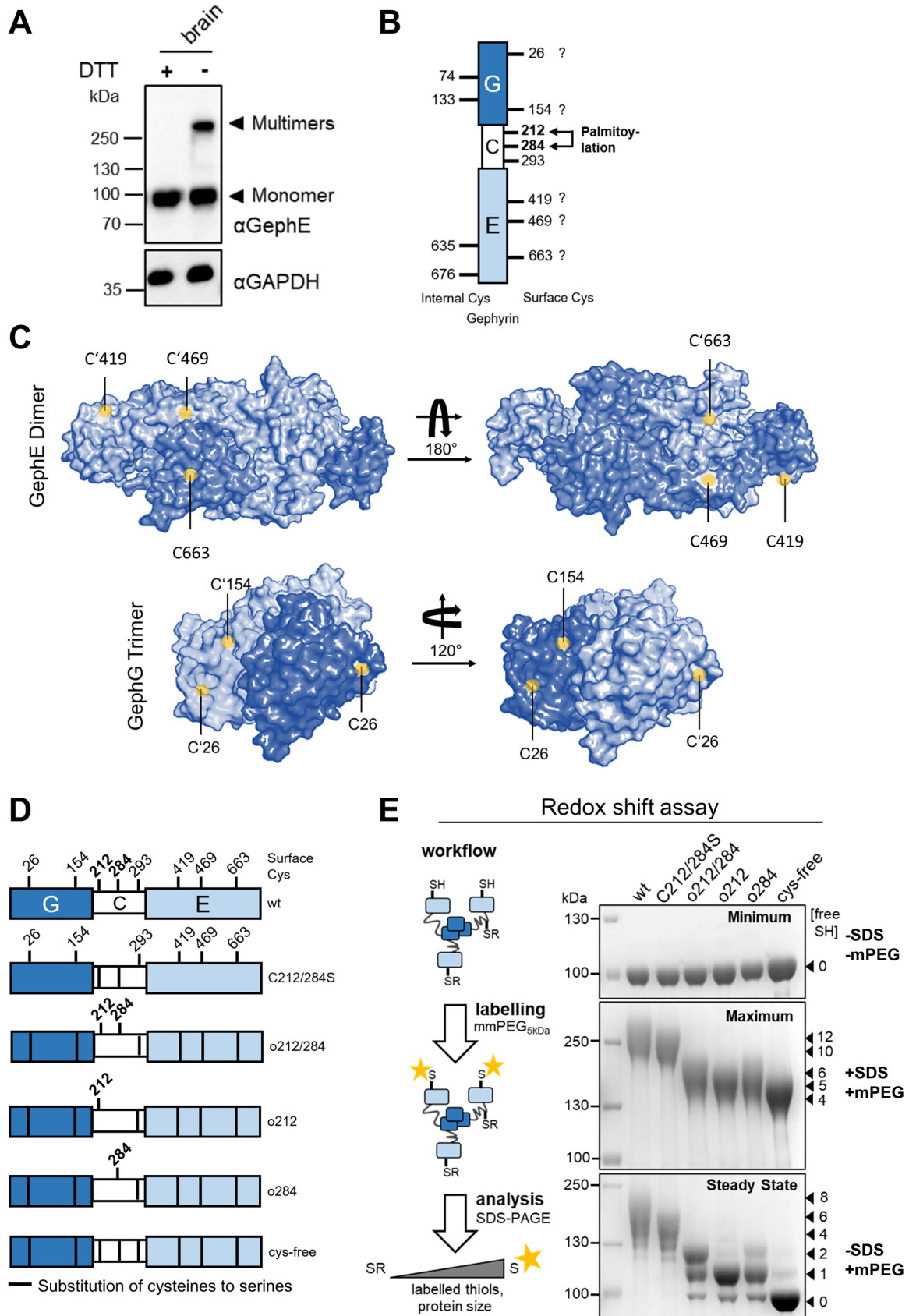


Figure 1: Role of surface-exposed cysteines of gephyrin.

Figure 1: Role of surface-exposed cysteines of gephyrin.

A) Representative Western Blot analysis of tissue lysates generated from mice, that have been perfused with NEM under reducing (DTT) or non-reducing conditions. Monomeric and multimeric gephyrin marked with arrows. **B)** Domain structure of gephyrin. G=G-domain; C=C-domain; E=E-domain. Positions of the eight potential surface-exposed and four internal cysteines are highlighted. **C)** Crystal structures of trimeric G-domain (PDB: 1IHC³⁴) and dimeric E-domain (PDB: 2FU3³³). Surface cysteines highlighted in yellow. **D)** Genetically engineered surface cysteine variants used in this study. Cysteines were substituted with serines at indicated positions. All internal cysteines remained unchanged. **E)** Redox Shift Assay with cysteine variants. Workflow: In this assay free thiols (-SH) are labelled with a maleimide-PEG-5kDa-tag (mmPEG) increasing the size in SDS-PAGE. Coomassie staining: Labelled, free-thiols cysteines indicated with arrows. As controls mmPEG and SDS were left out (Minimum) and both were added to additionally label the 4 internal cys (Maximum). For the steady state redox-status mmPEG but no SDS was used.

gephyrin species (Figure 1A). In brain lysates, the absence of a reductant resulted in bands corresponding to a molecular weight of approximately 300 kDa representing multimeric gephyrin species, whereas reduction of gephyrin led exclusively to monomeric gephyrin. The fractional abundance of multimeric gephyrin was approximately 20%. Based on this finding, we hypothesized that gephyrin cysteines are redox-sensitive and that thiol oxidation modulates intermolecular interactions, thereby influencing gephyrin multimerization, which is a crucial parameter for its synaptic function³². Thus, we wondered which surface cysteines of gephyrin could be important for multimerization.

Based on crystal structures of G- and E-domain^{33,34}, the identification of palmitoylation²⁵, as well as the disordered nature of the C-domain^{14,31} we assigned cysteines at position 26, 154, 212, 284, 293, 419, 469 and 663 as being surface-exposed (Figure 1 B and C). Interestingly, those cysteine residues are conserved in orthologues harboring a central nervous system (Figure S1), while other proteins such as Cnx1 in plants or bacterial orthologues lack those cysteine residues at their respective positions. To investigate the potential role of surface-exposed cysteines in redox regulation of gephyrin function, we generated cysteine variants by the substitution to serine (Figure 1D). Previously, Cys212 and 284 were identified as targets of palmitoylation²⁵. In this study, each of the other eight surface cysteines was individually substituted with serine²⁵. Overall, except for the palmitoylated Cys212 and 284, none of the other substitutions resulted in any noticeable effect on gephyrin cluster size, which suggested that they were individually dispensable²⁵. To test whether more than one cysteine outside the palmitoylation motif may have a functional role, we generated a gephyrin variant containing only Cys212 and 284 (o212/284) by replacing all other surface cysteines (Cys26, 154, 293, 419, 469, 663) with a serine. Additionally, we generated variants with remaining Cys212 (o212) and Cys284 (o284), respectively,

and one variant without any surface-exposed cysteine, which we called “cys-free”. Importantly, in all variants the four internal cysteines (Cys74, 133, 635, 676) remained unchanged.

The respective gephyrin variants were expressed and purified to homogeneity (Figure S2A). All variants showed similar secondary structure distribution as judged by CD-spectroscopy (Figure S2B). Besides that, we observed similar expression in COS7 cells (Figure S3A and B) and functional enzymatic activity, with the exception of “cys-free”, which displayed ~60 % enzymatic activity (Figure S3C).

With the purified variants we performed a redox-shift assay (Figure 1E) to determine free thiols by the attachment of a maleimide conjugate with a PEG-tag (mmPEG_{5kDa}), which requires an accessible, free thiol. Each labelled cysteine increased the size of gephyrin by approximal 10 kDa. Note that, internal Cys74, 133, 635 and 676 are accessible in the unfolded protein only (maximum shift). Thus, we confirmed that each individual cysteine among the eight predicted ones were indeed surface exposed, regardless of the presence of other cysteines (Figure 1E).

Based on the observed redox-dependent multimerization and redox-shift data, we anticipated a critical role for the eight surface-exposed cysteines in gephyrin. We focused on the scaffolding function of gephyrin in neurons. First, we performed Western blot and RT-PCR analysis demonstrating equal levels of expression for all variants (Figure S4). Afterwards, we expressed EGFP-tagged wt gephyrin, the variant lacking the palmitoylation sites (C212/284S), and our newly generated variants (o212/284 and cys-free) in hippocampal neurons (Figure 2A). Subsequently, we assessed synaptic gephyrin cluster size (Figure 2B). Overall, the expression of EGFP-tagged wt gephyrin resulted in synaptic gephyrin puncta sizes with a high degree of variability, possibly due to a number of different PTMs that can regulate its clustering¹⁸. The o212/284 variant did not exhibit a wt-like phenotype. The disturbance of synaptic clustering was similar to that observed in the C212/284S variant, as the synaptic cluster size was significantly reduced. This finding led us to conclude that the other surface cysteines serve an unidentified function, distinct from that of palmitoylation²⁵. Furthermore, this unknown function appears to be dependent on multiple cysteines, as single substitutions that were studied before did not show any significant impact on synaptic function²⁵. Moreover, the cysteine-free variant was in comparison to wt gephyrin significantly impaired, which was comparable to the o212/284 variant underlying the importance of all surface-exposed cysteines.

In summary, our data demonstrate that at least eight cysteines in gephyrin are surface exposed and play an important role in synaptic clustering (Figure 2C). Furthermore, the underlying mechanism, responsible for the multimerization of gephyrin at synapses relies on the cooperative action of multiple cysteines, which is in line with the identification of redox-dependent multimers found in native tissue.

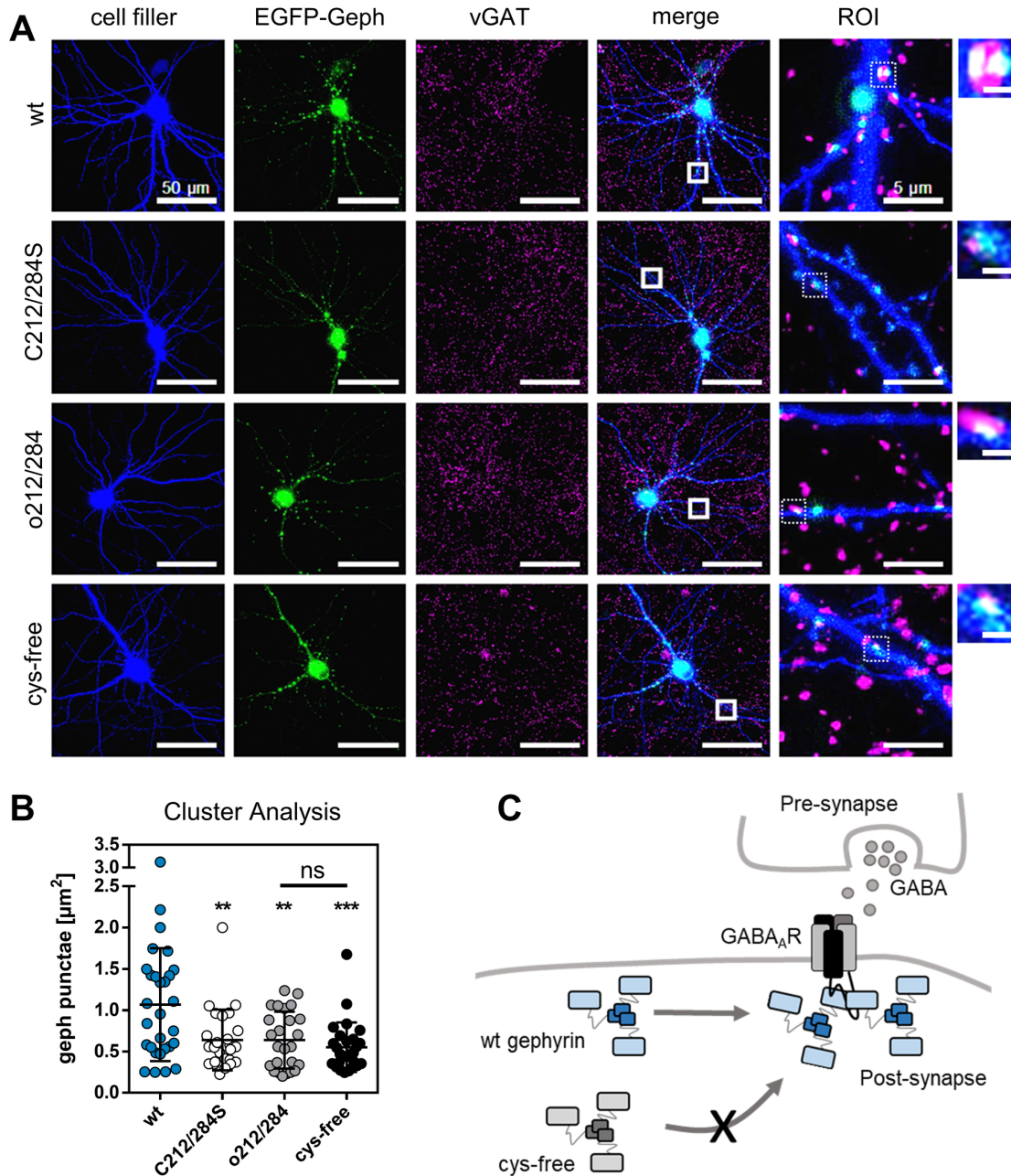


Figure 2: Role of the surface-exposed cysteines for gephyrin function.

A Primary, hippocampal neurons (DIV13) transfected with EGFP-gephyrin (green) and tdTomato (cell filler, blue) (scale bar 30 μm). Synaptic termini identified by vGAT staining. Dendrites enlarged (scalebar 5 μm) with increments of respective synaptic gephyrin clusters (scale bar 1 μm). **B** Cluster analysis of synaptic gephyrin. Significance tested by 1way ANOVA $F(3,103)=7.240$, $p=0.0002$. Bonferroni post-hoc test in comparison to wt: C212/284S: $p=0.0033$ (**); o212/284: $p=0.0035$ (**), cys-free: $p=0.0002$ (***). Bonferroni post-hoc test of o212/284 and cys-free $p=0.9368$ (ns). **C** Scheme of gephyrin clustering at GABAergic synapses. Cys-free gephyrin shows disturbed synaptic clustering.

3.4.2 Gephyrin undergoes reversible redox-dependent multimerization

Given that cysteine residues contribute to the regulation of gephyrin density at inhibitory synapses, and based on our observation of higher oligomer gephyrin species in brain extracts, we initially hypothesized that oxidation-induced disulfide bond formation may regulate gephyrin multimerization. However, we were unable to detect covalent gephyrin multimers in neuronal or non-neuronal cell culture systems, including HepG2, and neuroblastoma cells (Figure S5A-C). This prompted us to directly assess whether oxidative conditions *per se* can modulate gephyrin multimerization in a controlled *in vitro* setting. To this end, we treated recombinantly expressed gephyrin derived from *E. coli* with either the oxidant diamide or the reductant Tris-2-carboxyethyl-phosphine (TCEP) and analyzed the multimeric state of gephyrin using size exclusion chromatography (SEC). As a negative control, we used cysteine-free gephyrin. Upon oxidation, wt gephyrin displayed a pronounced shift toward higher-order species, consistent with an increased hydrodynamic radius and multimerization, whereas no such shift was observed for the cysteine-free variant (Figure 3A and B).

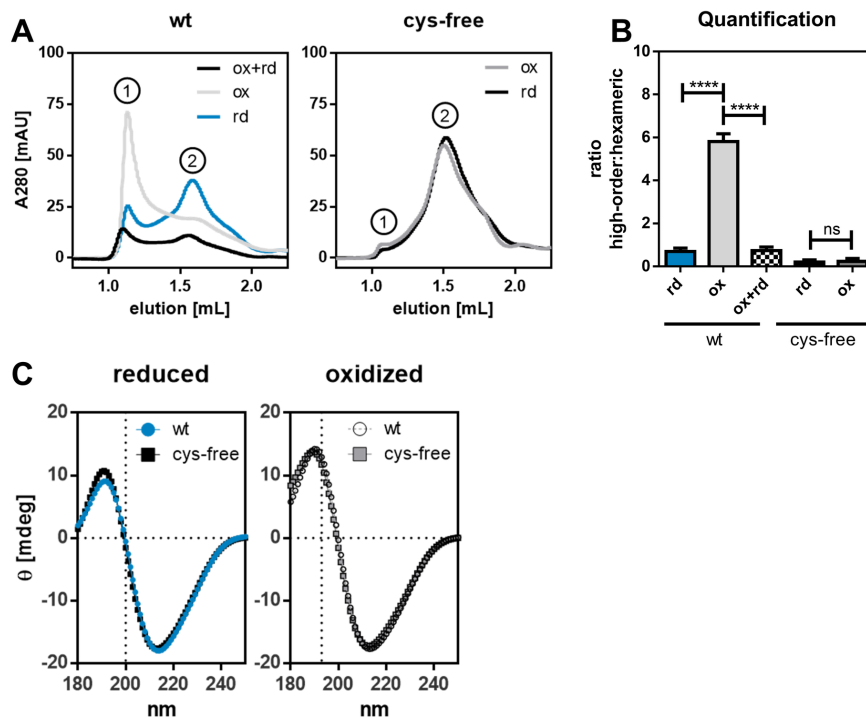


Figure 3: Reversible redox-dependent oligomerization of gephyrin *in vitro*

A-B) Size exclusion chromatography (SEC) with oxidized or reduced gephyrin wildtype (wt) or cysteine-free (cys-free) variant. **A)** Chromatograms with peaks of high-order multimers (1) or hexameric gephyrin (2). Reduced runs in blue (wt) or black (cys-free), oxidized in white (wt) or grey (cys-free) and oxidized peak collected and subsequently re-reduced in black (wt). **B)** Quantification of the ratio of the peak heights of three different runs. Significance tested by 1way ANOVA $F(4,10)=137.2$, $p<0.0001$, Bonferroni post-hoc test: $p<0.0001$ (****); $p>0.9999$ 05 (ns). **C)** CD Spectroscopy. Spectra of oxidized or reduced gephyrin wt or cys-free variant. Vertical dotted line indicates HT threshold of 800 mV.

Importantly, treatment with the reducing agent TCEP reversed this effect, restoring the elution profile to its pre-oxidation state. Similar results were obtained when diamide was replaced with H_2O_2 and TCEP with DTT (Figure S5D-E). Together, these findings demonstrate that oxidative conditions can reversibly promote gephyrin multimerization in a cysteine-dependent manner *in vitro*. While our data from brain tissue hinted towards the formation of disulfide-linked species under physiological conditions, the absence of detectable covalent multimers in cultured cells suggests that additional or alternative thiol-based modifications such as sulfenylation or sulfinylation may contribute to redox-dependent gephyrin organization in cells.

3.4.3 Gephyrin oxidation increases liquid-liquid phase separation

We identified that gephyrin multimerization was reversible and inducible by oxidation *in vitro* and we observed redox-dependent multimers *in vivo*. Next, we asked whether these oxidized, multimerized gephyrin species retain their functionality. Given that multimerization is a crucial feature of gephyrin, we sought to investigate the molecular consequences in respect to its biochemical properties, such as structure and receptor binding.

Firstly, we examined the secondary structure and binding capability of both wt gephyrin and the cysteine-free variant under oxidative and reductive conditions. Overall, we observed no alterations in the secondary structure, regardless of whether all cysteines were replaced with serines or if gephyrin underwent reduction or oxidation (Figure 3C). Similarly, we found no changes in the binding properties of gephyrin towards the glycine receptor β -ICD using isothermal titration calorimetry (ITC) experiments (Figure S6). For both, wt and cys-free variant, the interaction with the β ICD peptide occurred in a two-site binding mechanism (Figure S6). The stoichiometry observed indicated approximately one gephyrin trimer interacting with one β ICD peptide. The dissociation constant K_D was in the low micromolar range (wt = $0.041 \pm 0.010 \mu\text{M}$; cys-free = $0.063 \pm 0.018 \mu\text{M}$). Consequently, we concluded that cysteine-dependent oxidation does not alter the secondary structure or binding properties of gephyrin, while it changed its oligomeric state.

It would be plausible that oxidation-induced multimerization could lead to tighter packing of gephyrin clusters. Importantly, the quantity of gephyrin present at synapses and the protein density of clusters are crucial factors determining the number of

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receptor binding sites. Consequently, clustering can impact synaptic plasticity and neurotransmission. In line with this notion, we investigated in which manner oxidative processes would occur and be maintained, since gephyrin is a cytosolic protein and the cytosol represents a reductive environment. One possibility is the ability to form phase-separated droplets in the presence of a multimeric binding partner.

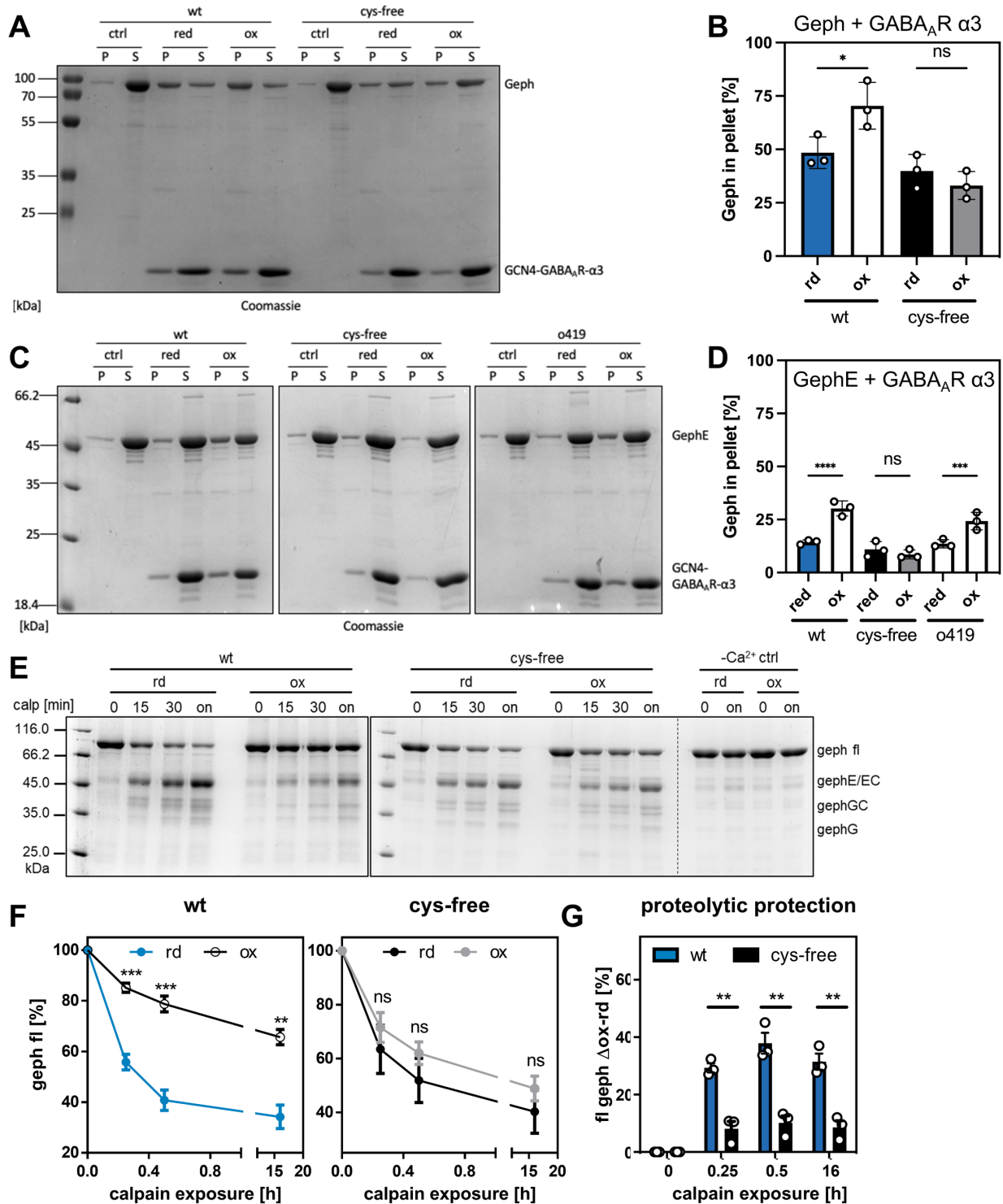


Figure 4: Sedimentation and calpain cleavage assays of oxidized and reduced gephyrin

Figure 4: Sedimentation and calpain cleavage assays of oxidized and reduced gephyrin

Oxidized (ox) or reduced (red) full length gephyrin (Geph) and isolated E-domain (GephE) wt and cysteine-free were used for sedimentation **A-D**) and calpain cleavage **E-G**) assays. n = 3 independent experiments, mean and standard deviation are displayed. **A-B**) Sedimentation assay using Geph + GCN4-GABAAR $\alpha 3$, condensation causes droplet formation (liquid-liquid phase separation), these droplets are sedimented by centrifugation. Sedimented proteins are recovered from the pellet (P), non-phase separated protein stays in the supernatant (S) fractions. As control oxidized gephyrin wt and cysteine-free alone were used. **A**) Coomassie Staining of fractions. **B**) Quantification of gephyrin band intensities. Relative abundance (%) normalized to sum of protein in pellet and supernatant of the corresponding fractions. Significance tested by 2way ANOVA F5,10=37.37, $p < 0.0001$. Tukey's multiple comparisons test: wt: $p = 0.0357$ (*); cys-free: $p = 0.8538 > 0.05$ (ns). **C-D**) Similar experiment with GephE and GCN4-GABAAR $\alpha 3$. Significance tested by 2way ANOVA F8,16=48.29, $p < 0.0001$. Tukey's multiple comparisons test: wt: $p < 0.0001$ (****); cys-free: $p = 0.8817 > 0.05$ (ns); oC419: $p = 0.0003$ (***). **E-G**) Exposure of gephyrin to the protease calpain for different times. **E**) Coomassie staining. Degradation products of gephyrin indicated at the different heights. fl=full-length; gephE=E-domain; gephEC=E-domain with C-domain fragments; gephG=G-domain, gephGC=G-domain with C-domain fragments. In the control, calcium was absent. **F**) Quantification of full-length (fl) gephyrin in each sample. Relative abundance (%) normalized to the start point 0. Significance between rd and ox determined by t-test and significance level alpha corrected by Bonferroni (/3): wt t1: $p = 0.0001$ (***); t2: $p = 0.0002$ (***); t3: $p = 0.006$; cys-free: t1: $p = 0.2535$ (ns); t2: $p = 0.1292$ (ns); t3: $p = 0.1843$ (ns). **G**) Comparison of proteolytic resistance. Relative abundance of fl geph in the reduced samples was subtracted from that of the oxidized ones (Δ_{ox-rd}). Significance tested by t-test and significance level alpha Bonferroni corrected (/3): t1: $p = 0.0022$ (**); $p = 0.0031$ (**); $p = 0.0033$ (**).

To investigate LLPS of gephyrin in relation to its redox-state, we used the GCN4 receptor model for GlyR, GABA_AR $\alpha 1$, and GABA_AR $\alpha 3$ ²⁹. While a substantial amount (~60 %) of gephyrin was recovered from the pellet fraction after co-incubation with GCN4-GlyR, no significant differences were observed when comparing oxidizing to reducing conditions (Figure S7A). However, when gephyrin was incubated with either GCN4-GABA_AR $\alpha 3$ (Figure 4A,B) or GCN4-GABA_AR $\alpha 1$ (Figure S7B), significantly more gephyrin was recovered from the respective pellet fractions under oxidizing compared to reducing conditions. Importantly, for all three model systems, gephyrin lacking surface exposed cysteine residues did not show redox-sensitive behavior (Figure 4A,B, Figure S7A,B).

Because recent work has highlighted the importance of SDII–SDII' interactions in driving gephyrin LLPS⁵⁷, we next examined whether any surface-exposed cysteine residues might directly contribute to this interface. Notably, Cys419 is positioned on the surface of SDII (Figure 1C).

To test its functional relevance, we purified three different gephyrin E-domain variants (GephE), wild type, a cysteine-free variant (C419/469/663S; “cys-free”), and a variant retaining only Cys419 (o419), and subjected them to LLPS assays using the three GCN4 receptor model systems described above. Consistent with our results for full-length gephyrin, oxidation selectively enhanced phase separation of GephE in the presence of GCN4-GABA_AR $\alpha 3$ (Figure 4C,D) and GCN4-GABA_AR $\alpha 1$ (Figure S7D),

but not with GCN4-GlyR (Figure S7C). Importantly, cys-free GephE failed to undergo phase separation under all conditions, whereas reintroduction of Cys419 alone was sufficient to restore LLPS capability. This finding was further potentiated under oxidizing conditions, particularly in combination with GCN4-GABA_AR α 3 (Figure 4C,D), suggesting that Cys419 plays a central role in mediating redox-sensitive modulation of SDII-driven gephyrin assembly.

Calpain-mediated cleavage is an important mechanism for the regulation of gephyrin clustering at synapses¹⁵. Besides calcium levels, the access to calpain cleavage sites determines the rate of proteolysis, which could be altered through redox-dependent multimerization. Therefore, the ability of calpain to cleave reduced or oxidized gephyrin was tested *in vitro* (Figure 4D-F). The sensitivity of oxidized gephyrin to calpain cleavage was significantly reduced by approximately two-fold, while there was no significant difference between reduced wt gephyrin and oxidized or reduced cysteine-free gephyrin (Figure 4E). Analyzing the difference in remaining full-length, undigested gephyrin between oxidized and reduced protein (Δ ox-rd), showed that wt gephyrin exhibited significant proteolytic resistance after oxidation even after short exposure towards calpain (Figure 4F). In contrast, the cysteine-free variant was equally digested as reduced wt gephyrin, regardless of whether it was in the reduced or oxidized state. In summary, our results show that structural properties of gephyrin that are influenced by its oligomeric state, such phase separation as well as proteolytic sensitivity, were redox-dependent.

3.4.4 H₂O₂ mediates synaptic clustering of gephyrin in neurons

To address the question of how gephyrin wt in comparison to the cys-free variant could be oxidized in neurons, we investigated H₂O₂, which is a commonly known oxidant emerging from different sources in the cell. Therefore, we expressed a cytosolic version of a D-amino acid oxidase (DAAO) with an mScarlet-tag in hippocampal neurons and tested the effect on endogenous gephyrin clusters (Figure 5A) or on recombinant cys-free and wt gephyrin (Figure 5B). DAAO enzyme produces H₂O₂ by the conversion of D-amino acids to the corresponding α -keto acids (Figure 5A), thus we treated our cells with D-Met. As a negative control, we generated a less active variant of DAAO by substituting Arg285 to Lys³⁵. Following cell treatment, we stained

for the presynaptic marker vGAT and analyzed its co-localization with endogenous gephyrin to identify synaptic gephyrin species.

Firstly, the ubiquitous and cytosolic localization of DAAO could be demonstrated (Figure 5C, full images in Figure S8A). D-methionine (D-Met) treatment caused a significant increase of synaptic cluster sizes by approximately 20% (from 0.1269 to 0.1521 μm^2) (Figure 5C and D). Additionally, the intensity of synaptic gephyrin was significantly increased by about 14% (from 1,364 to 1,559 a.u.) (Figure 5E). The

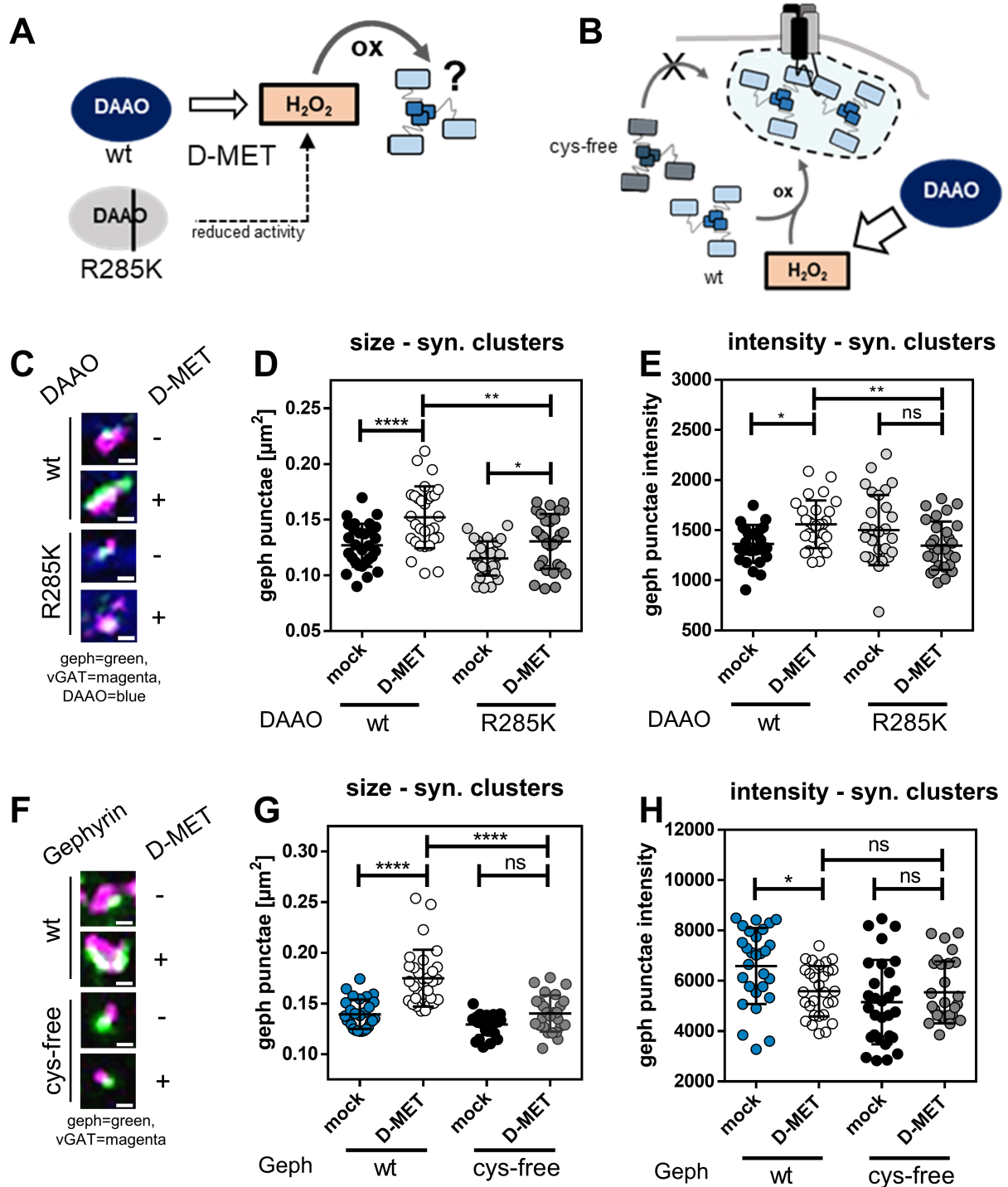


Figure 5: H_2O_2 drives gephyrin oligomerization at synapses

Figure 5: H₂O₂ drives gephyrin oligomerization at synapses

Expression of cytosolic mSclt-DAAO and mEGFP-Geph in primary, hippocampal neurons. Treatment at DIV14 with 5 mM D-MET for 1 h. Analysis of cluster sizes [μm^2], intensity [au] and co-localization with vGAT (=synaptic clusters) of gephyrin. **A)** Schematic description of H₂O₂ production of DAAO wt upon D-methionine acid treatment. DAAO mutant R285K is inactive. Can H₂O₂ oxidize gephyrin and influence its oligomerization? **B)** Schematic description of influence of DAAO-generated H₂O₂ on gephyrin clustering. Is cys-free gephyrin not influenced? **C-D)** mSclt-DAAO wt and R285K. Analysis of endogenous gephyrin clusters. **C)** Representative gephyrin clusters (Gephyrin: green, vGAT: magenta, DAAO: blue) (scale bar 0.5 μm). Synaptic termini identified by vGAT co-localization. **D)** Sizes of synaptic gephyrin. Significance tested by 1way ANOVA $F(3,113)=14.18$, $p<0.0001$. Bonferroni post-hoc test: wt vs. wt+D-MET: $p<0.0001$ (****); R285K vs R285K+D-MET: $p=0.0277$ (*), wt+D-MET vs R285K+D-MET: $p=0.0010$ (***). **E)** Intensity of synaptic gephyrin. Significance tested by 1way ANOVA $F(3,109)=4.431$, $p=0.0056$. Bonferroni post-hoc test: wt vs. wt+D-MET: $p=0.0204$ (*); R285K vs R285K+D-MET: $p=0.0808$ (ns), wt+D-MET vs R285K+D-MET: $p=0.0083$ (**). **F-H)** mSclt-DAAO wt and mEGFP-Geph wt and cys-free variants. Analysis of recombinant mEGFP-gephyrin clusters. **F)** Representative gephyrin clusters (Gephyrin: green, vGAT: magenta) (scale bar 0.5 μm). Synaptic termini identified by vGAT co-localization. **G)** Sizes of synaptic gephyrin. Significance tested by 1way ANOVA $F(3,107)=31.66$, $p<0.0001$. Bonferroni post-hoc test: wt vs. wt+D-MET: $p<0.0001$ (****); cys-free vs cys-free+D-MET: $p=0.1252$ (ns), wt+D-MET vs cys-free+D-MET: $p<0.0001$ (****). **H)** Intensity of synaptic gephyrin. Significance tested by 1way ANOVA $F(3,110)=5.608$, $p=0.0013$. Bonferroni post-hoc test: wt vs. wt+D-MET: $p=0.0212$ (*); cys-free vs cys-free+D-MET: $p=0.8567$ (ns), wt+D-MET vs cys-free+D-MET: $p>0.9999$ (ns).

expression of DAAO R285K variant in combination with D-Met treatment had a significant increase on gephyrin cluster size as well, but the effect size was smaller (Confidence Interval $[-0.011;-0.039]^{\text{wt}}$, $[-0.001;-0.030]^{\text{R285K}}$). The effect could be explained by the residual activity of the R285K mutant³⁵. However, no effect of the treatment in combination with the R285K mutant on the intensity of gephyrin was observed. Based on this observation, we conclude that the effects on gephyrin resulted mainly from the enzymatic activity of DAAO, the production of H₂O₂. Since increase of cluster size and intensity are signs of gephyrin recruitment to the synapse, we concluded that H₂O₂ is able to trigger synaptic multimerization of gephyrin in neurons. Next, we verified that H₂O₂ had a direct effect on the cysteines of gephyrin. Thus, we combined the expression of DAAO and subsequent D-Met treatment with the overexpression of mEGFP-tagged gephyrin (Figure 5F, full images in Figure S8B). As expected, overexpressed wt gephyrin showed a significant increase of about 25% (from 0.1393 to 0.1750 μm^2) in cluster size following D-Met treatment, while cys-free gephyrin showed no effect (Figure 5F and G). Strikingly, the intensity of synaptic mEGFP-gephyrin wt upon DAAO-mediated H₂O₂ production was decreased by -15% (from 6,591 to 5,588 a.u.), but that of cys-free gephyrin was unchanged (Figure 5H). This effect may originate from cysteine-dependent oxidation and/or impacts on mEGFP folding in oxidized mEGFP-gephyrin wt clusters that may be shielded from the reductive cytosol³⁶. In this set up, oxidation extended existing gephyrin clusters in size but did not increase the density within clusters.

In conclusion, we could show that DAAO-mediated H_2O_2 promotes synaptic gephyrin cluster formation. The mechanism involves surface cysteines of gephyrin since cysteine-free gephyrin did not react to cellular H_2O_2 formation.

3.4.5 Gephyrin oxidation promotes inhibitory synaptic transmission

To assess whether redox-dependent modulation of gephyrin clustering affects inhibitory synaptic transmission, we performed whole-cell patch clamp recordings to measure miniature inhibitory postsynaptic currents (mIPSCs). This approach was based on our biochemical findings indicating that gephyrin cysteine residues mediate redox-sensitive clustering, potentially influencing the availability of synaptic GABA_A receptors.

We conducted two sets of experiments to analyze functional consequences at the synapse. First, we compared mIPSCs in wild-type (wt) neurons and neurons expressing a cysteine-deficient gephyrin mutant (cys-free). Second, we manipulated intracellular reactive oxygen species (ROS) levels to modulate the redox state. To this end, neurons were transfected with either catalytically active D-amino acid oxidase (DAAO) and treated with D-methionine (D-Met) to elevate ROS levels (wt DAAO+D-MET), or with a catalytically inactive DAAO mutant (R285K).

Both experimental manipulations, gephyrin cysteine deletion and ROS level alteration, resulted in significant changes in mIPSC properties (Figure 6, Supplementary Figure 9). Particularly striking was the consistent and robust effect on mIPSC rise times observed across all experimental conditions. Specifically, mIPSC rise times were slower in neurons lacking gephyrin cysteine residues and faster in neurons with elevated ROS levels. The fastest rise times occurred in DAAO-overexpressing neurons, followed by R285K-expressing neurons (Figure 6G). WT neurons exhibited intermediate rise times, whereas neurons expressing the cysteine-deficient gephyrin displayed the slowest rise times.

These findings align with our biochemical data demonstrating that gephyrin multimerization is modulated by redox state, at least in part through redox reactions involving its cysteine residues. The graded, statistically significant distribution of mIPSC rise times supports a model in which both intact gephyrin cysteine residues and increased intracellular oxidation promote faster inhibitory synaptic responses. Overall, this relationship between redox state, gephyrin cysteine content, and mIPSC

Chapter 3

kinetics supports our hypothesis that gephyrin cysteines act as redox-sensitive switches that regulate synaptic localization and clustering, thereby modulating the functional efficacy of inhibitory synaptic transmission.

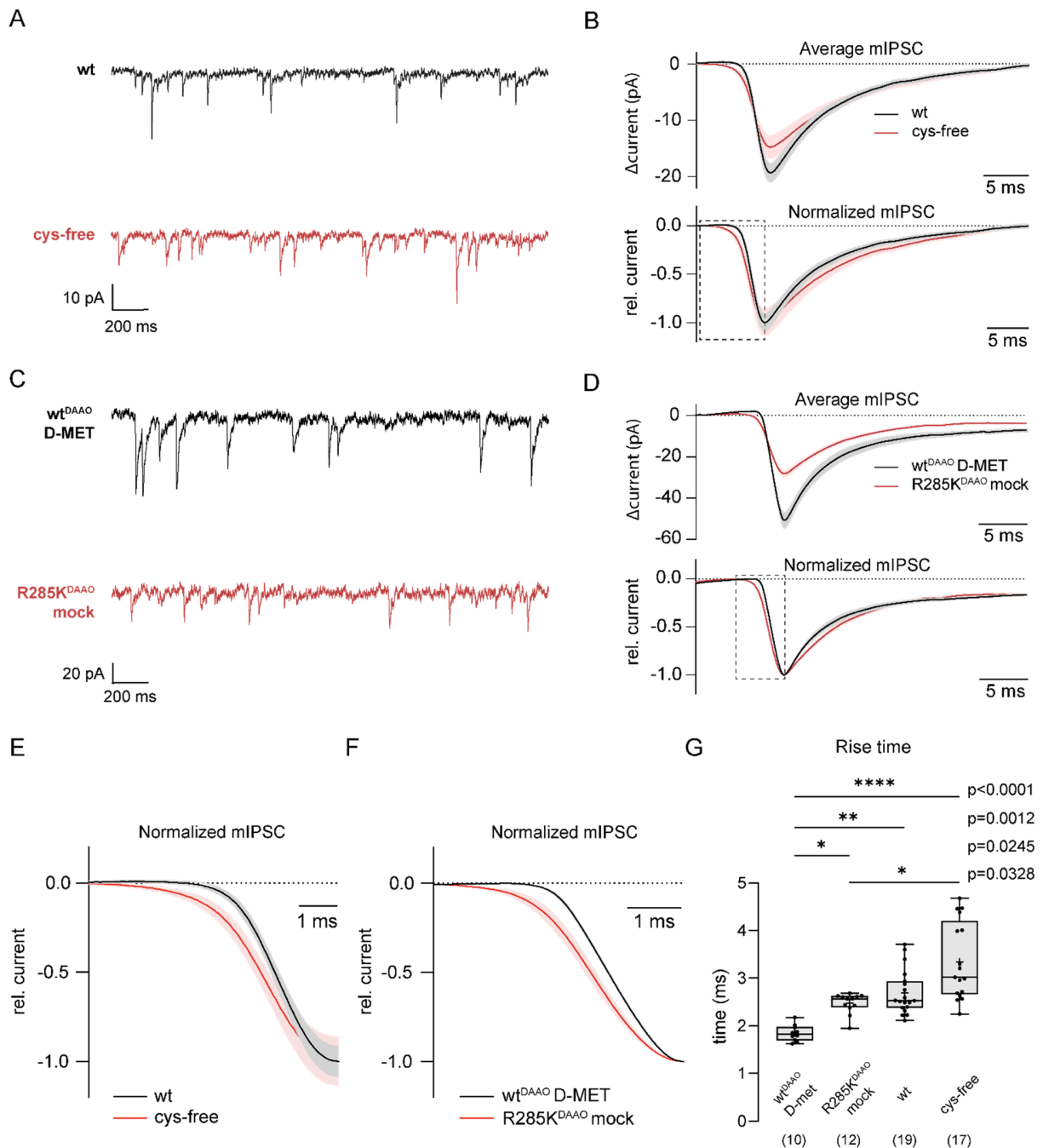


Figure 6: Gephyrin surface cysteines and intracellular H₂O₂ result in faster mIPSC rise times

Figure 6: Gephyrin surface cysteines and intracellular H₂O₂ result in faster mIPSC rise times

Miniature inhibitory postsynaptic currents (mIPSCs) recorded in primary cultures of murine hippocampal neurons **A, B** expressing mEGFP gephyrin wt and cys-free. **C, D** expressing wt DAAO + D-MET treatment (wtDAAO D-MET) and R285K + mock treatment (R285KDAAO mock). For the wt DAAO + D-MET group D-methionine (5 mM) was bath applied at least for 1h prior to the start of the recording. **A, C** Example mIPSC recordings from each experimental groups. **B, D** Mean ($\pm$ SEM) mIPSCs (top) and normalized mIPSCs (bottom). **E, F** Section (labelled by the red box in B and D) of the normalized mIPSCs showing the rising phase in higher time resolution. **G** Box plots showing the rise times of the mIPSCs of the different genotypes and treatment conditions. Each data point in G represents the mean of the mIPSCs recorded during a 50 s interval from a single neuron (260 ± 17 individual mIPSCs per neuron). All recordings were performed at 13–15 DIV. Glutamatergic input was blocked by CNQX and DL-AP5. Na⁺-mediated action potentials were blocked by TTX. Holding potential was -70 mV. Data were compared using the Kruskal-Wallis test followed by Dunn's post hoc multiple comparisons tests. In boxplots, horizontal lines indicate the data median, + signs indicate the means of the data, boxes indicate the interquartile range (25th to 75th percentile), and whiskers extend to the minimum and maximum values. Probability values (p) are reported as exact values, with sample sizes (n) provided in brackets.

3.5 Discussion

Our study provides new insight into the mechanism of gephyrin-dependent inhibitory synapse formation. First, we identified surface exposed cysteines *in vitro* and demonstrated their sensitivity to redox changes. As a result, we found that the multimerization of gephyrin was regulated by oxidation and reduction. We identified redox-dependent multimers *in vivo* and induced synaptic multimerization *in cellulo* through DAAO-mediated H₂O₂. Oligomerization is a crucial aspect of gephyrin scaffolds, significantly impacting the plasticity of inhibitory synapses^{32,37}. Consequently, the oligomeric state influences the number of available receptor binding sites and the sensitivity of gephyrin to proteolytic cleavage¹⁷. The structural basis of this oligomerization has only recently been resolved at high resolution by our group, revealing gephyrin E-domain filaments stabilized by SDII–SDII' contacts as the fundamental architecture driving both scaffold assembly and phase separation at inhibitory synapses⁵⁷. Changing the oligomeric state by oxidation enhanced clustering, while reduction reversed this process (Figure 7). In aggregate, our study provides a novel role of surface-exposed cysteines in gephyrin, which were found to be conserved in orthologues with a central nervous system while other orthologs in plants, yeast, fungi and prokaryotes lack those residues (Figure S1).

Comparing our data for redox-dependent multimerization of gephyrin with PSD95, the functional pendant at the excitatory PSD, multimerization by the formation of disulfide bridges was detected *in cellulo* and *in vivo*³⁸. Recently, the disruption of a cysteine linkage in PSD95 has been linked to an epileptic phenotype in rats³⁹, highlighting the importance of redox-regulation of neuronal scaffold proteins.

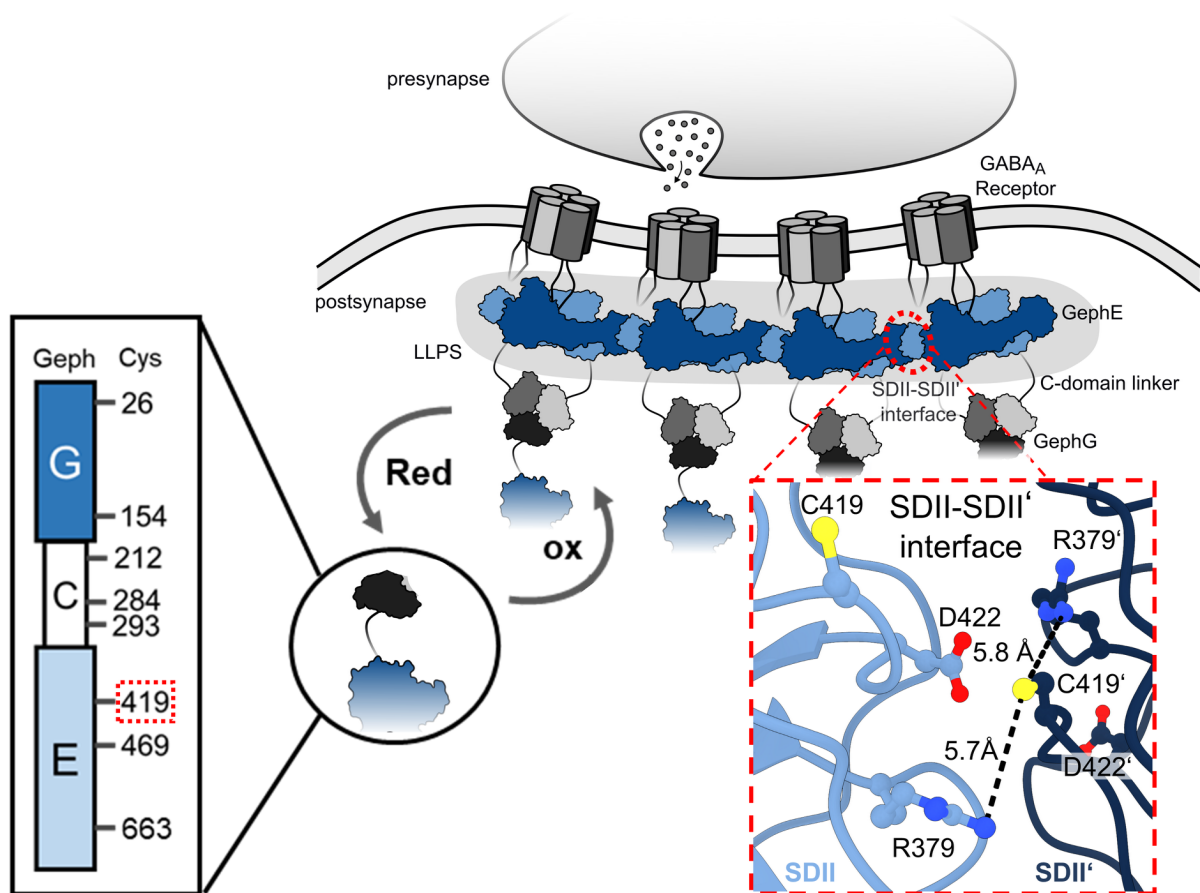


Figure 7: Redox-regulation of synaptic gephyrin clustering

Hypothesized model of redox-dependent gephyrin oligomerization at inhibitory GABAergic postsynapses. Gephyrin harbors eight surface-exposed cysteines that are redox-sensitive. ROS-mediated oxidation increases gephyrin oligomerization. This oxidation is reversible by reduction and allows for dynamic shuttling of gephyrin localization. LLPS (highlighted in grey) might offer an oxidative microdomain facilitating oxidation. Gephyrin oligomerization at synapses offers binding sites for GABA_ARs, ultimately strengthening inhibitory signal transmission. Detailed environment of Cys419 at the subdomain II interface is shown in the red box (PDB: 9GW9).

Cysteine oxidation in gephyrin could be reversed by reduction, which is essential for enabling dynamic changes and maintaining plasticity. Importantly, cysteine oxidation did not influence the secondary structure of gephyrin and its binding to the receptor, but it altered the density of gephyrin at the synapse, manipulating the availability of receptor binding sites. One possible explanation for the strong cysteine-dependent effects in the absence of detectable disulfide-linked multimers in cells is a local electrostatic mechanism involving Cys419. In the SDII–SDII' interface, the proximity of Arg379 may lower the pK_a of Cys419 (Figure 7), promoting formation of a negatively charged thiolate. This thiolate could stabilize the interface by strengthening ionic interactions with Arg379, analogous to the established Asp422–Arg379 interaction critical for LLPS⁵⁷. Furthermore, higher oxidation states of Cys419 would

introduce additional negative charge, potentially further enhancing interface stability and thereby promoting gephyrin multimerization and phase separation without requiring covalent crosslinking via disulfide-bridge formation. However, to which extend such a modification may be formed in brain tissue over time, requires future developmental studies.

Although, redox-dependent regulation of synaptic proteins has previously been investigated, important questions remain open such as the source of oxidation. Given that neurons are rich in mitochondria and mitochondria are located in close proximity to the synapse⁴⁰, we propose that mitochondria-derived ROS could serve as a source for oxidation. In this sense, gephyrin could provide the functional link between mitochondrial and neuronal activity. Highly active neurons require ATP production mainly derived from mitochondrial respiration being accompanied by the formation of mROS, which is rapidly converted to H_2O_2 ^{1,41,42}. H_2O_2 can reach the synapse and trigger gephyrin recruitment and network expansion, thereby recruiting additional receptors to further increase neurotransmission. Mitochondria are located in both pre-synaptic and post-synaptic regions, so it is conceivable that mROS could regulate the post-synapse from various angles and in reverse it can impact both sites of the synapse.

Besides mitochondria, there are additional origins of H_2O_2 , such as the NADPH-oxidase 2 (NOX2) pathway⁴³. NOX2 is highly abundant in cells throughout the CNS⁴⁴ and produces superoxide radicals in the extracellular environment, which are subsequently converted to H_2O_2 . This H_2O_2 can re-enter the cell through aquaporins. In a recent study, the effects of this pathway on inhibitory neurotransmission were investigated⁴. It was shown that communication of excitatory and inhibitory neurotransmission is mediated by ROS and nitric oxide, leading to the recruitment of GABA_AR-containing $\alpha 3$ subunits at inhibitory synapses. In contrast to our study, they identified the GABA_AR-associated protein (GABARAP) as the primary mediator of receptor recruitment, a protein that has been found to interact with gephyrin⁴⁵. Although, it is unclear, whether different ROS-mediated signaling mechanisms can co-exist, it highlights the importance of these mechanisms in regulating neuronal plasticity. It is important to note that the effects of ROS-mediated signaling on glycinergic synapses has not been studied. Furthermore, we did not answer the question whether gephyrin was directly oxidized by H_2O_2 or other oxidant species, which could be derived from H_2O_2 .

Another open question concerns the maintenance of oxidation processes in the reductive cytosol. In recent studies, LLPS has gained attention as a new principle of subcellular organization⁴⁶. For gephyrin it was recently demonstrated that it forms liquid droplets in the presence of a multimeric receptor model²⁹. Importantly, our data showed that oxidation of gephyrin strengthened LLPS with GABA_AR $\alpha 1$ and $\alpha 3$ but not with GlyR β model proteins. Neuroligin 2 (NLGN2) is a post-synaptic cell-adhesion molecule that can interact with pre-synaptic Neurexins and is exclusively found at GABAergic synapses⁴⁷. Local NLGN2 accumulation can promote the nucleation of gephyrin condensates³¹. Our data demonstrate that gephyrin oxidation increased condensation with GABA_AR model proteins, and future work could test whether oxidation could influence gephyrin nucleation (for example locally at NLGN2 contact sites). While our current study suggests that existing GABAergic synapses are strengthened by this process, work on gephyrin nucleation could be instructive as to whether the formation of new synapses could be promoted.

Prior studies investigated the relationship between the redox state of cysteines and the ability to undergo LLPS. One example is synapsin 1, which harbors two cysteines directly affecting LLPS in a redox-dependent manner, which is also relevant in aging⁴⁸. A study, that is mechanistically comparable to our work, was reported for oleosin, where the disulfide-bridge formation of artificially added cysteine residues on the protein surface triggered LLPS, while reduction was able to reverse this effect⁴⁹. In general, the concept of a reversible formation of phase-separated microdomains holds great potential and may be a key to understand redox-dependent processes in different cellular environments.

Building on the emerging view that SDII–SDII' interactions constitute a filamentous structural element underlying gephyrin assembly and LLPS⁵⁷, our observations further refine how this interface may be regulated at the molecular level. In this context, the positioning of Cys419 directly within the SDII surface suggests a mechanism by which redox-dependent modifications can influence intermolecular connectivity within gephyrin filaments and thus the entire network. Our LLPS data support this notion, as oxidation only in presence of Cys419 in combination with GABA_AR $\alpha 1$ and $\alpha 3$ model systems selectively enhanced phase separation, whereas no additional effect was observed with GlyR β , which binds with much higher affinity to gephyrin. Mechanistically, these findings suggest that oxidative modifications at Cys419 may directly tune the strength of the SDII–SDII' interface, thereby modulating

the overall connectivity of the gephyrin network. In particular, reversible oxidation of Cys419 could introduce additional negative charge, potentially reinforcing the proposed salt bridge between D422 and the opposing R379 across the SDII–SDII' interface⁵⁷.

A limitation of our study is the use of dissociated hippocampal cultures, which do not maintain their correct anatomic input-specificity. While prior studies have demonstrated that inhibitory synapses in molecular layer interneurons are strengthened upon ROS-induction through the recruitment of $\alpha 3$ -containing GABA_ARs in a gephyrin-independent manner^{3,4}, our findings in hippocampal neurons suggest the existence of an additional gephyrin-dependent mechanism. Interestingly, in this gephyrin-dependent mechanism, oxidation resulted in fast mIPSC rise times, which may reflect increased temporal precision of synaptic inhibition. Additionally, gephyrin oxidation in LLPS experiments suggests that both $\alpha 1$ - and $\alpha 3$ -containing GABA_ARs may be recruited by this mechanism.

Oligomerization as a function of the redox state of cysteines (including nitrosylation, persulfidation, sulfenylation and disulfide-formation) has been investigated previously^{1,48,50}. Such modifications may lead to functional multimers or harmful aggregates depending on the site and degree of oxidation thus reflecting the dose of ROS⁸. Imbalance in redox signaling is linked to aging and neurodegenerative diseases⁷. Our study identifies gephyrin as mediator between metabolic activity and inhibitory synapses using ROS as signaling mechanism.

3.6 Material and methods

DNA/expression constructs

Gephyrin P1 and cysteine variants in pQE80L (Qiagen) were cloned by Gibson assembly using EcoRI into pAAV_hSyn_mScarlet (a gift from Karl Deisseroth, Addgene plasmid #131001) and using EcoRI into pAAV_hSyn_mEGFP (generated from moxBFP, a gift from Erik Snapp, Addgene plasmid #68064; <http://n2t.net/addgene:68064>; RRID: Addgene_68064). Gephyrin was cloned using PCR and XhoI and KpnI into pEGFP-C2 (Clontech). The template for the generation of rat Gephyrin P1 constructs was in pQE80L⁵¹. The cysteine to serine substitutions were generated by site-directed mutagenesis. DAAO was cloned into pAAV_hSyn_mScarlet through PCR and Gibson Assembly using EcoRV and the

R285K mutation was introduced by site-directed mutagenesis. The plasmids pAdDeltaF6 (a gift from James M. Wilson, Addgene plasmid #112867; <http://n2t.net/addgene:112867>; RRID: Addgene_112867) and pAAV2/1 (a gift from James M. Wilson, Addgene plasmid #112862; <http://n2t.net/addgene:112862>; RRID: Addgene_112862) were used for virus production. The expression constructs of GlyR β ICD in pTYB was described previously^{52,53}. GCN4 constructs with GlyR, GABA α R α 1, and GABA α R α 3 were a kind gift from the laboratory of Prof. Mingjie Zhang, Hong Kong University of Science and Technology²⁹.

Recombinant expression and purification of gephyrin

E. coli BL21 Rosetta star was used for the expression of His-gephyrin and His-gephE. Cultures were grown at 37°C at 110 rpm and the expression induced at an OD₆₀₀ of 0.3 with 100 nM IPTG. After expression for 22 h at 18°C and 110 rpm, cells were harvested. To achieve reduced cysteines (free thiols), all solutions were supplemented with reductant until final storage of isolated proteins. The cell pellet was resuspended in lysis buffer (300 mM NaCl, 50 mM Tris/HCl, 5 mM beta-mercapto ethanol, 0.05% Tween20, 1 µg/mL lysozyme, 1x PI (*Roche*), pH 7.4) and stored at -80°C. Pellets were thawed in a water bath and all steps performed at 4°C. Cells were lysed through alternating ultrasound (3 min at 4°C, 30 s pulse, 30 s pulse, 40% amplitude) and pressure lysis (*EmulsiFlex*, 1,000-1,500 bar, 4°C) twice. The homogenate was centrifuged (45 min, 16,000 rpm, 4°C, JLA 16.250 rotor, Beckman-Coulter Centrifuge Avanti J25-01) and the supernatant subjected to Ni-NTA affinity purification (HIS-Select Nickel Affinity Gel (Sigma Aldrich)). All buffers for washing and elution were similar to the lysis buffer, contained no lysozyme, but contained 20 mM, 40 mM and 300 mM Imidazole, respectively. The eluate was further subjected to preparative size-exclusion chromatography (Superdex 200, 120 mL, *GE Healthcare*) using SEC buffer (300 mM NaCl, 50 mM Tris/HCl, 5 mM beta-mercapto ethanol, 0.05% Tween20, 5% glycerol, pH 7.4). The fractions of the peaks eluting around 60-70 mL (Trimers) and 45-60 mL (higher multimers) were collected and concentrated by an *Amicon* filter with a Cut-off of 50 kDa. To remove reductants, the concentrated protein was buffer exchanged by gel filtration (PD10 column) to storage buffer (300 mM NaCl, 50 mM Tris/HCl, pH 7.4) and stored at -80°C.

Purification of β ICD peptide from *E. coli*

For the β ICD peptide we used *E. coli* ER2566. Cultures grew at 37°C and 110 rpm until the OD₆₀₀ reached 0.3. Induction was induced by 250 μ M IPTG and expression performed for 20 h at 25°C and 110 rpm. Cells were harvested and stored at -80°C in ICD lysis buffer (300 mM NaCl, 50 mM Tris/HCl, 1 mM EDTA, 1x Protease Inhibitor (Roche), pH 7.4). Lysis of the thawed cells was performed by alternation of ultrasound (3 min at 4°C, 30 s pulse, 30 s pulse, 40% amplitude) and pressure lysis (*EmulsiFlex*, 1,000-1,500 bar, 4°C) twice. After centrifugation for 1 h at 16,000 x g (JLA 16.25 rotor) at 4°C, the supernatant was applied on a pre-equilibrated chitin matrix (*New England Biolabs*) and incubated 1.5 h at 4°C under shaking. The column was washed with 15 CV ICD buffer before 2.5 CV cleavage buffer (300 mM NaCl, 50 mM Tris/HCl, 1 mM EDTA, 50 mM DTT, pH 7.5) was applied and incubated for minimum 24 h at rt. The eluate was collected and another 2.5 CV cleavage buffer used for a second elution. The combined eluate was concentrated and filtered by *Amicon* filters with a cut-off of 10 kDa and 3 kDa. The buffer was exchanged to ITC buffer (300 mM NaCl, 50 mM Tris/HCl, pH 7.4) by dialysis over night at 4°C. The purified protein was stored at -80°C.

GlyR- β LD, GABA_AR- α LD and GABA_AR- α 3LD purification

GlyR- β LD, GABA_AR- α LD and GABA_AR- α 3LD were purified according to the protocol provided by Bai and colleagues²⁹. Recombinant proteins were expressed in *Escherichia coli* BL21-CodonPlus (DE3)-RL cells (Agilent Technologies) in LB medium at 16°C overnight and protein expression was induced by 0.25 mM IPTG at OD₆₀₀ between 0.6-0.8. Cultured cells were resuspended with the lysis buffer containing 50 mM Tris, pH 8.2, 1000 mM NaCl, 20 mM Imidazole, 1 mM PMSF and lysed with high pressure (*EmulsiFlex*, 1,000-1,500 bar, 4°C). Cell lysis was clarified by 18000 rpm centrifugation for 20 min. Lysis supernatant was incubated with Ni²⁺-NTA affinity beads for 10 min at room temperature. The beads were washed with the 10x volume of lysis buffer three times. The proteins were eluted with the elution buffer containing 50 mM Tris, pH 8.2, 500 mM NaCl, 250 mM Imidazole. Eluted proteins were purified using size-exclusion column (Superdex 200) in buffer containing 20 mM HEPES, pH 7.4, 200 mM NaCl (for GlyR- β LD) or 500 mM NaCl (for GABA_AR- α LD), 1 mM EDTA, 2 mM DTT. After the cleavage of the affinity tag by HRV 3C protease (overnight at 4°C), protein samples were diluted to around 100 mM NaCl and loaded to the ResourceS column to separate the remaining contaminating proteins and the cleaved affinity tag.

Proteins eluted from ResourceS were exchanged to the final buffer containing 50 mM Tris, 100 mM NaCl, 5 mM DTT or 1 mM TCEP, pH 7.5 using desalting column. Final proteins were concentrated to around 200 μ M and stored as aliquots at -80°C.

Redox shift assay

Typically, 10 μ g protein were prepared in a total volume of 20 μ L RSA buffer (50 mM Tris/HCl, 5 mM EDTA, pH 7.5) or denaturing RSA buffer (50 mM Tris/HCl, 5 mM EDTA, 2% SDS pH 7.5) and supplemented with 1 mM mmPEG5kDa or buffer as minimum controls. After incubation for 1 h in the dark at rt, samples were used for SDS PAGE. The expected shift size per labeled thiol is about 10 kDa. In denaturing conditions, internal cysteines are exposed to PEGylation additionally.

***In vitro* redox-modification**

Purified proteins were oxidized by incubation over night at 4°C with 1 mM Diamide or H₂O₂. Reduction was performed with 1 mM TCEP or DTT. In case the reagent would disturb in further assays, the chemical was removed by gel filtration using PD10 columns.

Sedimentation assay of *in vitro* phase separation

Prior the assay proteins were pre-cleared at 17,000 $\times g$ for 10 min at 25 °C. For sedimentation assays, proteins were mixed at designated combinations and conditions in 50 μ L final volume in assay buffer (25 mM Tris pH 7.4, 150 mM NaCl). After 10 min incubation at 25°C, the mixtures were centrifuged at 17,000 $\times g$ for 10 min at 4 °C. Right after centrifugation, the supernatant and pellet were separated. The pellet was brought to the same volume as the supernatant. Proteins recovered in supernatant and pellet were analyzed by SDS-PAGE with Coomassie Blue staining. The band intensities in SDS-PAGE gels were quantified by Image Lab software. Relative and absolute amounts of proteins in supernatant and pellet were calculated based on the relative band intensities. Data of three repeats were presented as means $\pm$ SD.

ITC

Isothermal titration Calorimetry (ITC) experiments were performed by using MicroCal Auto-ITC200 (*Malvern*) at 25 °C in ITC buffer (300 mM NaCl, 50 mM Tris/HCl, pH 7.4). We used an injection volume of 1-2 μ L, a spacing of 150 s between injections and an

initial delay of 60 sec. The reference power was 5 μ Cal/sec and the stirring speed 1000 rpm. The concentration of GlyR β ICD in the syringe was about 300-310 μ M, and the concentration of gephyrin in the sample cell was 31 – 35 μ M.

Parameters and curves were fitted and calculated by the software MicroCal Analysis and Origin7.

Size exclusion chromatography

Molecular masses of purified gephyrin and variants were detected by analytical size exclusion chromatography (SEC) using a Superose 6 increase 5/10 (*GE Healthcare*). Typically, 4 nmol pre-cleared protein in 50 μ L total sample volume were applied. Proteins were separated at 4°C and a flow rate of 0.1 mL/min in SEC buffer (50 mM Tris/HCl, 300 mM NaCl pH 7.4). Selected fractions of 100 μ L were collected and those of corresponding peaks combined and concentrated using *Amicon* filters with a 50 kDa cut-off. The combined protein was then further subjected to redox-modifications, cleared by centrifugation and re-applied on the column.

Calpain cleavage assay

A mastermix of 50 μ L contained 850 pmol redox-modified protein, 10 mM $\text{Ca}^{2+}\text{Cl}_2$, 1/2 U/mL calpain. The volume was adjusted using calpain buffer (300 mM NaCl, 50 mM Tris/HCl, pH 7.4). As a control, a calcium-free control was included because calpain is a calcium-dependent protease. At timepoints of 0, 5, 15, 30 min and overnight, 15 μ L samples were taken out and the digestion stopped by addition of SDS sample buffer and incubation for 5 min at 96°C. Cleavage products were afterwards analyzed by SDS-PAGE.

Circular dichroism spectroscopy

Determination of the secondary structure was performed by circular dichroism spectroscopy (CD spec). Typically, 0.3 mg/mL pre-cleared protein in CD buffer (10 mM $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, pH 7.5) was used and Far-UV spectra recorded with J-715 CD spectropolarimeter (*Jasco*) at 20°C in the range of 180-250 nm using quartz cuvette with 0.1 cm path length. The spectra were processed by the software Spectra Analysis version 1.53.07 of Spectra Manage version 1.54.0. We subtracted the buffer baseline

and smoothed three-times by the Savitzky-Golay algorithm with a convolution width of 21.

Virus preparation

Recombinant virus particles (AAV2/1) were produced in HEK293 cells and isolated by PEG/NaCl precipitation followed by chloroform extraction according to the previously established protocol ⁵⁴. The titers were determined by a Gel green® (*Biotium*) protocol ⁵⁵ and the fluorescence was detected (507 ± 5 nm excitation and 528 ± 5 nm emission) using a plate reader equipped with monochromators (Tecan Spark).

Culture of hippocampal neurons and expression of recombinant gephyrin

At E17.5, dissociated primary hippocampal cultures were prepared from C57BL/6NRj embryos. 90,000 cells were seeded on Poly-L-lysine coated 13-mm cover slips in 24-well plates. Neurons grew in neurobasal medium supplemented with B-27, N-2, and L-glutamine (*Thermo Fisher Scientific*). At 8 days *in vitro* (DIV), cells were transfected with 0.4 µg plasmid DNA per construct using lipofectamine 2000 (Life Technologies) according to the manufacturer's manual. Expression was performed for 5 days. Alternatively, at DIV10, cells were transduced with 2.5 x 10⁸ viral genome copies (GC). The virus particles were directly added to each individual well. At DIV14 the cells were either fixed or subjected to treatment. D-Methionine (D-Met) treatment was performed 1 h at 37°C. The medium of neurons was removed and replaced by minimal NB medium, supplemented with 5 mM D-Met. Mock samples were treated with the same volume of solvent solution (PBS). After treatments neurons were fixed and used for ICC.

Immunocytochemistry

Fixation was performed by replacing culture medium by 4% PFA in PBS. After incubation for 20 min at room temperature (rt), PFA was removed and cells washed twice with 50 mM NH₄Cl in PBS, each time 10 min at rt. Subsequently, cells were blocked for 1 h at rt with blocking solution (10% goat serum, 1% BSA, 0.2% TritonX100, in PBS). After washing cells with PBS for 5 min at rt, antibody incubation was performed for 1 h at rt. Antibodies were diluted in PBS. Afterwards, three washing steps

with PBS were performed, each 10 min at rt. Lastly, coverslips were mounted with Mowiol/Dabco and dried over night at rt. We used the following antibodies: anti-vesicular GABA transporter (vGAT) (1:1000, #131003, SYSY); anti-GABA_AR γ 2 (1:500, #224004, SYSY); anti-GephyrinE (3B11; 1:10, self-made); goat anti-mouse AlexaFluor 488 (1:500, #A-11029, *Invitrogen*), goat anti-rabbit AlexaFluor 647 (1:500, #A-21245, *Invitrogen*), goat anti-rabbit AlexaFluor 488 (1:500, #A-11075, *Invitrogen*).

Confocal microscopy and image analysis

Pictures were taken using the *Leica* TCS SP8 LIGHTNING upright confocal microscope with an HC PL APO CS2 63x/1.30 glycerol objective. The microscope was equipped with hybrid *Leica* HyD detectors and diode lasers with 405, 488, 522 and 638 nm. The LIGHTNING adaptive deconvolution of the mounting medium “Mowiol” was used, which is capable of theoretical resolutions down to 120 nm and 200 nm lateral and axial, respectively. Images were acquired with stacks of 3 μ m z-step size, 2048 x 2048 (144.77 x 144.77 μ m) and segmented and analyzed in an automated fashion using ImageJ/FIJI 1.53t. Therefore, the previously described macros⁵⁶ were used as a blueprint and adapted.

Perfusion of mice and sample preparation

Mice were anesthetized with Ketamine/Xylazine through intra-peritoneal injection. After surgical tolerance was achieved, the abdomen was opened and liver and heart uncovered. A canula was pricked into the bottom of the left heart chamber to inject PBS containing 50 mM NEM into the cardiovascular system. A drain was given by a cut above the liver. PBS was injected until the liver lost the red color and turned pale. After decapitation, liver, brain and spinal cord were removed and added to RIPA buffer (50 mM Tris/HCl pH 8.0, 150 mM NaCl, 0.1% SDS, 1% IGEPAL CA-630, 0.5% Na deoxycholate, 1 mM EDTA, freshly added protease inhibitor (*Roche*)) containing 50 mM NEM. The tissue was dounce homogenized with 1,200 rpm 20 times, sonicated 10 s at 4°C and 30% amplitude and subsequently centrifuged for 10 min at 4°C and 4,000 x g. The supernatant was transferred into a new tube and incubated for 1 h to let free thiols react with NEM. Afterwards, protein concentration was determined using BCA. Typically, 45 μ g protein were used for SDS-PAGE for WB analysis.

SDS-PAGE, Coomassie and Western Blot

Samples were supplemented with 1x sample buffer (5x: 250 mM Tris/HCl pH 6.8, 30% glycerol, 0.1% Bromophenol blue, 10% SDS) and incubated for 5 min at 95-98°C. We used either 5 mM β -ME for recombinant protein samples or 5 mM DTT for tissue samples or omitted it. Separation was performed with 5-12% SDS acrylamide gels using running buffer (3.029 g/L Tris, 14.4 g/L glycine, 0.1% SDS). Afterwards gels were either stained with coomassie (30% EtOH, 10% acetic acid, 0.25% Coomassie brilliant blue R250) or used for western blot. Therefore, the transfer was performed semi-dry using PVDF membranes and transfer buffer (3 g/L Tris, 24.15 g/L glycine pH 8.8, 10% MeOH). Blocking was performed for 1 h at rt under shaking in blocking solution (10% BSA in TBST). Primary and secondary antibodies (diluted in 1% BSA in TBST) were applied for 1 h at rt under shaking, with a wash step of 3 x 5 min with TBST (6.5 g/L Tris, 8.75 g/L NaCl pH 7.4, 0.05% Tween20) between primary and secondary antibody. After additional washing 3 x 5 min with TBST, detection was performed using ECL and the ChemiDoc™ Imaging System (*BioRad*). The following antibodies were used: anti-GephyrinE (3B11; 1:10, self-made); anti-GAPDH (1:1,000, G9545, *Sigma*), anti-mouse HRP-coupled (1:10,000, AP181P, *Sigma*), anti-rabbit HRP-coupled (1:10,000, AP187P, *Sigma*)

Electrophysiology

Miniature inhibitory postsynaptic currents (mIPSCs) were recorded using whole-cell patch clamp recordings in primary cultures of murine hippocampal neurons with various genotypes and subjected to different treatment conditions. Recordings were performed at 30°C between days 13 and 15 in vitro (DIV). Experimental procedures essentially followed protocols previously described in Liebsch et al. (2022)⁵⁶. Data were sampled at 20 kHz and low-pass filtered at 10 kHz with a four-pole Bessel filter. Offline, the recordings were smoothed by averaging the values in a ± 0.001 s interval (20 data points) around any given datapoint. The carbogenated (95% O₂ and 5% CO₂) extracellular saline contained (in mM): 125 NaCl, 21 NaHCO₃, 2.5 KCl, 2 MgCl₂, 1.2 NaH₂PO₄, 10 HEPES, 5 glucose, adjusted to pH 7.2 with NaOH. Glutamatergic input was blocked with 5×10^{-5} M DL-2-amino-5-phosphonopentanoic acid (DL-AP5; BN0086, Biotrend Chemikalien GmbH) and 10^{-5} M 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; C127, Sigma-Aldrich). Na⁺ mediated action potentials were blocked by 10^{-7} M tetrodotoxin (TTX; T-550, Alomone Labs). In the wt DAAO+D-MET group 5 mM

D- methionine (M9375, Sigma-Aldrich) was bath applied at least 1 h prior to the start of the recording. The electrode solution contained (in mM): 133 CsCl, 2 MgCl₂, 1 CaCl₂, 10 HEPES, 10 EGTA, adjusted with CsOH to pH 7.2. The liquid junction potential between intracellular and extracellular solution (6.3 mV) were calculated using the LJPcalc software (<https://swharden.com/LJPcalc>; arXiv:1403.3640v2; <https://doi.org/10.48550/arXiv.1403.3640>) and compensated. The holding potential was -70 mV. Because of the high intracellular chloride concentration, GABA_A receptor-mediated Cl⁻ currents were detected as inward currents. mIPSCs were analyzed offline from 50 s intervals taken after the recording had stabilized (~10 min after establishing the whole-cell configuration), using the Easy Electrophysiology analysis software (v2.6.4rcz, Joseph J Ziminski, www.easyelectrophysiology.com). Events were detected by crossing a threshold that was manually set and adjusted depending on the noise level of the signal. After the events were classified as mIPSCs by template-matching (correlation cutoff: 0.80; decay search period: 50ms; baseline search period: 10 ms), parameters including frequency, amplitude, rise and decay were determined. The recording-specific template was calculated by fitting the average of prior threshold-detected and manually reviewed events. This semi-automated detection procedure was verified by visual inspection.

Statistics and computation

The visualization and statistical analysis were performed with GraphPad Prism6 and R vers. 4.2.1. Standard deviation, individual data points and used tests are shown in the individual graph and in the captions respectively. Data points were identified as outliers when exceeding two-fold the standard deviation. If data was normalized, *student's* t-test was performed, and the significance level alpha corrected by the number of tests. Otherwise, we performed 1-way ANOVA and *Bonferroni* post-hoc tests. We analyzed: *N* = 6 individual mice for redox-dependent multimers; *N* = 3 technical replicates in the Moco activity assay; *N* = 3 technical replicates in SEC experiments; *N* = 3 technical replicates of gephyrin in ITC experiments; *N* = 3 technical replicates of gephyrin in co-sedimentation assays, *N* = 3 technical replicates of gephyrin in calpain cleavage assays; *N* = 10 cells per condition from 3 individual embryos of one neuron preparation in all experiments using hippocampal neurons. For Electrophysiology experiments, numerical data are presented as mean ± standard error of the mean (SEM). For pairwise comparisons of independent samples, Student's

t-test was applied for data with parametric distribution, while the Mann-Whitney *U* test was used for non-parametric data. Normality of data distribution was assessed using the Anderson–Darling test. For comparisons of more than two groups, Kruskal–Wallis tests were conducted followed by Dunn’s post hoc multiple comparisons tests. In boxplots, horizontal lines represent the median, + symbols denote the mean, boxes indicate the interquartile range (25th to 75th percentile), and whiskers extend to the minimum and maximum values. Exact *p*-values and sample sizes (*n*) are reported in the figures. Data analysis was performed using Spike2 and Prism 8 (GraphPad Software Inc.). Perplexity and ChatGPT were used for language style improvement. Final Figures were created with Adobe Illustrator (Adobe Inc.) and BioRender.

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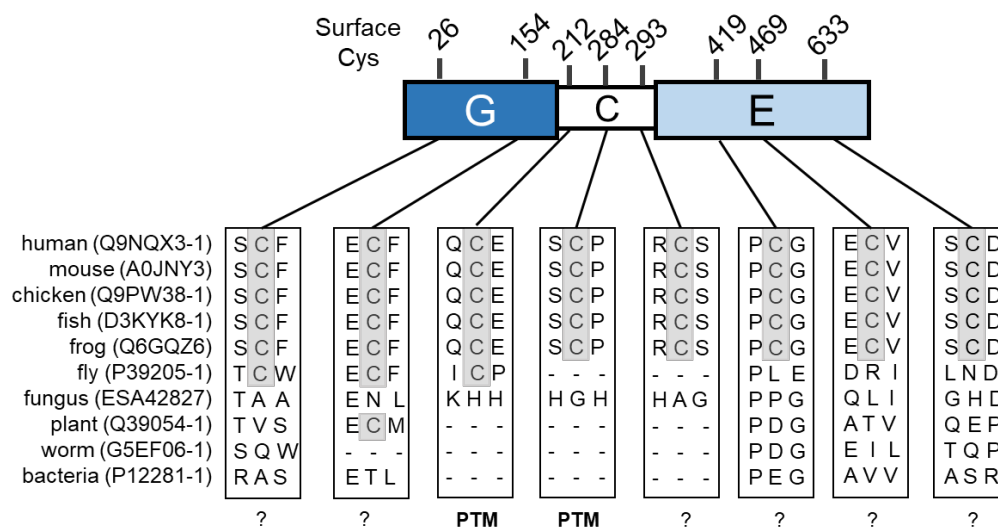
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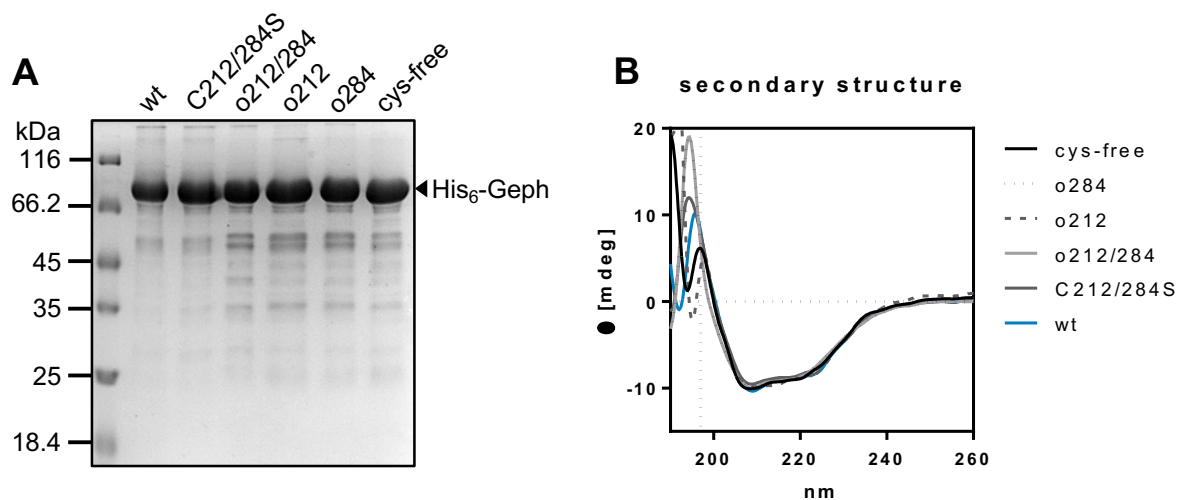
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3.8 Supplementary figures

**Figure S1: Alignment of gephyrin and homologues.**

Alignment of protein sequence of different species. Areas around the chosen cysteines of gephyrin depicted with boxes. Cys 212 and 284 have assigned functions (targets of palmitoylation), while the function of the others is unknown (?).

**Figure S2: His₆-Gephyrin derived from *E. coli*.**

His₆-Gephyrin variants recombinantly expressed and purified from *E. coli*. **(A)** Coomassie gel of purified proteins. **(B)** Analysis of secondary structure by CD Spec. Vertical dotted line indicates data points with HT values above 800 mV (machine threshold).

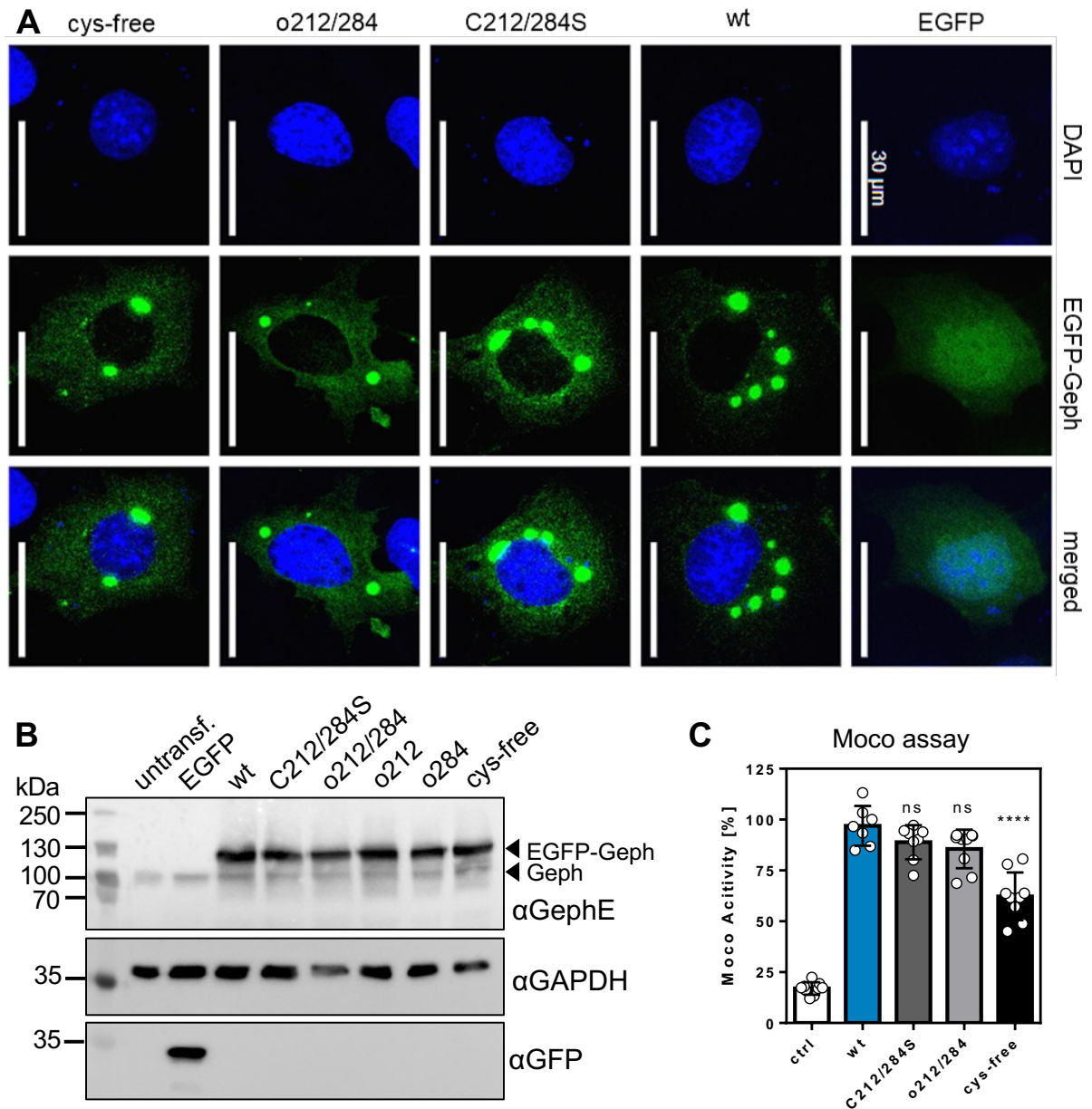


Figure S3: Expression of EGFP-Geph in COS7 and HEK293 cells.

EGFP-Gephyrin variants were recombinantly expressed in COS7 and HEK293 cells for 48 h. **(A)** Images of COS7 cells. Blue: DAPI, green: EGFP-Geph, scale bar of 30 μ m. **(B)** Expression in HEK293 cells. Protein amounts analyzed by Western blot. EGFP-Geph and endogenous gephyrin marked with arrows. **(C)** Moco assay with cysteine variants. Moco activity normalized to wt. Significance towards wt tested by student's t-test, alpha level corrected by three. C212/284S: $p=0.3288$ (ns); o212/284: $p=0.1026$ (ns); cys-free: $p<0.0001$ (****).

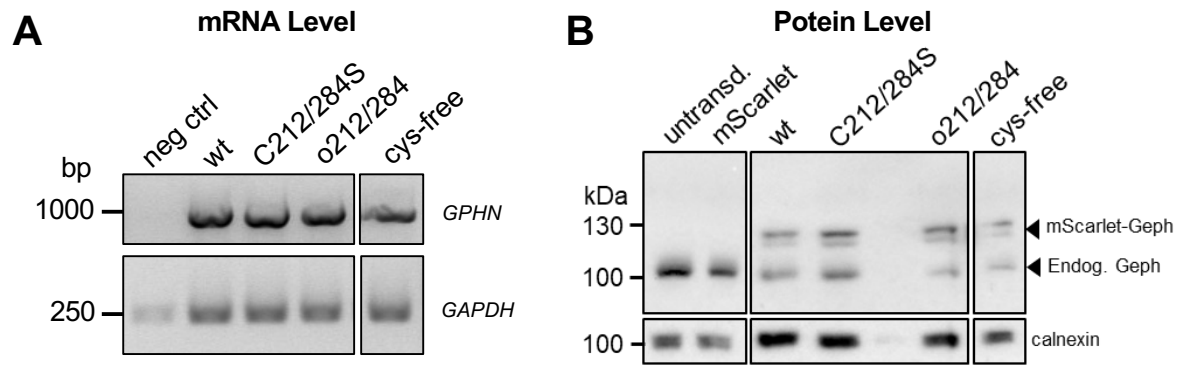


Figure S4: Expression of recombinant gephyrin in hippocampal neurons.

Expression of mScarlet-gephyrin via transduction in primary hippocampal neurons. **(A)** mRNA level determination. Isolated mRNA of mSclt-Geph was used for PCR. Primers were designed to exclusively bind to recombinant gephyrin. GAPDH used as a ctrl. **(B)** Western Blot of neuronal lysates. Endogenous and recombinant gephyrin species indicated by arrows.

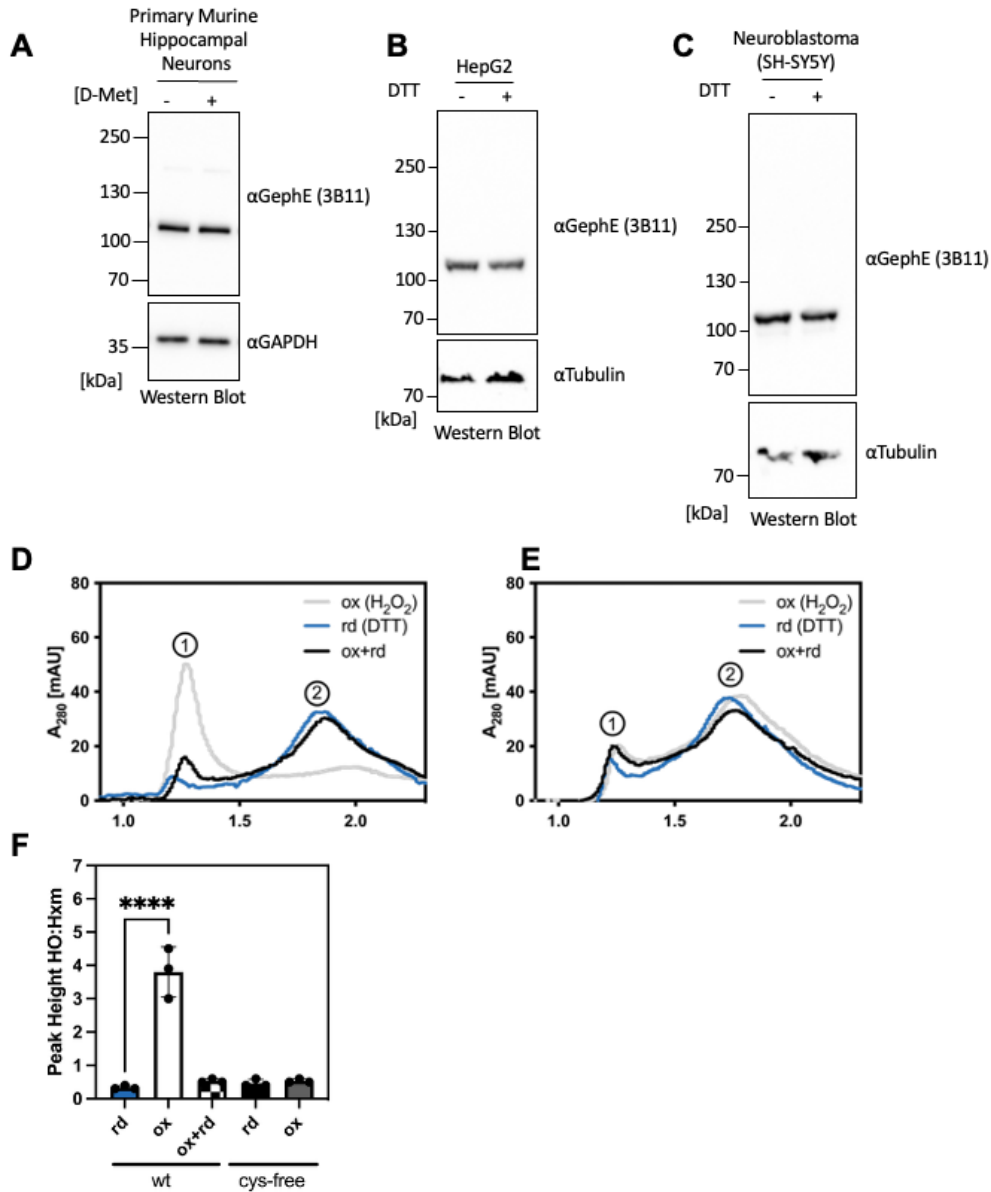


Figure S5: Western blot analysis of gephyrin in cultured cells and analysis of SEC fractions and ITC Data.

(A–C): Western blot analysis of endogenous gephyrin detected with 3B11 antibody does not show formation of covalent higher oligomers in cultured cells. (A) Primary hippocampal neurons were infected with AAV2/1 DAAO and treated with 5 mM D-Met (+) or not (-) to produce intracellular H₂O₂ like in Fig. S8. GAPDH as loading control. (B, C) HepG2 or neuroblastoma (SH-SY5Y) cells were treated with 150 μM H₂O₂ and after lysis treated with DTT (+) or not (-). Tubulin as loading control. (D, E): Size exclusion chromatography (SEC) with H₂O₂-oxidized (1 mM H₂O₂, 4°C, for 18 h) or reduced (1 mM DTT, 4°C, for 18 h) gephyrin wildtype (wt) (D) or cysteine-free (cys-free) variant (E). Chromatograms with peaks of high-order multimers (1) or hexameric gephyrin (2). Re-reduced runs in black (1 mM H₂O₂, 4°C, for 18 h and subsequently 5 mM DTT, 22 °C, 1 h). (F) Quantification of the ratio of the peak heights of three different runs. Significance tested by 1way ANOVA p<0.0001, Bonferroni post-hoc test: p<0.0001 (****); p>0.05 (ns).

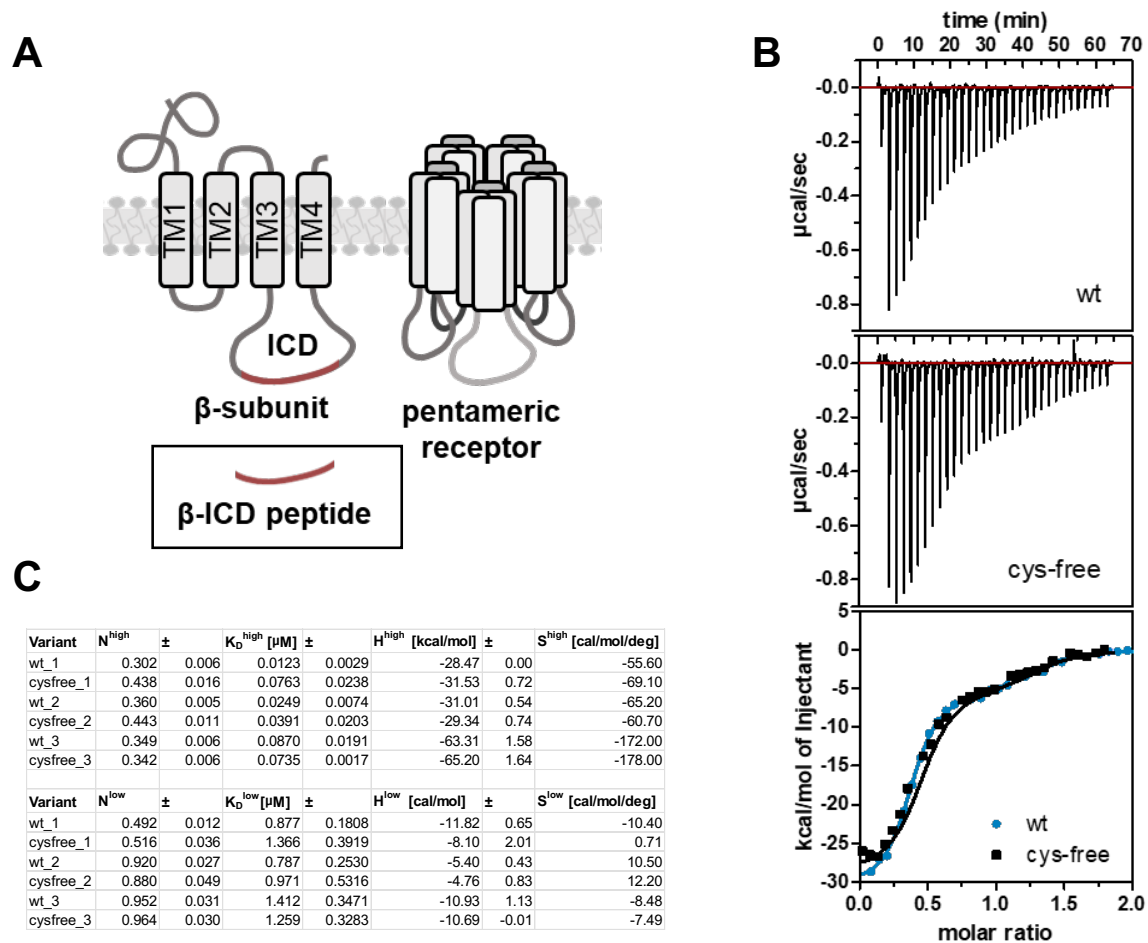


Figure S6: Analysis of ITC Data.

(A) Cartoon depicting glycine receptor β -ICD (GlyR β ICD peptide) peptide, which was used for ITC measurements. (B, C): Interaction of gephyrin wt or cys-free variant towards GlyR β ICD, parameters determined by mathematical fitting (two-site fit).

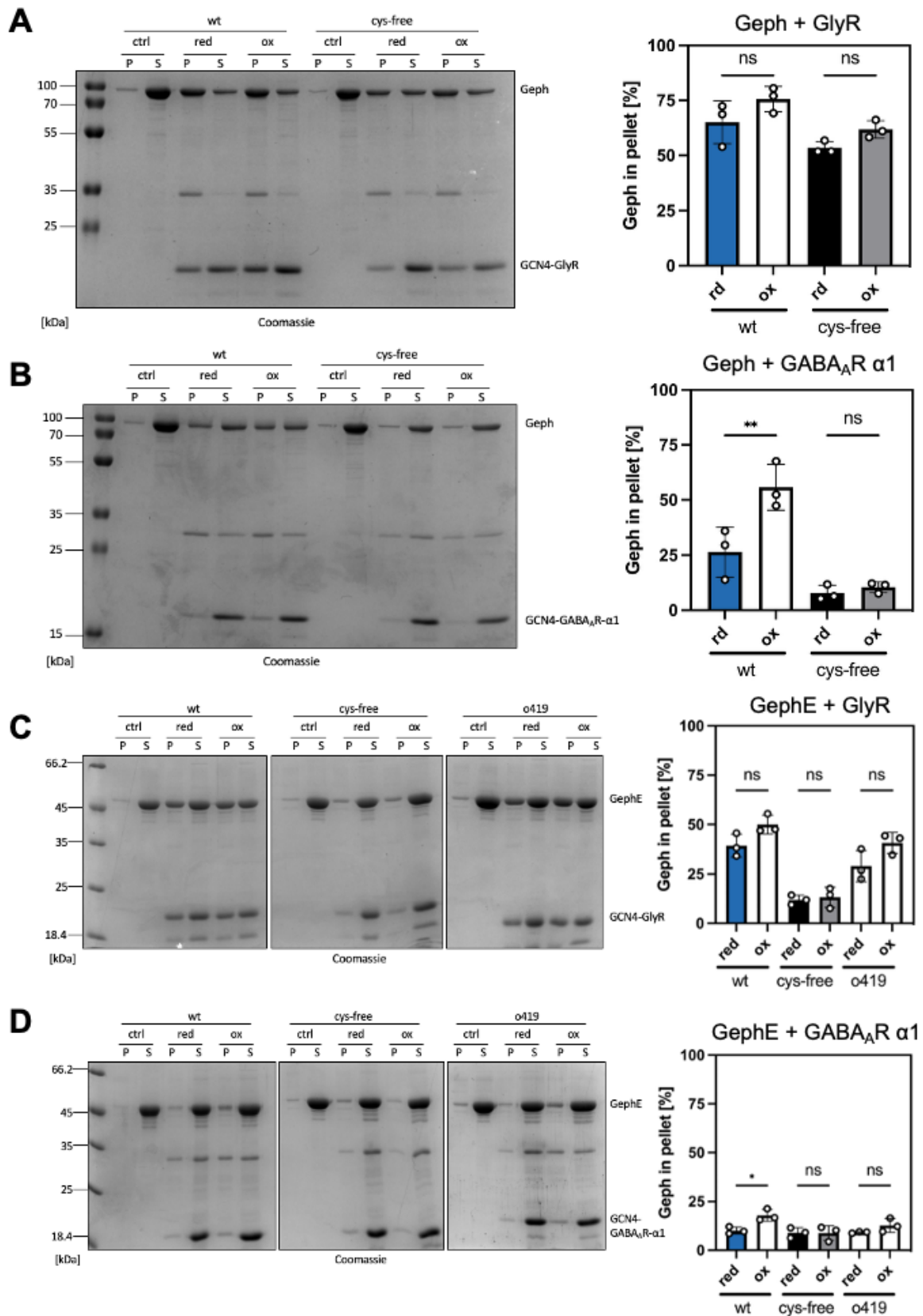


Figure S7: Liquid-liquid phase separation.

Figure S7: Liquid-liquid phase separation.

Oxidized (ox) or reduced (rd) gephyrin full length (Geph) or E-domain (GephE) wild type (wt), cysteine-free (cys-free) or only with Cys419 (oC419) were used for sedimentation. n = 3 independent experiments, mean and standard deviation are displayed. **(A)** Sedimentation assay using Geph + GCN4-GlyR, **(B)** Geph + GCN4-GABA_AR α1, **(C)** GephE + GCN4-GlyR or **(D)** GephE + GCN4-GABA_AR α1. Condensed proteins are recovered from the pellet (P) and dilute proteins from the supernatant (S) fractions. As control oxidized gephyrin wt and cysteine-free alone were used. Quantification of gephyrin band intensities. Relative abundance (%) normalized to sum of protein in pellet and supernatant of the corresponding fractions. Significance tested for: **(A)** by 2way ANOVA F5,10=87.63, p<0.0001. Tukey's multiple comparisons test: wt: p=0.3045>0.05 (ns); cys-free: p=0.5262>0.05 (ns); **(B)** by 2way ANOVA F5,10=23.65, p<0.0001. Tukey's multiple comparisons test: wt: p=0.0053 (**); cys-free: p=0.9970>0.05 (ns); **(C)** by 2way ANOVA F8,16=48.99, p<0.0001. Tukey's multiple comparisons test: wt: p=0.1085>0.05 (ns); cys-free: p=0.1260>0.05 (ns); oC419: p=0.0624>0.05 (ns); **(D)** by 2way ANOVA F8,16=6.121, p=0.0011. Tukey's multiple comparisons test: wt: p=0.0376 (*); cys-free: p=0.9245>0.05 (ns); oC419: p=0.7267>0.05 (ns)

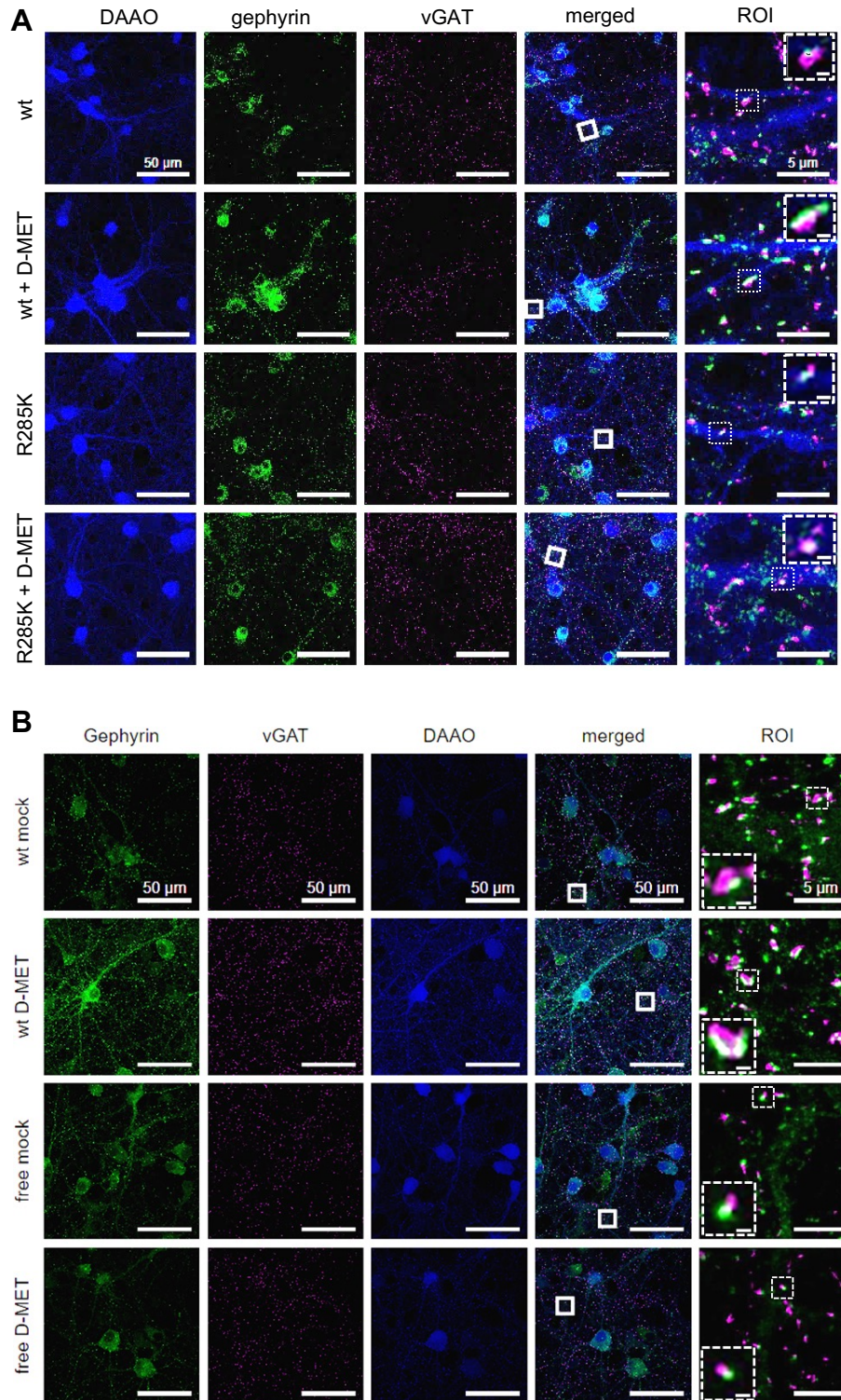


Figure S8: H₂O₂ drives gephyrin oligomerization at synapses.

Expression of cytosolic mSclt-DAAO and mEGFP-Geph in primary, hippocampal neurons. Treatment at DIV14 with 5 mM D-MET. Analysis of cluster sizes [μm^2], intensity [au] and co-localization with vGAT (=synaptic clusters) of gephyrin. **(A)** mSclt-DAAO wt and R285K. Analysis of endogenous gephyrin clusters. Gephyrin: green, vGAT: magenta, DAAO: blue; scale bar 30 and 0.5 μm . **(B)** mSclt-DAAO wt and mEGFP-Geph wt and cys-free variants. Analysis of recombinant mEGFP-gephyrin clusters. Gephyrin: green, vGAT: magenta; scale bar 30 and 0.5 μm .

Chapter 3

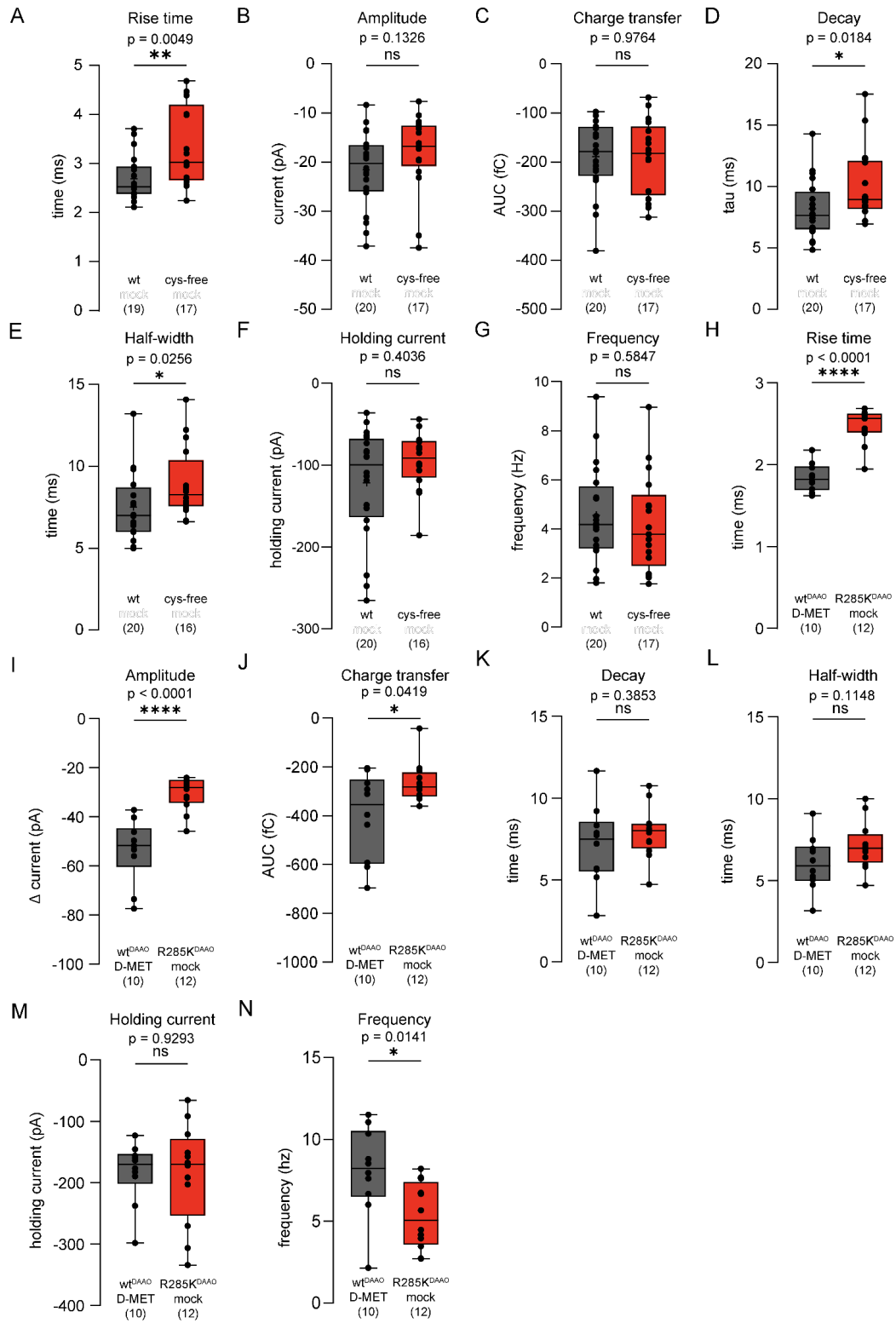


Figure S9: Supplementary data for Figure 6.

Figure S9: Supplementary data for Figure 6.

Box plots showing basic parameters of miniature inhibitory postsynaptic potentials (mIPSCs) recorded in primary cultures of murine hippocampal neurons. Each data point represents the mean of the mIPSCs recorded during a 50 s interval from a single neuron (260 ± 17 individual mIPSCs per neuron). All recordings were performed at 13–15 DIV. Glutamatergic input was blocked by CNQX and DL-AP5. Na⁺-mediated action potentials were blocked by TTX. Holding potential was -70 mV. In boxplots, horizontal lines represent the median, + symbols denote the mean, boxes indicate the interquartile range (25th to 75th percentile), and whiskers extend to the minimum and maximum values. Probability values (p) are reported as exact values, with sample sizes (n) provided in brackets. Student's t-test: t-test. Mann-Whitney U test: U test. **(A-G)** wt mock and cys-free mock. **(H-N)** wt DAAO+D-MET and R285K mock. **(A, H)** Rise time. **(B, I)** Amplitude. **(C, J)** Charge transfer. **(D, N)** Decay time constant. **(E, K)** Half-width. **(F, M)** Holding current. **(G, N)** Frequency. Wt Mock vs Cys-free mock: **(A)** Rise time: wt mock: $2,7 \pm 0,1$ ms; cys-free mock: $3,3 \pm 0,2$ ms. U test. **(B)** Amplitude: wt mock: -22 ± 2 pA; cys-free mock: -18 ± 2 pA. U test. **(C)** Charge transfer: wt mock: -189 ± 17 fC; cys-free mock: -188 ± 18 fC. t-test. **(D)** Decay time constant: wt mock: 8 ± 1 ms; cys-free mock: 10 ± 1 ms. U test. **(E)** Half-width: wt mock: $7,5 \pm 0,5$ ms; cys-free mock: $9 \pm 0,5$ ms. U test. **(F)** Holding current: wt mock: -122 ± 15 pA; cys-free mock: -95 ± 9 pA. U test. **(G)** Frequency: wt mock: $4,5 \pm 0,4$ Hz; cys-free mock: $4,2 \pm 0,5$ Hz. t-test. wt DAAO+D MET vs R285K: **(H)** Rise time: wt DAAO+D-MET: $1,8 \pm 0,1$ ms; R285K: $2,5 \pm 0,1$ ms. U test. **(I)** Amplitude: wt DAAO+D-MET: -54 ± 4 pA; R285K mock: -30 ± 2 pA. t-test. **(J)** Charge transfer: wt DAAO+D-MET: -402 ± 56 fC; R285K mock: -264 ± 24 fC. t-test. **(K)** Decay time constant: wt DAAO+D-MET: 7 ± 1 ms; R285K mock: $8 \pm 0,5$ ms. t-test. **(L)** Half-width: wt DAAO+D-MET: $6 \pm 0,5$ ms; R285K mock: $7 \pm 0,5$ ms. t-test. **(M)** Holding current: wt DAAO+D-MET: -183 ± 16 pA; R285K mock: -186 ± 24 pA. t-test. **(N)** Frequency: wt DAAO+D-MET: 8 ± 1 Hz; R285K mock: $5 \pm 0,6$ Hz. t-test.

Chapter 4: Gephyrin filaments represent the molecular basis of inhibitory postsynaptic densities

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4.1 Author contributions

Arthur Macha, Filip Liebsch, Emanuel E. W. Bruckisch, Nele Burdina, Elmar Behrmann and Günter Schwarz designed the study. Arthur Macha, Filip Liebsch, Emanuel E. W. Bruckisch, Nele Burdina, Imke von Stülpnagel, Konrad Benting, Monika Gunkel, Elmar Behrmann and Günter Schwarz wrote and corrected the manuscript. Emanuel H. W. Bruckisch performed protein purifications and *in vitro* phase separation experiments. Arthur Macha, Nele Burdina, Konrad Benting and Monika Gunkel purified protein, performed SEC for cryo-EM, acquired and analyzed cryo-EM data. Arthur Macha performed structural model building. Arthur Macha and Imke von Stülpnagel performed ITC experiments. Filip Liebsch performed murine hippocampal neuron culture experiments.

4.2 Abstract

The multifunctional protein gephyrin clusters inhibitory receptors at the postsynaptic membrane in the CNS. Gephyrin has been proposed to form the inhibitory postsynaptic density by liquid-liquid phase separation, involving a complex interplay between receptor binding and oligomerization via its conserved G- and E-domains. Here we show by single particle cryo-EM analysis that dimerization promotes the formation of gephyrin filaments in which two E-domain dimers are linked by Z-shaped interfaces formed between two subdomains II (SDII) of adjacent dimers. Deletion of SDII, introduction of two epilepsy-causing pathogenic variants, or neutralization of an opposing charge in the interface abolish the formation of filaments, *in vitro* phase separation, and synaptic receptor clustering in hippocampal neurons. In conclusion, this work identifies gephyrin E-domain filaments as the structural foundation underlying gephyrin both phase separation and receptor clustering at inhibitory postsynaptic densities.

4.3 Published manuscript with introduction, results, discussion, material and methods and references

The fully published version of this chapter is included below in its original *nature communications* format, comprising the continuous sections Introduction, Results, Discussion, Materials and Methods, and References.



Gephyrin filaments represent the molecular basis of inhibitory postsynaptic densities

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The multifunctional protein gephyrin clusters inhibitory receptors at the postsynaptic membrane in the CNS. Gephyrin has been proposed to form the inhibitory postsynaptic density by liquid-liquid phase separation, involving a complex interplay between receptor binding and oligomerization via its conserved G- and E-domains. Here we show by single particle cryo-EM analysis that dimerization promotes the formation of gephyrin filaments in which two E-domain dimers are linked by Z-shaped interfaces formed between two subdomains II (SDII) of adjacent dimers. Deletion of SDII, introduction of two epilepsy-causing pathogenic variants, or neutralization of an opposing charge in the interface abolish the formation of filaments, *in vitro* phase separation, and synaptic receptor clustering in hippocampal neurons. In conclusion, this work identifies gephyrin E-domain filaments as the structural foundation underlying gephyrin both phase separation and receptor clustering at inhibitory postsynaptic densities.

Gephyrin (Geph) is the major scaffold protein at inhibitory synapses in the central nervous system (CNS)^{1–3}. It directly interacts with the intracellular domains (ICDs) of the β -subunit of glycine receptors (GlyR) as well as with various subunits of γ -aminobutyric acid type A receptors (GABA_ARs)^{4–8}. These interactions facilitate the incorporation of inhibitory receptors into the postsynaptic density (PSD), which involves many other interacting proteins such as tubulin, collybistin and neuroligin^{9–13}. The presence of a sufficient number of inhibitory receptors at the synaptic site is essential for efficient inhibitory signal transmission between neurons^{14,15}.

Gephyrin was initially identified as a 93 kDa protein associated with GlyR^{16–18}. It is comprised of an N-terminal G-domain (GephG), an E-domain (GephE), and a flexible central linker region, referred to as the C-domain (GephC) (Fig. 1a)^{4,19,20}. Structural analyses showed that isolated GephG forms trimers^{19,21}, while GephE forms dimers^{4,20,22}. Biochemical studies suggest that full-length Geph is a trimer, mediated by the central GephG trimer^{9,16,20}. The interaction with the ICDs of inhibitory receptors occurs at the dimerized GephE, in which the two GephE subunits form two C-shaped hydrophobic binding pockets^{23,24}. *In vitro* interaction studies using gephyrin trimer and a peptide derived

from the ICD of the GlyR- β subunit (GlyR-loop) revealed biphasic binding kinetics, characterized by distinct high-affinity and low-affinity interactions with dissociation constants (K_D) in the low nanomolar to low micromolar range^{4,20}. The ICDs of GABA_AR α 1, GABA_AR α 3, GABA_AR β 2, and GABA_AR β 3 subunits bind to the same binding pocket as the GlyR-loop, but with approximately 20–50 times lower affinity^{5,7}.

Based on the distinct oligomerization properties of the G- and E-domains of gephyrin, a hexagonal lattice has been proposed as the structural basis for the inhibitory postsynaptic density^{22,25}. While such an ordered lattice has neither been observed *in vitro* nor *in vivo*, a complex network of receptors at the synaptic cleft has been identified by cryo-electron tomography that appears to be supported by a thin electron-dense layer that would agree with a network of Geph²⁶. Furthermore, structural and functional data revealed that localized variations in protein density of both receptors and scaffolding proteins can lead to the formation of highly stable nanodomains within the PSD^{27–30}. On a conceptual level, liquid-liquid phase separation (LLPS) has been proposed as an underlying principle of gephyrin clustering and inhibitory synapse formation and linked to the unstructured and charged GephC domain based on *in vitro* assays^{31,32}. However, the

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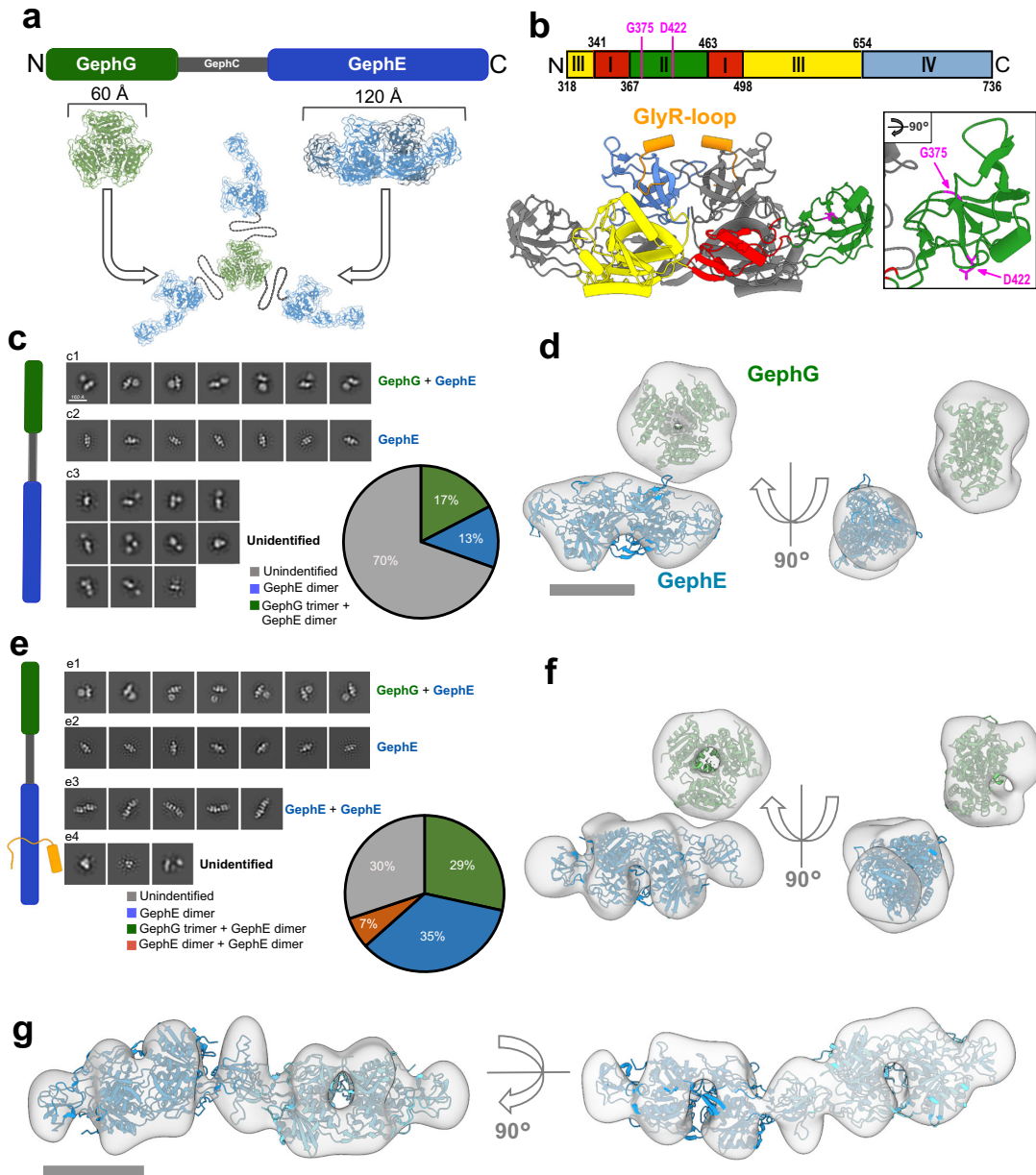


Fig. 1 | GlyR-loop stabilizes GephE dimerization within gephyrin trimer.
a Gephyrin trimer domain structure overview showing G-domain (GephG), C-domain (GephC) and E-domain (GephE). The respective crystal structures of GephG (green, PDB: 1JLJ) and GephE (blue, PDB 2FU3) are shown in cartoon representation with faded surfaces and size indicators. A graphical representation of the full-length gephyrin trimer is depicted (middle panel). **b** Cartoon representation of isolated GephE dimer in complex with GlyR-loop (PDB: 2FTS), highlighting subdomain (SD) division (color code: SDI: red; SDII: green; SDIII: yellow; SDIV: blue) and position of GlyR-loop (orange). The second monomer is shown in gray. Positions of G375 and D422 in SDII are highlighted (magenta). **c** Representative 2D class averages of isolated Geph trimer following negative stain SPA showing GephE with GephG (c1), isolated GephE (c2) and undefinable densities (c3). The pie chart indicates the relative distribution of particles from classes with GephE + GephG (c1), GephE dimer (PDB 2FU3, blue) and GephG trimer (PDB: 1JLJ, green) shown in cartoon representation were rigid-body fitted into the respective densities in the map. Scale bar: 50 Å. **d** Surface representation of final map using particles from classes with GephE + GephG (c1), GephE dimer (PDB 2FU3, blue) and GephG trimer (PDB: 1JLJ, green) shown in cartoon representation were rigid-body fitted into the respective densities in the map. Scale bar 50 Å. **e** Representative 2D class averages of isolated Geph trimer in complex with GlyR-loop after negative stain SPA showing GephE with GephG (e1), isolated GephE (e2), complex of two GephE dimers (e3) and undefinable densities (e4). The pie chart indicates the relative distribution of particles from classes with GephE + GephG (e1), GephE dimer (PDB 2FU3, blue) and GephG trimer (PDB: 1JLJ, green) shown in cartoon representation were rigid-body fitted into the respective densities in the map. Scale bar 50 Å. **f** Surface representation of final map using particles from classes with GephE + GephG (e1). GephE dimer (PDB 2FU3, blue) and GephG trimer (PDB: 1JLJ, green) shown in cartoon representation were rigid-body fitted into the respective densities in the map. Scale bar 50 Å. **g** Surface representation of final map using particles from classes with GephE + GephG (e1). GephE dimer (PDB 2FU3, blue) and GephG trimer (PDB: 1JLJ, green) shown in cartoon representation were rigid-body fitted into the respective densities in the map. Scale bar 50 Å. **h** Surface representation of final map using particles from classes with GephE + GephG (e1). GephE dimer (PDB 2FU3, blue) and GephG trimer (PDB: 1JLJ, green) shown in cartoon representation were rigid-body fitted into the respective densities in the map. Scale bar 50 Å. **i** Surface representation of final map using particles from classes with GephE + GephG (e1). GephE dimer (PDB 2FU3, blue) and GephG trimer (PDB: 1JLJ, green) shown in cartoon representation were rigid-body fitted into the respective densities in the map. Scale bar 50 Å.

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molecular mechanism that enables Geph to self-assemble at the PSD remained elusive for nearly three decades, and neither the hexagonal lattice nor the LLPS hypothesis have been able to fully explain known pathogenic variants of Geph, as highlighted by Geph^{G375D} and Geph^{D422N}^{14,33}, which harbor mutations outside the known receptor and oligomerization sites (Fig. 1b). In addition to its function in the CNS and spinal cord, Geph also harbors an enzymatic function in molybdenum cofactor biosynthesis^{34–36}.

Here we elucidated a hitherto unknown molecular interface formed between subregions of GephE, which not only rationalizes two known pathogenic patient variants carrying point mutations in this subregion, but also allowed the rational design of a third variant again disrupting Geph oligomerization. Our negative stain EM data, in combination with functional data, revealed that the presence of the GlyR-loop stabilized intramolecular GephE dimerization within full-length Geph. Subsequent cryo-EM data of the Geph-GlyR-loop complex uncovered that Geph is able to form flexible filaments, in which dimerized GephE domains are connected via their subdomain II (SDII). Geph variants either lacking SDII or carrying known pathogenic amino acid exchanges consistently lose filament-forming capability, are unable to engage in LLPS *in vitro*, and are deficient in synapse formation *in vivo*. Moreover, the structural data allowed predicting a complementary third variant showing the same kind of loss in filament formation and phase separation. Collectively, our structural and functional studies highlight Geph filament formation as an underlying molecular mechanism of Geph-mediated postsynaptic clustering of inhibitory neurotransmitter receptors.

Results

GlyR-loop stabilizes GephE dimerization within full-length Geph

Given that the full-length structure of gephyrin remains unknown, we carried out single-particle analyses (SPA) of isolated wild-type gephyrin trimers (Geph^{WT}). For this, Geph^{WT} was overexpressed in *E. coli* and purified to homogeneity using affinity chromatography combined with analytical scale size-exclusion chromatography (aSEC) (Supplementary Fig. 1a), and then prepared for negative stain EM. Micrographs showed particles of varying sizes (Supplementary Fig. 2a) with a high degree of heterogeneity; however, common architectural principles could be identified after 2D classification (Fig. 1a, c). A subset of class averages, accounting for about 17% of particles, showed an elongated bilobed structure adjacent to a round structure. Based on the molecular dimensions of the GephG (PDB 1JLJ) and GephE (PDB 1JLJ) crystal structures, these shapes likely represent a GephG trimer and GephE dimer, respectively (Fig. 1c1). A second subset of class averages, accounting for about 13% of particles, showed a single elongated bilobed structure, likely representing a GephE dimer without a resolved GephG trimer (Fig. 1c2). A third subset of class averages showed structures of varying shapes and sizes that could not be confidently assigned to any gephyrin domain (Fig. 1c3). Approximately 70% of all particles fell into this heterogeneous group of class averages, suggesting that the majority of gephyrin trimers adopted heterogeneous conformations, consistent with structural flexibility observed in previous SAXS and AFM studies²⁰. The particle subset showing putative GephG and GephE densities in close proximity (Fig. 1c1, green) was further processed in 3D (Supplementary Fig. 2b), yielding an intermediate-resolution negative stain EM density map that features two distinct, disconnected densities that were confirmed as GephE and GephG by rigid-body fitting known crystal structures (PDB 2FU3 and PDB 1JLJ) indicating the presence of stable GephE dimers and GephG trimers in our sample (Fig. 1d and Supplementary Fig. 2c). Neither density corresponding to the third copy of GephE nor density that might correspond to GephC could be identified.

We next investigated if the addition of GlyR-loop, as a mimic for receptor ICD binding, affects the architecture of full-length Geph^{WT}. For this we added a 3-fold excess of GlyR-loop to Geph^{WT} and

subsequently isolated the complex by aSEC before staining for negative stain EM (Supplementary Figs. 1a and 3). Micrographs appeared similar to those obtained for the sample without the GlyR-loop (apo-Geph), showing particles of varying sizes (Supplementary Fig. 3a), which, after processing resulted in visually similar 2D class averages as before (Fig. 1e). However, in contrast to apo-Geph, the relative number of particles contributing to the heterogeneous group of class averages was decreased more than two-fold (Fig. 1e1–4), implying that the presence of the GlyR-loop reduced heterogeneity by stabilizing Geph in defined conformations. Again, the particle subset showing GephG and GephE in close proximity (Fig. 1e1) was further processed in 3D, revealing a structure composed of two distinct densities that was similar to the one obtained for apo-Geph (Fig. 1f and Supplementary Fig. 3b). However, while nominal at the same resolution and only 50% more particles contributed to the reconstruction, the structure was much better defined, particularly in the GephE region (Fig. 1d, f). Still, stain granularity prevented the direct observation of GlyR-loop binding.

Interestingly, 2D classification revealed an additional subset of 2D class averages that appeared to comprise linear assemblies of two or more GephE dimers (Fig. 1e3). We also processed this particle subset showing linear assemblies (Fig. 1e3) in 3D, resulting in an elongated negative stain EM density that allowed rigid-body fitting of two GephE dimers, which appeared to be linked via their respective SDIIs (Fig. 1g and Supplementary Fig. 3c).

In conclusion, our SPA analysis of full-length Geph revealed high structural heterogeneity, which was decreased following the incubation with GlyR-loop. The presence of GlyR-loop, moreover, triggered the formation of linear assemblies that appear to comprise two or more directly interacting GephE dimers.

Full-length Geph forms flexible filaments at high protein concentrations

To uncover the molecular basis for the linear assemblies of Geph observed in negative stain EM, we investigated full-length Geph^{WT} trimers complexed with the GlyR-loop by cryo-EM. Under identical sample preparation conditions as used for negative stain EM, except at a 100-fold increased protein concentration, we obtained micrographs that were densely packed with particles (Supplementary Fig. 4a). Subsequent unsupervised 2D classification resulted in a set of high-quality 2D class averages featuring distinct secondary structure elements (Fig. 2a). While some 2D class averages were centered on the GephE dimer, others were centered on a Z-shaped structural feature connecting two GephE dimers (Fig. 2a and Supplementary Fig. 4b). 2D class averages centered on the GephE dimer showed weak adjacent density, indicating that these GephE dimers are likely incorporated into larger linear assemblies, or filaments, in agreement with our negative stain EM data (Figs. 1e3 and 2a). Therefore, we re-extracted these particles using a box size twice as big as before and repeated the unsupervised 2D classification. The resulting 2D class averages confirmed the presence of slightly curved GephE filaments comprising GephE dimers interconnected by a Z-shaped interface (Fig. 2a and Supplementary Fig. 4c). We additionally observed 2D class averages showing GephE dimers in close proximity to a smaller bilobed density, which could be identified as a side view of the GephG trimer based on *in silico* 2D back-projections of the GephG crystal structure (Fig. 2a, b). However, subsequent 3D reconstruction of the GephE filament failed due to the crowdedness of these grids and a high degree of preferential orientation of the particles (Supplementary Fig. 4d).

Given that Geph complexed with the GlyR-loop eluted in SEC homogeneously at the expected elution volume of the trimeric Geph-GlyR complex (Supplementary Fig. 1a), we concluded that the observed GephE filaments must have been formed during the cryo-EM sample grid preparation. As the vitrification process itself has been shown to only minimally affect the sample, especially with regard to

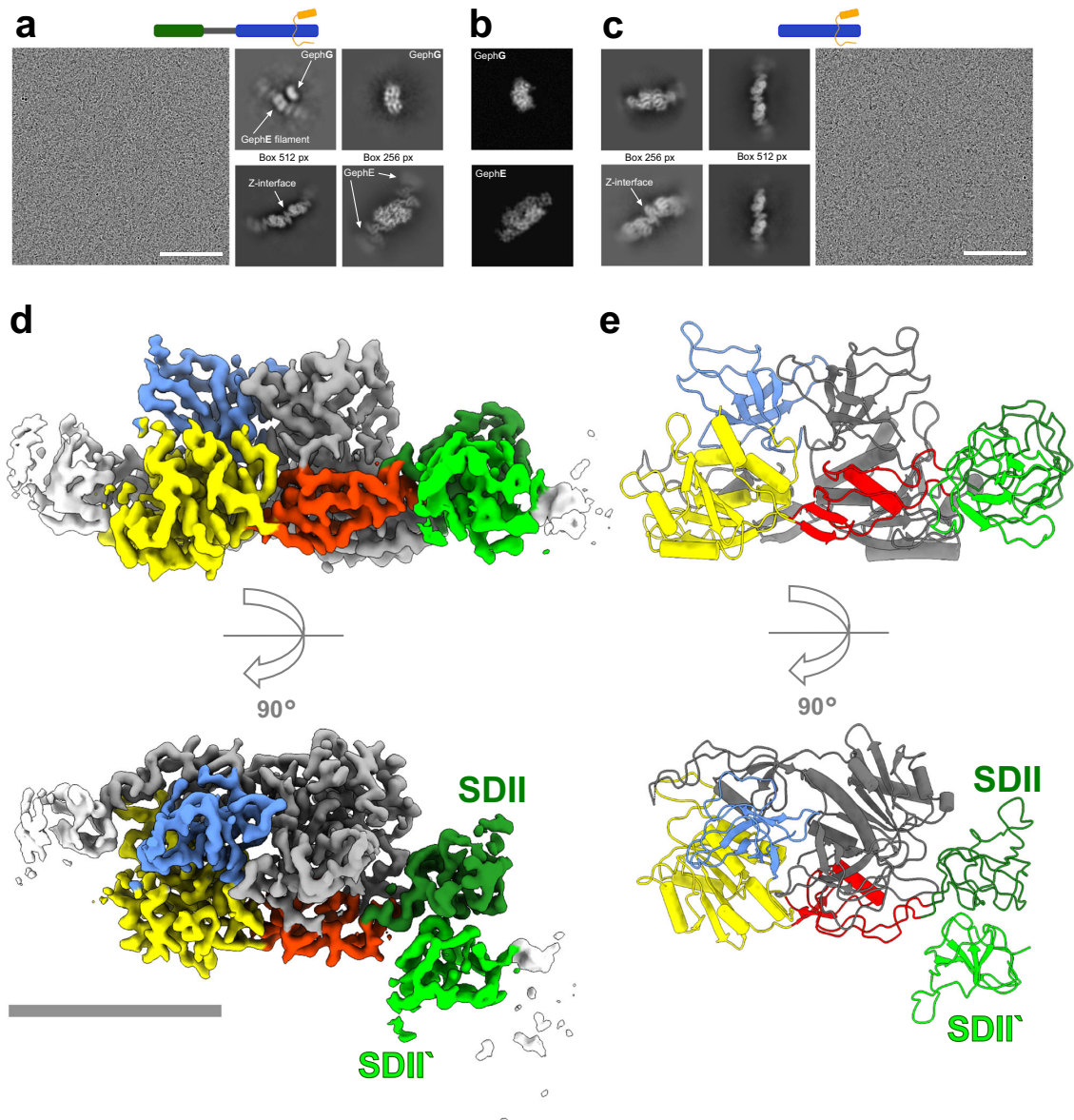


Fig. 2 | Cryo-EM SPA of gephyrin reveals formation of filaments facilitated by GephE SDII interactions. **a** Representative micrograph (scale bar 100 nm) of Geph trimer in complex with GlyR-loop with representative 2D class averages depicting GephG and GephE incorporated into filaments through a Z-shaped interface (arrow). Densities corresponding to GephE and GephG are highlighted in the 2D class average pictures, respectively (arrow). **b** Representative 2D back-projections of GephG (PDB: 1JLJ) and GephE (PDB: 2FU3). **c** Representative micrograph (scale bar 100 nm) with GephE in complex with GlyR-loop with representative 2D class averages depicting GephE incorporated into filaments through a Z-shaped interface (arrow). **d** Surface representation of cryo-EM map of GephE dimer inserted into

filaments with highlighted SDI (red), SDII (dark green), SDIII (yellow), SDIV (blue) from one GephE monomer and SDII from adjacent GephE dimer (SDII', light green). The second GephE monomer is colored in gray. Regions of the structure not used for model building are shown in white. Scale bar 50 Å. **e** Final structure of GephE, shown on a cartoon representation incorporated into filaments, is built into the map shown in (d), highlighting the SDs positions and the SDII-SDII' interface. Cartoon bar diagrams illustrate the Geph constructs used in this experiment (green: GephG, gray: GephC, light green: SDII, red: position of amino acid substitution, orange: GlyR-loop).

processes on the time-scale of protein oligomerisation³⁷, we hypothesize that the blotting step might have induced the oligomerisation process, possibly due to a rapid increase in local protein concentration as observed for other proteins³⁸.

Because in our negative staining SPA elongated assemblies of GephE dimers were only observed when Geph was complexed with the

GlyR-loop, but not for apo-Geph, we also prepared cryo-EM samples in the absence of the GlyR-loop. Resulting micrographs were of comparable quality, also showing densely packed particles (Supplementary Fig. 5a). Interestingly, despite the absence of the GlyR-loop, data processing revealed 2D class averages that were similar to those observed in the presence of the GlyR-loop, showing GephE filaments alone or in

close proximity to a smaller bilobed density (Supplementary Fig. 5b), implicating that potentially the increased concentration might overcome the necessity for GlyR-loop binding observed during negative stain EM.

GephE filaments are connected via an interlocking interface of neighboring SDIIs

In order to confirm that neither GephG nor the C-linker is involved in the formation of the filaments, and aiming to reduce the crowdedness of the sample on the grid, we prepared cryo-EM samples of isolated GephE (residues 327–736) using identical experimental conditions as for full-length Geph before (Supplementary Fig. 6). Again, 2D class averages revealed linear, but flexible assemblies of GephE dimers connected by a Z-shaped interface (Fig. 2c and Supplementary Fig. 6c).

To mitigate the effects of preferred orientation, we acquired an additional dataset at a 25° tilt of the sample stage (Supplementary Fig. 6b). In this dataset, several micrographs showed highly reduced particle crowdedness and clearly illustrated the distribution and proportions of filaments on the micrographs (Supplementary Fig. 6b1). Processing of combined datasets yielded extended particle orientations (Supplementary Fig. 6d), which allowed for an ab-initio 3D reconstruction that revealed a complete GephE dimer with additional density on one side, corresponding to the SDII of an adjacent GephE dimer in the filament (hereafter referred to as SDII') (Supplementary Fig. 6e). Further processing yielded a final map at a global resolution of 3.5 Å, showing clear side-chain density in most areas (Supplementary Fig. 6f). However, density for the SDII-SDII' interface remained fragmented, which we attributed to conformational flexibility in agreement with the various degrees of curvature observed for the 2D class averages of the filament (Fig. 2c and Supplementary Fig. 6c, d). Using a motion-based deep generative model for continuous heterogeneity³⁹, the flexibility of SDII and the SDII-SDII' interface to the adjacent GephE dimer was confirmed (Supplementary Fig. 6g).

In order to improve the resolution in the SDII-SDII' interface region, we used in silico-generated 3D structures based on the deformation flow field from the flexibility analysis to initiate a 3D classification. This yielded one class with a poorly resolved SDII-SDII' interface (35% of particles) and two classes with improved resolution at the SDII-SDII' interface (65% of particles, Supplementary Fig. 6h), of which the latter subset of particles was further refined. Global refinement and subsequent local refinement focussing on the area encompassing the core part of GephE dimer including the SDII-SDII' interface yielded a map with continuous density at the SDII-SDII' interface and a resolution of approximately 3.0 Å in the core part of the GephE dimer and 3.5 to 4.0 Å in the SDII-SDII' interface (Fig. 2d; Supplementary Figs. 6i, j and 7a), allowing us to build an almost complete atomic model into the density map following additional deep-learning based⁴⁰ map sharpening (Fig. 2e and Supplementary Fig. 6j). In our atomic model chain A comprises all four subdomains of GephE (Fig. 2e). Due to the highly fragmented density of chain B in the SDII region, the respective amino acid residues (Ala370–Pro459) were not built into our atomic model (Fig. 2e). For SDII' (chain C) we utilized a predicted structure of SDII as a starting template for building the atomic model^{41,42}.

Overall, our structure of the GephE dimer highly agrees with previous crystal structures, especially in the regions of SDI, SDIII and SDIV⁴. The here identified SDII-SDII' interface features a prominent groove on the SDII side that interlocked with a complementary bulge on the SDII' side (Fig. 2e and Supplementary Fig. 7b, c).

Plasticity of the SDII-SDII' interface supports GephE filaments flexibility

While our 3D sorting strategy could alleviate the fragmentation of the SDII-SDII' interface density, likely caused by hinge-like motions at the two loops connecting SDI and SDII, the lower resolution of this area in

our final reconstruction implied the presence of remaining structural flexibility. To further characterize potential heterogeneity of the SDII-SDII' interface we re-processed the particles from the initial 3D sorting (Supplementary Figs. 6h and 8). For this, particles that did not show a well-resolved SDII-SDII' interface region ("fragmented"), and particles that showed a well-resolved SDII-SDII' interface region ("defined"), were individually refined and then subjected to a motion-based deep generative model for continuous heterogeneity in order to visualize the main modes of SDII-SDII' interface movement (Supplementary Fig. 8a, b). Notably, the consensus position of the SDII' in the structure from the "fragmented" subset was shifted by approximately 9.8° upward as compared to the previously identified, higher resolved, SDII' position (Fig. 3a and Supplementary Fig. 8c). SDII and SDII' from the "defined" subset showed a movement of up to 3.9° and 9.5° for SDII and SDII', respectively (Fig. 3b and Supplementary Fig. 9a). In contrast, the movement of the interface with the uptilted SDII' showed an increased movement of up to 9.9° for SDII and 20.9° for SDII' (Fig. 3c and Supplementary Fig. 9b). Potentially, the later "fragmented" particle population (Supplementary Fig. 8a) might represent a less stable state before SDII and SDII' fully interlock.

To follow up on the observed mobility of the SDII, we investigated whether the incorporation of GephE into filaments changes the position of the SDII in relation to the other domains of the protein. When comparing SDII positions in the crystal structure of human GephE either without or with bound GlyR-loop (PDB: 2FU3, 2FTS) we found that SDII in our structure is positioned further away from the core part of the protein compared to the GephE crystal structure without bound GlyR-loop (Fig. 3d). This difference was smaller if comparing to the GlyR-loop bound GephE crystal structure, implying a GlyR-loop dependent shift in the SDII positioning might be related to filament formation as it promotes a positioning of the SDII more similar to the positioning observed in GephE filaments (Fig. 3e and Supplementary Fig. 9d). A similar effect was observed in the crystal structures of the GephE plant orthologue, Cnx1E, that shows a shift of SDII towards the filament-promotion angle when the cofactor was bound (Fig. 3f, g and Supplementary Fig. 9e, f)^{43,44}.

A single GlyR-loop is able to occupy both binding sites within one GephE dimer

Despite saturation of the sample with the GlyR-loop, the consensus structure showed only fragmented density in the receptor binding sites (Figs. 1b and 2d). We hypothesized that this might be caused by the GlyR-loop adopting multiple conformations within the binding pocket^{4,45}. In order to better resolve the GlyR-loop density, we carried out a 3D classification of all particles of GephE irrespective their SDII conformation using a mask focusing on the core part of the GephE dimer, including both receptor-binding sites (Supplementary Fig. 6f; Sub-Fig. 10). The subset of particles showing the most continuous density for the GlyR-loop was further processed by local refinement excluding the SDII subdomains (Supplementary Fig. 10a). This yielded a final map that after low-pass filtration to 6 Å showed a continuous density spanning both binding pockets on the GephE dimer (Fig. 4a; Supplementary Fig. 10a). 3D classification resulted in classes that either showed GlyR-loop density in both binding sites or a complete absence of GlyR-loop density, suggesting that in the case of binding, a single GlyR-loop is simultaneously occupying both binding sites within one GephE dimer (Fig. 4a, b; Supplementary Fig. 10b). Importantly, this finding rationalizes previous biochemical data reporting the binding of one GlyR-loop to one GephE dimer^{4,23,31,45}. Moreover, machine-learning-based structure predictions⁴⁶ of the most probable position of a single GlyR-loop within the GephE dimer are in line with the observed density for GlyR-loop in our reconstruction (Fig. 4c–e and Supplementary Fig. 11), and predict an extension of the GlyR-loop core binding motive (R394-Y412) towards the C-terminal end, enabling the occupation of both binding sites simultaneously (D413-L426, Fig. 4d, e). This agrees

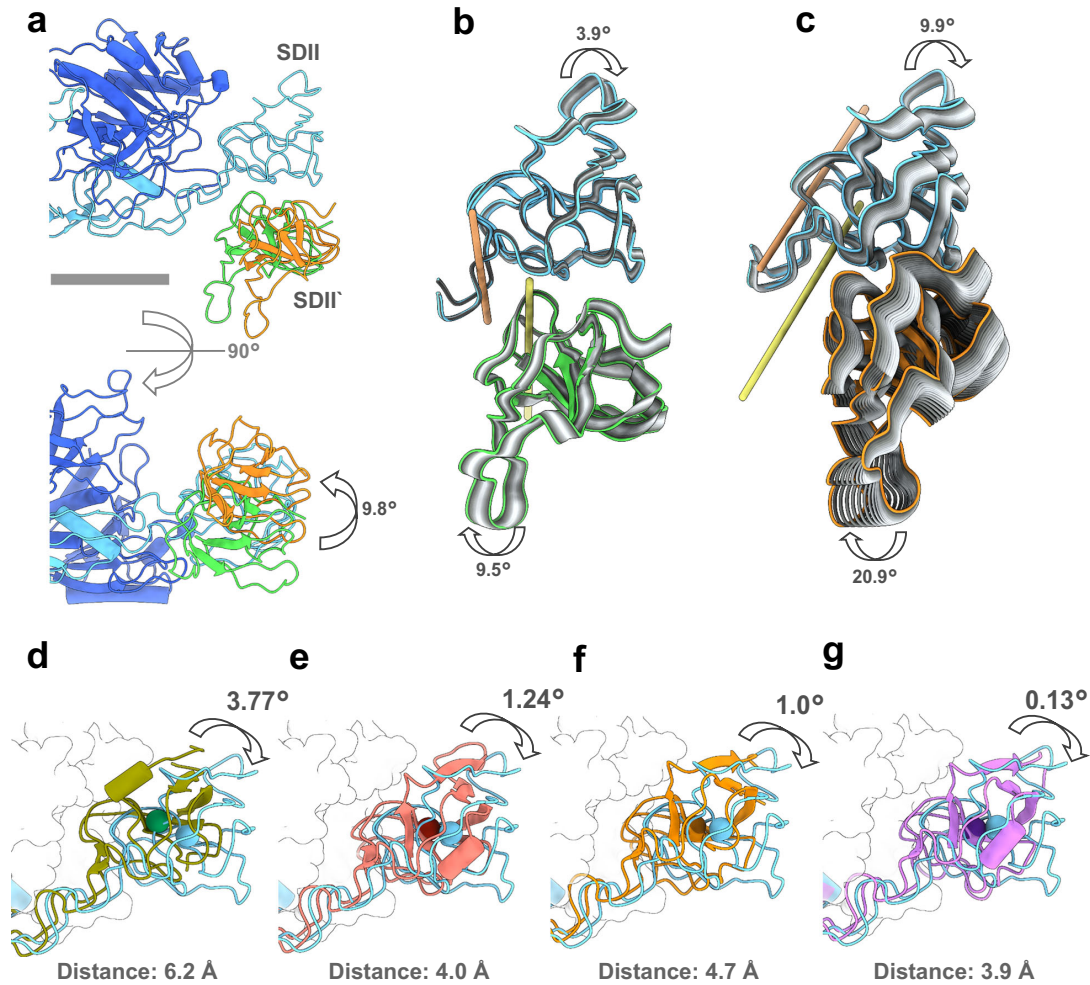


Fig. 3 | The SDII-SDII' interface allows for motion between GephE dimers.
a Overlay of the alternative SDII' binding position (orange) within the SDII-SDII' interface with the main position observed for SDII' in the interface (green). For orientation, SDI and SDII of one GephE subunit (light blue), and SDII and SDIV of the second GephE monomer (dark blue) in the dimer are shown. The shift between the SDII' positions is indicated. For further information, see Supplementary Fig. 8. Scale bar is 25 Å. **b, c** Visualization of main trajectories depicting possible degrees of freedom of SDII (blue) and SDII' (green or orange) in the main (**b**) and alternative (**c**) binding positions based on motion-based deep generative modeling. Strongest deviation from the consensus position is highlighted in color, the course of

movement shown in shades of gray, and the difference between the extreme positions indicated in degrees. For further information see Supplementary Figs. 8 and 9d–g) Structural comparison of the SDII position in the GephE dimer incorporated into filaments in the presence of GlyR-loop (blue) with the crystal structure without GlyR-loop (PDB: 2FU3, olive (**d**), the GephE crystal structure bound to GlyR-loop (PDB: 2FTS, red (**e**), the Cnx1E crystal structure without Moco-AMP (PDB: 5G2R, dark orange (**f**), and the Cnx1E crystal structure bound to Moco-AMP (PDB: 6Q32, purple (**g**)). Differences in SDII positions are indicated in distances and angular changes. For further information, see Supplementary Fig. 9.

with previous in vitro studies showing that residues 413–426 are required for high-affinity binding of the GlyR-loop to GephE dimer²³.

SDII is crucial for Geph filament formation, LLPS, and clustering at synapses

To confirm that the observed filament formation is solely dependent on SDII interactions, we generated a full-length Geph variant lacking the SDII domain (Geph^{ΔSDII}, deleted residues 371–459) and purified the protein in complex with GlyR-loop to homogeneity similarly to the wild-type protein previously. Structural analysis revealed dimeric and trimeric particles, but no signs of filament formation as seen with wild-type protein, confirming that also in the presence of GephC and GephG filament formation requires SDII (Supplementary Fig. 12). Importantly,

binding studies with GlyR-loop showed that both full-length Geph^{ΔSDII} as well as GephE^{ΔSDII} maintained similar binding affinities as compared to the wild-type protein, indicating that the deletion of SDII does not interfere with receptor binding and GephE dimerization (Supplementary Fig. 1c–f).

Next, we asked whether the deletion of SDII would abolish Geph LLPS in vitro, which has been hypothesized to represent the molecular basis of Geph clustering^{31,32,47}. For this, we mixed the GlyR- and GABA_AR-mimetic ICD proteins GlyR-LD, GABA_AR-α1LD, and GABA_AR-α3LD as described previously³¹ with GephE variants and performed sedimentation experiments. In the presence of GlyR-LD, approx. 10 times more GephE^{WT} was detected in the pellet fraction as compared to the sample without GlyR-LD, thus demonstrating strong

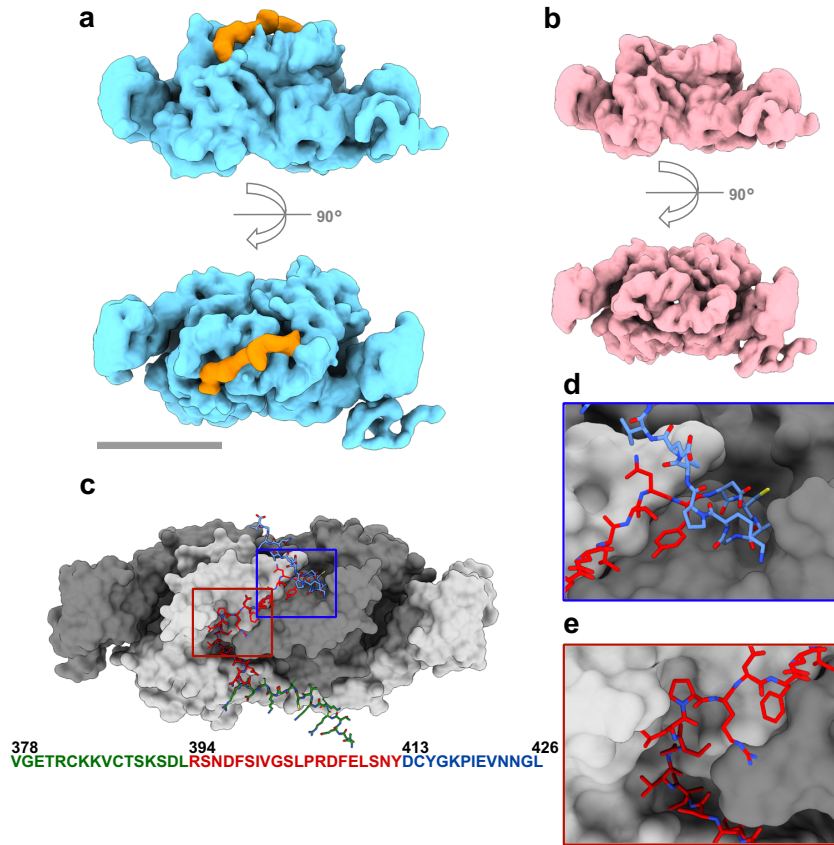


Fig. 4 | GlyR-loop occupies both binding sites within one GephE dimer simultaneously. **a** Surface representation of GephE dimer in complex with GlyR-loop lowpass filtered to 6 Å (light blue) with highlighted density depicting the position of GlyR-loop (orange). **b** Surface representation of GephE dimer without GlyR-loop lowpass filtered to 6 Å. For further information, see Supplementary Fig. 10). **c** GephE dimer in complex with GlyR-loop modeled after structure prediction. GephE dimer is shown in surface presentation with highlighted GephE monomers

(light gray, dark gray). GlyR-loop is shown in stick presentation with color-coded amino acid sequence. Amino acid sequence of modeled GlyR-loop with respective positions is depicted beneath. **d** Magnification of GlyR-loop binding site one occupied by the core binding motive of GlyR-loop (red). **e** Magnification of second GlyR-loop binding site occupied by the C-terminally extended binding motive of GlyR-loop (blue).

LLPS formation (Fig. 5a, b). In contrast, Geph^{ASDII} did not sediment in the presence of GlyR-LD, as the band in the pellet fraction was similar to the control without GlyR-LD (Fig. 5a, b). Similar results were obtained for GABA_AR-α1LD and GABA_AR-α3LD, with again the amount of Geph^{ASDII} within the pellet fraction being unaffected by the presence or absence of the respective receptor ICD model proteins (Supplementary Fig. 1g, h). This data collectively demonstrated that SDII is required for Geph LLPS.

To confirm the physiological relevance of SDII-mediated Geph filament formation for postsynaptic Geph clustering, we expressed mScarlet-tagged gephyrin constructs in dissociated murine hippocampal neurons lacking endogenous gephyrin. This was achieved by infecting hippocampal cultures from floxed gephyrin mice⁴⁸ with recombinant adeno-associated viruses (rAAVs) encoding Cre recombinase and subsequently with rAAVs encoding Cre-dependent mScarlet-Geph or mScarlet-Geph^{ASDII}, before the onset of synapse formation^{49,50}. After 14 days in vitro, mScarlet-Geph formed frequent somatic and dendritic clusters, which co-localized with pre-synaptic vesicular GABA transporter (vGAT) and post-synaptic GABA_ARs (GABA_AR-γ2), both markers of GABAergic synapses. In contrast, mScarlet-Geph^{ASDII} was diffusely distributed and did not form postsynaptic clusters (Fig. 5c, d), despite the fact that Geph^{ASDII} retained its

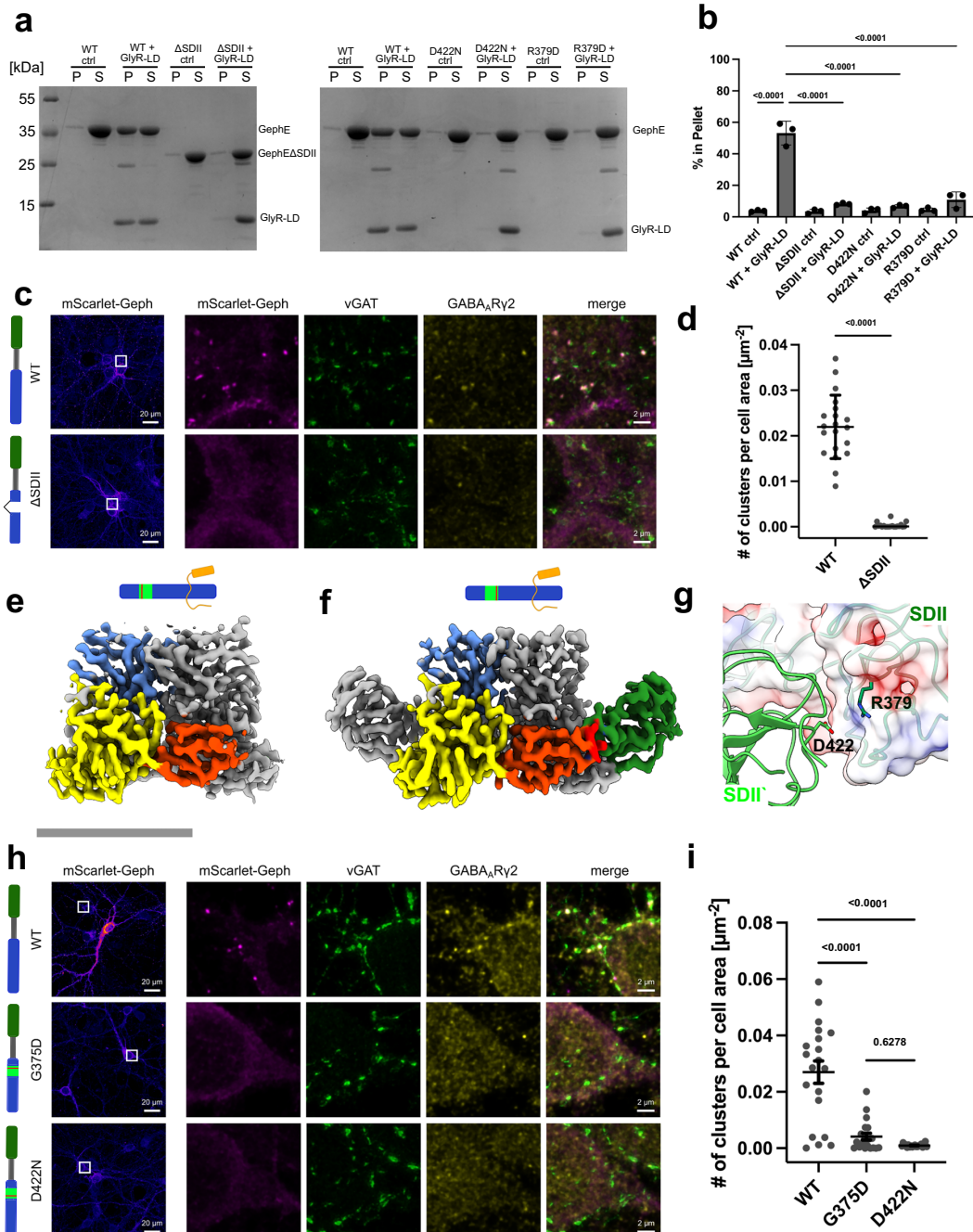
receptor-binding but lost its filament formation ability. Overall, these experiments demonstrate that the SDII subdomain-mediated filaments are a prerequisite for the formation of postsynaptic clusters at GABAergic synapses.

Loss of Geph filaments provides the molecular basis of epileptic encephalopathy in two patients

After showing that SDII is required for Geph filament formation, LLPS and receptor clustering, we asked whether we can disclose the mechanistic basis of epileptic encephalopathy in previously identified pathogenic variants of Geph, Geph^{G375D} and Geph^{D422N}, which are both located within SDII and in case of Geph^{G375D} shows a strong impairment in LLPS formation^{14,31,33}.

Similar to Geph^{WT}, we purified Geph^{G375D} to high homogeneity and added the GlyR-loop in a five-fold molar excess before cryo-EM sample preparation (Supplementary Fig. 1b). The resulting micrographs were of high quality, showing numerous evenly distributed particles (Supplementary Fig. 13a). Unsupervised 2D classification yielded high-quality class averages showing dimeric structures from various angles, but no indications of filament formation. Moreover, the observed structures were more compact than those observed for Geph^{WT} (Supplementary Fig. 13b). Further 3D processing yielded a

Chapter 4



final map of the GephE dimer with a global resolution of 3.2 Å, which, while showing density for the GlyR-loop, was lacking density for the SDII region in both subunits (Fig. 5e and Supplementary Fig. 13c, d). Given that neither 3D sorting nor local refinement allowed us to recover density in the SDII region, we hypothesize that the SDII in GephE^{G375D} is unable to fold properly. This finding is supported by GephE^{G375D} aSEC experiments showing a larger hydrodynamic radius than GephE^{WT}

(Supplementary Fig. 1b). In conclusion, GephE^{G375D} exhibits an unstructured SDII, thus leading to a loss of Geph filament formation.

Next, we expressed and purified the second pathogenic Geph variant, GephE^{D422N}, and prepared it for cryo-EM. The resulting micrographs were of high quality, showing numerous evenly distributed particles (Supplementary Fig. 14a). Unsupervised 2D classification yielded high-quality class averages showing dimeric structures from

Fig. 5 | Proper SDII folding and build-up of Gephyrin filaments are essential for synaptic cluster formation and LLPS. **a** Representative SDS-PAGE of sedimentation experiment showing the amount of protein present in pellet (P) and supernatant (S) fractions of GephE variants (WT, ΔSDII, D422N, R379D) either without GlyT-LD (ctrl) or with (+GlyR-LD). **b** Quantification of band intensities in the pellet fraction; $n = 3$ presented as means $\pm$ SD. Data were analyzed by one-way ANOVA ($F_{9,20} = 47.78$, $p = 1.42 \cdot 10^{-11}$), Tukey's post hoc test; p -values are indicated. **c** Representative images of gephyrin^{floxed/floxed} murine hippocampal neurons infected with mScarlet-Geph^{WT} or mScarlet-Geph^{ΔSDII} (pseudo-colored fire LUT, magenta) and immunostained against vesicular GABA transporter (vGAT, green) and GABA_AR $\gamma 2$ subunit (yellow). **d** Quantification of gephyrin clusters from c per cell area; $n = 20$ images per condition from four independent cultures. Each data point represents cluster density per acquired image, means $\pm$ SD are indicated. Data were analyzed by unpaired, two-tailed Welch's two-sample t -test ($t(19.3) = 13.8$, $p = 1.92 \cdot 10^{-11}$). **e** Surface representation of GephE^{G375D} dimer highlighting the positions of SDI (red), SDIII (yellow) and SDIV (blue) of one monomer.

The second monomer is shown in gray. Scale bar 50 Å. **f** Surface representation of GephE^{D422N} dimer highlighting the positions of SDI (red), SDII (green) SDIII (yellow) and SDIV (blue) of one monomer. The second monomer is shown in gray. Scale similar to (f). **g** Close up depiction of the SDII interface highlighting D422 situated in SDII' and R379 in close proximity situated in SDII. **h** Representative images of gephyrin^{floxed/floxed} murine hippocampal neurons infected with mScarlet-Geph^{WT}, mScarlet-Geph^{G375D} or mScarlet-Geph^{D422N} (pseudo-colored fire LUT, magenta) and immunostained against vesicular GABA transporter (vGAT, green) and GABA_AR $\gamma 2$ subunit (yellow). **i** Quantification of gephyrin clusters from h per cell area; $n = 20$ images per condition from four independent cultures. Each data point represents cluster density per acquired image; means $\pm$ SD are indicated. Data were analyzed by one-way ANOVA ($F_{2,57} = 34.94$, $p = 1.25 \cdot 10^{-39}$), Tukey's post hoc test; p -values are indicated. Cartoon bar diagrams illustrate the Geph constructs used in this experiment (green: GephG, gray: GephC, light green: SDII, red: position of amino acid substitution).

various angles, but no indications of filament formation. Different from GephE^{G375D}, the observed structures were similar to those observed for GephE^{WT}, including folded SDIIs (Supplementary Fig. 14b, c). Further 3D processing yielded a final map of the GephE dimer with a global resolution of 3.1 Å, which showed density for the SDII region in both subunits, allowing for C2 symmetric 3D reconstruction (Fig. 5f and Supplementary Fig. 14d, e). Importantly, while GephE^{D422N} showed a completely folded SDII, no filament formation was evident either during 2D or 3D processing (Fig. 5f and Supplementary Fig. 14c). Therefore, the substitution of Asp422 by Asn appears to fully abolish SDII-based filament formation. Close inspection of the SDII-SDII' interface in our GephE^{WT} structure identified a possible ion bridge between Asp422 and Arg379 of the neighboring GephE dimer (Fig. 5g). To investigate the potential importance of this hypothesized ion bridge, we created a variant in which Arg379 was substituted with Asp (GephE^{R379D}) to reverse the charge and thus disrupt the hypothesized ion bridge. Similar to GephE^{D422N}, GephE^{R379D} showed completely folded SDIIs but no filament formation, demonstrating that this ion bridge is a crucial component of the filament interface (Supplementary Fig. 15).

Next, we corroborated that for both the known patient mutant GephE^{D422N} and the mutant GephE^{R379D} the inability to form filaments affects their ability to undergo LLPS in vitro. Indeed, both mutant variants exhibited strongly reduced LLPS formation comparable to Geph^{ΔSDII} (Fig. 5a, b).

Finally, to corroborate that the inability to form filaments also has physiological relevance, we expressed either mScarlet-tagged Geph^{G375D} or Geph^{D422N} constructs in dissociated murine hippocampal neurons lacking endogenous gephyrin. Similar to mScarlet-Geph^{ΔSDII}, mScarlet-Geph^{G375D} and mScarlet-Geph^{D422N} were diffusely localized throughout hippocampal neurons and did not form any postsynaptic clusters (Fig. 5h, i).

Combined, our structural and functional studies of Geph disclosed a hitherto unknown type of oligomerization of GephE dimers, leading to Geph filament formation. This interaction is stabilized by a single GlyR-loop bound to both receptor binding sites of a GephE dimer. Deleting the filament forming SDII or introducing either known pathogenic mutations or targeted disruption of an ion-bridge, all prohibited GephE filament formation, caused loss in LLPS and impaired synapse formation in neurons.

Discussion

Our structural and functional data uncovered GephE dimer filament formation as the underlying molecular mechanism for the formation of Geph-containing postsynaptic densities. Negative stain EM data revealed pronounced conformational heterogeneity of full-length gephyrin, consistent with previous studies, which suggest structural variability in purified Geph trimers ranging from so-called 'closed' to

'open' states²⁰. The addition of the GlyR-loop not only reduced overall heterogeneity, but also led to the formation of filamentous assemblies of Geph. Cryo-EM data revealed that these filaments are mediated by a hitherto unknown SDII-SDII' interface, which was corroborated by structural data derived both from full-length gephyrin and various GephE variants. Our observation that a single GlyR-loop could bridge two GephE subunits by occupying both previously identified GlyR-loop binding sites simultaneously highlights the important role of receptor binding in GephE dimerization and filament formation: While previous studies have shown that multiple receptor binding sites increase affinity towards gephyrin²⁴, the here observed binding of a single peptide bridging both receptor sites in GephE would support a receptor-mediated stabilization of GephE dimers, thus probably lowering the concentration threshold required for filament formation. Notably, gephyrin variants with either a deleted SDII or with single residue substitutions in SDII derived either from epileptic patients or designed based on our atomic model were collectively unable to form GephE filaments, to engage in LLPS or to form postsynaptic clusters. Neither deletion of SDII nor the pathogenic amino acid substitutions affected Geph's ability to bind the receptor drastically, with only minor loss in affinity observed^{14,33}. This finding highlights that the underlying cause of the pathological loss of gephyrin function is most likely the inability to form Geph filaments. Given that filament formation requires a critical interplay of GephE dimers, the lack of filament formation by Geph^{G375D} variant further explains its dominant nature in the patient with the hemizygous *GPHN* mutation¹⁴.

Previous electron tomography (ET) has uncovered that the PSD of inhibitory synapses forms a thin layer of 5-15 nm width underneath the postsynaptic membrane⁵¹. Cryo-ET revealed a mesophasic organization of inhibitory receptors in the postsynaptic membrane, with most receptors having four or fewer neighbors assembling into short, curved and presumably branched assemblies²⁶. Our structural observation that Geph is able to form flexible filaments, with trimeric GephG forming the basis for GephE dimer-linked filaments and/or branching points, aligns well with the Cryo-ET data. In addition, an underlying filament network at inhibitory PSD was identified in previous ET studies of GABAergic synapses, revealing filaments with a length of approx. 24 to 32 nm²⁹, which would correlate to filaments consisting of two to three GephE dimers, agreeing with what has been observed in our study. In contrast to the previously proposed hexagonal lattice model, relying on GephG trimer and GephE dimer interactions, gephyrin filaments not only help to understand the observed regular assemblies of inhibitory receptors in the postsynaptic membrane, but also provide further understanding of the mode of action of pathogenic gephyrin variants^{14,33}. Our data lend themselves to the conclusion that a hexagonal lattice is neither a requirement nor a precursor for gephyrin filaments, given that gephyrin filaments were also observed for isolated GephE dimers. However, based on studies from human

patients with different forms of neuropsychiatric disorders harboring gephyrin deletions in either GephG, GephC, or GephE, we conclude that proper oligomerization of both GephG and GephE is required for synapse formation⁵². Future studies are needed to structurally resolve the GephG in gephyrin full-length filaments. One possible role is generating branching points that connect gephyrin filaments, thus incorporating elements of the hexagonal lattice model.

Multiple lines of studies have shown specific organization of receptors and PSD protein throughout the synapse, indicating regulatory mechanisms controlling protein density^{26,29,30,53}. Receptor ICD binding stabilizes the GephE dimer, thereby fostering filament formation and PSD stabilization. Our data also suggests that in the absence of receptors, Geph retains its ability to self-organize but only at elevated protein concentration. While we did not observe filament formation during SEC experiments, neither in the presence nor in the absence of the GlyR-loop, we detected filaments also in the absence of the GlyR-loop during cryo-EM. In negative stain EM, we only observed filaments in the presence of GlyR-loop. Geph concentration is a main difference between these three experimental approaches, with SEC having the lowest, negative stain-EM an intermediate, and cryo-EM the highest concentration, especially when accounting for a concentration increase due to the blotting process during grid preparation³⁸. Considering an ice thickness of approximately 200 Å and a particle density ranging from 300 to 1000 particles per micrograph, as estimated from our data, a Geph concentration between 0.2 mM and 0.7 mM can be assumed⁵⁴.

According to high-resolution light microscopy of postsynaptic densities, it was estimated that an inhibitory PSD contains approximately 200–300 gephyrin molecules^{12,28}. Based on an average synapse area of 0.07 μm^2 and a thickness of 0.015 μm , this corresponds to an estimated gephyrin concentration between 0.34 mM and 0.51 mM within the inhibitory PSD, which agrees well with the gephyrin concentration estimated on our cryo-EM grids⁵¹. Additionally, it can be assumed that the length of gephyrin filaments correlates with increased or decreased local protein concentrations in the PSD^{26,53,55}. Local increase in receptor and gephyrin concentration likely influences the formation of nanodomains within the synapse, which are characterized by elevated receptor and PSD protein concentrations, which is an underlying prerequisite for LLPS at the postsynapse^{30,53,55}.

A structural comparison of apo- and filament-GephE revealed that binding to GlyR-loop alters the position of SDII closer to the position observed in GephE filaments. This observation suggests a GlyR-ICD-induced conformational change rendering SDII more prone to engage in GephE filament formation. This agrees with the previous X-ray structure of GephE, which identified SDII as the most mobile part of GephE^{4,8}. As SDII harbors one half of the substrate binding site for gephyrin's enzymatic function, the conversion of molybdopterin-adenylate into molybdenum cofactor⁴⁴, this activity may also impact SDII position and thus filament formation. Additionally, a negatively charged and partially disordered region in SDII, opposite to the here identified filament-forming SDII-interface, has been found crucial for LLPS formation, probably impacting the conformational position of SDII within GephE dimers as well³¹.

The concept of LLPS as an underlying mechanistic principle in PSD formation has been put forward in recent years^{31,32,56,57}. LLPS often requires increased local protein concentration, intrinsically unstructured protein motifs and a defined window of ion strength^{31,58–61}. Although the unstructured GephC could not be resolved in our cryo-EM study, we can exclude its direct participation in the protein-protein interaction. It rather enabled the alignment of various GephE dimers derived from different Geph trimers and may play a regulatory role in the prohibition of phase separation at lower concentrations^{62,63}. Given that an SDII-deficient Geph variant was unable to form filaments, to engage in LLPS and to form synapses, we conclude that SDII-dependent GephE filaments contribute strongly to the underlying mechanistic principle of Geph LLPS³¹. Furthermore, in line with

previous studies that identified a lack in LLPS for the pathogenic GephE^{G375D} variant³¹, we could now resolve the underlying cause as a loss in SDII folding, thus prohibiting GephE filament formation while receptor binding remained largely uncompromised. Finally, in the pathogenic variant Geph^{D422N} a critical salt bridge within the SDII interaction site has been abolished underlying the functional importance of a precise alignment of both SDII domains during GephE filament formation. In this regard, receptor binding was slightly reduced, but gephyrin's enzymatic function remained unchanged³³.

In summary, our data collectively support the concept of LLPS-driven subcellular compartmentalization and identified GephE filament formation as the underlying molecular principle that assembles gephyrin into the PSD of inhibitory synapses^{58,64}.

Methods

Ethics statement

We complied with all relevant ethical regulations for animal testing and research. Experiments were approved by the local research ethics committees (Germany, Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, reference 2021.A450).

Expression constructs

6His-tagged Geph, GephE WT and GlyR β -loop-intein expression vectors have been described before^{14,23}. 6His-tagged GephE^{G375D} and GephE^{D422N} expression vectors have been described before^{14,33}. For the 6His-tagged Geph Δ SDII (residues: 1-383, GSGSG-linker, 473-751) variant, the previously described 6His-tagged Geph construct was used as template²³. Deletion of SDII (residue: 383-473) and insertion of GSGSG-linker was achieved by Gibson Assembly using DpnI restriction enzyme and the following primers (fw-GGCTCAGGTTCCGACCCATCGGC-CACGACATTAA; rv-CAACCTACCCCATTCAGGCTCAGGTTCCGG A). For the 6His-tagged GephE^{R379D} variant, the previously described 6His-tagged GephE WT construct was used as template²³. Side-directed mutagenesis for GephE^{R379D} was achieved by PCR with the following primers (fw-CTATGCTGTTGATGCTGCTGATGGT; rv-ACCATCAGCAG-CATCAACAGCATAG) followed by DpnI restriction enzyme digest. GlyR-DL and GABAAR- α 1/3-DL were kindly provided by the Zhang lab³¹.

Protein expression and purification

All gephyrin variants used in this study were recombinantly expressed in *E. coli* BL21 (DE3) Rosetta cells in 1x LB medium containing 0.1 mg/ml ampicillin. Prior to expression, cells were grown until an OD₆₀₀ of 0.3–0.4 was reached. His-tagged gephyrin variants were expressed for 16 h at 18 °C. His-tagged GephE WT was expressed for 5 h at 25 °C, whereas GephE variants were expressed for 16 h at 18 °C. Protein expression was induced by the addition of isopropyl- β -D-thiogalactopyranoside (IPTG) to an end-concentration of 250 μM in the cell culture. After protein expression, cells were harvested by centrifugation at 4000 $\times g$ and resuspended in lysis buffer (50 mM Tris/HCl, pH 7.5, 250 mM NaCl, 5 mM β -mercaptoethanol, 1x protease inhibitor (cOmplete, Roche, Basel, Switzerland) and lysozyme from chicken egg (Sigma-Aldrich) and stored at –20 °C for further use. For purification, the cells were lysed mechanically using EmulsiFlex (Avestin, Ottawa, Canada) and ultrasound for 3 $\times$ 30 s at 45% amplitude. Cytosolic extract was separated from cell debris by centrifugation at 50,000 $\times g$ for 45 min. Prior to Ni-NTA chromatography, the cell extracts were supplemented with imidazole to 20 mM final concentration. The cell extract was applied to 10 ml Ni-NTA resin, and proteins were washed with 10 column volumes (CV) of lysis buffer and 10 CV of lysis buffer containing 40 mM imidazole. Proteins were eluted with 3 CV lysis buffer containing 300 mM imidazole. All proteins were further purified via size exclusion chromatography (SEC) (Superdex 16/600 prep grade, GE Healthcare) using protein buffer (25 mM Tris/HCl, pH 7.5, 250 mM NaCl, 5 mM β -mercaptoethanol, 5% glycerol). All purification steps were performed at 4 °C. GlyR- β loop was expressed

and purified as stated previously²³. GlyR-βLD, GABA_AR-αLD and GABA_AR-α3LD were purified according to the protocol provided by Bai and colleagues³¹

Isothermal titration calorimetry (ITC)

All experiments were performed with a MicroCal Auto-ITC200 (Malvern, Malvern, United Kingdom) in ITC buffer (25 mM Tris pH 7.5, 250 mM NaCl, 5 mM β-mercaptoethanol, 5% glycerol). The sample cell was filled with gephyrin or GephE variants at a concentration of 20–30 μM. GlyR-βloop was filled into the syringe at a concentration of 250–350 μM. Experiments were conducted at 37 °C with an injection volume of 1.5–2 μl, a set reference power of 5 μcal/s, spacing time of 180–300 s and a stirring speed of 750 rpm. Data was analyzed using Origin 7 (OriginLab Corporation), and binding parameters were derived by applying a one-site or two-site binding model.

Sedimentation assay of in vitro phase separation

For sedimentation assays, proteins were mixed at designated combinations and conditions in a 100 μl final volume in assay buffer (25 mM Tris, pH 7.5, 150 mM NaCl). After 10 min incubation at room temperature, the mixtures were centrifuged at 16,000 × *g* for 10 min at 25 °C. Right after centrifugation, the supernatant and pellet were separated. The pellet was brought to the same volume as the supernatant. Proteins recovered in supernatant and pellet were analyzed by the Bradford assays or SDS-PAGE with Coomassie Blue staining. The band intensities in SDS-PAGE gels were quantified by ImageJ software. Relative and absolute amounts of proteins in supernatant and pellet were calculated based on the input amounts and relative band intensities. Data from three repeats were presented as means ± SD.

Negative stain sample preparation and data collection

For negative stain EM, all samples were SEC purified prior to grid preparation using either a Superdex 6 increase 10/300 GL or Superose 6 increase 10/300 GL column with grid buffer (25 mM Tris pH 7.5, 250 mM NaCl, 25 mM Arg/Glu, 5 mM β-mercaptoethanol, 1.5% sucrose). For grid preparation, protein samples were diluted to a final concentration of 0.005 mg/ml using grid buffer. Continuous carbon grids (Quantifoil, Cu 200 mesh) were subjected to glow discharge using a Zepto Plasma Cleaner (Diener) for 30 s to clean the surface and render it hydrophilic. 3 μl protein solution was incubated on the grid for approximately 1 min at room temperature, and then stained with uranyl formate as described elsewhere⁶⁵. The grids were stored in the dark in a dry atmosphere until further use. EM data were acquired using a Talos L120C (Thermo Fisher Scientific) electron microscope equipped with a LaB₆ emitter operated at 120 kV. Images were collected automatically using EPU (version 2.12.1.2782REL, Thermo Fisher Scientific) on Ceta16M CMOS detector with a calibrated pixel size of 1.86 Å per pixel. Defocus values were set to range from −0.3 to −2.0 μm.

Cryo-EM sample preparation and data collection

Prior to grid preparation, all samples were isolated using a Superdex 6 increase 10/300 GL column with grid buffer. Afterwards, samples were diluted to a concentration of 0.5 mg/ml using the grid buffer. Protein samples were applied to a glow-discharged UltrAufoil R1.2/1.3 Au300 (Quantifoil Micro Tools GmbH), incubated for 5 s, blotted for 3.5 s and flash-frozen in liquid ethane using a Vitrobot Mark IV device (Thermo Fisher Scientific) set to 100% humidity at 21 °C. Grids were stored under liquid nitrogen conditions until usage. Cryo-EM data were acquired using a Titan Krios G3i (Thermo Fisher Scientific) electron microscope operated at 300 kV with either 0° or 25° stage tilt. Images were collected automatically using EPU (version 2.12.1.2782REL, Thermo Fisher Scientific) on a Falcon III direct electron detector with a calibrated pixel size of 0.862 Å px^{−1}. Movies were collected in counting mode with a total dose of approximately 30–40 e/Å² distributed among 42 frames. Defocus values ranged from −0.5 to −2.6 μm.

EM image processing

Image processing was performed using cryoSPARC (version 4.4)⁶⁶. Movie stacks were first corrected for drift and beam-induction motion, and then used to determine defocus and other CTF-related values. Only high-quality micrographs with low drift metrics, low astigmatism and good agreement between experimental and calculated CTFs were further processed. On these high-quality micrographs, putative particles were automatically picked based on expected protein diameter, extracted, and subjected to reference-free 2D classification. Representative 2D classes were then used for a template-based picking approach, particles extracted again, subjected to reference-free 2D classification to exclude artefacts, and subsequent 3D classification using C1 symmetry to identify high-quality particles. 3D classification was performed with a target resolution of 6 Å in order to ensure that secondary-structure elements are also included as a feature in the classification algorithm. Particles showing protein-like density features, especially features resembling secondary structure elements, were further refined using the non-uniform refinement strategy. Subsequent data processing workflows differ from this point onwards between the different datasets (For more information, see supplementary information).

Atomic model building

The atomic model was built starting from the gephyrin E domain dimer crystal structure (PDB ID 2FTS) and the gephyrin amino acid sequence (UniProt Q9NQX3). First, Coot (version 0.9.8.02)⁶⁷ was used to semi-manually fit amino acids into a single subunit of the EM density map. Next, Phenix (version 1.21)⁶⁸ was used to generate a non-crystallographic symmetry (NCS)-related second subunit and both subunits were then simultaneously refined against the 3D density map. This process was iterated until the fit to the density map and geometric parameters converged. The final atomic model accounts for residues 31 to 556. For further details and statistics, see Supplementary Table 1. Molecular visualization and analysis were done using UCSF ChimeraX (version 1.9)⁶⁹

In the final atomic model, the following amino acid sequences were reduced to their respective C-alpha: ChainA/LEU 432-ILE 447, ChainA/VAL 574-ASP 580, ChainB/VAL 574-ASP 580, ChainC/LEU 432-ILE 447.

DNA constructs

mScarlet-Gphn C4c from pAAV-hSyn-fDIO-mScarlet-Gphn_C4c (Addgene plasmid # 194974; RRID:Addgene_194974) and pAAV-hSyn-fDIO-mScarlet-Gphn_P1 (Addgene plasmid # [194972]; RRID:Addgene_194972) were initially subcloned into pGP-AAV-syn-FLEX-jGCaMP7s, in which the WPRE was removed using HindIII and NotI (pGP-AAV-syn-FLEX-jGCaMP7s-WPRE was a gift from Douglas Kim & GENIE Project (Addgene plasmid # 104491; RRID:Addgene_104491)) using NEBuilder HiFi DNA Assembly (NEB). Since one of the AAV2 ITR sites was destroyed in the process, the inserts mScarlet-Gphn C4c and mScarlet-Gphn_P1, including loxP and lox2272 sites, were subcloned into the backbone of pAAV-hSyn-fDIO-mScarlet-Gphn_P1 (Addgene plasmid # 194972; RRID:Addgene_194972) using HindIII and Eco47III. His-tagged Gphn C4c (P2) ΔSDII in pQE80 was generated by site-directed mutagenesis using forward GGCTCA GGTCCGGACCCATCGGCCACGACATTAA and reverse TCCGGAA CCTGAGCCTGGGAATGGGGTAGGTTG primers. His-tagged Gphn (P1) D422N in pQE80 was generated by site-directed mutagenesis using forward TCGGGTGCTAATGCAGTAGTG and reverse CACTACTGCAT-TAGCACCACA primers. His-tagged Gphn (P1) G375D in pQE80 was generated by site-directed mutagenesis using forward CATCAG-TAAAGATGACTATGCTGTTCCG and reverse CGAACAGCATAGT CATCTTTTACTGATG primers. Gphn C4c (P2) ΔSDII, Gphn (P1) D422N, and Gphn (P1) G375D were subcloned into the backbone of pAAV-hSyn-FLEX-mScarlet-Gphn C4c or pAAV-hSyn-FLEX-mScarlet-Gphn P1 using

XhoI and BshTI restriction sites. pAAV-CamKIIa-moxBFP-P2A-Cre was generated in two steps. First, pAAV-CamKIIa-moxBFP-Cre was generated by subcloning Cre from pAAV.CMV.H1.eGFP-Cre.WPRE.SV40 (a gift from James M. Wilson (Addgene plasmid # 105545; RRID:Addgene_105545)) into pAAV-CamKIIa-moxBFP-WPRE (Addgene plasmid # 194976; RRID:Addgene_194976). In the second step, the P2A site was inserted in between moxBFP and Cre via site-directed mutagenesis using forward GGCGACGTGGAGGAGAACCCCGCCCGCCGG CAGCATGTCCGGAGAGCAAAAGCTG and reverse primers TCTCTCC ACGTCGCGCGCTGCTTCAGCAGGCTGAAGTTGGTGGCGCCGCTGCC CTTGTACAGCTCGTCCATGC. The plasmids pAdDeltaF6 (Addgene plasmid # 112867; RRID:Addgene_112867) and pAAV2/1 (Addgene plasmid # 112862; RRID:Addgene_112862) were gifts from James M. Wilson. All DNA constructs were confirmed by Sanger sequencing (Eurofins), and the integrity of AAV2 ITR sites was confirmed by analytical SmaI restriction digest.

Primary hippocampal cultures

Floxed gephyrin mice⁴⁸ were a gift from Ralph A. Nawrothski. Floxed gephyrin mice were back-crossed to C57BL/6NRj. Mice were housed at 22 ± 2 °C, with a humidity of 55 ± 10%, on a 12 h light-dark cycle with free access to food and water.

Dissociated primary hippocampal cultures were prepared from homozygous gephyrin floxed embryos (E17.5) of either sex, which were obtained from a total of four homozygous parental pairs. Per preparation, all embryonic hippocampi obtained from one pregnant mouse were pooled prior to cell dissociation. After dissociation, 65,000 cells were seeded on Poly-L-lysine-coated 13-mm cover slips. Cultures were grown in 0.5 ml per well neurobasal medium supplemented with B-27, N-2, GlutaMAX[™] (Thermo Fisher Scientific), and Pen/Strep. Recombinant AAV1 particles were diluted in Neurobasal medium with supplements before they were added to the cultures. Cultures were infected after 4 days in vitro (DIV) with 1 × 10⁸ viral genome copies (GC) of moxBFP or moxBFP-P2A-Cre and after 8 DIV with 5 × 10⁸ GC of either AAV-hSyn-FLEX-mScarlet-Gphn C4c. On DIV 11, 50% media was exchanged for fresh neurobasal media with supplements.

rAAV2/1 preparation

Recombinant AAV2/1 particles were prepared in HEK293T cells (DSMZ no. ACC 635) according to the protocol by Kimura and colleagues⁷⁰. Viral particles were precipitated with PEG/NaCl and subsequently cleared via chloroform extraction⁷⁰. Purity of viral preparations was assessed with SDS-PAGE, and AAV titers were determined using Gel green[®] (Biotium) protocol⁷¹. Fluorescence was measured at 507 ± 5 nm excitation and 528 ± 5 nm emission using a plate reader equipped with monochromators (Tecan Spark).

Immunocytochemistry

Cells were fixed at DIV14 with 4% formaldehyde in PBS for 15 min and quenched with NH₄Cl in PBS at room temperature (RT). All subsequent incubation and wash steps (PBS) were performed at RT. After blocking/permeabilization for 1 h with 10% goat serum, 1% BSA, 0.2% Triton X-100 in PBS, the following primary antibodies (Synaptic Systems) were used: anti-vesicular GABA transporter (vGAT) (1:1000, #131003) for inhibitory presynaptic terminals; anti-GABA_A R γ2 (1:500, #224004) for postsynaptic GABA_ARs. The following secondary antibodies were used: goat anti-rabbit AlexaFluor 488 (1:500, #A-11034, Thermo Fisher Scientific) and goat anti-guinea pig AlexaFluor 647 (1:500, #ab150187, Abcam). Coverslips were mounted with Mowiol/Dabco.

Confocal microscopy and image analysis

Image stacks [0.3-μm z-step size, 3432 × 3432 (144.77 × 144.77 μm)] were acquired on a Leica TCS SP8 LIGHTNING upright confocal microscope with an HC PL APO CS2 63×/1.30 glycerol objective,

equipped with hybrid detectors (Leica HyD) and the following diode lasers: 405, 488, 552, and 638 nm. LIGHTNING adaptive deconvolution using “Mowiol” setting was applied. Images were segmented and analyzed in an automated fashion using ImageJ/FIJI 1.53c (<https://imagej.net/software/fiji/>), and the code is available online at GitHub (https://github.com/Fillieb/quantitative_synapse_analysis).

Statistics and reproducibility

Micrographs shown for the cryo-EM experiments are representative for the respective dataset. Statistical analyses were performed using R version 4.2.2 (2022-10-31 ucrt), readxl v. 1.4.1, rstatix v. 0.7.1, tidyverse v. 1.3.2 in Rstudio environment (<https://www.rstudio.com/>), Platform: x86_64-w64-mingw32/x64 (64-bit). Exact *p*-values can be found in the source data.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Unless otherwise stated, all data supporting the results of this study can be found in the article, supplementary, and source data files. Plasmids have been made available via Addgene [<https://www.addgene.org/>]. The EM maps for Geph^{WT}, Geph^{E^{G37SD}}, Geph^{E^{D422N}}, and Geph^{E^{R379D}} have been deposited in the EMDB under accession codes EMD-51644, EMD-54820, EMD-54821, EMD-54824. The raw electron microscopy imaging data are available on request due to their large size. Atomic coordinates for Geph^{WT} have been deposited in the Protein Data Bank under the accession code 9GW9. Source data are provided with this paper.

Code availability

The code used for the image analysis is available at GitHub [https://github.com/Fillieb/quantitative_synapse_analysis].

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Author contributions

A.M., E.B., and G.S. designed the research study; A.M., F.L., E.H.W.B., I.v.S., and K.B. conducted experiments; A.M., F.L., E.H.W.B., N.B., K.B., and M.G. acquired data; A.M., F.L., E.H.W.B., N.B., M.G. analyzed data; A.M. performed structural model building; A.M., F.L., E.B., and G.S. wrote the manuscript with input from all coauthors.

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Competing interests

The authors declare no competing interests.

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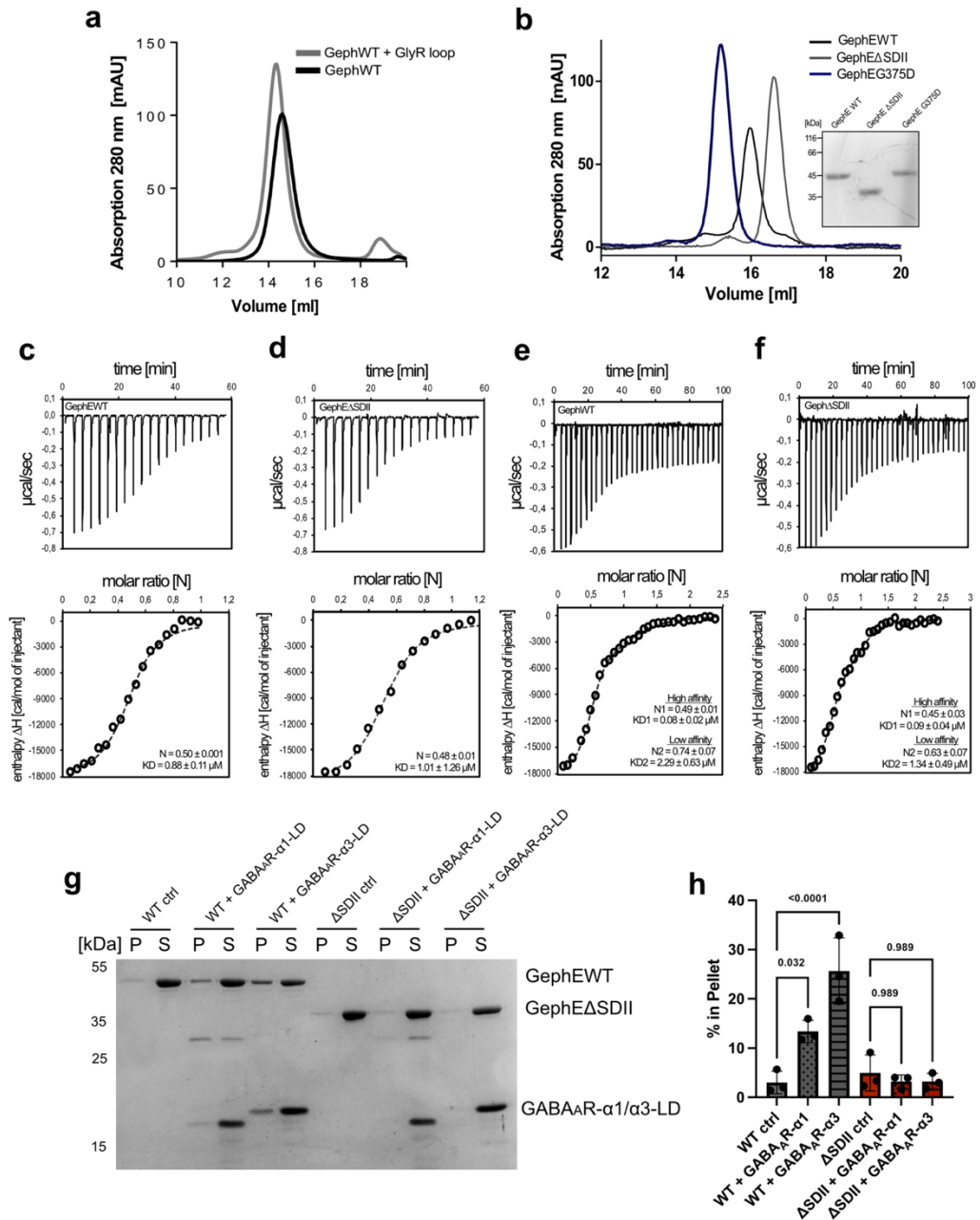
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4.4 Supplementary figures



Supplementary Figure 1: Biochemical analysis of gephyrin variants.

a) Representative SEC chromatograms of Geph^{WT} with and without GlyR-loop.

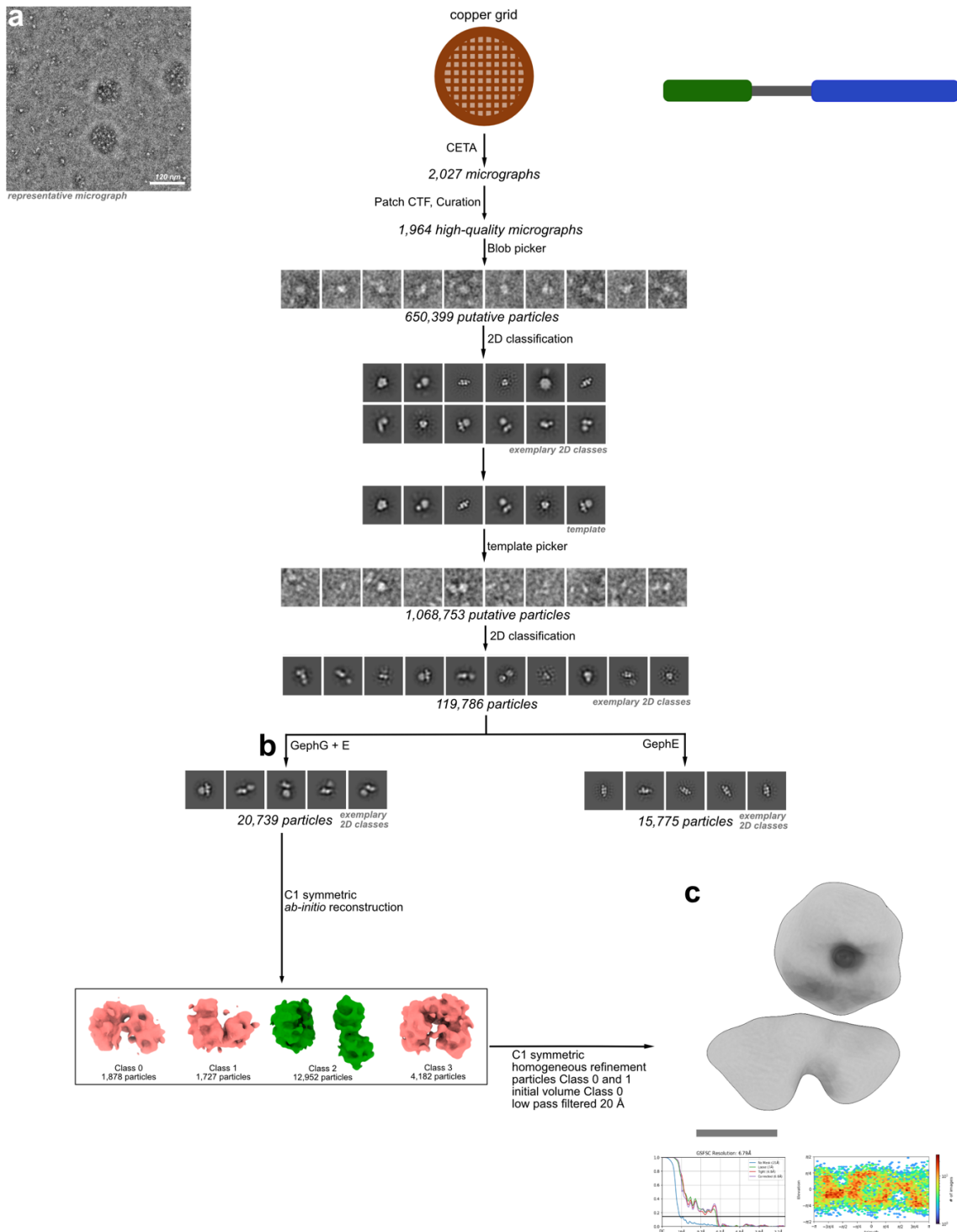
b) Representative SEC chromatograms of Geph^{EWT}, Geph^{EG375D} and Geph^{EΔSDII} (added for size comparison). Insert shows SDS-PAGE of respective peak fractions.

c-f) Representative ITC experiments of Geph^{EWT} (c), Geph^{EΔSDII} (d), Geph^{WT} (e), Geph^{ΔSDII} (f) titrated with GlyR-loop. Raw isotherms are depicted in the upper panel. Respective peak integrations and two-side fit are shown in lower panel with values for binding stoichiometry (N) and dissociation constant (K_D) and respective fitting error following either a one side fit (c, d) or two side fit (e, f) depicting a high and low affinity binding event.

g) Representative SDS-PAGE of sedimentation experiment using GephE and GABA_AR homolog protein (GABA_AR-α1-LD, GABA_AR-α3-LD) showing the amount of protein present in pellet (P) and supernatant (S) fractions.

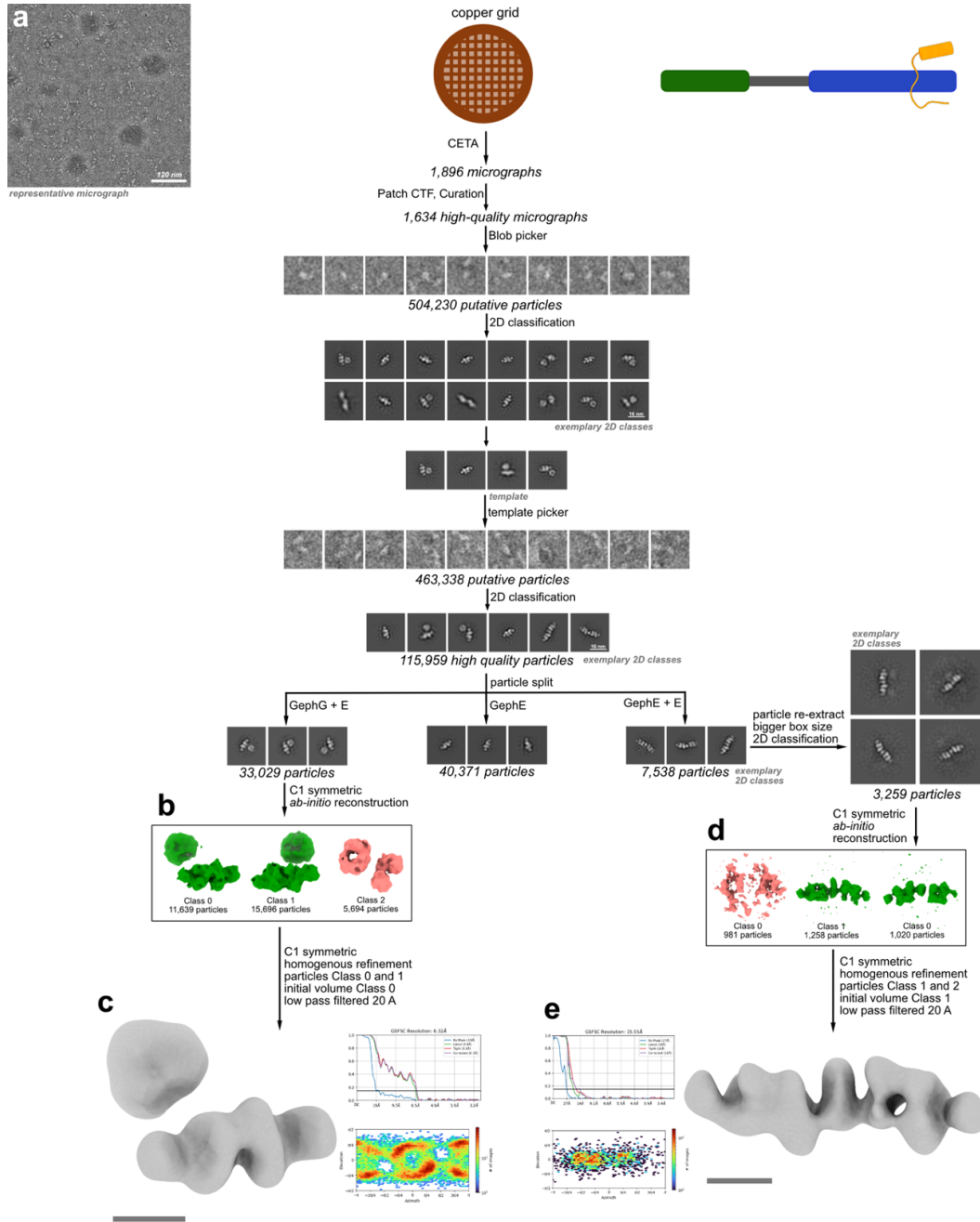
h) Quantification of relative GephE amount present in the pellet fraction. Data from three different batches of experiments were presented as means $\pm$ SD. Data were analysed by one-way ANOVA ($F_{5,12} = 20.34$, $p = 1.76 \cdot 10^{-5}$), Tukey's post hoc test; $n=3$, p values of selected comparisons are indicated.

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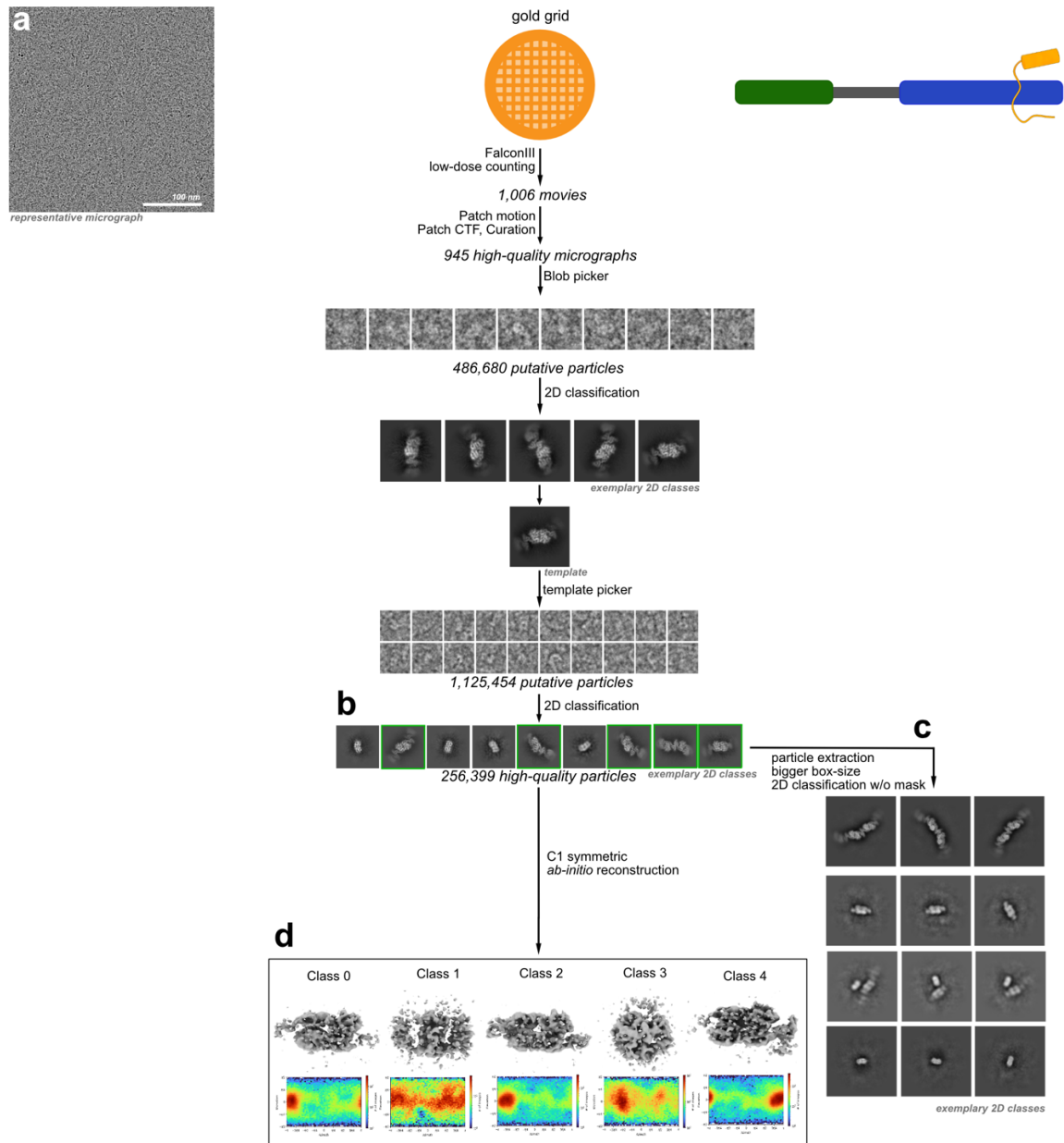
Supplementary Figure 2: Overview of Geph^{WT} negative stain data processing workflow. For the dataset micrographs were picked with good CTF scores and sufficient signal to noise ratio (a). Particles were first picked using a blob picker, and then subjected to unsupervised 2D classification. For 3D reconstruction of gephyrin including both G and E domains (GephG + E) particles from classes showing sharp outlines were used. GephG + E particles were further used for unsupervised *ab-initio* classification sorting them into four distinct populations (b). From these the particles of one class (shown in green) depicting two distinct densities were subjected to homogenous refinement algorithm with C1 symmetry resulting in a map at approx. 6.8 Å. Shown is the final map low pass filtered to 20 Å (c). Scale bar 50 Å. The cartoon bar diagram illustrates which Geph construct was used in this experiment (green: GephG, grey: GephC, light green: SDII).

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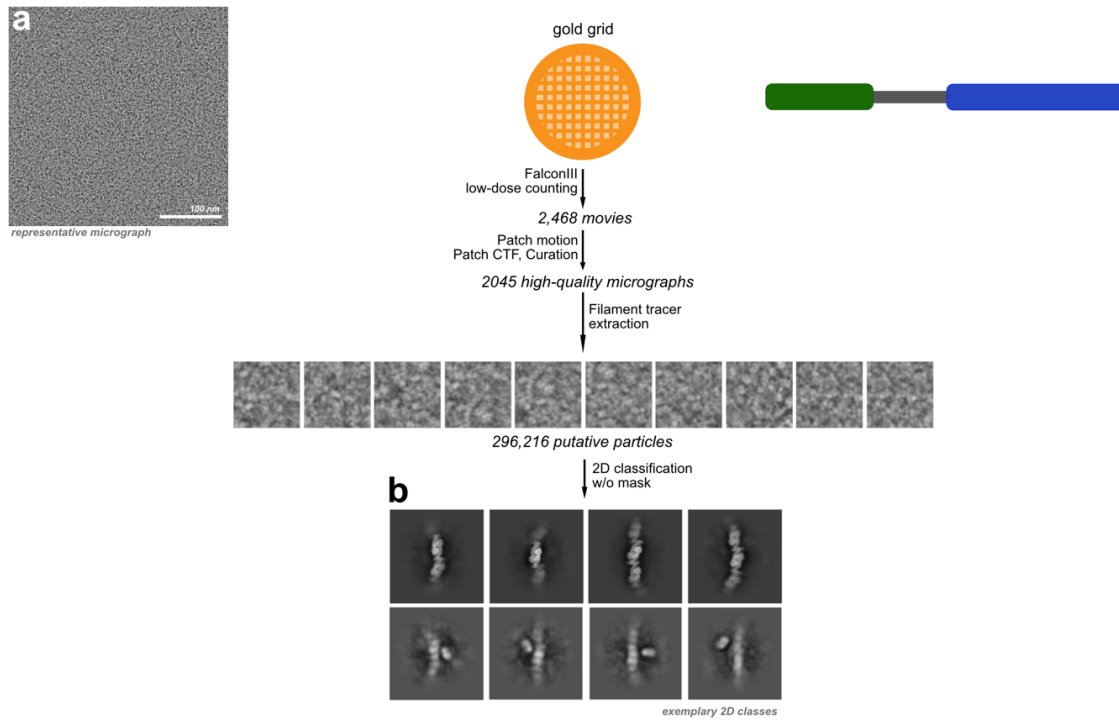
Supplementary Figure 3: Overview of Geph^{WT} in complex with GlyR-loop negative stain data processing workflow. For the dataset micrographs were picked with good CTF scores and sufficient signal to noise ratio (a). Particles were first picked using a blob picker, and then subjected to unsupervised 2D classification continuing with a second supervised 2d classification. For 3D reconstruction of gephyrin including both G and E domains (GephG + E) particles from classes showing sharp outlines were used. GephG + E particles were further used for unsupervised ab-initio classification sorting them into three distinct populations (b). From these the particles of class 0 and 1 (shown in green) depicting two distinct densities were subjected to homogenous refinement algorithm with C1 symmetry resulting in a map at approx. 6.3 Å. Shown is the final map low pass filtered to 20 Å (c). For 3D reconstruction of gephyrin depicting two E domains dimer (GephE + E) particles from classes showing sharp outlines were used. GephE + E particles were further used for unsupervised ab-initio classification sorting them into three distinct populations (d). From these the particles of two classes (shown in green) depicting two distinct densities were subjected to homogenous refinement algorithm with C1 symmetry resulting in a map at approx. 15.6 Å. Shown is the final map low pass filtered to 20 Å (e). Scale bar 50 Å. The cartoon bar diagram illustrates which Geph construct was used in this experiment (green: GephG, grey: GephC, light green: SDII, orange: GlyR-loop).

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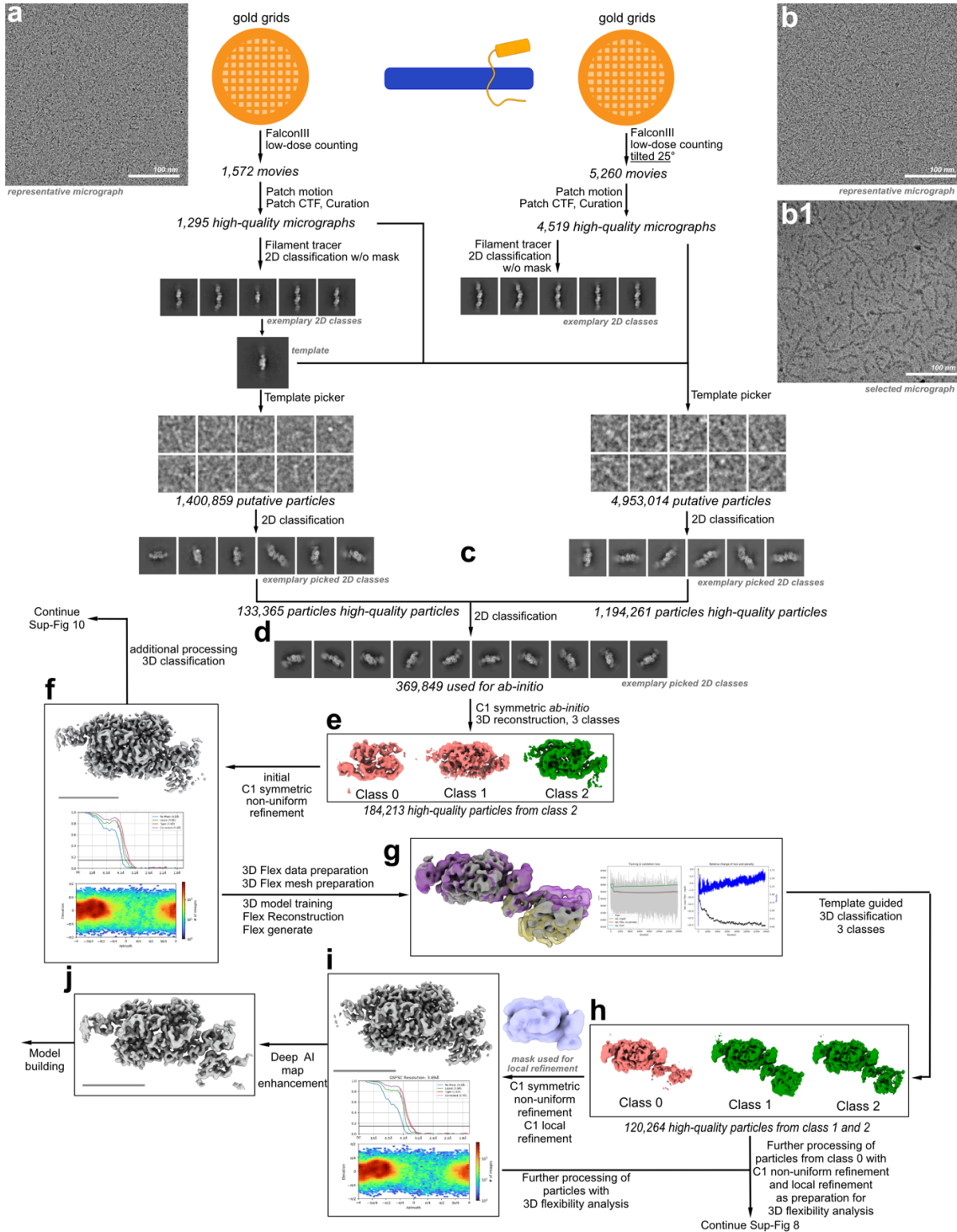
Supplementary Figure 4: Overview of Geph^{WT} in complex with GlyR-loop cryo-EM data processing workflow. For the dataset micrographs were picked with good CTF scores and sufficient signal to noise ratio (a). Particles were first picked using a blob picker, and then subjected to unsupervised 2D classification continuing with a second supervised 2D classification. Resulting 2D classes depicting gephyrin domains either isolated or incorporated into filaments (b). Re-extraction of particles with bigger box-size (512 px) and re-classification depicted the characteristic of gephyrins domains being incorporated into filaments or being isolated on the micrographs (c). Particles from highlighted 2D classes (b, green box) were subjected to unsupervised *ab-initio* classification sorting them into 5 classes. Reconstruction yielded poor classes with highly fragmented maps and mostly high degree of preferred orientation (d). The cartoon bar diagram illustrates which Geph construct was used in this experiment (green: GephG, grey: GephC, blue: GephE, orange: GlyR-loop).

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Supplementary Figure 5: Overview of Gephyr^{WT} without GlyR-loop cryo-EM data processing workflow. For the dataset micrographs were picked with good CTF scores and sufficient signal to noise ratio (a). Particles were first picked using a blob picker and extracted using 512 px box-size and then subjected to unsupervised 2D classification. The resulting 2D class averages depicting gephyrin molecules being incorporated into filaments (b). The cartoon bar diagram illustrates which Geph construct was used in this experiment (green: GephG, grey: GephC, blue: GephE).

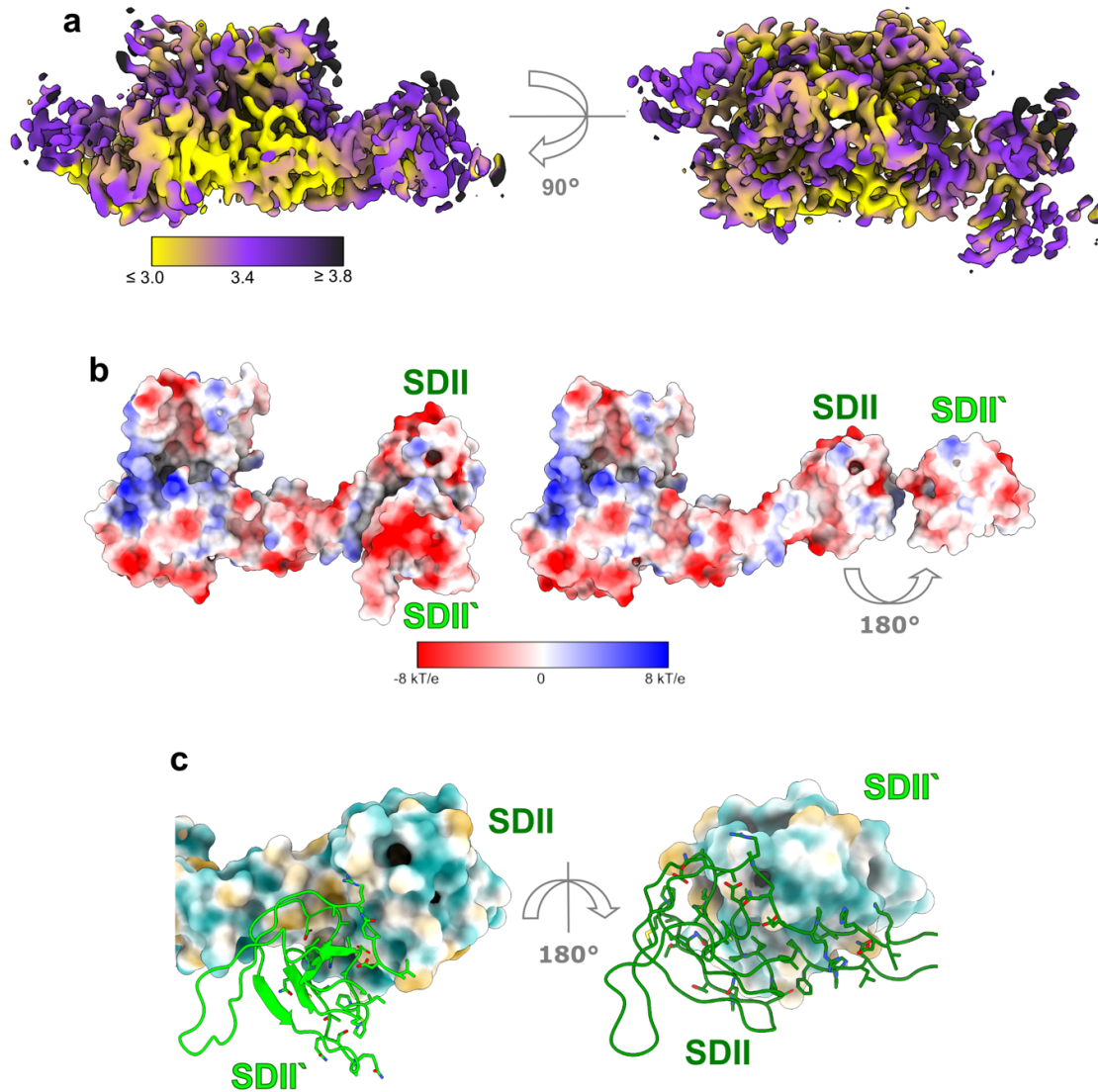
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Supplementary Figure 6: Overview of GephE^{WT} in complex with GlyR-loop cryo-EM data processing workflow. For analysis an un-tilted dataset was combined with a dataset recorded at 25° stage tilt to increase the orientations of particles on the micrographs. Subsequently, micrographs were picked with good CTF scores and sufficient signal to noise ratio (a, b). While crowding prevented the clear identification of filaments on most micrographs, several showed clearly visible filaments (b1). Both datasets yielded 2D class averages of good quality depicting GephE incorporated into filaments (c). In a final 2D classification step high-quality particles from both datasets were combined and high-quality particles were picked for ab-initio classification (d). Particles were classified into four classes using unsupervised ab-initio classification of which one class showed coherent density map which included the filament interface (e). Particles from this class were subjected to unsupervised non-uniform refinement which resulted in a map with a global resolution of 3.6 Å (f). The Map and particles were

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further used for 3D flexibility reconstruction which yielded density maps showing the SDII interface and adjacent GephE dimer in various positions (g). Two most excessive positions (yellow, purple) and a position occupying a position in between (grey) were chosen for template guided 3D classification of particles used in initial non-uniform refinement (h). 3D classification showed two classes with strong density depicting the filament interface and adjacent GephE dimer and one with weak density (h). Particles from two classes with strong density for the filament interface were further used for non-uniform refinement and subsequent local-refinement utilizing a mask covering the core part of one GephE dimer and the SDII-SDII' interface (i). The resulting map showed high resolution in the core part of the protein and coherent density at the SDII-SDII' interface with a global resolution of 3.7 Å (i). As a final step the resulting map was subjected to deep AI map enhancement for map sharpening which was finally used for atomic model building (j). Scale bars 50 Å. The cartoon bar diagram illustrates which Geph construct was used in this experiment (blue: GephE, orange: GlyR-loop).



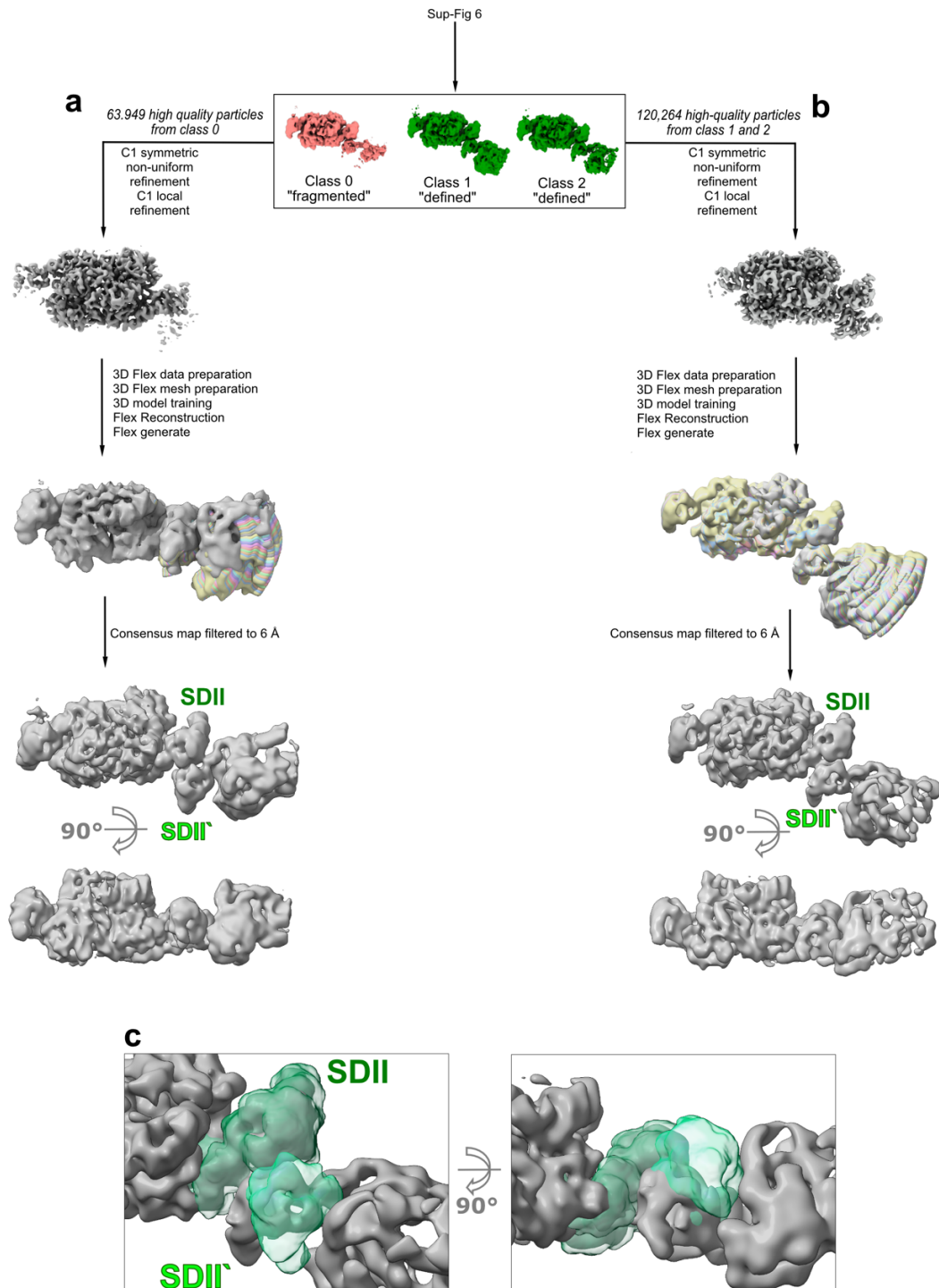
Supplementary Figure 7: Characterization of gephyrin filament interface.

a) Local resolution of final map of GephE dimer (reconstruction from Sup-Figure 6) incorporated into filaments. Colour code indicates resolution [Å].

b) Electrostatic potential distribution of one GephE monomer with filament interface in surface presentation. For better visualization of electrostatic potential of the filament interface, SDII' was turned 180° (right panel). Colour code indicates electrostatic potential [kT/e].

c) Hydrophobicity distribution of GephE interface SDII in surface presentation with SDII' in cartoon presentation (light green, left side) and SDII' in surface presentation with SDII in cartoon presentation (dark green; right side).

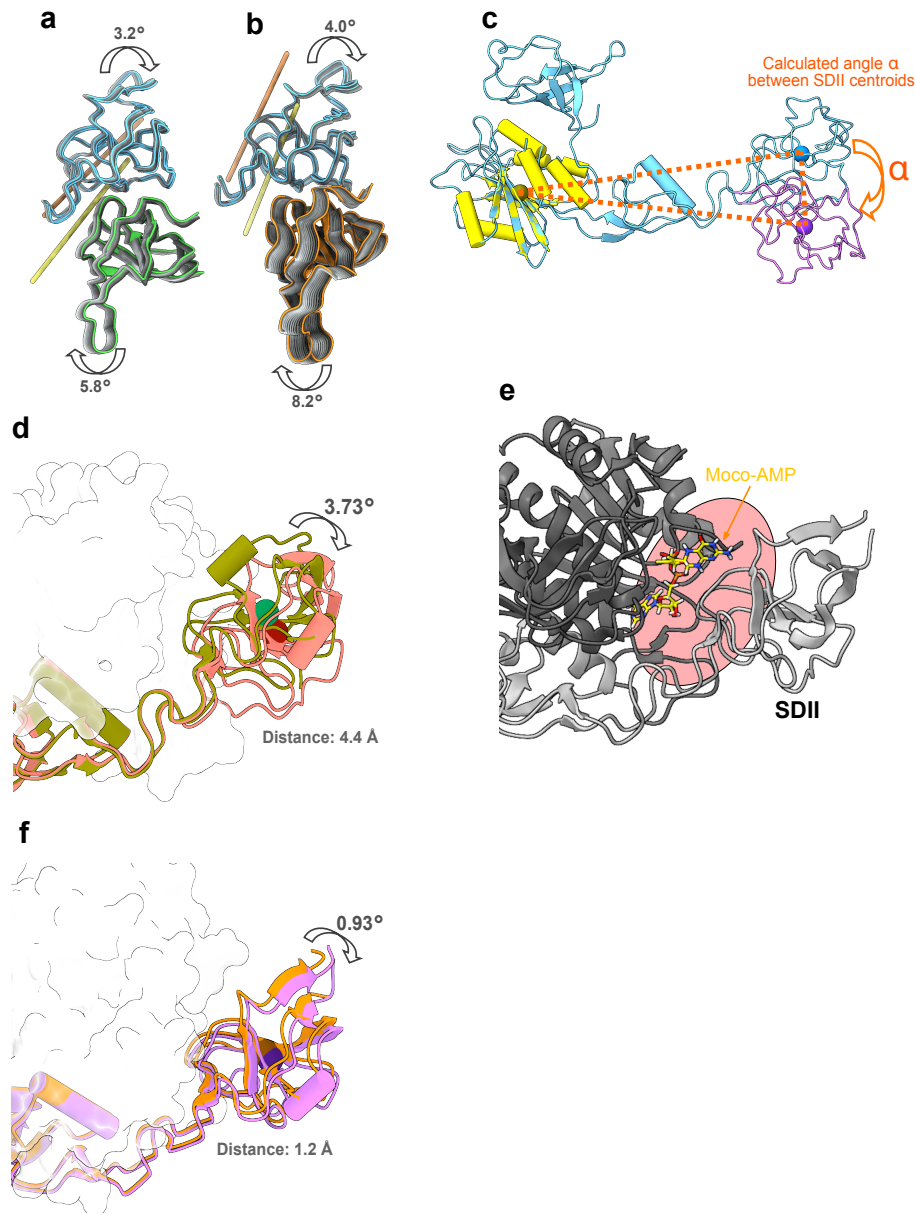
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Supplementary Figure 8: Workflow motion-based deep generative modelling of GephE incorporated into filaments.

a, b) Analysis workflow of particles from ab-initio classification depicting either weak (a) or strong (b) density in GephE filament interface region. Colours of overlaying density maps indicating different interface conformations.

c) Alignment of consensus maps depicting the positions of SDII and SDII'. Consensus map from b is shown in surface representation (grey) with overlaying positions of SDII and SDII' from a (green).



Supplementary Figure 9: Analysis of SDII flexibility.

a, b) Visualization of secondary trajectories depicting possible degrees of freedom of SDII (blue) and SDII' (green or orange) in the main (a) and alternative (b) binding positions based on motion-based deep generative modelling. Strongest deviation from the consensus position is highlighted in colour, the course of movement shown in shades of grey, and the difference between the extreme positions indicated in degree.

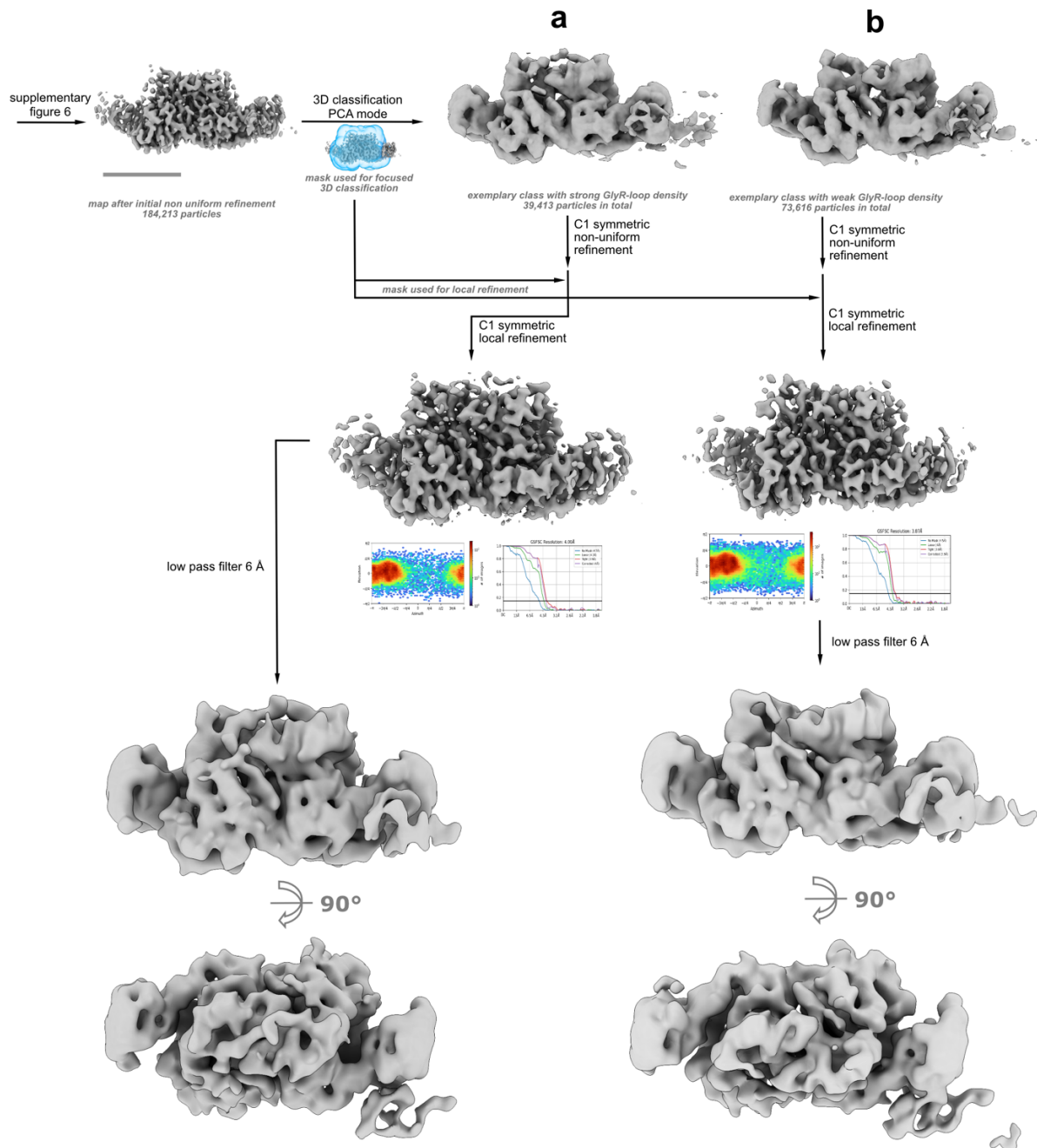
c) Visualization of the process by which the positional shift between the SDIIs from different models was measured. Respective atomic structures were aligned to the secondary structure elements of SDIII (selection marked in yellow). The distances between the centroids of SDIII (yellow), SDII (blue) and SDII in the shifted position (purple) were then measured, and by applying trigonometry the angle α was calculated as a proxy for the positional shift between the SDIIs. Note that in this panel for better visualization SDI, SDIII and SDIV from the GephE monomer of shifted position (purple) were removed and change in SDII position was overexaggerated.

d) Alignment of GephE with bound GlyR-loop (rosa, PDB 2FTS) and GephE without GlyR-loop (olive, PDB 2FU3). Changes in angular position and distance between SDII centroids are indicated.

e) Depiction of Moco-AMP (stick representation, yellow) in the active centre of the GephE dimer. GephE monomers are shown different shades of grey. The pink region highlights the active centre relevant for Moco biosynthesis.

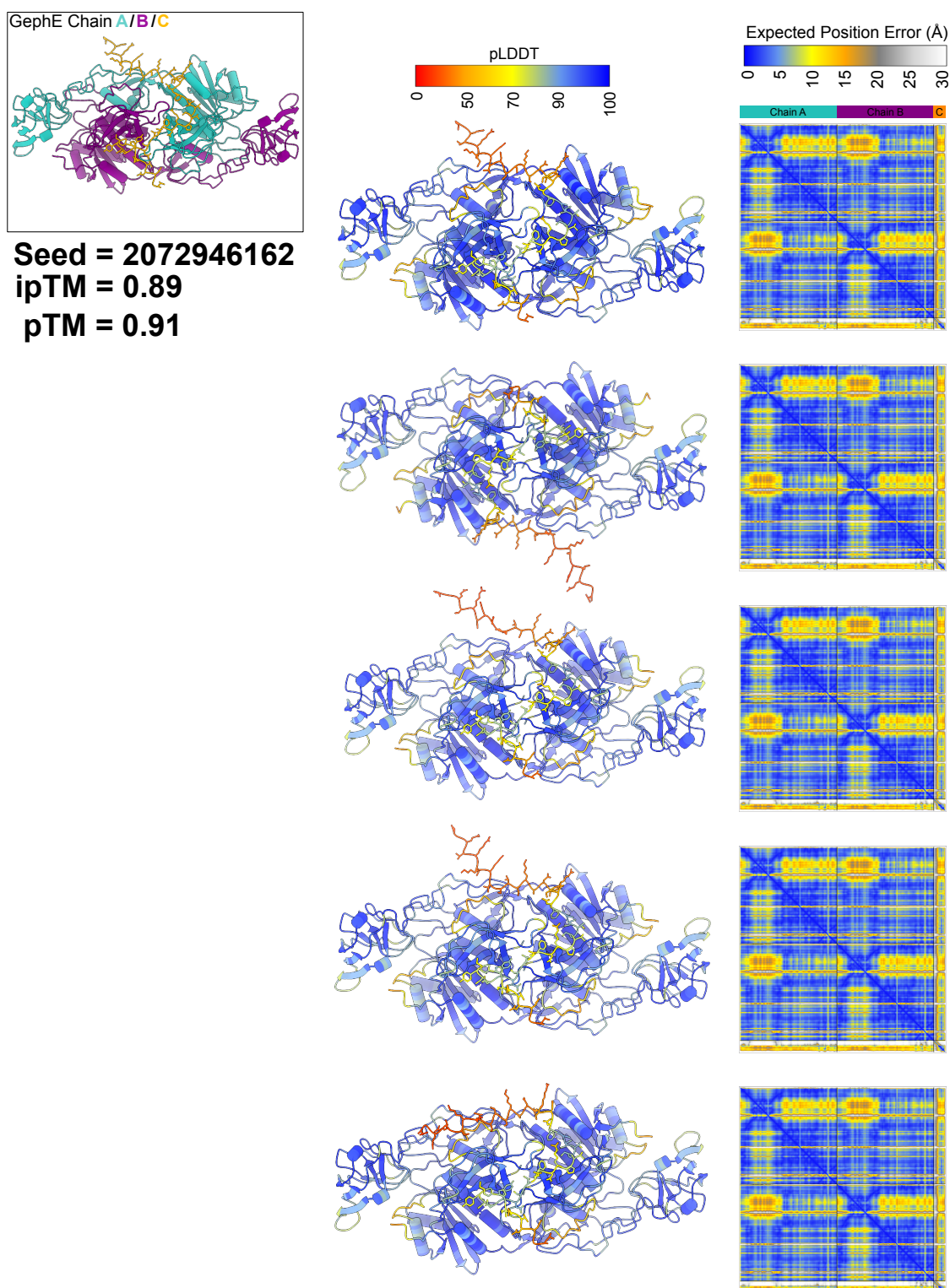
f) Alignment of Cnx1E with bound Moco-AMP (purple, PDB 6Q32) and Cnx1E without Moco-AMP (orange, PDB 5G2R). Changes in angular position and distance between SDII centroids are indicated.

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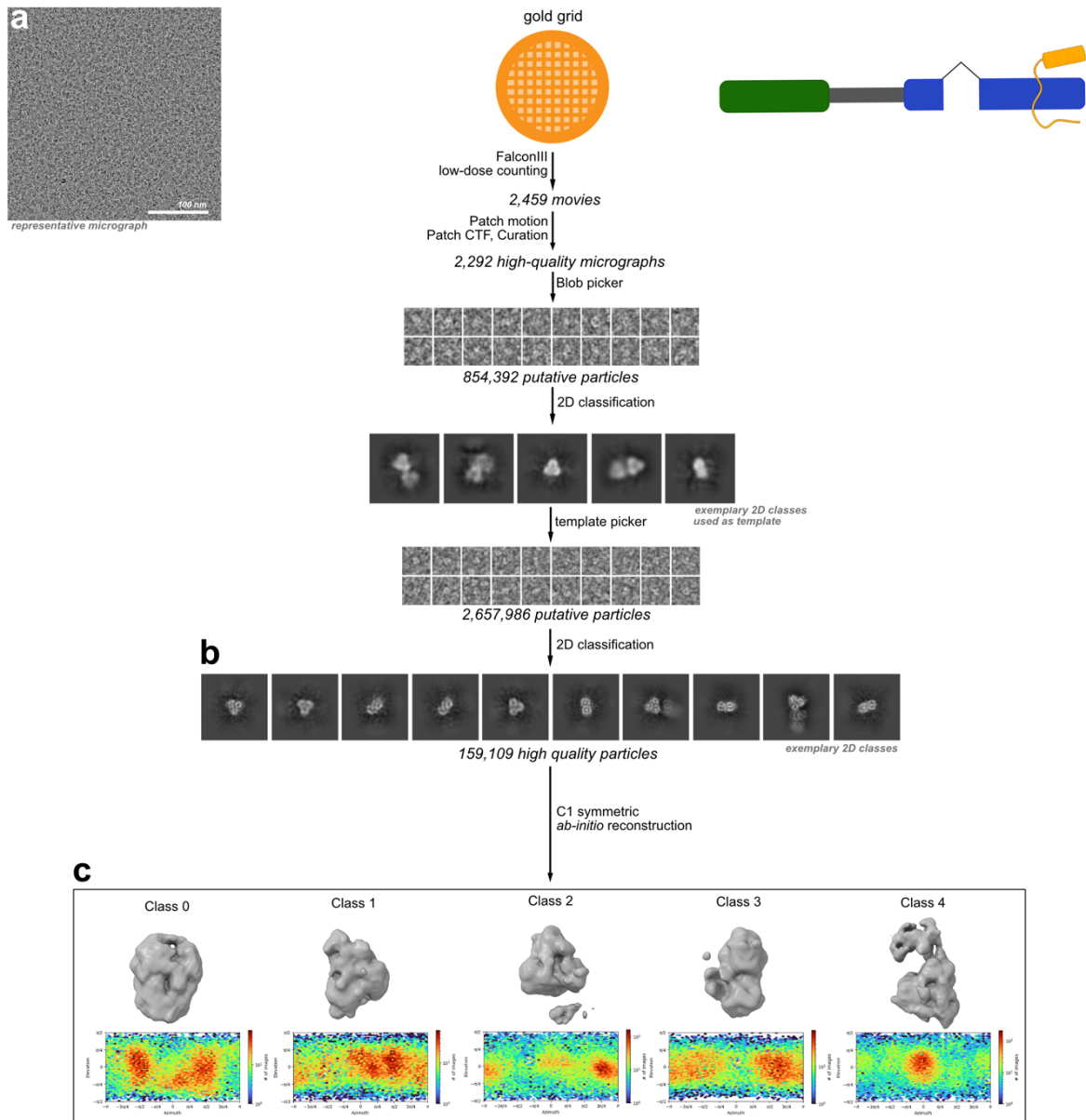
Supplementary Figure 10: Workflow of particle processing to elucidate position of GlyR-loop bound to GephE dimer. Particles from initial reconstruction which were used for initial 3D reconstruction (see Sup-Figure 6) were subjected to 3D classification utilizing a mask focussing on the core part of GephE dimer. Particles from classes showing strong density for GlyR-loop (a) were separately processed from particles with weak or no density for GlyR-loop (b). Both sets of particles were subjected to non-uniform and local refinements. Local refinements were performed with mask focussing on core part of the dimer. The resulting maps were low pass filtered to 6 Å. Scale bar 50 Å.

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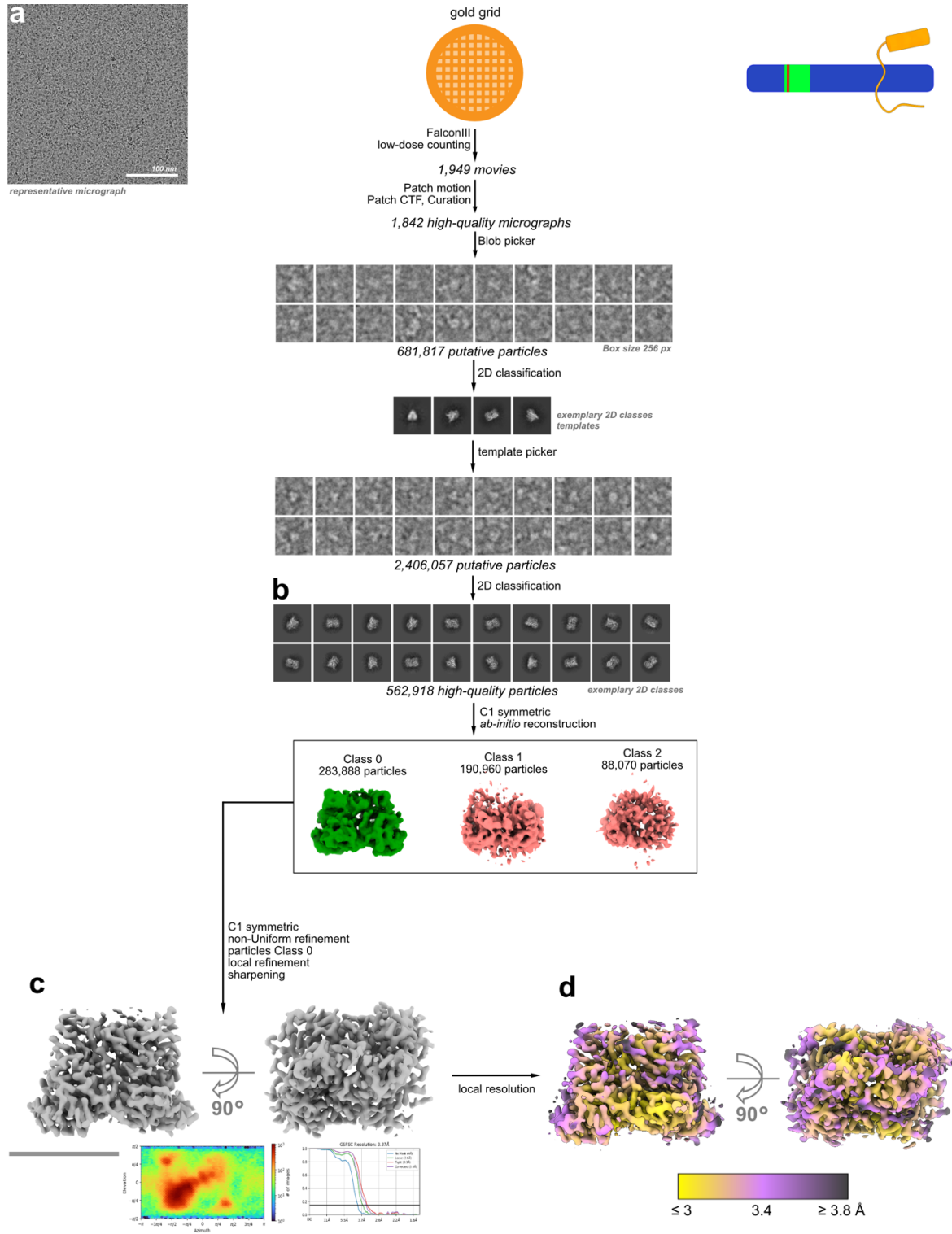
Supplementary Figure 11: Overview of AlphaFold 3 quality scores of GephE dimer in complex with GlyR-loop. Shown are the top five prediction models with predicted aligned error (PAE) and predicted local distance difference test (pLDDT). All structures were aligned as shown in the insert. GephE dimer (Chain A, B) and GlyR-loop (Chain C) are shown in cartoon and in stick representations respectively.

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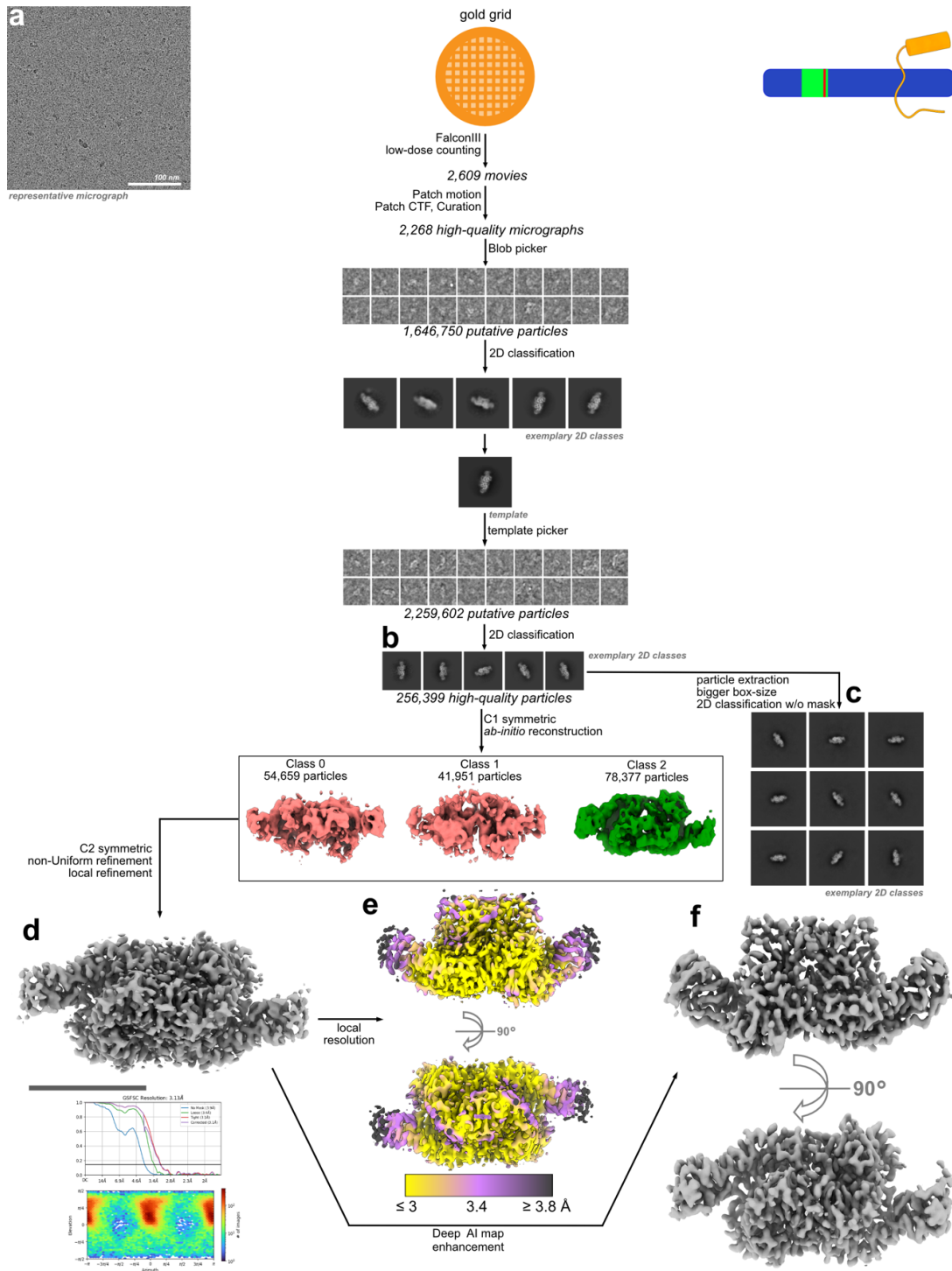
Supplementary Figure 12: Overview of Geph^{ASDII} with GlyR-loop cryo-EM data processing workflow. For the dataset micrographs were picked with good CTF scores and sufficient signal to noise ratio (a). Particles were first picked using a blob picker and subjected to unsupervised 2D classification followed by a second iteration of supervised 2D classification. The resulting 2D class averages depicting isolated particles from various angles along the rotational long axis of the protein (b). High quality particles were used for unsupervised ab-initio reconstruction classifying them into 5 classes. None of the resulting ab-initio classes depicted a map of high quality which could be further processed to high resolution. The cartoon bar diagram illustrates which Geph construct was used in this experiment (green: GephG, grey: GephC, blue: GephE, orange: GlyR-loop).

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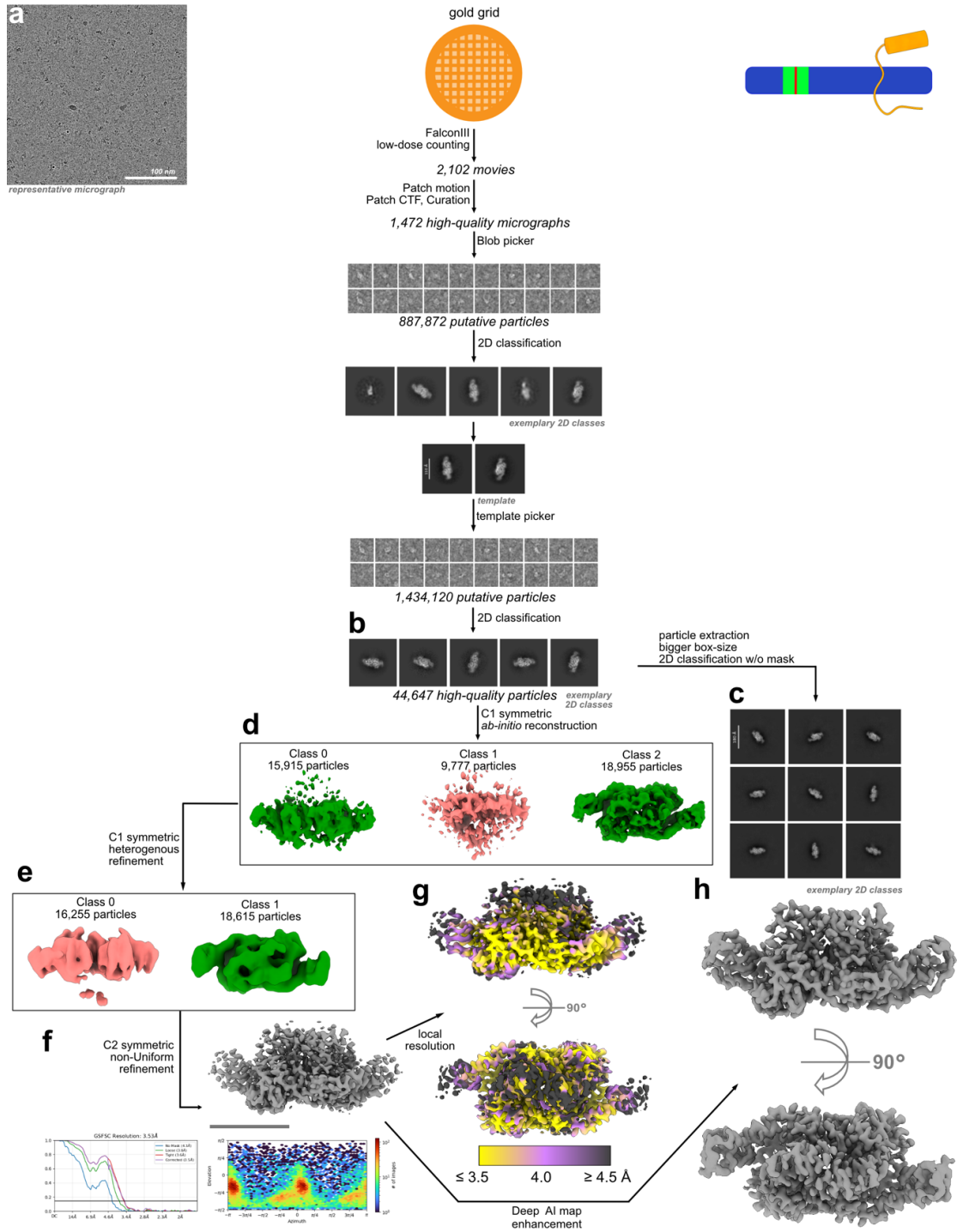
Supplementary Figure 13: Overview of GephE^{G375D} with GlyR-loop cryo-EM data processing workflow. For the dataset micrographs were picked with good CTF scores and sufficient signal to noise ratio (a). Particles were first picked using a blob picker and subjected to unsupervised 2D classification. The resulting 2D class averages depicting isolated particles from various angles (b). High quality particles were used for unsupervised ab-initio reconstruction classifying them into three classes. Particles from best ab-initio class, showing cohesive map and sufficient particle orientations, were further used for non-uniform and local refinement (c). Final map had a local resolution of approx. 3 – 3.8 Å (d). The cartoon bar diagram illustrates which Geph construct was used in this experiment (blue: GephE, light green: SDII, red: position of amino acid substitution, orange: GlyR-loop).

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Supplementary Figure 14: Overview of GephED^{D422N} with GlyR-loop cryo-EM data processing workflow. For the dataset micrographs were picked with good CTF scores and sufficient signal to noise ratio (a). Particles were first picked using a blob picker and subjected to unsupervised 2D classification followed by a second iteration of supervised 2D classification. The resulting 2D class averages depicting isolated particles from various angles along the rotational long axis of the protein (b). For comparison high quality particles were re-extracted with bigger box-size (512 px) and subjected to 2D classification. Resulting 2D class averages depicted highly isolated particles (c). High quality particles were used for unsupervised ab-initio reconstruction classifying them into three classes. Particles from best ab-initio class, showing cohesive map, were further used for non-uniform and local refinements with applied C2 symmetry (d). Final map had a local resolution of approx. 3 – 3.8 Å (e). For further map sharpening final map was subjected to deep AI map enhancement to adjust for minor anisotropy in the map (f). The cartoon bar diagram illustrates which Geph construct was used in this experiment (blue: GephE, light green: SDII, red: position of amino acid substitution, orange: GlyR-loop).

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Supplementary Figure 15: Overview of GephE^{R379D} with GlyR-loop cryo-EM data processing workflow. For the dataset micrographs were picked with good CTF scores and sufficient signal to noise ratio (a). Particles were first picked using a blob picker and subjected to unsupervised 2D classification followed by a second iteration of supervised 2D classification. The resulting 2D class averages depicting isolated particles from various angles along the rotational long axis of the protein (b). For comparison high quality particles were re-extracted with bigger box-size (512 px) and subjected to 2D classification. Resulting 2D class averages depicted highly isolated particles (c). High quality particles were used for unsupervised ab-initio reconstruction classifying them into three classes (d). Particles from best ab-initio class, showing cohesive map, were further used for heterogenous refinement with two classes (e). Particles from heterogenous refinement class 1 were used for further non-uniform refinement with applied C2 symmetry (f). Final map had a local resolution of approx. 3.5 – 4.5 Å (g). For further map sharpening final map was subjected to deep AI map enhancement to adjust for minor anisotropy in the map (h). Scale bar 50 Å. The cartoon bar diagram illustrates which Geph construct was used in this experiment (blue: GephE, light green: SDII, red: position of amino acid substitution, orange: GlyR-loop).

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Supplementary Table 1: Statistics and additional information of the refinement and model building process

Geph ^{EW} -GlyR loop		untilted	25° tilt
Sample conditions			
Sample concentration	0.5 mg/ml		
GlyR-loop	3x fold excess		
Sample preparation	vitrification		
Grid type	UltraUfoil R1.2/1.3 (300 mesh)		
EM data collection			
Microscope	Titan Krios G3i		
Voltage (kV)	300		
Spherical aberration Cs (mm)	2.7		
Condenser C2 aperture size (μm)	70		
Objective aperture size (μm)	100		
Camera	Falcon III		
Pixel size (Å)	0.862		
Total dose (electron*Å ⁻²)	31		
Exposure time (sec)	40		
Number of frames	42		
Exposures per hole	2		
Energy filter	None		
Defocus range (μm)	-2.6 to -0.5		
# micrographs collected	1,572	5,260	
% micrographs used	82	86	
EM data processing			
Software	cryoSPARC v4.4		
Picked particles	1,400,859	4,953,014	
Particles after 2D classification	133,365	1,194,261	
Particles after 3D sorting	120,264		
Resolution (FSC 0.143, Å)	3.6		
Model building and refinement			
Software for building	Coot 0.9.8.92		
Residues build	327-736		
Software for refinement	PHENIX 1.21-5207		
Composition (#)			
Chains	3		
Atoms	6105 (Hydrogens: 0)		
Residues	Protein: 819		
Water	0		
Ligands	0		
Bonds (RMSD)			
Length (Å) (# > 4σ)	0.005		
Angles (°) (# > 4σ)	1.024		
Ramachandran plot (%)			
Outliers	0.86		
Allowed	12.70		
Favored	86.44		
Rotamer Outliers (%)	0.0		
MolProbity score	1.99		

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Geph^{WT}-GlyR loop		
Sample conditions		
Sample concentration	0.005 mg/ml	
GlyR-loop	3x fold excess	
Sample preparation	Negative stain w/ uranyl formate	
Grid type	Continuous carbon (200 mesh)	
EM data collection		
Microscope	Talos L120C	
Voltage (kV)	120	
Spherical aberration Cs (mm)	2.7	
Condenser C2 aperture size (μm)	70	
Objective aperture size (μm)	100	
Camera	Ceta16M	
Pixel size (Å)	1.86	
Total dose (electron*Å ⁻²)	40	
Exposure time (sec)	1	
Number of frames	N/A	
Exposures per hole	1	
Energy filter	None	
Defocus range (μm)	-2.0 to -0.3	
# micrographs collected	1,896	
% micrographs used	86	
EM data processing		
Software	cryoSPARC v4.4	
Picked particles	504,230	
Particles after 2D classification	115,959	
Particles after 3D sorting	GephG+E: 27,335	GephE+E: 2,278
Resolution (FSC 0.143, Å)	GephG+E: 6.3	GephE+E: 15.5

Geph^{WT}	
Sample conditions	
Sample concentration	0.005 mg/ml
GlyR-loop	none
Sample preparation	Negative stain w/ uranyl formate
Grid type	Continuous carbon (200 mesh)
EM data collection	
Microscope	Talos L120C
Voltage (kV)	120
Spherical aberration Cs (mm)	2.7
Condenser C2 aperture size (μm)	70
Objective aperture size (μm)	100
Camera	Ceta16M
Pixel size (Å)	1.86
Total dose (electron*Å ⁻²)	40
Exposure time (sec)	1
Number of frames	N/A
Exposures per hole	1
Energy filter	None
Defocus range (μm)	-2.0 to -0.3
# micrographs collected	2,027
% micrographs used	97
EM data processing	
Software	cryoSPARC v4.4
Picked particles	650,399
Particles after 2D classification	119,786
Particles after 3D sorting	12,952
Resolution (FSC 0.143, Å)	6.8

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<i>Geph^{WT}</i>-GlyR loop	
Sample conditions	
Sample concentration	0.5 mg/ml
GlyR-loop	3x fold excess
Sample preparation	vitrification
Grid type	UltrAUfoil R1.2/1.3 (300 mesh)
EM data collection	
Microscope	Titan Krios G3i
Voltage (kV)	300
Spherical aberration Cs (mm)	2.7
Condenser C2 aperture size (μm)	70
Objective aperture size (μm)	100
Camera	Falcon III
Pixel size (Å)	0.862
Total dose (electron*Å ⁻²)	31
Exposure time (sec)	40
Number of frames	42
Exposures per hole	2
Energy filter	None
Defocus range (μm)	-2.6 to -0.5
# micrographs collected	1,006
% micrographs used	94
EM data processing	
Software	cryoSPARC v4.4
Picked particles	486,680
Particles after 2D classification	256,399

<i>Geph^{WT}</i>	
Sample conditions	
Sample concentration	0.5 mg/ml
GlyR-loop	none
Sample preparation	vitrification
Grid type	UltrAUfoil R1.2/1.3 (300 mesh)
EM data collection	
Microscope	Titan Krios G3i
Voltage (kV)	300
Spherical aberration Cs (mm)	2.7
Condenser C2 aperture size (μm)	70
Objective aperture size (μm)	100
Camera	Falcon III
Pixel size (Å)	0.862
Total dose (electron*Å ⁻²)	31
Exposure time (sec)	40
Number of frames	42
Exposures per hole	2
Energy filter	None
Defocus range (μm)	-2.6 to -0.5
# micrographs collected	2468
% micrographs used	83
EM data processing	
Software	cryoSPARC v4.4
Picked particles	296,216

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<i>Geph^{ASDII}- GlyR loop</i>	
Sample conditions	
Sample concentration	0.5 mg/ml
GlyR-loop	3x fold excess
Sample preparation	vitrification
Grid type	UltrAUfoil R1.2/1.3 (300 mesh)
EM data collection	
Microscope	Titan Krios G3i
Voltage (kV)	300
Spherical aberration Cs (mm)	2.7
Condenser C2 aperture size (μm)	70
Objective aperture size (μm)	100
Camera	Falcon III
Pixel size (Å)	0.862
Total dose (electron*Å ⁻²)	31
Exposure time (sec)	40
Number of frames	42
Exposures per hole	2
Energy filter	None
Defocus range (μm)	-2.6 to -0.5
# micrographs collected	2459
% micrographs used	93
EM data processing	
Software	cryoSPARC v4.4
Picked particles	854,392
Particles after 2D classification	159,109

<i>Geph^{G375D}- GlyR loop</i>	
Sample conditions	
Sample concentration	0.5 mg/ml
GlyR-loop	3x fold excess
Sample preparation	vitrification
Grid type	UltrAUfoil R1.2/1.3 (300 mesh)
EM data collection	
Microscope	Titan Krios G3i
Voltage (kV)	300
Spherical aberration Cs (mm)	2.7
Condenser C2 aperture size (μm)	70
Objective aperture size (μm)	100
Camera	Falcon III
Pixel size (Å)	0.862
Total dose (electron*Å ⁻²)	31
Exposure time (sec)	40
Number of frames	42
Exposures per hole	2
Energy filter	None
Defocus range (μm)	-2.6 to -0.5
# micrographs collected	1949
% micrographs used	94
EM data processing	
Software	cryoSPARC v4.4
Picked particles	2,406,057
Particles after 2D classification	562,918
Particles after 3D sorting	283,888
Resolution (FSC 0.143, Å)	3.4

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<i>Geph^{D422N}- GlyR loop</i>	
Sample conditions	
Sample concentration	0.5 mg/ml
GlyR-loop	3x fold excess
Sample preparation	vitrification
Grid type	UltrAUFoil R1.2/1.3 (300 mesh)
EM data collection	
Microscope	Titan Krios G3i
Voltage (kV)	300
Spherical aberration Cs (mm)	2.7
Condenser C2 aperture size (μm)	70
Objective aperture size (μm)	100
Camera	Falcon III
Pixel size (Å)	0.862
Total dose (electron*Å ⁻²)	31
Exposure time (sec)	40
Number of frames	42
Exposures per hole	2
Energy filter	None
Defocus range (μm)	-2.6 to -0.5
# micrographs collected	2609
% micrographs used	87
EM data processing	
Software	cryoSPARC v4.4
Picked particles	2,259,602
Particles after 2D classification	256,399
Particles after 3D sorting	78,377
Resolution (FSC 0.143, Å)	3.1

<i>Geph^{R379D}- GlyR loop</i>	
Sample conditions	
Sample concentration	0.5 mg/ml
GlyR-loop	3x fold excess
Sample preparation	vitrification
Grid type	UltrAUFoil R1.2/1.3 (300 mesh)
EM data collection	
Microscope	Titan Krios G3i
Voltage (kV)	300
Spherical aberration Cs (mm)	2.7
Condenser C2 aperture size (μm)	70
Objective aperture size (μm)	100
Camera	Falcon III
Pixel size (Å)	0.862
Total dose (electron*Å ⁻²)	31
Exposure time (sec)	40
Number of frames	42
Exposures per hole	2
Energy filter	None
Defocus range (μm)	-2.6 to -0.5
# micrographs collected	2,102
% micrographs used	70
EM data processing	
Software	cryoSPARC v4.4
Picked particles	2,259,602
Particles after 2D classification	1,434,120
Particles after 3D sorting	18,615
Resolution (FSC 0.143, Å)	3.5

Chapter 5: General discussion

Disruption of the coordinate network between excitatory and inhibitory signaling, as observed in neurological disorders such as epilepsy and hyperekplexia, profoundly affects central nervous system function. Inhibitory neurotransmission is primarily driven by gamma-aminobutyric acid type A receptors (GABA_ARs) and glycine receptors (GlyRs). Proper localization and stabilization of these receptors at inhibitory synapses is essential for their function and is orchestrated by the cytosolic scaffold protein gephyrin, which anchors many subtypes of inhibitory receptors through interactions with their intracellular loops/domains (Kim *et al*, 2006; Fritschy *et al*, 2008; Maric *et al*, 2011).

Recent work has suggested that interactions between receptor intracellular domains and the gephyrin E-domain can drive liquid–liquid phase separation (LLPS), leading to the formation of protein-dense assemblies at inhibitory postsynapses (Bai *et al*, 2021). In this context, this thesis investigated the molecular mechanisms underlying gephyrin LLPS, with a focus on structural determinants, regulatory modifications, and higher-order network assembly.

The work presented here integrates three complementary aspects of gephyrin LLPS. First, a patient-derived mutation within the G-domain (G134R) revealed a critical role of this domain as a connectivity node that governs higher-order oligomerization and phase separation. The disruption of G-domain trimerization impairs network formation and provides a mechanistic link between altered LLPS behavior and severe neurological phenotypes (Chapter 2). Second, redox-dependent post-translational modifications, particularly reversible cysteine oxidation, are identified as regulatory elements that modulate gephyrin multimerization, condensate stability, and receptor-binding capacity. These findings demonstrate that LLPS is not solely driven by intrinsic protein interactions but is dynamically tuned by the cellular environment (Chapter 3). Third, structural analyses uncover a filament-based organization of gephyrin assemblies, in which receptor binding promotes the formation of extended E-domain filaments that are essential for LLPS and synaptic clustering (Chapter 4). Together, these findings provide a structural and mechanistic framework for understanding how gephyrin forms higher-order assemblies at inhibitory synapses.

5.1 Structural reinterpretation of inhibitory postsynapses

The structural organization of inhibitory postsynapses has undergone continuous refinement over the past two decades, reflecting both advances in experimental methodologies and an evolving understanding of gephyrin as a scaffold protein. Early models were primarily derived from biochemical and structural properties of isolated domains, whereas more recent concepts incorporate dynamic behavior, heterogeneous interactions, and emerging biophysical principles such as LLPS.

The first widely adopted structural model of gephyrin organization was the hexagonal lattice model, which was based on the intrinsic oligomerization properties of gephyrin (Kneussel & Betz, 2000). In this model, E-domain dimers form the sides of a hexagon, while G-domain trimers define the vertices, resulting in a planar and highly ordered lattice (Figure 7 left). While this arrangement provided an intuitive framework for synaptic organization, it was largely derived from logical extrapolation of domain behavior and has not been experimentally validated. Moreover, such a structure would impose significant steric constraints, leading to a rigid, flat, and highly symmetrical assembly that is difficult to reconcile with the dynamic nature of synapses.

Subsequent advances in imaging challenged the feasibility of this strictly planar architecture. Single-molecule localization microscopy and electron tomography studies revealed that the iPSD has a thickness of approximately 10–20 nm, which would not readily accommodate an extended planar lattice (Specht *et al*, 2013; Tao *et al*, 2018). In addition, considering Brownian motion and the crowded intracellular environment, a perfectly ordered and symmetric lattice would likely lack the flexibility required for stable yet adaptable synaptic assemblies.

This led to the development of more dynamic models, most prominently the “slot model”, which accounts for the temporal plasticity of synaptic components (Figure 7 left). Time-lapse imaging studies demonstrated that synapses can persist for days while their molecular composition is continuously remodeled on timescales ranging from seconds to hours (Rubinski & Ziv, 2015). Within this framework, gephyrin provides discrete binding “slots” that can be transiently occupied by receptors and interacting proteins. Experimental support for this model comes from single-particle tracking and quantum dot studies, which revealed alternating phases of receptor immobilization and exchange within the same synaptic compartment (Renner *et al*, 2017; Specht *et al*, 2013). Furthermore, fluorescence recovery after photobleaching (FRAP) experiments

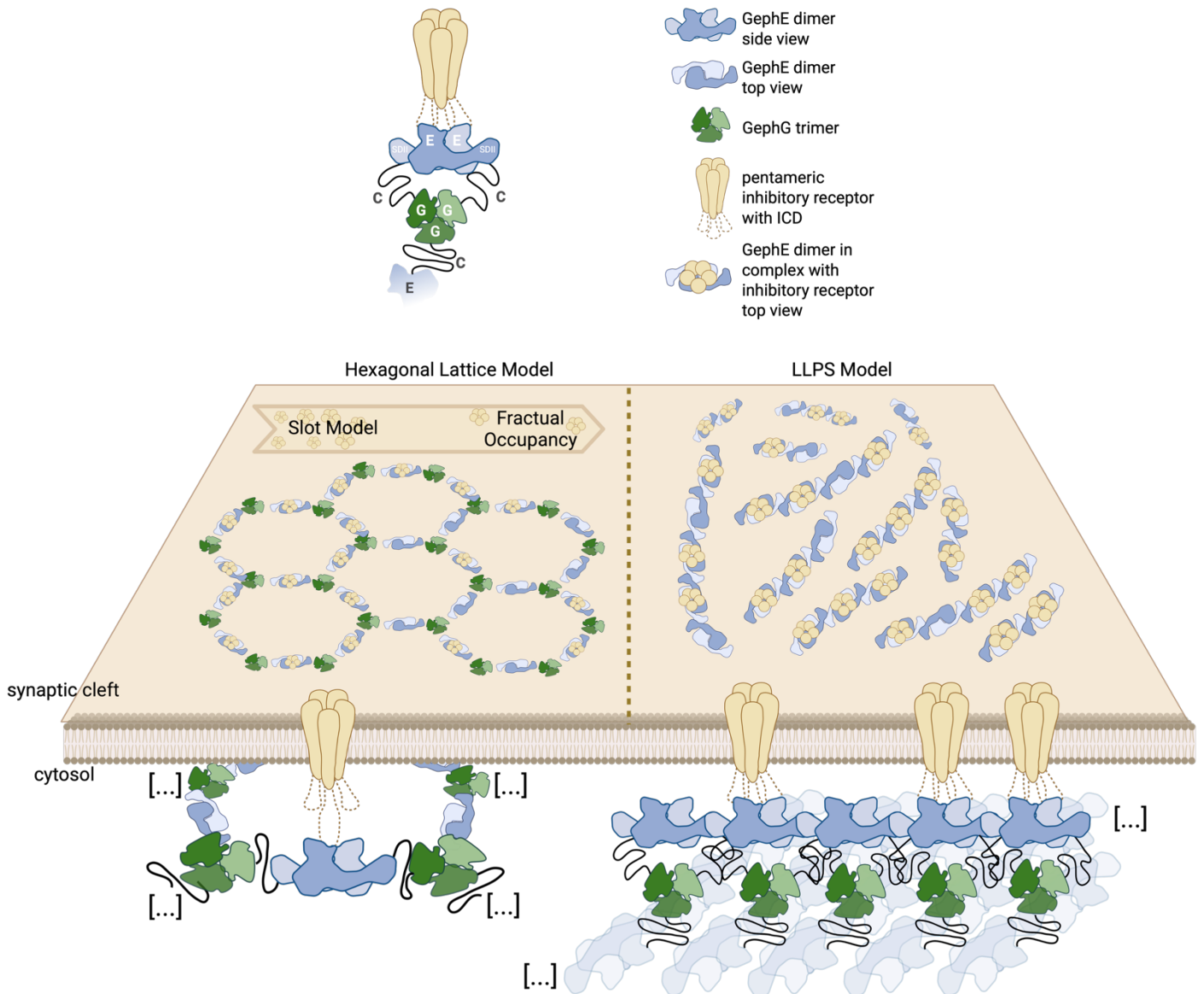


Figure 7: Comparison of models for gephyrin postsynaptic density organization.

The left panel illustrates the hexagonal lattice model originally proposed by Kneussel and Betz, including later refinements that account for binding to pentameric ligand-gated ion channels. In this model, gephyrin forms a flat, two-dimensional lattice, with E- and G-domains arranged within the same planar level.

The right panel depicts the filament-based LLPS model, in which multiple separate E-domain filaments are interconnected by a distinct layer of G-domain assemblies. This organization may extend into additional layers of E-domain filaments, giving rise to a three-dimensional, multilayered condensate architecture. (Figure created with BioRender)

showed that approximately 40% of the gephyrin pool is exchanged within 30 minutes, corresponding to recovery time constants of roughly two hours (Calamai *et al*, 2009). Similar dynamic behavior has been observed in hippocampal slice preparations and *in vivo* systems such as zebrafish larvae (Vlachos *et al*, 2013; Chow *et al*, 2017).

While the slot model introduced an essential dynamic component, it still largely retained the assumption of an underlying scaffold organization based on alternating G- and E-domain interactions. At the same time, biochemical and structural studies began

to reveal limitations of this framework. The central C-domain was shown to inhibit E-domain dimerization *in vitro*, suggesting that full lattice formation may be restricted in the cytosolic context (Bedet *et al*, 2006; Sander *et al*, 2013). Consistently, single-molecule localization microscopy indicated that cytosolic gephyrin predominantly exists in a trimeric state in cells, supporting the idea that G-domain assemblies are favored under basal conditions (Patrizio *et al*, 2017). In contrast, E-domain dimerization remains essential for high-affinity receptor binding (Kim *et al*, 2006; Maric *et al*, 2014). Importantly, there is no evidence that G-domain trimers dissociate in cells, and biophysical studies have shown that G-domain trimerization is more stable than E-domain dimerization (Schwarz *et al*, 2001; Herweg & Schwarz, 2012; Sander *et al*, 2013). Together, these observations highlight an intrinsic asymmetry in gephyrin oligomerization and further argue against a rigid, fully symmetric lattice.

A further conceptual advance was introduced with the notion of fractional occupancy, which describes how binding sites within a scaffold are not fully saturated but instead occupied in a sub-stoichiometric manner (Figure 7 left) (Hein *et al*, 2015). This concept is particularly relevant for gephyrin, where binding partners display a wide range of affinities. The GlyR β subunit interacts with gephyrin with relatively high affinity (K_D 14 nM–2 μ M), whereas interactions with GABA_AR subunits are generally weaker and span the micromolar range (K_D α 1/3 with 5-17 μ M and β 2 with 17-55 μ M) (Grünewald *et al*, 2018; Maric *et al*, 2014, 2011; Mukherjee *et al*, 2011; Tretter *et al*, 2011; Kowalczyk *et al*, 2013). It is worth noting that GABA_ARs engage gephyrin through a greater number of interacting subunits than GlyRs, and this increased multiplicity of contacts could partially compensate for the individually weaker binding affinities through cooperative effects. Beyond mere compensation, however, the overall weaker GABA_AR–gephyrin interaction may be functionally significant in its own right: lower affinity intrinsically favors faster exchange kinetics and greater receptor mobility. GABAergic synapses have been more extensively shown to undergo multiple forms of plasticity, including presynaptic, postsynaptic, structural, and chloride-dependent forms of regulation (Flores & Méndez, 2014). Consistent with this interpretation, existing evidence supports subunit-specific differences in synaptic mobility, with several GABA_AR subtypes appearing less stably scaffolded than GlyR complexes, a property that directly reflects their weaker gephyrin-binding affinities (Maric *et al*, 2014). In combination with the differing strengths of G-domain trimerization and E-domain dimerization, this results in a heterogeneous interaction landscape composed of both

strong and weak interactions. Such a mixture is thought to enable the formation of stable local clusters that remain interconnected through more transient interactions, thereby preventing the isolation of highly stable but disconnected assemblies.

First indications of a more complex structural organization were observed in electron tomography studies, where filamentous architectures were detected within inhibitory synapses (Linsalata *et al*, 2014). However, the molecular basis of these assemblies remained unclear. With the emerging recognition of LLPS as a conceptual framework for postsynaptic organization, new interpretations were developed. It was shown that gephyrin, particularly through its E-domain interactions, has an intrinsic propensity to undergo LLPS, leading to the formation of condensed protein assemblies enriched in receptors (Figure 7 right) (Bai *et al*, 2021). These assemblies appear to be interconnected, potentially via contributions from both the G-domain and the C-domain, although the precise role of the C-domain remains under debate (Lee *et al*, 2024; Bai *et al*, 2021).

Building on these findings, structural work presented in Chapter 4 of this thesis demonstrates that gephyrin E-domain dimers assemble into filaments through specific interactions between subdomain II (SDII) regions. Disruption of this interface abolishes filament formation, LLPS, and synaptic receptor clustering, establishing a direct structural link between filament assembly and postsynaptic density formation (Macha *et al*, 2025). This raises the question of whether LLPS drives filament formation or whether filaments constitute the structural basis of LLPS. Rather than being mutually exclusive, these processes may represent two aspects of the same underlying phenomenon, where filamentous assemblies and phase-separated condensates coexist and reinforce each other. The locally increased protein concentrations observed in our structural analyses ($\sim 0.2\text{--}0.7\text{ mM}$) support the idea that a critical threshold for condensate formation is reached. Consistently, molecule counting estimates $\sim 130\text{--}190$ gephyrin molecules at GABAergic and $\sim 300\text{--}600$ at glycinergic synapses correspond to similar local concentrations of $\sim 0.2\text{--}0.3\text{ mM}$ and $\sim 0.5\text{--}1.0\text{ mM}$, respectively, given an approximate thickness of 14 nm and an area of $\sim 0.07\text{ }\mu\text{m}^2$ (Specht *et al*, 2013; Patrizio *et al*, 2017; Nusser *et al*, 1998; Tao *et al*, 2018; Liu *et al*, 2020). These values further support that gephyrin operates within a concentration regime permissive for LLPS *in vivo*.

Importantly, the work presented in this thesis extends this structural framework by introducing additional levels of connectivity. In Chapter 2, the G-domain is identified

as a critical and indispensable node that governs higher-order interconnectivity, one that acts in concert with, but is not redundant to, E-domain-mediated filament formation. A central finding is that the E-domain filaments alone are insufficient to drive LLPS *in vivo*; rather, G-domain trimerization constitutes a required second axis of connectivity that bridges filaments into a cohesive, phase-separating network. This is compellingly demonstrated by the G134R variant, in which G-domain trimerization is selectively disrupted: despite the presumed integrity of E-domain filaments, network formation and LLPS are abolished, causally establishing G-domain-mediated inter-filament connectivity as a non-redundant requirement for condensate assembly in the cellular context. In parallel, regulatory contributions of the C-domain are proposed to further modulate network organization, as described in Chapter 3. These findings suggest that gephyrin assemblies are not limited to linear filaments but instead form branched and interconnected networks. Within this context, LLPS can be understood as the emergent property of a percolated system in which multivalent interactions give rise to a network spanning the synaptic space and forming a dense yet dynamic molecular scaffold.

Together, these observations support a fundamental shift in the conceptualization of inhibitory postsynapses. Rather than static, planar lattices, gephyrin assemblies are better described as dynamic, filamentous, and interconnected networks capable of undergoing LLPS. This framework reconciles structural observations with the dynamic behavior of synapses and provides a mechanistic basis for understanding how mutations and regulatory modifications can profoundly alter synaptic organization. The implications of this network-based perspective, particularly in the context of percolation and system-wide connectivity, will be discussed in the following section.

5.2 Molecular principles of gephyrin LLPS and percolated networks

LLPS provides a framework to understand how gephyrin organizes inhibitory postsynapses beyond classical scaffold models (Bai & Zhang, 2022). In contrast to stoichiometric type of interactions in dilute solutions, LLPS arises when favorable molecule–molecule interactions outweigh molecule–solvent interactions, leading to the formation of a dense phase that coexists with a surrounding dilute phase (Alberti *et al*, 2019). Importantly, this separation does not imply static assemblies; rather, molecules continuously exchange between the condensate and the surrounding environment at

thermodynamic equilibrium. A key advantage of such a mechanism lies in its ability to locally concentrate molecules without requiring uniformly high cellular abundance. If molecular enrichment were solely governed by diffusion and specific binding, concentration gradients would necessitate large overall protein quantities to achieve sufficient local accumulation. In contrast, phase separation allows for differences in concentration between phases that can span several orders of magnitude, enabling efficient and spatially restricted enrichment. This principle is particularly relevant for synaptic compartments such as the PSD, where high local concentrations must be achieved within extremely confined volumes (Chen *et al*, 2020).

To further conceptualize the process of condensation, LLPS can be extended to the framework of percolation theory. The term “percolation,” derived from the Latin *percolare* (“to filter through”), describes the emergence of connectivity within a system as individual components become increasingly linked. In statistical physics, percolation theory addresses how the addition of nodes (p) or interactions leads to a transition from small, disconnected clusters to a system-spanning network. This transition occurs at a critical threshold ($p > p_c$), above which a connected “spanning cluster” emerges. Below this threshold, only finite clusters exist, whereas near the critical point, networks display highly heterogeneous connectivity (Shante & Kirkpatrick, 1971; Saberi, 2015). This can be envisioned through an electrical honeycomb analogy (Figure 8A): below the percolation threshold ($p < p_c$), the system consists of multiple disconnected clusters, represented by distinct colors, none of which spans the entire network. Were these clusters to conduct electrical current, the circuit would remain open and the light bulb would not illuminate. Once a single cluster percolates across the system ($p > p_c$), shown in orange, a continuous conductive pathway is established, closing the circuit and allowing current to flow, and the light bulb turns on. As further nodes would be added and connectivity increases beyond the critical threshold, the remaining isolated clusters would be progressively incorporated into the spanning network, until ultimately the entire system coalesces into a single, fully connected cluster, represented by a uniform color.

Biological condensates exhibit properties consistent with the above described percolated systems. Rather than behaving as simple liquids, they are best described as dense, viscoelastic materials in which molecular motion is heterogeneous. Molecules within the “condensed phase” alternate between more mobile “m” states and transiently confined “c” states, reflecting their incorporation into an underlying

interaction network (Figure 8B). This view has been experimentally supported in reconstituted postsynaptic assemblies and stress granule systems, where adaptive single-molecule tracking revealed that molecules spend substantial time in motion-restricted states due to transient trapping within a multivalent network (Shen *et al.*, 2023). These networks arise from a combination of strong, specific interactions and weaker, multivalent contacts, resulting in high local enrichment and sub-diffusive dynamics. This distinction is illustrated in Figure 8C, which contrasts two condensate architectures: on the right, only weak multivalent interactions are present, allowing

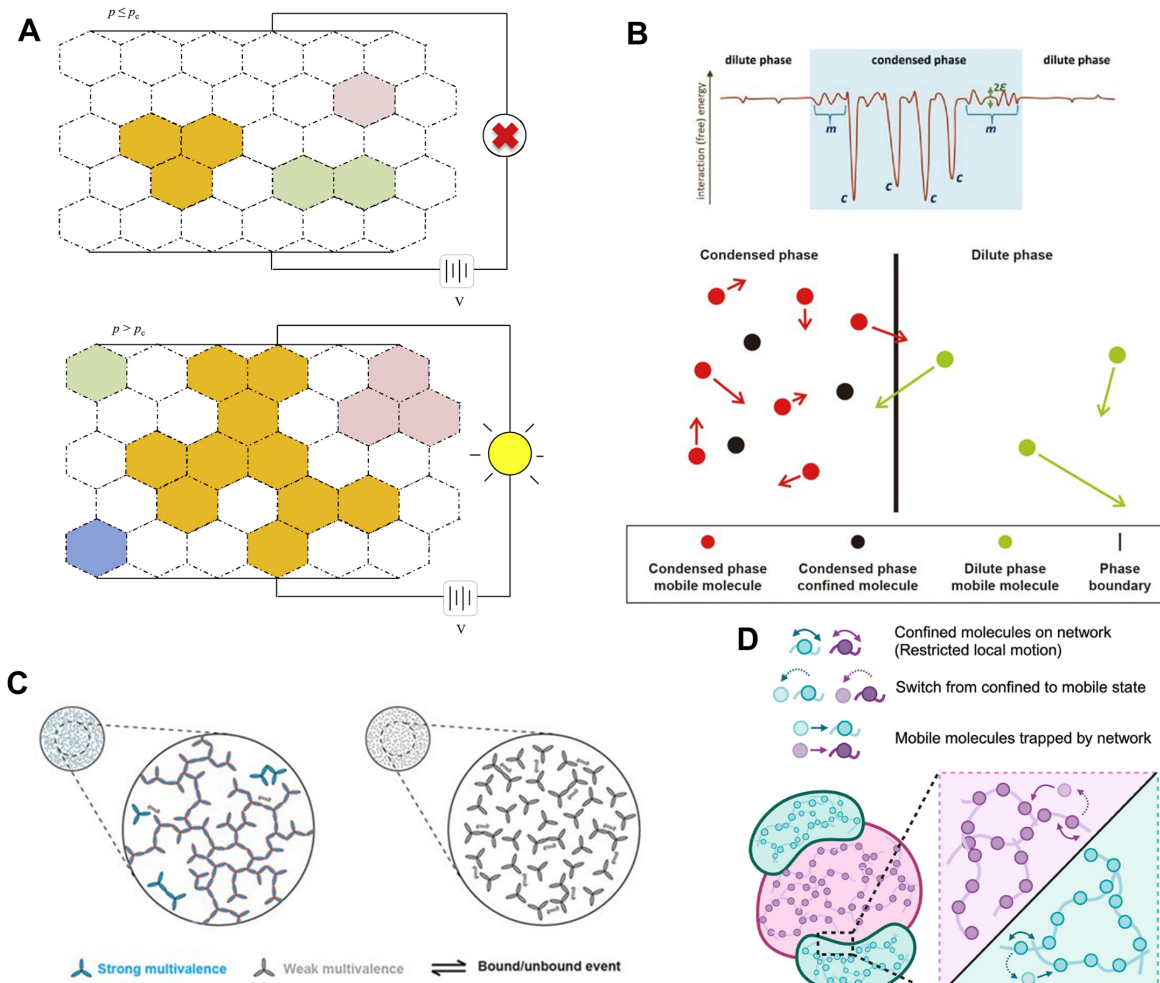


Figure 8: Conceptual framework of percolation and network organization in biomolecular condensates.

A) Illustration of a percolation process using an electrical circuit analogy. A voltage source and bulb are connected via a honeycomb lattice composed of insulating elements, in which a fraction p of sites is occupied by conductive hexagons. Upon reaching a critical threshold (p_c), a continuous, system-spanning conductive cluster emerges, enabling current flow and resulting in illumination of the bulb (adapted from Saberi, 2015). **B)** Schematic representation of the free energy landscape associated with phase separation. Mobile (m) and confined (c) states (adapted from Shen *et al.*, 2023). **C)** Depiction of how a combination of strong and weak multivalent interactions facilitates the formation of system-spanning networks (adapted from Shen *et al.*, 2023). **D)** Schematic illustrating demixing of coexisting condensates driven by network formation (adapted from Zhu *et al.*, 2024).

molecules to transiently contact a broad range of binding partners and exchange rapidly between bound and unbound states. On the left, the superposition of additional strong multivalent interactions, depicted in blue, renders the network percolated, molecules become progressively trapped within the mesh, the frequency of discrete binding and unbinding events is reduced, and mobility is globally suppressed. Importantly, slow fluorescence recovery as observed in single molecule studies in such systems (Triller & Choquet, 2008) does not necessarily reflect globally slow diffusion but can instead arise from repeated binding and release within a percolated network.

Such principles have also been demonstrated for excitatory postsynapses, where multivalent interactions between core scaffold proteins such as PSD-95, Shank, GKAP, Homer, and receptor subunits give rise to a percolated network that defines the structural and functional properties of the ePSD (Shen *et al*, 2023). Perturbations at specific nodes of this network, for example through competing peptides or splice variants that disrupt key interactions, can selectively alter connectivity at different hierarchical levels, emphasizing the modular and tunable nature of percolated systems (Jia *et al*, 2025).

Extending this framework to inhibitory synapses, several lines of evidence suggest that gephyrin assemblies likewise operate as percolated networks. Notably, at mixed synapses containing both excitatory and inhibitory components, phase-separated assemblies remain spatially segregated even when strong artificial interactions are introduced. In this context, forced mislocalization of gephyrin to excitatory postsynapses via a PSD-95–targeting intrabody with high affinity ($K_D \approx 4$ nM) failed to overcome this segregation, indicating that the intrinsic organization of these condensates is stabilized by cumulative forces exceeding even strong binary interactions (Zhu *et al*, 2024). This observation implies that gephyrin-based assemblies are not maintained by isolated high-affinity binding events but rather by a dense, multivalent network of interactions that collectively generate a robust and self-organizing scaffold that builds a distinct and separate condensate (Figure 8D). This is again underscored by the architecture of GABA_ARs themselves, which comprise multiple subunits each capable of binding gephyrin, and while these interactions are individually weak, their cooperative and multivalent engagement reinforces the percolated network, amplifying scaffold stability beyond what any single binding event could achieve.

Within this context, gephyrin can be understood as a system composed of distinct interaction modules with different functional roles. Strong interactions, such as G-domain trimerization and E-domain-mediated filament formation, act as high-valency nodes or “stickers”, whereas more flexible and dynamic regions are “spacers”, including the weaker interacting IDR of the C-domain. Together all of them facilitate network connectivity (Figure 9). Notably, the distinction between “stickers” and “spacers” is not restricted to intrinsically disordered regions but can also exist within folded domains, where specific subregions mediate interaction hotspots while others primarily serve structural roles (Choi *et al*, 2020). In light of the structural findings

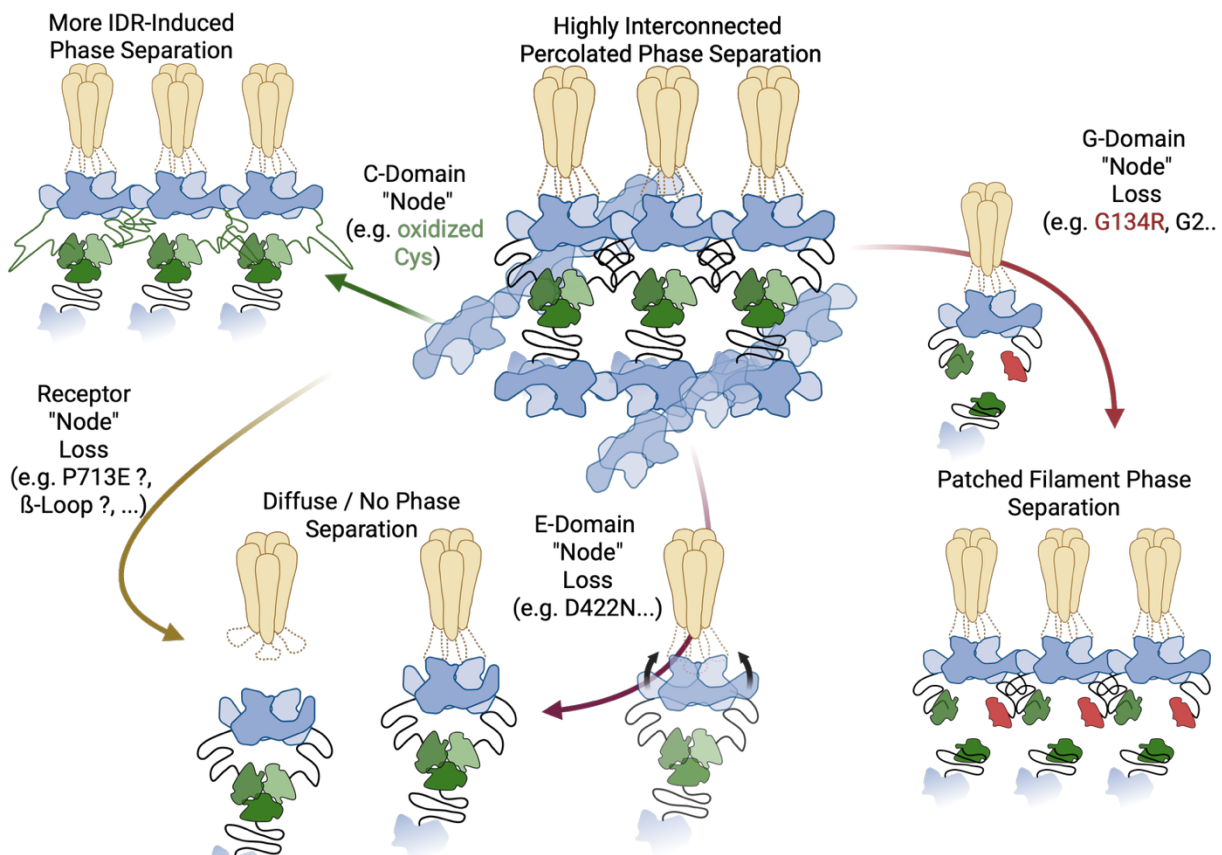


Figure 9: Schematic overview of regulatory nodes controlling gephyrin percolation behavior. A conceptual model illustrating potential regulatory mechanisms that modulate percolation within gephyrin-based networks. The central panel depicts a highly interconnected, percolated network of gephyrin in association with inhibitory receptors, representing the functional state underlying robust condensate formation. Four key nodes influencing this filament-based organization are highlighted. In the upper left, the C-domain, with its intrinsically disordered regions, is shown to enhance network connectivity by promoting IDR-driven LLPS, for example through redox-dependent modulation such as oxidation described in this work. On the right, disruption of the G-domain node, as observed for the G134R variant or the G2 splice isoform, reduces interconnectivity and results in a more fragmented, patch-like filament organization. The lower panels illustrate conditions in which LLPS is fully abolished. Loss of the E-domain node, for example in the D422N variant, prevents filament formation entirely, thereby disrupting higher-order assembly. Similarly, absence of receptor-mediated interactions, as in control conditions lacking receptor input, leads to loss of network connectivity. This scenario can potentially be recapitulated by gephyrin variants deficient in receptor binding, such as P713E. (Figure created with BioRender)

presented in this thesis in Chapter 4, the E-domain dimer may therefore largely function as a structural “spacer”, while defined interfaces such as the SDII-mediated contacts act as key “sticker” hotspots driving filament formation and network assembly. This framework can be extended to a higher hierarchical level, in which the entire flexible E-domain filament itself assumes the role of a spacer, while the comparatively strong, trimerized G-domain serves as the dominant sticker, a conceptualization supported by the dramatically altered network properties observed for the G134R variant described in Chapter 2.

The importance of the gephyrin network connectivity is highlighted by the effects of disrupting individual nodes. Loss of the receptor as a another linker or loss of the capacity to bind the receptor, such as with the gephyrin variant P713E, which was created in a structure-guided approach (Kim *et al*, 2006), likely also influence the connectivity. Loss of G-domain trimerization, as observed for the G134R variant, abolishes higher-order oligomerization and LLPS, demonstrating that this interaction represents a critical organizing element. The strong and persistent nature of G-domain trimerization (Herweg & Schwarz, 2012), together with evidence that trimers are maintained both *in vitro* and in cells, suggests that it provides a stable core that promotes efficient engagement of E-domain interactions; i.e. trimerization happens first and facilitates the dimer formation. This hierarchical organization ensures that gephyrin reaches the critical connectivity required for LLPS and filament maturation under physiological conditions, i.e. the trimers could be build first and help the dimers and filament-fragments find themselves. Disruption of the E-domain node as demonstrated by variants that disrupt filament formation (G375D, D422N) or that cannot dimerize (gephyrin G483R/R523E/A532R also termed “RER”, (Saiyed *et al*, 2007)) fully disable LLPS and synapse formation (Bai *et al*, 2021; Macha *et al*, 2025). The fusion of G- and E-domains within a single polypeptide, connected by a flexible C-domain, may thus represent an evolutionary optimization that simultaneously enables improved metabolic function in Moco biosynthesis and precise control over postsynaptic scaffold assembly (Belaidi & Schwarz, 2013). In the context of Moco biosynthesis, the length of the flexible linker likely reflects the structural requirement to bridge the C-terminal end of the G-domain to the N-terminus of the E-domain under product-substrate channeling constraints. Yet with emerging insight into the regulation of LLPS, the long linker may serve an additional function, providing the spatial separation between the

trimeric core and filamentous assemblies necessary for dynamic regulation without steric interference.

The central C-domain emerges as a key regulatory element within this framework. As an IDR, it is essential for gephyrin condensation and can be functionally substituted by heterologous IDRs, indicating that it primarily acts as a “charged spacer” modulating phase behavior rather than as a classical self-associating domain (Lee *et al*, 2024). At the same time, segments of the C-domain can inhibit E-domain-driven phase separation *in vitro*, suggesting that intramolecular interactions provide a regulatory mechanism that restrains spontaneous condensation (Bai *et al*, 2021). Integrating these findings, the C-domain can be viewed as a dynamic regulator that tunes the balance between network assembly and inhibition. In this scenario, the G-domain not only serves as an interconnecting node between E-domain filaments but may also exert allosteric control over C-domain conformation, modulating transitions between compact, shielded states that limit LLPS and more extended conformations in which the C-domain becomes exposed. In the latter state, favorable electrostatic interactions between the C-domain and other regions, together with an increase in effective valency, can promote network formation and drive the system towards percolation.

The conformational heterogeneity has been reported in earlier structural observations indicating a polydisperse ensemble of gephyrin states (Sander *et al*, 2013). The flexibility of the C-domain may further allow it to respond to local synaptic conditions, such as receptor density, thereby defining the spatial boundaries of condensates and preventing uncontrolled growth. In this context, interfaces of condensates are known to exhibit distinct energetic and structural properties compared to the dense phase, and the C-domain may contribute to regulating these boundary effects (Alberti *et al*, 2025). A recent study that solidly reproduced the filament formation shown in Chapter 4 of this thesis advances on certain aspects of the C-domain (Ortiz-López *et al*, 2026). Via cryo-EM they show that parts of the C-domain close to the E-domain, can exist in conformations that block parts of the receptor loop pocket (named “in”-states). When gephyrin is found in filaments, this C-domain part was exclusively found in the opposite “out”-state, leaving the receptor loop pocket free for interaction with the receptor, thus enabling interconnection with this node. This multifunctionality of the C-domain is further supported by its role as a platform for extensive post-translational modifications. Notably, the C-domain harbors numerous

phosphorylation sites whose modification would directly alter its charge distribution and, consequently, its interactions with the G- and E-domains, providing, for the first time, a mechanistic framework for understanding how phosphorylation governs scaffold assembly and function through the lens of LLPS. It was demonstrated that phosphorylation within the C-domain directly modulates gephyrin's capacity to undergo LLPS, with distinct serine residues in two autoinhibitory segments exerting opposing effects (Bai *et al*, 2021). Modification of S268 or S270 residing in the first segment, deepened autoinhibition and attenuated phase separation with GABA_AR- α 3 subunits, consistent with reduced receptor clustering at the synapse. In contrast, phosphomimetic substitution at S305, promoted condensation, likely because the introduced negative charge counteracts the local positive charge of that region and thereby relieves its suppressive effect on the E-domain's secondary receptor-binding surface. The opposing outcomes of phosphorylation at these sites recast the C-domain as a regulatory rather than a simple inhibitory element: the net balance of converging kinase activities on distinct residues continuously tunes the condensation propensity of gephyrin–receptor complexes and, by extension, the density of inhibitory receptors at the postsynaptic membrane. Furthermore, post-translational modification in the C-domain includes redox-sensitive cysteine residues, which will be discussed in the following section.

5.3 Regulation of network assembly: cysteine-dependent modulation

Gephyrin network assembly is subject to multiple layers of regulation, particularly within the C-domain, including phosphorylation, alternative splicing, interactions with proteins such as collybistin and DLC, as well as proteolytic cleavage by calpain (Ramming *et al*, 2000; Fuhrmann *et al*, 2002; Zacchi *et al*, 2014; González, 2019; Burdina *et al*, 2025). However, in the context of LLPS and higher-order network formation, this section focuses on the role of oxidative modifications of eight surface-exposed cysteine residues in gephyrin.

Notably, one of these residues, Cys419, is positioned within SDII of the E-domain and is directly exposed at the interface implicated in filament formation (Figure 10B) (Macha *et al*, 2025). This spatial distribution suggests that cysteine modifications may influence both local interactions and global network connectivity.

A primary mechanism by which cysteines can regulate protein assembly is through the formation of disulfide bonds. Initially, it was hypothesized that gephyrin may form intermolecular disulfide bridges between neighboring molecules, thereby stabilizing higher-order assemblies and potentially reinforcing condensate integrity. This hypothesis was supported by the observation that gephyrin isolated under non-reducing conditions from murine brain tissue exhibited higher-order oligomers that could be reversed upon chemical reduction (Chapter 3). However, further investigation revealed that stable disulfide bond formation could not be conclusively demonstrated in cellular systems or under defined *in vitro* conditions. Despite this finding, oxidation of gephyrin consistently led to pronounced functional effects: synaptic clusters expanded, became more resistant to proteolytic degradation, and displayed increased receptor-binding capacity, ultimately enhancing inhibitory signaling. These findings indicate that redox regulation of gephyrin does not only rely on stable covalent crosslinking but also on more subtle and dynamic modifications.

Indeed, cysteine residues are subject to a wide range of oxidative modifications beyond disulfide formation, including S-sulfonylation, S-palmitoylation, S-nitrosylation, S-sulfhydration, and S-glutathionylation, while further oxidation to sulfinic and sulfonic acid states represents irreversible modifications (Figure 10A) (Chung *et al*, 2013; Filipovic, 2025). Several of these modifications have already been described for gephyrin, particularly within the C-domain. For example, palmitoylation of selected residues promotes membrane association via lipid anchoring, whereas nitrosylation

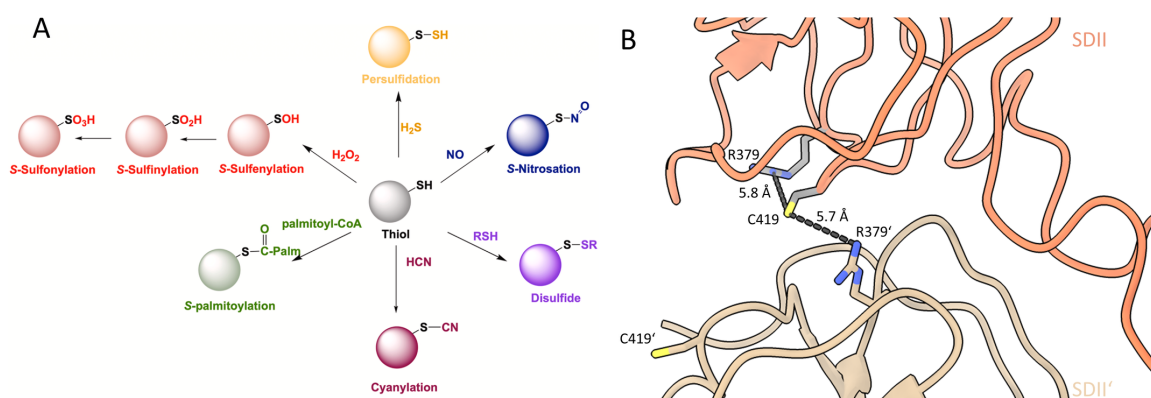


Figure 10: Cysteine modifications and local cysteine environment within gephyrin SDII. **A)** Schematic overview of major regulatory cysteine post-translational modifications and the biological signals that induce them. H₂O₂: hydrogen peroxide; NO: nitric oxide; H₂S: hydrogen sulfide; RSH: any thiol, including glutathione; HCN: hydrogen cyanide; palmitoyl-CoA: palmitoyl coenzyme A (adapted from Filipovic, 2025). **B)** Structural representation of the spatial arrangement of Cys419 relative to Arg379 and Arg379' at the SDII–SDII' interface. The distances highlight the close proximity of Cys419 to these residues, whereas the corresponding Cys419' of the other SDII' is located beyond 5 Å and these distances are therefore not depicted (PDB: 9GW9).

was found to inhibit palmitoylation and has been observed at multiple sites, including residues within the G- and E-domains in pathological contexts (Dejanovic *et al*, 2014; Dejanovic & Schwarz, 2014; Seneviratne *et al*, 2016; Yang *et al*, 2022). These findings highlight the complexity of cysteine-based regulation and its potential to integrate diverse cellular signals.

Redox regulation has also been described for excitatory synaptic scaffolds. Oxidative modifications have been shown to affect proteins such as PSD-95 and related membrane-associated guanylate kinase (MAGUK) family members, influencing their clustering and synaptic function (Hsueh *et al*, 1997; Lee & Kim, 2020). Conversely, reduction of such modifications in the case of PSD-95 can be mediated by enzymes such as protein disulfide isomerase (PDI) (Lee & Kim, 2020). Given that PDI is canonically an endoplasmic reticulum-resident enzyme, the precise mechanisms by which it accesses and tunes cytosolic substrates such as PSD-95 remain to be resolved, highlighting the broader complexity of compartment-specific redox regulation at the synapse. These observations nonetheless emphasize that redox-dependent modulation may constitute a more general organizational principle of synaptic scaffolding, and that a fuller mechanistic understanding of both inhibitory and excitatory systems warrants continued investigation.

The work presented in Chapter 3 of this thesis demonstrates that oxidation directly alters gephyrin behavior both *in vitro* and in neuronal systems. Oxidized wild-type gephyrin forms higher-order assemblies detectable by analytical size-exclusion chromatography, which can be reversed upon reduction, whereas cysteine-free variants fail to form such complexes. Strikingly, oxidation strongly enhances the propensity of gephyrin to undergo LLPS, while cysteine-free variants show markedly reduced phase separation. These findings suggest that specific oxidative modifications of cysteine residues modulate the interaction landscape of gephyrin. One possible mechanism is that oxidation increases the polarity of cysteine side chains, for example through sulfenylation, thereby creating a more hydrophilic microenvironment that can influence intermolecular interactions and promote molecular crowding. Similar effects have been described for other systems, such as the *Arabidopsis thaliana* protein ENOLASE2, where oxidation enhances oligomerization propensity (Liu *et al*, 2022).

To further dissect the structural basis of cysteine based redox regulation in gephyrin, we mapped cysteine contributions to specific domains, revealing a central role for Cys419 within the SDII interface of the E-domain. Variants of purified gephyrin

E-domain lacking this residue fail to undergo LLPS, whereas constructs in which Cys419 is the only remaining cysteine retain phase separation capability, which is further enhanced upon oxidation. This identifies Cys419 as a critical determinant of LLPS within the E-domain. From a structural perspective, this effect can be rationalized within the SDII–SDII' interface. In the cryo-EM structure shown in Chapter 4 of this thesis, the distance between the sulfur atom of Cys419 and the guanidinium group of Arg379 and Arg379' falls within a range that allows electrostatic interactions to influence the protonation state of the thiol (beyond 5 Å through longer-range electrostatic interactions via partial peptide atom charges; Figure 10B) (Madzellan *et al*, 2012). Such interactions can lower the pK_a of the cysteine, favoring formation of the negatively charged thiolate under physiological conditions. Further oxidation of thiolate to sulfenylated or sulfonylated states would introduce additional negative charges, potentially strengthening these interactions even further. The increased electronegativity and altered geometry of these modifications may allow a closer approach to Arg379 compared to the unmodified thiol, thereby enhancing stabilization of the filament interface. This provides a mechanistic explanation for the increased LLPS propensity observed upon oxidation. Within this context, Cys419 could form stabilizing electrostatic interactions with Arg379 from the opposing SDII, analogous to previously described interactions involving Asp422 that are essential for LLPS (Chapter 4) (Macha *et al*, 2025).

Beyond local effects at the filament interface, cysteine-dependent modulation may also influence higher-order network organization through emergent properties. In the context of the sticker–spacer framework, initial oligomerization events mediated by G- and E-domain interactions may expose additional regions, particularly within the C-domain, that can further contribute to LLPS (as described in the previous section). This behavior is consistent with an “emergent sticker” model, in which new interaction sites become functionally relevant only upon network formation and increased local concentration. Similar mechanisms have been described for *Arabidopsis thaliana* protein auxin response factor 19 (ARF19), where oligomerization exposes IDRs that subsequently drive phase separation (Choi *et al*, 2020).

Having established cysteine-based redox regulation of gephyrin LLPS, a key question concerns the physiological sources of reactive oxygen species (ROS) that could enact such regulation at inhibitory synapses. One prominent source is mitochondria-derived ROS (mROS). Synapses are particularly rich in mitochondria,

which are indispensable not only for axon differentiation, but also for calcium storage and buffering, sustaining neuronal polarity and the rapid metabolic recovery that effective neurotransmission demands (Faitg *et al*, 2021; Duarte *et al*, 2023). Beyond these structural and homeostatic roles, mitochondrially produced ATP is equally critical for a broad range of neuronal processes, including the maintenance of ion gradients and membrane potential, vesicle fusion, and neurotransmitter reuptake (Nicholls & Budd, 2000). Within the framework developed in Chapter 3, elevated mROS levels would generate a more oxidizing local environment, favoring the stabilization of gephyrin condensates and thereby strengthening inhibitory inputs at precisely those synapses subject to heightened demand, a form of activity-dependent feedback regulation. Intriguingly, this logic may extend beyond the inhibitory synapse itself, as neighboring excitatory synapses represent an additional potential source of oxidative signals. In support of this, studies on NADPH oxidase 2 (NOX2) have demonstrated that ROS generated in response to excitatory synaptic activity can initiate a transsynaptic signaling cascade, engaging kinases at inhibitory synapses rather than directly oxidizing their molecular targets (Larson *et al*, 2020). Together, these observations suggest that gephyrin LLPS may serve as a redox-sensitive integrator of both intrinsic mitochondrial output and intercellular oxidative crosstalk between excitatory and inhibitory compartments. Although initial experiments employing antimycin A, an inhibitor of respiratory chain complex III (ubiquinol–cytochrome *c* oxidoreductase), to acutely elevate intracellular ROS provided encouraging preliminary evidence for this model, technical inconsistencies precluded definitive conclusions, and these experiments remain an important avenue for future investigation.

Beyond extrinsic sources of oxidative signals, emerging evidence suggests that phase-separated condensates may themselves become autonomous generators of ROS, pointing toward a self-reinforcing feedback mechanism. Recent work on the presynaptic protein synapsin has demonstrated that condensate formation gives rise to steep electrical potential differences at the condensate–solvent interface, electrical responses that are entirely absent when the same protein exists in a dispersed single-phase state (Hoffmann *et al*, 2023). This interfacial electric field is not merely a passive electrostatic consequence of condensation, but carries direct chemical reactivity: such fields have been shown to drive spontaneous redox reactions both *in vitro* and in living cells, promoting the oxidation of hydroxide ions and generating solvated electrons alongside hydroxyl radicals, whose recombination subsequently yields hydrogen

peroxide (Dai *et al*, 2023), one of the most biologically prominent ROS and a species also produced by mitochondria during normal respiratory activity. Transposing this logic onto gephyrin, one can envisage a self-amplifying cycle: initial physiological ROS, whether derived from heightened mitochondrial activity at active synapses or from NOX2-dependent excitatory-to-inhibitory crosstalk, promotes oxidative modification of surface-exposed cysteine residues (most critically Cys419), lowering the energetic barrier for LLPS and nucleating gephyrin condensate formation. Once a condensate is established, the resulting interfacial electric field would itself generate hydrogen peroxide locally, further oxidizing gephyrin cysteines and reinforcing condensate stability in a feed-forward manner. This coupling between condensate physics and redox chemistry would allow the inhibitory synapse to translate transient oxidative signals into a durable structural remodeling of the postsynaptic scaffold, providing a mechanism by which synaptic activity is not merely sensed but actively consolidated through the emergent properties of phase separation itself.

Taken together, these findings highlight cysteine oxidation as a versatile and dynamic regulator of gephyrin network assembly. Rather than acting through static crosslinking, oxidative modifications modulate interaction strength, electrostatic properties, and conformational states, thereby fine-tuning the balance between filament formation, network connectivity, and phase separation. In combination with the structural and percolation-based principles discussed above, redox regulation provides an additional layer of control that enables inhibitory synapses to rapidly adapt to changing physiological conditions.

5.4 From molecular disruption to disease mechanisms

A growing number of disease-associated variants in gephyrin have been identified, several of which are addressed within this thesis. These mutations provide important mechanistic insight into how perturbations at different levels of the gephyrin scaffold network can give rise to neurological phenotypes such as epilepsy. Notably, the phenotype of the variants discussed here derives not primarily from disrupted receptor binding itself but rather of interference with higher-order assembly, thereby highlighting the importance of network formation and LLPS for synaptic function.

Within the E-domain, two disease-associated mutations located in SDII have been characterized (Dejanovic *et al*, 2015; Macha *et al*, 2022). The G375D variant

leads to a complete unfolding of the SDII region, while D422N disrupts a critical ionic interaction within the SDII–SDII' interface (Chapter 4) (Macha *et al*, 2025). Both mutations were initially assumed to impair receptor binding; however, the structural and mechanistic analyses presented in this thesis reveal a different underlying cause. In both cases, filament formation is abolished, and consequently, the ability of gephyrin to undergo LLPS is severely impaired. This demonstrates that disruption of higher-order assembly, rather than loss of receptor interaction *per se*, is sufficient to compromise inhibitory synapse organization.

A complementary mechanism is observed for the G134R variant located within the G-domain (Chapter 2). Receptor binding remains unaffected, yet higher-order oligomerization and LLPS are disrupted due to impaired G-domain trimerization. In this context, receptors can still be recruited and clustered, albeit in a less efficient and less stable manner. This is exemplified by the G134R patient, where pharmacological treatment with benzodiazepines was sufficient to alleviate symptoms, indicating that residual receptor populations remain functional and can be modulated to restore inhibitory signaling. This finding underscores the role of the G-domain as a central connectivity node within the gephyrin network. These observations suggest that direct loss of receptor binding would likely have more severe, potentially lethal consequences, whereas perturbations of higher-order assembly may still allow partial functionality.

Differences in phenotypic severity among mutations within the E-domain further highlight the importance of network integrity. The G375D mutation predominantly affects GABAergic synapses, as glycinergic symptoms such as hyperekplexia or hypertonia were not observed in the patient. In contrast, the D422N variant leads to a more severe clinical presentation, including early onset, developmental delay, treatment-resistant seizures, and motor abnormalities, suggesting that both GABAergic and glycinergic transmission are compromised. A likely explanation may be found in the genetic context: while G375D and G134R are heterozygous and thus retain one wild-type allele, D422N is effectively pseudo-homozygous due to nonsense-mediated decay (NMD) of the second allele (Macha *et al*, 2022). Consequently, no functional wild-type gephyrin is present to support scaffold formation. Nevertheless, the other two heterozygous variants exhibit dominant-negative effects on synaptic clustering, yet these effects may be more pronounced at GABAergic synapses, where network stability appears more sensitive to perturbation. In contrast, glycinergic

transmission may be partially preserved through the remaining wild-type protein. This is in line with previous studies of gephyrin variants associated with schizophrenia and autism spectrum disorders, which reported impaired GABAergic but largely preserved glycinergic transmission in heterozygous contexts (Lionel *et al*, 2013). In the absence of any wild-type protein, as in the case of D422N, scaffold formation is globally compromised, leading to more severe phenotypes affecting multiple inhibitory pathways.

The disease variants discussed above not only deepen our mechanistic understanding of gephyrin network assembly but also open speculative yet tantalizing avenues for therapeutic intervention. The emerging appreciation that percolated condensate networks can be disrupted by node-breaking peptides (Jia *et al*, 2025) or that proteins can be artificially redirected between condensates using engineered intrabodies (Zhu *et al*, 2024) suggests that the biophysical properties of LLPS systems may themselves be pharmacologically tractable. Translated to gephyrin-associated epilepsies, one could envision a conceptually novel strategy: rather than targeting receptor binding or ion channel activity, therapeutic agents could be designed to artificially crosslink gephyrin G- or E-domain interfaces, compensating for the weakened oligomerization caused by pathological variants and thereby restoring the network connectivity required for productive LLPS. In this context, designed ankyrin repeat proteins (DARPin) represent a particularly compelling scaffold, a class of small, potent, and highly specific protein therapeutics with rapidly expanding translational potential (Plückthun, 2015). Strikingly, the DARPin "27F3" has recently been mapped with structural precision to a binding site within the gephyrin E-domain (Ortiz-López *et al*, 2026), establishing a proof-of-principle that the very interfaces governing gephyrin condensation are accessible to engineered protein binders. Whether such molecules could be harnessed to stabilize pathological gephyrin assemblies, fine-tune condensate properties, or even serve as carriers for targeted delivery to inhibitory postsynaptic densities remains an open and exciting question, one that places gephyrin LLPS squarely at the intersection of condensate biology and the next generation of precision neurotherapeutics.

5.5 Closing remarks

The work presented in this thesis provides new insight into three central aspects of gephyrin LLPS and establishes a conceptual framework for understanding the structural organization of inhibitory postsynapses.

First, the identification and characterization of the G134R variant highlight the G-domain as a critical regulatory node within the gephyrin network. While previous studies have primarily focused on the E-domain as the main driver of receptor binding and clustering, this work demonstrates that G-domain trimerization is essential for higher-order connectivity and LLPS. Within the framework of percolation theory, the G-domain can be interpreted as a key node that determines whether the system reaches the threshold required for forming a system-spanning network. Future approaches

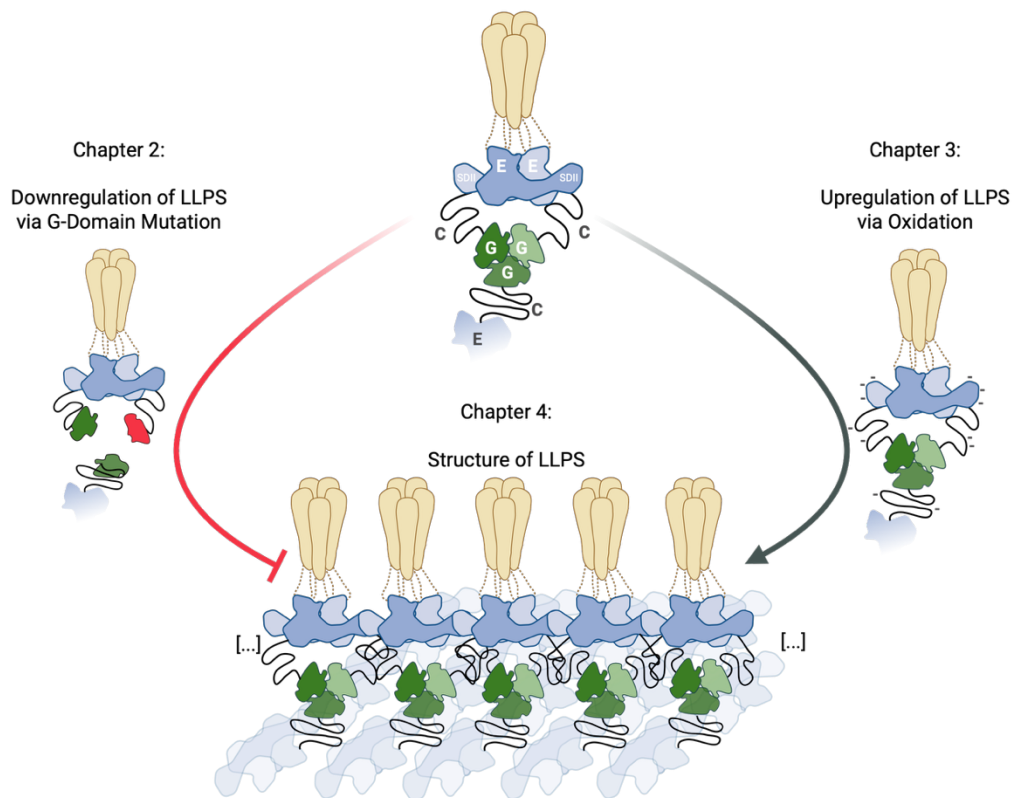


Figure 11: Final integrative model of gephyrin LLPS: G-domain dependent inhibition, cysteine driven upregulation, and filament architecture.

Schematic summary of the three research chapters presented in this thesis. The central model depicts a gephyrin G-domain trimer connected to E-domain dimers, highlighting the SDII region and in complex with a pentameric ligand-gated ion channel.

On the left, findings from Chapter 2 are illustrated: disruption of G-domain trimerization, as observed for the G134R variant, reduces network connectivity and leads to a global downregulation of LLPS. On the right, results from Chapter 3 are summarized: cysteine oxidation at multiple sites enhances LLPS, indicated here by the introduction of additional negative charges. At the bottom, the structural outcome described in Chapter 4 is shown: gephyrin assembles into filamentous structures mediated by SDII–SDII' interactions, which are interconnected via the G-domain. This higher-order organization is further stabilized under oxidative conditions, linking regulatory mechanisms to the structural basis of inhibitory postsynaptic assembly. (Figure created with BioRender)

aiming to systematically alter oligomerization valency, for example by replacing the G-domain with defined dimeric, trimeric, or higher-order oligomerization modules, will allow direct testing of how connectivity controls condensate formation and synaptic organization, while also separating structural roles from metabolic functions such as Moco synthesis.

Second, this thesis establishes cysteine-dependent redox regulation as an additional and highly tunable layer of control over gephyrin assembly. Oxidative modifications were shown to directly influence higher-order complex formation and LLPS behavior, with a particularly prominent role for Cys419 within the SDII interface. These findings demonstrate that even single residues can critically modulate phase behavior, likely by altering local interaction properties and filament stability. Future work will be required to define the exact oxidative states involved and to resolve how these modifications mechanistically influence filament formation and network connectivity at the structural level.

Finally, this work advances the understanding of the structural basis underlying gephyrin LLPS. While phase separation is often associated with weak, transient interactions and intrinsically disordered regions, the results presented here demonstrate that robust, concentration-dependent filament formation within the E-domain provides a structural backbone for network assembly. These filaments likely create an interconnected scaffold that is ideally suited to concentrate and stabilize inhibitory receptors. An important open question remains how these filaments are crosslinked into higher-order networks, whether primarily via G-domain-mediated interactions or through additional interfaces within the E-domain, representing a key direction for future structural and biophysical studies.

Taken together, this thesis integrates G-domain-mediated connectivity, cysteine-dependent regulation, and filament-based structural organization into a unified framework of gephyrin LLPS. By linking these principles to synaptic function and disease, this work contributes to a deeper understanding of inhibitory postsynapse organization and highlights LLPS as a central mechanism underlying gephyrin function in both health and disease. In this way, the study of a single protein becomes a window into the physical principles that organize life on earth.

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