Scuola Internazionale Superiore di Studi Avanzati

GEPHYRIN REGULATES TRANS-SYNAPTIC SIGNALING AT GABAERGIC CONNECTIONS IN THE HIPPOCAMPUS

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NOTES

The work described in this dissertation was carried out at the International School for Advanced Studies (SISSA), Trieste, between October 2006 and October 2010, and was published in the following papers. All work reported here, with the exception of pair recordings from interconnected hippocampal neurons (carried out by Rocco Pizzarelli) and the pull-down/co-immunoprecipitation experiments (carried out by Roberta Antonelli and Paola Zacchi) arises from my own experiments and data analysis.

Zacchi, P., Dreosti, E, Visintin, M, Moretto-Zita, M, Marchionni, I, Cannistraci, I, **Kasap, Z**, Betz, H, Cattaneo, A, Cherubini, E (2008) Gephyrin selective intrabodies as a new strategy for studying inhibitory receptor clustering. *J Mol Neurosci* **34**:141-148.

Marchionni, I.*, **Kasap, Z***, Mozrzymas, J.W, Sieghart, W, Cherubini, E, Zacchi, P (2009) New insights on the role of gephyrin in regulating both phasic and tonic GABAergic inhibition in rat hippocampal neurons in culture. *Neuroscience* **164**:552-562.

Kasap Varley, Z.*, Pizzarelli, R*, Antonelli, R, Stancheva, S.H, Kneussel, M, Cherubini, E, Zacchi, P (2010) Gephyrin regulates GABAergic and glutamatergic synaptic transmission in hippocampal cell cultures. *(Submitted).*

* Equally contributed

ABBREVIATIONS

AP2: Clathrin-adaptor protein 2

ASD: Autism spectrum disorders

BGT-1: Betaine/ γ-aminobutyric acid Transporter

BIG2: brefeldin A-inhibited GDP/GTP exchange factor 2

CA 1/3: *cornu ammonis* regions 1/3

EDTA: Ethylenediaminetetraacetic acid

EPSC: Excitatory Postsynaptic Current

GABA: γ-aminobutyric acid

GABARAP: GABAA receptor associated protein

GAD: glutamic acid decarboxylase

GAT: γ-aminobutyric acid transporter

GODZ: Golgi-specific DHHC zinc finger domain protein

HAP1: Huntingtin-associated protein 1

KIF: Kinesin family

KO: Knock Out

IACT: Intracellular Antibody Capture Technology

IPSP: Inhibitory Postsynaptic Potential

mIPSC: Miniature Inhibitory Postsynaptic Current

NMDA: N-Methyl-D-Aspartate

PKA: protein kinase A

PKC: protein kinase C

Plic-1: protein linking integrin-associated protein to cytoskeleton-1

scFv: single chain fragment variable

THDOC: 3α,21dihydroxy-5α-pregnan-20-one

TM: transmembrane domain

VGAT: Vesicular GABA transporter

VGLUT: Vesicular glutamate transporter

ABSTRACT

Gephyrin is the central component of the postsynaptic scaffold at inhibitory synapses, ensuring receptor accumulation in precise apposition to presynaptic release sites. Synapses are highly dynamic structures, with receptors constantly moving in and out of postsynaptic sites. The mechanisms regulating synaptic organization are thus crucial for an efficient and reliable synaptic transmission. My thesis focuses on the role of gephyrin in regulating GABAergic transmission.

To study gephyrin function, I used intracellular single chain antibody fragments against gephyrin (scFv-gephyrin) which could remove endogenous gephyrin from its subcellular location, leading to an overall loss of gephyrin clusters. Transfecting hippocampal neurons in culture with scFv-gephyrin led to a reduced density of synaptic γ2-subunit containing $GABA_A$ receptors. This effect was associated with a decrease in the amplitude and frequency of mIPSCs, and a slow-down in their onset kinetics. Using an ultrafast agonist application system which mimics synaptic events, I found that the slow onset of GABA-evoked currents was due to a slower entry of the receptors into the desensitized state. Hence, hampering gephyrin function affects the gating properties of $GABA_A$ receptors. Disruption of gephyrin clusters also altered the $GABA_A$ mediated tonic conductance, an effect that could be attributed to a reduced GABAergic innervation.

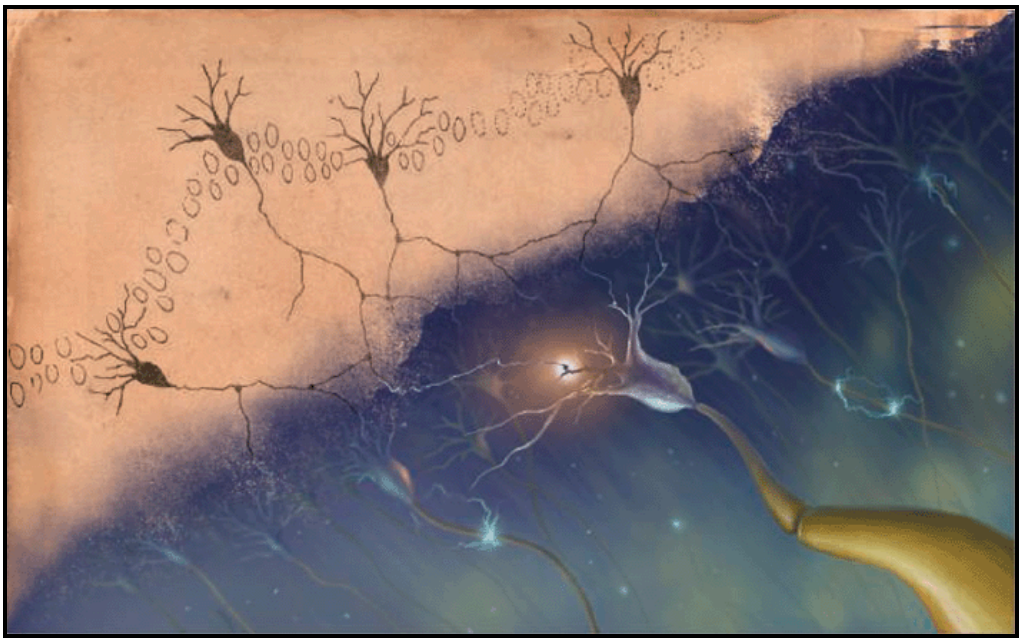
Gephyrin ablation led to a reduction in the density of the vesicular GABA transporter, VGAT. Moreover, pair recordings from interconnected neurons revealed a reduction in amplitude and in the number of successes as well as an increase in paired-pulse ratio and in the coefficient of variation of $GABA_A$-mediated synaptic currents in scFv-gephyrin transfected neurons, indicating a reduced probability of GABA release. Gephyrin may exert this trans-synaptic action through the neuroligin/neurexin complex. Consistent with this hypothesis, I found that upon scFv-transfection, the neuroligin isoform known to be preferentially localized at GABAergic synapses (neuroligin 2) was significantly reduced. Furthermore, in molecular biology experiments, gephyrin was found to immunoprecipitate neuroligin 2 from rat brain lysates, indicating the formation of a complex between these two proteins. Co-expression of neuroligin 2 with scFv-gephyrin

was able to rescue the reduction in GABAergic innervation, suggesting that gephyrin can regulate GABA release through neuroligin 2.

Neuroligins can localize at both GABAergic and glutamatergic synapses, and modulate the excitatory/inhibitory (E/I) balance within the neuronal network. Interestingly, scFv-gephyrin transfection resulted in a significant reduction in glutamatergic innervation, as revealed by the decrease in the density of the vesicular glutamate transporter, VGLUT, as well as by the reduction in the frequency of mEPSCs. Rescue experiments with the co-expression of neuroligin 2 and scFv-gephyrin did not reverse the effect of scFv-gephyrin on glutamatergic innervation, suggesting that it was not due to a homeostatic compensatory mechanism. Based on the observation that gephyrin can co-immunoprecipitate neuroligin 1 (the isoform enriched at glutamatergic synapses), it is possible that gephyrin modulates both GABAergic and glutamatergic synapses *via* neuroligins. However, whether gephyrin regulates glutamate release *via* neuroligin 1 remains to be elucidated.

Overall, interfering with gephyrin clustering at the post-translational level has revealed new insights on the role of this scaffold protein in GABAergic synapses and has prompted further investigation into the function of gephyrin in regulating the E/I balance possibly through neuroligins.

INTRODUCTION



Bullock et al., 2005

1. SYNAPTIC TRANSMISSION

The human brain is made up of a vast network of more than 100 billion individual neurons. The current view that neurons are the functional signaling units in the nervous system dates back to the end of the 19th century when Ramón y Cajal postulated his "neuron doctrine", following a series of exquisite neurohistological studies (López-Muñoz et al., 2006). Cajal envisioned neurons as highly polarized cells that receive signals through their dendrites, relay them through their long axons and communicate with each other at specific points of apposition called synapses, a term introduced by Charles Sherrington in 1897 based on the Greek word 'to clasp' (Foster, 1897). Although essentially correct, this is an oversimplified view of synaptic transmission in the mammalian brain. There are actually two functionally and structurally distinct forms of synapses: *chemical*, which are between two neurons physically separated by a 'synaptic cleft' and are mediated by neurotransmitters released from a presynaptic neuron acting on postsynaptic receptor proteins, and *electrical*, which are mediated through gap junctions that bridge the cytoplasm of presynaptic and postsynaptic neurons (Kandel et al., 2000).

In addition, the unidirectional signal propagation inferred by Cajal and his contemporaries was challenged by findings revealing that information is relayed also retrogradely from the postsynaptic neuron to the presynaptic one *via* small molecules and neuromodulators (Nusbaum et al., 2001). The current 'bidirectional' view of signal transfer across synapses is essential for numerous processes ranging from synapse assembly to synaptic plasticity, and to higher brain functions like learning and memory (Jessell and Kandel, 1993).

2. INHIBITORY TRANSMISSION

Excitatory and inhibitory inputs contribute to set the resting membrane potential of the cell, which in turn determines whether the neuron will reach the threshold for producing an action potential. Excitatory inputs depolarize the neuron, bringing it closer to the threshold for firing an action potential, whereas inhibitory inputs hyperpolarize the neuron, bringing the membrane potential away from the action potential threshold. Once the action potential is initiated, it propagates along the axon and triggers exocytosis of synaptic vesicles and the release of neurotransmitters from the axon terminal. The properties of the postsynaptic receptors binding the neurotransmitter determine whether the resulting synaptic potential is excitatory or inhibitory. In the adult central nervous system, the main inhibitory neurotransmitter is GABA, whereas excitatory transmission is mediated by glutamate.

2.1 GABA AS A NEUROTRANSMITTER

In 1967, Krnjevic & Schwartz demonstrated that GABA has a hyperpolarizing effect on cerebral cortical neurons that imitates the potential change during synaptic inhibition (Krnjević and Schwartz, 1967). GABA is now considered the main inhibitory neurotransmitter in the adult brain, with 17-20% of neurons in the brain being GABAergic (Mody and Pearce, 2004). GABA is synthesized from glutamate by glutamic acid decarboxylase (GAD), an enzyme that catalyzes the decarboxylation of glutamate to GABA and CO_2. In mammals, GAD exists in two isoforms, GAD65 and GAD67,

encoded by two different genes, *Gad1* and *Gad2* (Erlander et al., 1991). Of particular significance is GAD65, which is concentrated in the nerve terminals to a much greater degree than GAD67, where it synthesizes GABA for neurotransmission purposes (Martin and Rimvall, 1993). Once released from the presynaptic terminal, GABA acts on ionotropic $GABA_A$ receptor channels permeable to chloride and bicarbonate (Schofield et al., 1987) and metabotropic $GABA_B$ receptors that are coupled to ion channels via guanine nucleotide-binding proteins and second messengers (Kaupmann et al., 1997). GABA is then cleared from the synaptic cleft by high affinity Na^+/Cl^- coupled transporters (GAT) localized on axon terminals and astrocytes (Conti et al., 2004), or is degraded to succinate by GABA-glutamate transaminase and succinate semialdehyde dehydrogenase.

2.2 PHASIC AND TONIC GABAERGIC INHIBITION

In the adult central nervous system, $GABA_A$-receptors mediate two types of inhibition: phasic and tonic (Farrant and Nusser, 2005). The first consists of fast inhibitory postsynaptic potentials (IPSPs) regulating point-to-point communication between neurons. The second consists of a persistent inhibitory conductance that plays a crucial role in regulating the membrane potential and cell excitability (Semyanov et al., 2004) (Figure 1).

Phasic inhibition is mediated by postsynaptic $GABA_A$ receptors located in direct apposition to the presynaptic release sites. Following GABA release from the presynaptic terminal, these receptors are activated by a high concentration of GABA (~3 mM) for a brief period of time (~100 μs). Channel opening leads to an inward flux of Cl^- resulting in an inhibitory postsynaptic potential (IPSP) (Mozrzymas et al., 2003a). This type of inhibition is crucial for information transfer and network synchronization (Cobb et al., 1995).

Tonic inhibition, on the other hand, results from the persistent activation of extrasynaptic $GABA_A$ receptors (localized away from the synapses) by a low concentration of ambient GABA. In order to detect such low concentrations of GABA for prolonged periods of time, extrasynaptic $GABA_A$ receptors should have a high affinity for GABA and should

exhibit a low desensitization rate (Farrant and Nusser, 2005; Semyanov et al., 2004). Tonic inhibition was first identified in rat cerebellar granule cells, where application of $GABA_A$ receptor antagonists bicuculline and gabazine revealed a reduction in the holding current and background noise (Brickley et al., 1996; Kaneda et al., 1995). Since then tonic inhibition has been detected in several brain regions, including the dentate gyrus, the thalamus, cortical layer 2/3, as well as the CA1 and the CA3 hippocampal regions (Glykys and Mody, 2007). The amount of GABA in the extracellular space originates from various sources, the first of which is spillover from vesicular GABA release (Mitchell and Silver, 2000).

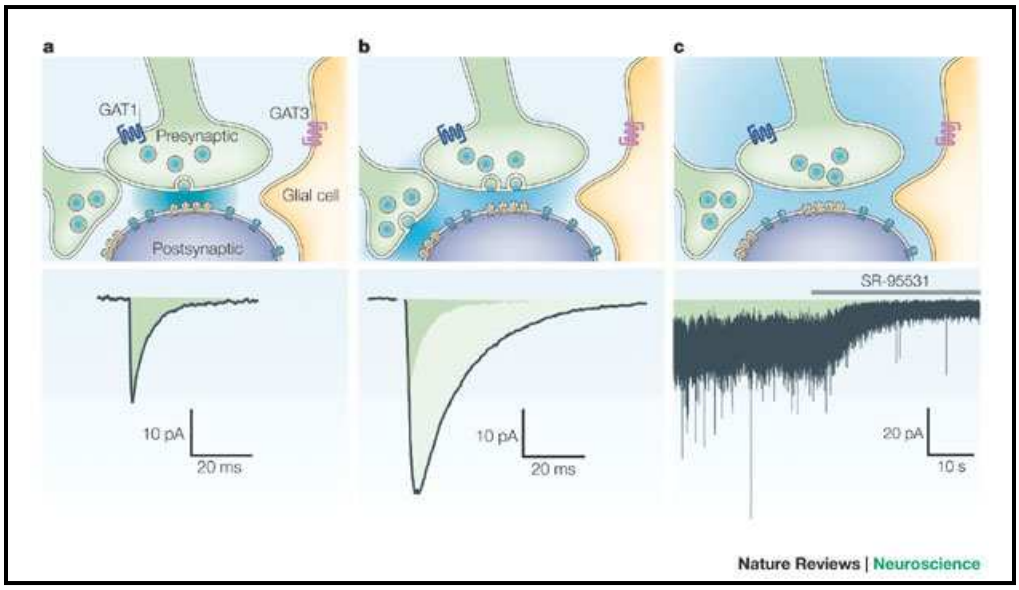


Figure 1. **Modes of $GABA_A$ receptor activation.** *(a)* Miniature inhibitory postsynaptic currents (mIPSCs) recorded in the presence of the sodium channel blocker tetrodotoxin result from the release of a single vesicle from the presynaptic terminal. mIPSCs activate only those postsynaptic $GABA_A$ receptors that are clustered in the membrane immediately beneath the release site (yellow). The averaged waveform of mIPSCs are shown below. *(b)* Action potential-dependent or evoked release of multiple vesicles from several terminals promotes GABA spillover, and activates both synaptic and extrasynaptic receptors. The current record shows the larger and much slower averaged waveform of evoked IPSCs. *(c)* A low concentration of ambient GABA tonically activates high-affinity extrasynaptic receptors. Application of the $GABA_A$ antagonist gabazine (SR-95531) blocks tonic channel activity, causing a change in the holding current and a reduction in current variance. (Farrant and Nusser, 2005)

As already mentioned, GABA is cleared from the synaptic cleft by specific transporters, but a low concentration of the neurotransmitter remains to act on high affinity extrasynaptic $GABA_A$ receptors. In addition, action-potential independent GABA release from astrocytes and reversed transport from GABA transporters (Attwell et al., 1993) have been reported as sources of ambient GABA.

The physiological role of each type of inhibition is only beginning to be understood. Phasic inhibition, apart from preventing over-excitation of neurons, is essential for rhythm generation in neuronal networks (Cobb et al., 1995) and for setting the time window during which synaptic inputs evoke action potentials (Pouille and Scanziani, 2001). On the other hand, tonic inhibition causes a persistent increase in the neuron's input conductance and modulates neuronal gain (Semyanov et al., 2004). Both types of inhibition control neuronal excitability under physiological and pathological conditions. It should therefore come as no surprise that GABAergic function is fine-tuned at multiple levels.

$GABA_A$-mediated IPSPs are characterized by a large variability that is generated by various factors acting at presynaptic, cleft and postsynaptic levels.

2.3 PRESYNAPTIC MODULATION OF GABAERGIC INHIBITION

Once the action potential reaches the axon terminal, it depolarizes the membrane which in turn activates voltage gated calcium channels. Ca^{+2} entry through these channels triggers the machinery for neurotransmitter release (Nicholls et al., 2001). Neurotransmitters are contained in small vesicles at the nerve terminal and released via exocytosis, which is the fusion of vesicles with the presynaptic plasma membrane. Vesicle exocytosis is spatially restricted to active zones and temporally regulated by Ca^{+2}, triggered within a few hundred microseconds after Ca^{+2} influx (Goda and Südhof, 1997). Once the fusion-pore opens and neurotransmitter is released, the vesicles are endocytosed and refilled to be used in a new round of exocytosis. The total number of vesicles participating in the vesicle trafficking during prolonged stimulation is referred to as "the recycling pool", while those that are docked to the membrane and have been primed for release comprise the "readily releasable pool". Using fluorescent FM1-43

dyes which allow labeling endocytosed vesicles, it was estimated that only ~25 vesicles are present in the recycling pool, one third of which are in the readily releasable population (Murthy and Stevens, 1999).

After the synaptic vesicles dock at the active zone, two types of fusion events may occur; full collapse fusion where vesicles flatten into the plasma membrane, or partial fusion where vesicles form a transient fusion pore and are quickly recycled (also called "kiss-and-run"). At hippocampal synapses, kiss-and-run was initially considered as the prevalent mode of vesicle fusion (Aravanis et al., 2003) as it allows fast and reliable synaptic transmission during high-frequency stimulation. However, a recent study using pH-sensitive quantum dots revealed that full fusion and kiss-and-run can both be predominant under different conditions (Zhang et al., 2009). In this study, kiss-and-run was the principal mode of fusion under high activity demand, but it gave way to full collapse fusion as stimulation continued, and under steady-state stimulation at low rates. Nevertheless, fast partial fusion and reuse of readily releasable pool of vesicles would reduce synaptic variability due to vesicle volume or neurotransmitter content, particularly during burst firing found in hippocampal neurons.

Early work by Fatt and Katz on the frog neuromuscular junction revealed that acetylcholine contained in synaptic vesicles is released from nerve terminals in multimolecular packets, called 'quanta' (Fatt and Katz, 1952). The quantal theory of neurotransmitter release extends to all neurotransmitters in the brain, and provides a theoretical framework to understand the stochastic properties of synaptic transmission. Accordingly, synaptic efficacy (E), which is the mean amplitude of unitary postsynaptic currents, is governed by the quantal content (i.e. mean number of quanta released per action potential) and by quantal size (i.e. magnitude of response to a single quantum). While the quantal size (Q) depends on both presynaptic and postsynaptic factors, the quantal content is directly related to the number of release sites (N) and the probability of release (P) at each site (Cherubini and Conti, 2001). Changes in either Q, N or P will therefore modulate the strength of a synaptic connection.

At GABAergic synapses, GABA release is mediated not only by vesicular but also non-vesicular release. Non-vesicular GABA release through the reversed action of GABA transporters under physiological conditions was recently demonstrated (Wu et al., 2007).

GAT-1 (the neuronal isoform of the GABA transporter) was shown to have a reversal potential close to the resting membrane potential of neurons, and could reverse during action potentials. Although the *in vivo* relevance of this phenomenon is yet to be clarified, transporter-mediated GABA release could act as a brake to prevent runaway excitation during high frequency firing when vesicular fusion begins to fail.

Probability of GABA release (*P*) is modulated by various factors ranging from calcium release from intracellular stores, to multivesicular release and presynaptic autoreceptors. Presynaptic ryanodine-sensitive calcium stores contribute to GABA release in resting conditions leading to miniature inhibitory postsynaptic currents (mIPSCs) in hippocampal pyramidal cells (Savić and Sciancalepore, 1998) and cerebellar Purkinje cells (Bardo et al., 2002), as well as during evoked neurotransmitter release at cerebellar basket cell-Purkinje cell synapses (Galante and Marty, 2003).

The amplitude distributions of evoked synaptic potentials in central synapses can be fitted with binomial distributions, and the number of peaks is believed to correspond to the number of release sites (Redman, 1990). Moreover, the lack of evidence for double-sized events has led to the assumption that one site corresponds to one quantum release. This hypothesis was challenged at cerebellar stellate-basket cell synapses, where multivesicular release was observed in IPSC recordings (Auger et al., 1998). Furthermore, synapses can have more than one functional release site (Kondo and Marty, 1998), or vesicles can be released asynchronously long after the arrival of the action potential (Lu and Trussell, 2000), contributing to the variability in synaptic efficacy.

GABA release can be modulated by presynaptic $GABA_A$ and $GABA_B$ autoreceptors, which are located on the axon terminals close to release sites. Presynaptic $GABA_A$ and $GABA_B$ receptors are activated by GABA spillover from synaptic cleft or by ambient GABA in the extracellular space. In the hippocampal mossy fibers, presynaptic $GABA_A$ receptor activation increases membrane conductance which produces a shunting effect, raising the threshold for evoking an action potential (Ruiz et al., 2003). Conversely, in immature hippocampal neurons (where GABA exerts a depolarizing action due to high intracellular $[Cl^-]$), $GABA_A$ receptor activation enhances transmitter release (Safiulina et al., 2006; Xiao et al., 2007). Activation of $GABA_B$ receptors, on the other hand, reduces GABA release by inhibiting voltage-dependent calcium channels (Poncer et al., 1997).

This mechanism has been shown to play an important regulatory role in immature hippocampal mossy fiber-CA3 synapses which, immediately after birth, are mainly GABAergic (Safiulina and Cherubini, 2009).

2.4 GABA TRANSIENT IN THE SYNAPTIC CLEFT

Once released from the presynaptic terminal, GABA diffuses through the synaptic cleft and binds postsynaptic receptors. Using theoretical modeling and experiments modifying $GABA_A$ receptor gating, the synaptic GABA transient was estimated to be ~100 μs (Mozrzymas et al., 2003a). This means that $GABA_A$ receptors are activated in non-equilibrium conditions, with synaptic currents outlasting the duration of GABA transient. It is therefore conceivable that GABA concentration and its rate of clearance from the synaptic cleft influence GABAergic currents (Barberis et al., 2004).

The neurotransmitter concentration in the synaptic cleft depends on the number of neurotransmitter molecules within each vesicle, and by the fraction of content that is released after a fusion event. Electron microscopy revealed synaptic vesicles to be ~40 nm in diameter, but it is still unclear whether this value varies significantly from one vesicle to the other (Takamori et al., 2006). The neurotransmitter concentration profile in the synaptic cleft is further complicated by multivesicular release, which would account for 30% of IPSCs recorded from interneuron-interneuron synapses in the molecular layer of the cerebellum (Auger et al., 1998).

Neurotransmitters released from vesicles are cleared from the synaptic cleft by diffusion and reuptake. Diffusion is influenced by the overall “tortuosity” of the extracellular space, which is determined by the hindrance due to the geometry of the synaptic cleft and connectivity of the extracellular space, as well as by the extracellular volume fraction, which is the relative volume in which the neurotransmitters are moving (Nicholson and Syková, 1998). Studies using dextran, a polysaccharide that increases the viscosity of the extracellular space, revealed that reducing the coefficient of diffusion of GABA in the synaptic cleft increases the amplitude of mIPSCs, an effect that was more pronounced for small amplitude events, indicating a crucial role for GABA synaptic clearance in variability of mIPSCs (Barberis et al., 2004; Perrais and Ropert, 2000).

GABA transporters actively remove GABA from the extracellular space, modulating phasic and tonic inhibition as well as GABA spillover. Of the four high affinity Na^+/Cl^- coupled transporters identified to date (GAT-1, GAT-2, GAT-3 and BGT-1), GAT-1 is the most copiously expressed in the brain, localizing mainly at GABAergic axon terminals and distal astrocytic processes (Conti et al., 2004). Experiments using selective GAT-1 antagonists revealed that blocking GAT-1 increases the decay of evoked IPSCs ((Engel et al., 1998; Thompson and Gähwiler, 1992), as well as tonic inhibition (Keros and Hablitz, 2005; Petrini et al., 2004; Semyanov et al., 2003). GAT-1 knockout mice exhibited a severe impairment of GABA clearance from the extracellular space, effecting not only tonic but also phasic inhibition particularly during sustained neuronal activity (Bragina et al., 2008).

2.5 POSTSYNAPTIC MODULATION OF GABAERGIC INHIBITION

As discussed above, synaptic efficacy is governed by postsynaptic factors, namely the functional properties, distribution and modulation of postsynaptic receptors. $GABA_A$ receptors are pentameric complexes assembled from 19 subunits (α1–6, β1–3, γ1–3, ε, δ, θ, π and ρ1–3), resulting in a highly heterogeneous array of receptor subtypes with distinct physiological and pharmacological properties (Whiting, 1999).

2.5.1 $GABA_A$ receptor structure and subtypes

$GABA_A$ receptors are members of the pentameric Cys-loop receptor superfamily of ligand-gated ion channels, which also includes nicotinic acetylcholine receptors, glycine receptors and $5HT_3$ serotonin receptors. Members of this superfamily are very similar in their sequence and structure. Each subunit consists of a large extracellular N-terminus, four α-helical transmembrane domains (TM1-TM4) with a large cytoplasmic loop between TM3 and TM4, and a short extracellular C-terminus (Figure 2). N-terminus contains the binding site for ligand and various drugs, while TM2 forms the hydrophilic lining of the ion channel. The large cytoplasmic loop includes multiple sites for protein-

protein interaction and phosphorylation (Michels and Moss, 2007; Olsen and Sieghart, 2009).

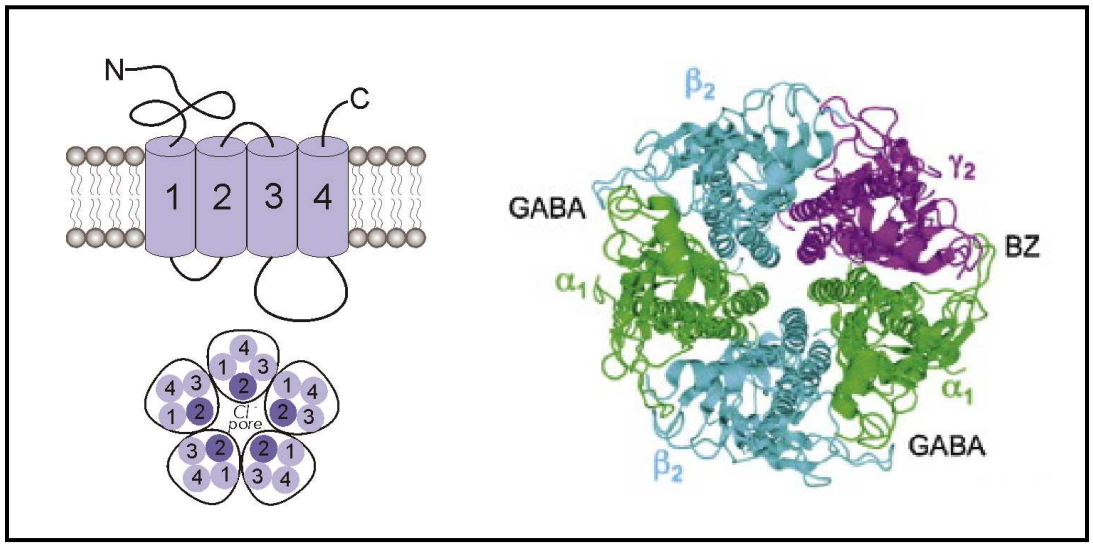


Figure 2. **$GABA_A$ receptor structure** *(Left)* Receptor subunits consist of four transmembrane domains (TM1-4). TM2 lines the pore of the chloride channel. *(Right)* α1β2γ2 is the most abundant subtype. GABA binding sites are between the α and β subunits, while benzodiazepines bind between the α and γ subunits (D'Hulst *et al.*, 2009)

$GABA_A$ receptor subunit families could in theory give rise to thousands of different receptor subtypes, but studies revealed that only a limited number of combinations actually exist on the neuronal cell surface. The working list of native $GABA_A$ receptors currently includes 26 members, but this list will undoubtedly expand as more experimental data will be provided (Olsen and Sieghart, 2008). The most abundant $GABA_A$ receptors in the brain are composed of two α, two β and one γ subunit, with α1β2γ2 subtype representing the largest portion, followed by α2β3γ2 and α3β3γ2 (Whiting, 2003). The less common δ and ε subunits may substitute for the γ subunit, and θ for the β subunit (Sieghart et al., 1999). The ρ subunits, initially identified in the retina and referred to as $GABA_C$ receptors, are pharmacologically different from traditional $GABA_A$ receptors, in that they are relatively insensitive to bicuculline and other $GABA_A$ receptor modulators (e.g. benzodiazepines and general anesthetics). They are, however,

considered as part of the $GABA_A$ receptor family, as they are closely related to $GABA_A$ receptors in terms of sequence, structure and function (Olsen and Sieghart, 2008).

The diversity of $GABA_A$ receptors at the cell surface is governed by mechanisms that control the assembly and transport of defined subunit combinations. $GABA_A$ receptors are assembled in the endoplasmic reticulum, which acts as a checkpoint to ensure efficient assembly of functional receptor complexes (Kittler et al., 2002). Studies on heterologous cell lines expressing various $GABA_A$ receptor subunits revealed that the cell surface is limited to the combinations $\alpha\beta$ and $\alpha\beta\gamma2$, while most single subunits and $\alpha1\gamma2$, $\beta2\gamma2$ combinations are retained in the ER, where they are rapidly degraded (Connolly et al., 1996a; Gorrie et al., 1997) The $\beta3$ subunit, on the other hand, can form homomeric receptors that can access the cell surface, due to the key residues in its N-terminal domain which were shown to mediate the selective assembly of $GABA_A$ receptors (Connolly et al., 1996b; Taylor et al., 1999). The significance of N-terminal domains for oligomerization and $GABA_A$ receptor subunit assembly was further demonstrated for α and $\gamma2$ subunits. N-terminal residues 58-67 of α subunits were shown to mediate their interaction with β but not γ subunits, while residues 80-100 were identified as the $\gamma2$ subunit-binding domain (Klausberger et al., 2001; Taylor et al., 2000). Similarly, two different regions within the N-terminal of $\gamma2$ were found to mediate $\gamma2$ subunit assembly with $\alpha1$ and $\beta3$ subunits (Klausberger et al., 2000).

Further complexity in the number of functional $GABA_A$ receptors is provided by alternative splicing of primary gene transcripts. One of the most widely studied is the $\gamma2$ subunit, which has two splice variants differing in an eight-amino acid stretch within the intracellular loop (Whiting et al., 1990). The long variant ($\gamma2L$) has the consensus sequence for phosphorylation by protein kinase C, and transgenic mice knockout for the short variant ($\gamma2S$) exhibit increased affinity for benzodiazepine agonists (Quinlan et al., 2000). In addition, a recent study demonstrated that $\gamma2S$ can also act as an accessory protein, modulating both $GABA_AR$ pharmacology and kinetics (Boileau et al., 2010).

The heterogeneity of $GABA_A$ receptors has crucial functional consequences, as the pharmacological and physiological properties of $GABA_A$ receptors depend on their subunit composition. In particular, the kinetics of different $GABA_A$ receptors determine

the time course of synaptic response, essential for synaptic integration. In the cerebellum, fast desensitizing responses were shown to be mediated by α2β2/3γ2 receptors, whereas slow desensitizing responses were generated by α3β2/3γ2 receptors (Devor et al., 2001). Similarly, the age-dependent decrease in deactivation kinetics of cerebellar granule cells IPSCs and their increased sensitivity to furosemide was attributed to the enhanced expression of α6 subunits (Tia et al., 1996).

2.5.2 $GABA_A$ receptor distribution

The expression of different $GABA_A$ receptor subtypes among brain regions and cell types has been studied extensively, using various methods ranging from *in situ* hybridization (Wisden et al., 1992) to immunohistochemistry (De Blas, 1996) and immunoprecipitation (McKernan and Whiting, 1996). These studies have revealed a highly heterogeneous distribution of each subunit, with differentially overlapping expression profiles throughout the brain.

α1, β2, and γ2 are considered the most abundant $GABA_A$ receptor subunits in the brain (Pirker et al., 2000). α2 subunits are largely located in forebrain and cerebellum, while α3 are highly expressed in the cortex (Christie et al., 2002). The expression of α4 subunits is relatively limited mainly to thalamic and hippocampal regions, where they are co-expressed with either γ2 or δ subunits at synaptic and extrasynaptic regions, respectively (Sur et al., 1999). α4β2δ subtypes are exclusively extrasynaptic. These receptors have a high sensitivity to GABA and desensitize little, making them ideal for mediating tonic inhibition. (Brown et al., 2002; Mtchedlishvili and Kapur, 2006). α5-containing $GABA_A$ receptors, likewise, are localized primarily to extrasynaptic sites of pyramidal neurons in the CA1 and CA3 regions of the hippocampus, where they mediate tonic inhibition (Caraiscos et al., 2004). In addition, it has recently been shown that α5-subunits located at synaptic sites contribute to the slow phasic inhibition of CA1 pyramidal neurons (Vargas-Caballero et al., 2010; Zarnowska et al., 2009). α6 subunits are present exclusively in cerebellar granule cells (Nusser et al., 1999), where they partner with δ-subunit-containing receptors to mediate tonic conductance (Brickley et al.,

2001; Rossi and Hamann, 1998). Of the β subunits, β2 is widely expressed in the brain, and knockout of the gene encoding for this subunit results in a 50% loss of $GABA_A$ receptors in mouse brain (Sur et al., 2001). In the hippocampus, the β1 subunit is located in the CA1 region and in the dentate gyrus while the β3 accumulates in the CA3 area and dentate gyrus (Christie et al., 2002). γ2-subunits co-assemble with other subunits to form 75-80% of $GABA_A$ receptors, and are required for normal channel conductance and postsynaptic clustering (Essrich et al., 1998; Günther et al., 1995). γ2 subunits in association with α1, α2 or α3 form the predominant $GABA_A$ receptor subtype mediating phasic inhibition. δ-subunits are expressed in the cerebellum and several forebrain areas, in association with α4 or α6 subunits (Olsen and Sieghart, 2008) and in the dentate gyrus (Glykys et al., 2008; Nusser and Mody, 2002). As mentioned above, δ-subunit containing $GABA_A$ receptors located on perisynaptic and extrasynaptic sites contribute to tonic inhibition (Nusser et al., 1998; Wei et al., 2003).

2.5.3 $GABA_A$ receptor pharmacology and modulation

$GABA_A$ receptors possess binding sites for a variety of drugs, including benzodiazepines and barbiturates currently used for the treatment of neuropsychiatric disorders. Benzodiazepines exert anxiolytic, sedative, anticonvulsant and hypnotic effects by allosterically modulating $GABA_A$ receptor currents (Hattori et al., 1986). Benzodiazepines enhance the affinity of $GABA_A$ receptors for GABA and increase the probability of channel opening (Rogers et al., 1994). While GABA binds to the interface between α and β subunits, benzodiazepines bind to a pocket on the α/γ interface (Ernst et al., 2003). γ2 subunit is essential for benzodiazepine binding, as γ2 knockout mice exhibit a 94% loss of benzodiazepine-binding sites (Günther et al., 1995). However benzodiazepines such as diazepam act on α1, α2, α3 and α5-containing $GABA_A$ receptors, each mediating a different effect of the drug. This subtype-specific action of diazepam was studied on mice with a knock-in point mutation on the drug binding site of each α subunit gene, rendering the respective subunit insensitive to diazepam (Rudolph and Möhler, 2004). These studies have revealed that α1βγ2 receptors mediate the

sedative but not anxiolytic effect of zolpidem (McKernan et al., 2000; Rudolph et al., 1999), whereas the anxiolytic action is largely mediated by α2 and, under conditions of high receptor occupancy, also by α3-containing $GABA_A$ receptors (Dias et al., 2005; Löw et al., 2000).

Barbiturates also potentiate the action of GABA, leading to sedative, hypnotic and anesthetics effects. These substances increase the mean channel open duration but have no effect on channel conductance or channel open probability (Sieghart, 1995). At higher concentrations (>50 μM) such as those used in anesthesia, they directly open $GABA_A$ receptors in the absence of the agonist (Franks and Lieb, 1994). In addition at millimolar concentrations, pentobarbital inhibits $GABA_A$ receptor function, possibly through a low-affinity open channel block mechanism (Rho et al., 1996).

Alcohol at high concentrations is also known to potentiate $GABA_A$ receptor function, by increasing the probability and duration of channels opening, channel bursts and bursts duration thus reducing the time spent in the closed state (Tatebayashi et al., 1998). δ-subunit containing receptors (usually associated with α4 and α6 subunits) have been shown to be highly selective to ethanol (Sundstrom-Poromaa et al., 2002; (Wallner et al., 2003). As δ-subunits are extrasynaptic, a recent model has emerged with ethanol selectively enhancing tonic, rather than phasic GABAergic transmission (Lobo and Harris, 2008). Although the enhancing effect of high concentrations of alcohol on $GABA_A$ receptors is well documented, the results obtained with lower concentrations remain controversial (Lobo and Harris, 2008).

The activity of $GABA_A$ receptors can be influenced by a number of endogenous modulators, including kinases, neurosteroids, protons and zinc. $GABA_A$ receptors are phosphorylated by protein kinase A (PKA) and protein kinase C (PKC) at conserved serine residues in the major intracellular loop of β and γ2 subunits (Brandon et al., 2002). Phosphorylation may affect $GABA_A$ receptor channel kinetics and desensitization rate (Hinkle and Macdonald, 2003). The effect of phosphorylation largely depends on the subunit composition, as demonstrated by the differential modulation of β subunits by PKA. While β3 phosphorylation enhances GABA-activated response, β1 phosphorylation reduces it. The β2 subunit is not affected by PKA (McDonald et al., 1998). Moreover, the functional effect of phosphorylation on GABAergic transmission is region-specific. For

example, in hippocampal pyramidal cells, intracellular delivery of PKA, but not of PKC, reduces the amplitude of mIPSCs, whereas in dentate gyrus granule cells PKC but not PKA enhances the peak amplitude of mIPSCs (Poisbeau et al., 1999). On the other hand, γ2-subunit phosphorylation has been shown to play a role in synaptic plasticity at CA1 inhibitory synapses. Calcineurin, which is a Ca^{2+}/calmodulin-dependent phosphatase, can be recruited to form a complex with the basally phosphorylated γ2 subunit. Selective dephosphorylation of γ2 following calcineurin binding is necessary and sufficient for the induction of long term depression at individual CA1 synapses (Wang et al., 2003).

The transition metal ion Zn^{+2}, contained in mossy fiber terminals (from which it can be released along with glutamate upon nerve stimulation), inhibits $GABA_A$ receptors (Smart et al., 1994). The inhibition of miniature IPSCs by zinc occurs *via* an allosteric modulation of receptor gating, including binding, desensitization and conformational change (Barberis et al., 2000). $GABA_A$ receptors composed of α and β subunits have much higher sensitivity for zinc as compared to αβγ subtypes (Hosie et al., 2003). αβ receptors were identified on the extrasynaptic sites of hippocampal pyramidal neurons, where they contribute to tonic inhibition. It has been therefore proposed that zinc mainly modulates tonic inhibition through γ-subunit lacking extrasynaptic receptors (Mortensen and Smart, 2006). Evidence in favor of a modulatory role of endogenously released zinc on $GABA_A$-mediated IPSCs was provided by Ruiz *et al*, who demonstrated that chelating zinc with either calcium-saturated EDTA or N,N,N',N'-tetrakis (2-pyridylmethyl)ethylenediamine enhances the amplitude of IPSCs evoked by granule cells but not stratum radiatum stimulation (Ruiz et al., 2004).

Activation of $GABA_A$ receptors leads to an efflux of HCO_3^-, causing changes in the pH level near the channel pore (Kaila, 1994). Hydrogen ions were shown to affect the amplitude and time course of mIPSCs in hippocampal neurons by modulating the gating properties of the channel. Increasing pH led to a strong enhancement of desensitization and binding rates (Mozrzymas et al., 2003a). Modulation of $GABA_A$ receptors by protons is highly subtype-specific; while protons increase α1β1 and α1β1δ-mediated currents, they decrease α1β1γ2Sδ, and have no effect on α1β1γ2Sδ-mediated currents (Krishek et al., 1996).

Endogenously synthesized steroids, such as 3α,21dihydroxy-5α-pregnan-20-one (THDOC), are positive allosteric modulators of $GABA_A$ receptors. They potentiate inhibitory postsynaptic currents by slowing down their deactivation process (Zhu and Vicini, 1997). δ-subunit containing $GABA_A$ receptors exhibit enhanced sensitivity to neurosteroids (Wohlfarth et al., 2002). Studies on δ-subunit knockout mice revealed that, at physiologically relevant concentrations, the likely site of action of THDOC was on the tonic conductance mediated by δ subunit-containing $GABA_A$ receptors (Stell et al., 2003). Moreover, the anxiolytic and anesthetic effects of THDOC were blunted in these mice (Mihalek et al., 1999). Changes in neurosteroid levels may contribute to neurological disorders, such as epilepsy. They have been implicated in a variety of psychological conditions, including panic attacks, major depression, postpartum depression, premenstrual tension, and schizophrenia (Herd et al., 2007).

2.5.4 $GABA_A$ receptor kinetics

The time course of synaptic currents is shaped by two major factors: the synaptic agonist transient and the gating properties of postsynaptic receptors. As discussed above, the time course of GABA transient is extremely short (~ 100 μs), which implies that postsynaptic $GABA_A$ receptors are activated in non-equilibrium conditions. Studies on the gating properties of $GABA_A$ receptors should therefore be carried out under conditions mimicking the synaptic agonist transient. Such temporal resolution can be achieved by applying the agonist directly to the cell using an ultrafast perfusion system, which can exchange solutions within 80-250 μs (Jonas, 1995). Combined with gating model simulations, current responses to ultrafast agonist applications can describe the precise gating properties of $GABA_A$ receptors.

Several models have been proposed to describe the gating properties of ligand-gated ion channels. The basic scheme proposed by Del Castillo and Katz (1957) for acetylcholine receptors involves binding of the ligand to the receptor, which leads to a conformational transition from bound-closed to bound-open states (Del Castillo and Katz, 1957). $GABA_A$ receptor gating is more complex as it involves sequential binding of two GABA molecules to the receptor, therefore both singly- and doubly-bound conformational states

should be taken into account (Bormann and Clapham, 1985). The kinetic model that presents a good compromise between simplicity and sufficiency to describe $GABA_A$-receptor mediated macroscopic currents is the one proposed by Jones and Westbrook (1995):

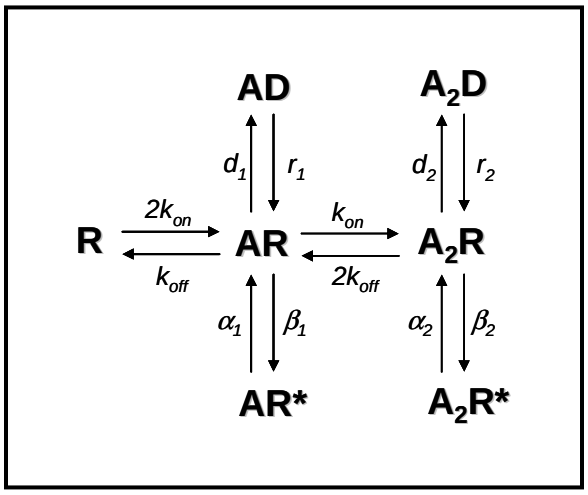


Figure 3. **Kinetic model proposed by Jones and Westbrook (1995)** The receptor (R) can bind two molecules of agonist (A), reaching the doubly bound closed state (A_2R). From this state, the receptor can open or desensitize (A_2R^* or A_2D, respectively). The singly bound open and desensitized states are also present (AR* and AD, respectively).

According to this model, the unbound receptor (R) can bind one or two agonist molecules (A) to reach a singly-bound (AR) or doubly-bound (A_2R) closed state. After agonist binding, the receptor can open (AR and A_2R^*) or desensitize (AD and A_2D). Desensitization is characterized by a ligand-bound, non-conductive state that has a high probability of reopening before the agonist can dissociate. The deactivation kinetics are therefore strongly influenced by desensitization, as the channel oscillates between open and long desensitized states before unbinding, prolonging the duration of deactivation (Jones and Westbrook, 1995).

All conformational states are functionally coupled to each other; hence the current responses are influenced by all the rate constants (Mozrzymas et al., 2003b). However, certain experimental protocols can be used to dissect each rate constant, as demonstrated by several studies (Barberis et al., 2000; Mozrzymas et al., 1999; Petrini et al., 2003).

For instance, the agonist binding rate is proportional to GABA concentration (k_{on} x [GABA]), so at low [GABA] the binding step becomes much slower than the conformational change that leads to channel opening. Therefore at low concentrations of GABA (<100 μM), the current onset would be largely governed by k_{on}, while at saturating GABA concentrations, conformational change would be rate limiting. Using this scheme, it was demonstrated that Zn^{+2} slows down the onset kinetics of GABA-induced currents by decreasing both k_{on} and β2 rate constants (Barberis et al., 2000). Moreover, increasing the pH was shown to enhance the binding rate of $GABA_A$ receptors, which was evident by the pH-dependent increase in amplitude at low GABA concentrations (Mozrzymas et al., 2003a).

The kinetics of the desensitization process are elucidated by long applications of saturating GABA concentrations, which reveal a biphasic process with two time constants, τ_{fast} and τ_{slow}. However, synaptic currents and currents evoked by a brief pulse of agonist are likely affected by the fast component alone. Besides its impact on deactivation, rapid desensitization (which occurs in a millisecond timescale) may also affect current amplitude and onset, as the receptors preferentially enter into the desensitized state (Mozrzymas et al., 2003b). Interestingly, low concentration of GABA would 'trap' $GABA_A$ receptors in a partially bound, slowly absorbing desensitized state, thus reducing the number of receptors available for subsequent opening in response to synaptic GABA release (Mozrzymas et al., 2003b). This phenomenon would be relevant for tonic inhibition by ambient GABA, and represents a potent mechanism for IPSC modulation.

2.5.5 $GABA_A$ receptor trafficking

GABAergic neurotransmission depends on the correct localization of $GABA_A$ receptors at synaptic and extrasynaptic sites. The trafficking of $GABA_A$ receptors to and from the neuronal membrane is highly dynamic, and involves several steps including the assembly in the endoplasmic reticulum, transport and insertion at the membrane, endocytic internalization and sorting, postsynaptic clustering as well as lateral diffusion within the plasma membrane (Arancibia-Cárcamo and Kittler, 2009; Kneussel and Loebrich, 2007;

Triller and Choquet, 2005) (Figure 4). The interplay between these processes determines the number of receptors available for ligand binding, and is modulated by numerous $GABA_A$ receptor-associated proteins (Lüscher and Keller, 2004).

Newly synthesized $GABA_A$ receptor subunits are assembled in the endoplasmic reticulum, in association with chaperone proteins immunoglobulin heavy chain binding protein (BiP) and calnexin (Connolly et al., 1996a). Chaperone proteins retain misfolded and unassembled receptors in the endoplasmic reticulum, thus regulating which receptor subtypes are to be transported to the plasma membrane. Further control is provided by the ubiquitin-proteasome system in the endoplasmic reticulum, which targets unassembled subunits (Saliba et al., 2008). Ubiquitin-like protein Plic-1 was shown to directly interact with the intracellular loop of all α and β subunits and promote $GABA_A$ receptor accumulation at the cell surface (Bedford et al., 2001). Further studies revealed that Plic-1 increases the stability of ubiquitinated $GABA_A$ receptors in the endoplasmic reticulum, resulting in an increase in the number of receptors that are available for membrane insertion (Saliba et al., 2008). Interestingly, increasing neuronal activity was shown to reduce $GABA_A$ receptor ubiquitination and enhance receptor stability on the plasma membrane, supporting a regulatory role for ubiquitination on synaptic efficacy and plasticity (Saliba et al., 2007).

The vesicular trafficking of $GABA_A$ receptors from the endoplasmic reticulum to the *trans*-Golgi network and plasma membrane involves several proteins including BIG2 (brefeldin A-inhibited GDP/GTP exchange factor 2), which binds the intracellular loops of all β subunits of $GABA_A$ receptors (Charych et al., 2004). Electron microscopy studies have revealed that BIG2 is localized within the *trans*-Golgi network as well as on vesicle-like structures in the dendritic cytoplasm, sometimes colocalizing with $GABA_A$ receptors, suggesting a role in the exocytosis of assembled receptors to the cell surface (Charych et al., 2004).

Another protein implicated in the surface transport of $GABA_A$ receptors is GABARAP ($GABA_A$ receptor associated protein), which was shown to interact with the γ2-subunits of $GABA_A$ receptors (Wang et al., 1999). Its role in intracellular $GABA_A$ receptor transport was suggested on the basis of its interaction with the cytoskeleton, and with *N*-ethylmaleimide-sensitive factor (NSF), a protein critical for intracellular membrane

trafficking events (Kittler et al., 2001). GABARAP was found on Golgi membranes and intracellular vesicles, as well as in the perinuclear cytoplasm and proximal dendrites colocalizing with γ2-subunit containing $GABA_A$ receptors (Kittler et al., 2001; Leil et al., 2004). However, even though overexpression of GABARAP with $GABA_A$ receptors increases the number of receptors on the membrane surface (Chen et al., 2005; Leil et al., 2004), the lack of GABARAP expression at synapses suggests that its main role is in the intracellular transport of $GABA_A$ receptors rather than postsynaptic receptor anchoring (Kneussel et al., 2000). Furthermore, GABARAP-knockout mice exhibited no changes in the expression and distribution of the γ2-subunit, indicating that GABARAP is not critically important for $GABA_A$ receptor trafficking (O'Sullivan et al., 2005). It may, however, play a role in the regulated delivery of $GABA_A$ receptors to the neuronal surface, as it was shown to be a central player in the delivery of $GABA_A$ receptors to the membrane following long term depression induced by NMDA receptor activation (Marsden et al., 2007).

Post-translational attachment of the fatty acid palmitate (palmitoylation) to cysteine residues on the γ2-subunits also affects the membrane targeting and intracellular trafficking of $GABA_A$ receptors, although the exact mechanism remains unknown (Fang et al., 2006; Keller et al., 2004; Rathenberg et al., 2004). Palmitoylation of the γ2 subunit is mediated by a Golgi-specific DHHC zinc finger domain protein (GODZ), which interacts directly with the intracellular domain of the γ2 subunits. This interaction seems necessary for the transport and accumulation of $GABA_A$ receptors at synapses, as well as the normal function of GABAergic synapses (Fang et al., 2006).

$GABA_A$ receptors on the membrane surface undergo clathrin-dependent endocytosis, a process shown to be mediated by the direct binding of the clathrin-adaptor protein (AP2) to the β and γ2 subunits of $GABA_A$ receptors (Herring et al., 2003; Kittler et al., 2000; Kittler et al., 2008). Blocking this interaction increased the number of surface receptors and the amplitude of miniature IPSCs, underlining the importance of AP2-dependent endocytosis for regulating synaptic strength (Kittler et al., 2000; Kittler et al., 2005).

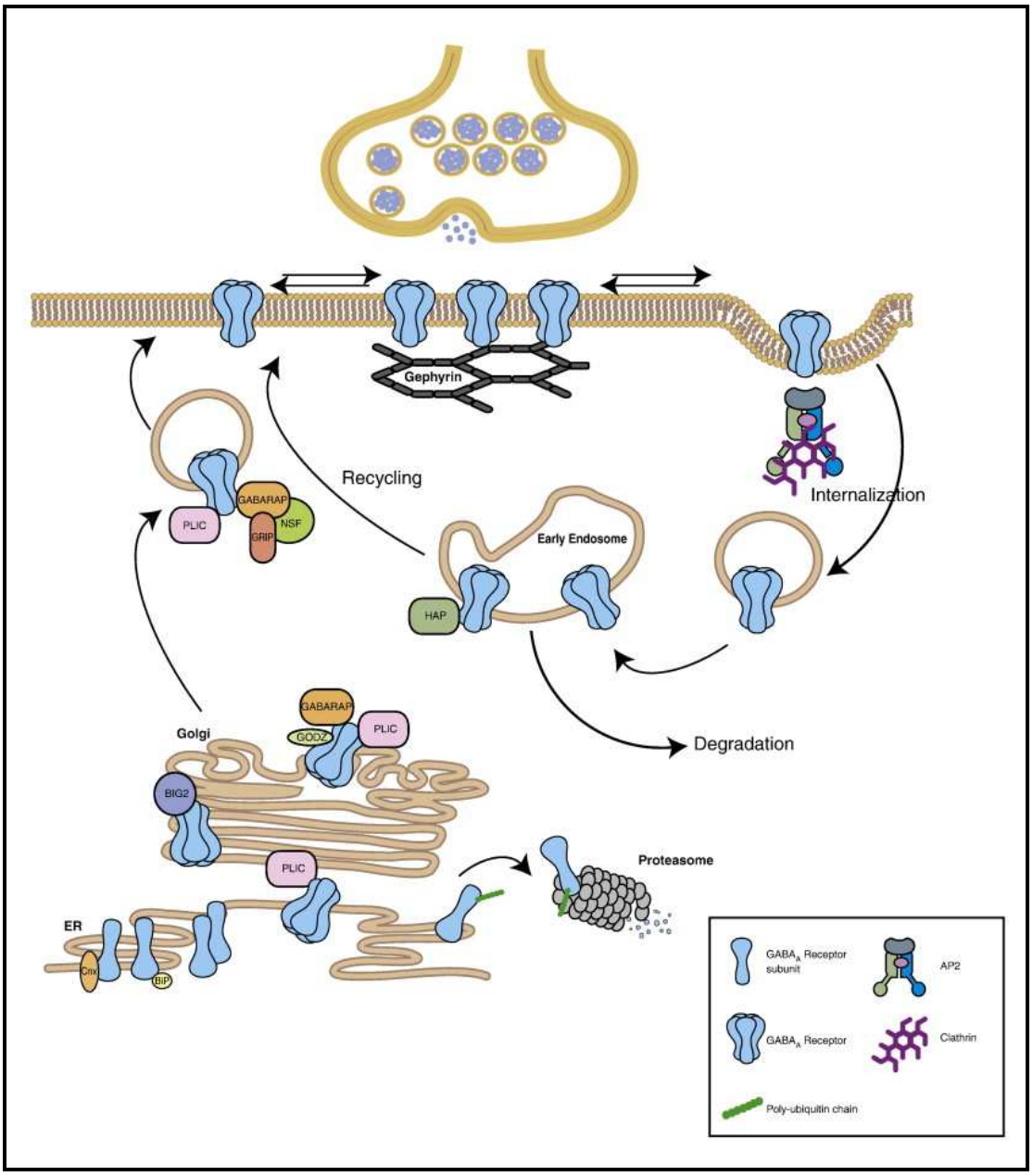


Figure 4. **GABA$_A$ receptor trafficking.** GABA$_A$ receptors are assembled in the endoplasmic reticulum (ER). Unassembled receptor subunits are subject to unibquitination and proteasomal degradation. Correctly assembled GABA$_A$ receptors interact with several proteins in the Golgi which facilitate their transport to the cell surface. Once inserted in the plasma membrane, GABA$_A$ receptor can laterally diffuse in and out of synaptic sites. Gephyrin scaffold stabilizes the receptors at synapses. GABA$_A$ receptors on the membrane surface undergo clathrin-dependent endocytosis. Once endocytosed, GABA$_A$ receptors are either recycled back to the cell membrane or targeted for lysosomal degradation. (Arancibia-Carcamo & Kittler, 2009)

Moreover, the AP2-binding sites on the β and γ2 subunits are regulated by phosphorylation, which reduces binding to AP2 and modulates cell-surface stability of $GABA_A$ receptors (Kittler et al., 2005; Kittler et al., 2008). Once endocytosed, $GABA_A$ receptors are either recycled back to the cell membrane or targeted for lysosomal degradation. Huntingtin-associated protein 1 (HAP1) was shown to play a pivotal role in the fate of endocytosed receptors, as it binds to the β-subunit and inhibits $GABA_A$ receptor degradation, enhancing receptor recycling to the plasma membrane (Kittler et al., 2004). Recently it was demonstrated that HAP1 acts as an adaptor that links $GABA_A$ receptors to the kinesin family (KIF) microtubule motors, mediating the transport of receptors to synapses (Twelvetrees et al., 2010). The disruption of HAP1-KIF5 complex may lead to altered synaptic inhibition and disrupt the excitatory/inhibitory balance in Huntington's disease.

In addition to intracellular vesicular trafficking, the number of $GABA_A$ receptors at synapses is further modulated by lateral diffusion between synaptic and extrasynaptic sites (Renner et al., 2008; Triller and Choquet, 2005). This phenomenon was largely revealed by single particle tracking experiments, using small fluorophores, latex beads and photostable quantum dots, which allowed visualization of individual receptor molecules in real time. Accordingly, a dynamic view of the postsynaptic organization has emerged, where receptors continuously move on the neuronal plasma membrane and are transiently stabilized at synaptic sites by interactions with scaffold proteins and cytoskeleton (Renner et al., 2008; Triller and Choquet, 2008). At GABAergic synapses, lateral mobility of $GABA_A$ receptors was studied using both electrophysiological and fluorescence imaging techniques (Jacob et al., 2005; Lévi et al., 2008; Thomas et al., 2005). Following an irreversible block of synaptic $GABA_A$ receptors, the amplitude of miniature IPSCs recovered rapidly within 10 minutes, an effect associated with the diffusion of unblocked extrasynaptic receptors into the synaptic membrane domain (Thomas et al., 2005). Using fluorescent bungarotoxin labeling, it was further demonstrated that $GABA_A$ receptors were largely inserted at extrasynaptic sites, after which they could directly access the synaptic sites (Bogdanov et al., 2006). A recent study using γ2-subunits directly labeled with quantum dots revealed that the lateral diffusion of $GABA_A$ receptors on the membrane can be modulated by neuronal activity,

providing a rapid modification in receptor numbers at synapses (Bannai et al., 2009). Excitatory activity reduced the dwell time of $GABA_A$ receptors at synapses and increased their synaptic and extrasynaptic diffusion coefficients, which was paralleled by a translocation of $GABA_A$ receptors to the extrasynaptic membrane, resulting in a reduction in the amplitude of miniature IPSCs.

The confinement of $GABA_A$ receptors at synapses depends on the rather crowded nature of postsynaptic sites which act as a brake to slow down their diffusion rate. Fluorescent recovery after photobleaching (FRAP) imaging of GFP-tagged $GABA_A$ receptors revealed that synaptic receptors have a three-fold lower rate of mobility as compared to their extrasynaptic counterparts (Jacob et al., 2005). This effect was due to the scaffold protein gephyrin, which is the main component of glycinergic and GABAergic postsynaptic organization (Fritschy et al., 2008; Kneussel and Betz, 2000). Interestingly, the gephyrin scaffold itself is dynamically regulated by synaptic activity, emphasizing the role of receptor-scaffold interactions in providing a rapid control of receptor number at synapses (Hanus et al., 2006; Maas et al., 2006). The properties of the gephyrin scaffold and its role in the clustering of $GABA_A$ receptors are presented in the following sections.

3. POSTSYNAPTIC ORGANIZATION OF GABAERGIC SYNAPSES

Despite the constant turnover of postsynaptic proteins, an efficient and reliable synaptic transmission requires receptors to be accumulated precisely in front of presynaptic release sites. A mechanism is therefore needed to anchor receptors at postsynaptic sites and maintain the stability of synapses. This is provided by scaffold proteins that mediate the clustering of receptors and link them to the cytoskeleton as well as to other proteins that can modulate receptor function and intracellular signaling cascades.

The major component of the inhibitory postsynaptic organization is gephyrin, which was initially identified as a 93-kDa protein co-purified with glycine receptors (Pfeiffer et al., 1982). It was later shown to be enriched at both glycinergic and GABAergic synapses (Triller et al., 1987). Numerous studies in the last twenty years have revealed gephyrin to be an essential part of inhibitory postsynaptic organization, in line with its ubiquitous

expression in the mammalian central nervous system (Kirsch and Betz, 1993; Prior et al., 1992; Waldvogel et al., 2003).

Gephyrin binds with high affinity to the β-subunit of glycine receptors, and is essential for glycine receptor clustering in various neuronal tissues including the spinal cord, the hippocampus and the retina (Feng et al., 1998; Fischer et al., 2000; Lévi et al., 2004; Meyer et al., 1995). Gephyrin was shown to act as a cargo adaptor for long distance microtubule-based transport of glycine receptors to and from distal neurites (Maas et al., 2006; Maas et al., 2009), and to stabilize glycine receptors at synaptic sites once they are inserted in the neuronal membrane by confining their later movements (Ehrensperger et al., 2007; Meier et al., 2001). The function of gephyrin on GABAergic synapses is more complicated due to the heterogeneity of $GABA_A$ receptor subtypes and the subunit-specific clustering of $GABA_A$ receptors by gephyrin-independent mechanisms (Kneussel et al., 2001; Lévi et al., 2004). Nevertheless, gephyrin does play a major role in $GABA_A$ receptor clustering as revealed by the drastic impairment of synaptic $GABA_A$ receptor clustering in the absence of gephyrin (Essrich et al., 1998; Kneussel et al., 1999a; Marchionni et al., 2009; Yu et al., 2007).

In addition to its role in the synaptic organization, gephyrin is also involved in the biosynthesis of the molybdenum cofactor (Moco), a highly conserved molecule required for the activity of molybdenum enzymes that are essential for the survival of all organisms from bacteria to eukaryotes (Stallmeyer et al., 1999). Targeted disruption of the gephyrin gene in mice leads to a lethal phenotype shortly after birth, and neonates display deficits in Moco biosynthesis, as well as impaired glycine and $GABA_A$ receptor clustering (Feng et al., 1998; Kneussel et al., 1999a).

3.1 GEPHYRIN STRUCTURE AND CLUSTER FORMATION

Gephyrin is made up of a G- and an E-domain, which are homologous to bacterial MogA and MoeA proteins that are involved in Moco biosynthesis, linked by a central domain which contains binding sites for several gephyrin interactors (Fritschy et al., 2008). The crystal structures of both G- and E-domains have been obtained, revealing valuable information about gephyrin clustering and binding to glycine receptors (Kim et al., 2006;

Sola et al., 2001; Sola et al., 2004). The N-terminal G-domain was shown to form trimers whereas the C-terminal E-domain can form dimers, leading to a hexagonal lattice formation onto which receptors can be anchored (Fritschy et al., 2008; Kneussel and Betz, 2000) (Figure 5). Impairing the oligomerization of G- and E-domains disrupts hexamer formation and abolishes gephyrin clustering at synaptic sites (Saiyed et al., 2007). Moreover, insertion of a splice cassette C5' into the G-domain was shown to interfere with gephyrin trimerization, leading to a depletion of both gephyrin and glycine receptor clusters from postsynaptic sites in hippocampal neurons (Bedet et al., 2006). However, gephyrin aggregation and postsynaptic clustering might represent different mechanisms, as a chimeric gephyrin containing two E-domains was shown to form intracellular aggregates but the aggregates were rarely found at postsynaptic sites (Lardi-Studler et al., 2007). Thus, gephyrin targeting to synapses might involve post-translational modifications or interaction with the receptors themselves, as shown for $GABA_A$ receptors, where targeted deletion of the γ2 subunit leads to the disruption of gephyrin clusters (Essrich et al., 1998; Li et al., 2005a).

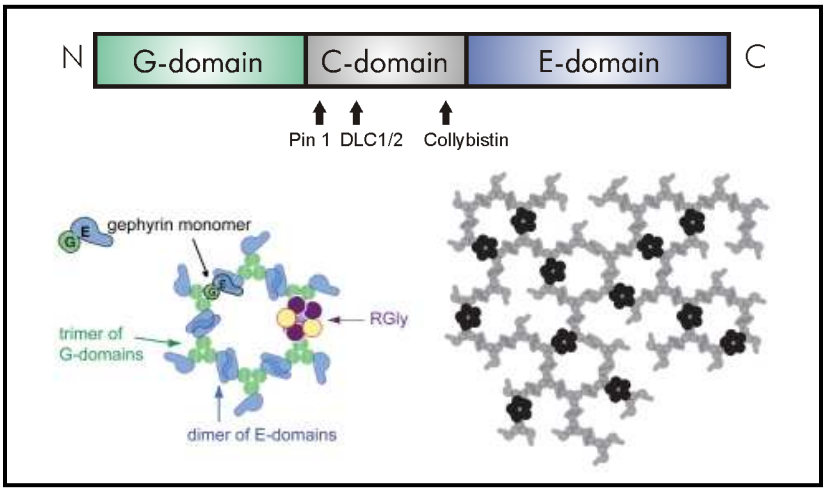


Figure 5. **Gephyrin domain structure and aggregation.** *(Top)* Gephyrin is made up of a G- and an E-domain, linked by a central C-domain. The binding sites for Pin1, DLC1/2 and collybistin are depicted by arrows. *(Bottom)* E- and G-domains are able to dimerize and trimerize, respectively, which would lead to a hexagonal lattice formation underneath the postsynaptic membrane. Glycine receptors (in black) would be anchored to the gephyrin scaffold through the binding of the β subunit with the E-domain of gephyrin. (Dumoulin *et al.*, 2010)

The E-domain contains the high-affinity binding site for the large cytoplasmic loop connecting transmembrane segments 3 and 4 of the glycine receptor β-subunit (Kim et al., 2006; Schrader et al., 2004; Sola et al., 2004). The direct binding of gephyrin to $GABA_A$ receptor subunits remained elusive until Tretter *et al.* (2008) provided evidence for *in vitro* binding of gephyrin to the intracellular domain of the α2 subunit (Tretter et al., 2008). A very recent report confirmed this result, and further identified a nineteen amino acid stretch at the start of the gephyrin E-domain as the α2 binding site (Saiepour et al., 2010). Interestingly, this site was distinct from the glycine receptor binding site, allowing simultaneous binding of gephyrin to both glycine and $GABA_A$ receptors which might explain the colocalization of glycine and $GABA_A$ receptors at GABAergic synapses (Lévi et al., 2004). Direct binding of gephyrin to $GABA_A$ receptor subunits other than α2 has not been demonstrated yet, which points toward an indirect interaction via bridging molecules.

3.2 GEPHYRIN-ASSOCIATED PROTEINS

Several gephyrin-interacting proteins have been identified to date, linking gephyrin to the cytoskeleton, motor protein complexes, signal transduction mechanisms and phosphorylation-dependent processes (Arancibia-Cárcamo and Kittler, 2009; Fritschy et al., 2008).

When ectopically expressed in human embryonic kidney cells (HEK 293), gephyrin forms large intracellular aggregates that trap glycine receptor β-subunits and other gephyrin-interacting partners (Kneussel et al., 1999b; Zita et al., 2007). The translocation of these aggregates to submembrane regions was observed upon co-expression of collybistin II, a brain-specific GDP/GTP exchange factor that activates the Rho-like GTPase Cdc42 (Kins et al., 2000). Collybistin exists in three isoforms (CB I-III), differing in their C-termini and by the presence of an N-terminal *src* homology 3 (SH3) domain, which negatively regulates gephyrin targeting to the plasma membrane (Harvey et al., 2004). Recent studies revealed that the activation of collybistin isoforms harboring the SH3 domain is mediated by the interaction of collybistin with the cell adhesion protein neuroligin 2 and the α2 subunit of $GABA_A$ receptors, although other interactors

likely exist (Poulopoulos et al., 2009; Saiepour et al., 2010). Both neuroligin 2 and α2 could form a tripartite complex with gephyrin and collybistin, tethering collybistin-gephyrin to the plasma membrane and acting as a nucleation point for the postsynaptic scaffold. The structural and functional consequences of SH3 domain activation remain to be elucidated. Nevertheless, gene ablation studies revealed the importance of collybistin for the formation and maintenance of gephyrin and γ2-subunit containing $GABA_A$ receptor clusters at synapses in the hippocampus and amygdala regions (Papadopoulos et al., 2007; Papadopoulos et al., 2008). Furthermore, collybistin deficiency led to increased anxiety and impaired spatial learning in mutant mice, with significant changes in the hippocampal GABAergic inhibition, network excitability and synaptic plasticity *in vivo (J*edlicka et al., 2009; Papadopoulos et al., 2007). Interestingly, in some brain regions of the collybistin knockout mice (e.g. the cerebellum), the impairment in gephyrin clustering was not reflected by a reduction in $GABA_A$ receptor clusters, pointing towards region-specific and gephyrin-independent mechanisms for $GABA_A$ receptor clustering (Kneussel et al., 2001; Papadopoulos et al., 2007).

Gephyrin provides a direct link with the cytoskeleton *via* its binding to polymerized tubulin (Kirsch et al., 1991). Further evidence for the role of gephyrin in microtubule-based transport was provided by its interaction with dynein and kinesin motor complexes (Fuhrmann et al., 2002; Maas et al., 2006; Maas et al., 2009). Gephyrin was shown to act as an adaptor protein between glycine receptor carrying vesicles and microtubule motor complexes (namely the dynein light chain and kinesin family 5), mediating the transport of gephyrin-glycine receptor complexes to and from synapses (Maas et al., 2006; Maas et al., 2009). A similar role for gephyrin in the intracellular transport of $GABA_A$ receptors has not been established yet. In addition to its association with the microtubules, gephyrin also interacts with several proteins of the actin cytoskeleton. Yeast two-hybrid screening revealed two gephyrin-interacting proteins that regulate actin polymerization, namely profilins 1 and 2 (Mammoto et al., 1998). Gephyrin was shown to form a complex with profilins and microfilament adaptors of the mammalian enabled (Mena)/vasodilator stimulated phosphoprotein (VASP) family, which are essential for submembranous actin filament generation and organization (Giesemann et al., 2003). The functional role of gephyrin-microfilament interactions is still not clear, although disruption of the actin

cytoskeleton resulted in a loss of small gephyrin clusters from immature neurons, suggesting an early role of the actin cytoskeleton in gephyrin scaffold formation (Bausen et al., 2006).

Other proteins identified as gephyrin-interacting partners include the rapamycin and FKBP12 target 1 (RAFT1), the peptidyl-prolyl isomerase NIMA interacting protein 1 (Pin1), and the glutamate receptor interacting protein 1 (GRIP1) (Sabatini et al., 1999; Yu et al., 2008a; Zita et al., 2007). RAFT1 mediates the *in vivo* effects of the immunosuppressant rapamycin and acts as an important regulator of messenger RNA translation (Sabatini et al., 1999). Through its interaction with RAFT1 and the concomitant signal transduction pathway, gephyrin might mediate translational control at synaptic sites. On the other hand, Pin1 was shown to bind to the C-domain of phosphorylated gephyrin and trigger conformational changes in the gephyrin molecule, enhancing its binding to the β-subunit of glycine receptors (Zita et al., 2007). The precise role of GRIP1 in GABAergic transmission remains unclear, despite its direct interaction with gephyrin and GABARAP. GRIP1 was observed at GABAergic synapses in association with gephyrin and $GABA_A$ receptors (Li et al., 2005b; Yu et al., 2008b). Although GRIP1 knockout mice did not exhibit any changes in the number of $GABA_A$ receptors on the cell surface (Hoogenraad et al., 2005), the regulated delivery of $GABA_A$ receptors to synapses following long term depression induced by NMDA receptor activation was mediated by GRIP1 and its binding partner GABARAP (Marsden et al., 2007).

3.3 $GABA_A$ RECEPTOR CLUSTERING

$GABA_A$ receptors form clusters at both synaptic and extrasynaptic sites. These clusters are important for the proper functioning of the synapses. As discussed above, while gephyrin is pivotal for glycine receptor clustering, its role in $GABA_A$ receptor clustering remains elusive (Lévi et al., 2004). Studies from cultured hippocampal neurons obtained from gephyrin knockout mice reported either a complete (Kneussel et al., 1999a) or a reduced loss (Lévi et al., 2004) of α2 and γ2-subunits clusters. Similarly, knocking down gephyrin with RNA interference reduced (but did not abolish) synaptic α2 and γ2

subunits clusters (Essrich et al., 1998; Jacob et al., 2005; Yu et al., 2007). These findings suggest that gephyrin is not essential for the initial clustering of $GABA_A$ receptors at synapses, but rather plays a role in the stabilization and maintenance of GABAergic synapses. It is worth noting that α1 and α5 subunits clusters were not affected in the hippocampus and spinal cord of gephyrin knockout animals (Kneussel et al., 2001; Lévi et al., 2004), indicating that gephyrin-independent mechanisms may also exist.

Interestingly, synaptic localization of gephyrin appears to depend on $GABA_A$ receptor clustering as demonstrated by a reduction in gephyrin immunoreactivity in the absence of γ2 subunits (Alldred et al., 2005; Li et al., 2005a; Schweizer et al., 2003). Likewise, the targeted deletion of α1 or α3 subunits led to a disruption in the synaptic targeting of gephyrin, which formed large cytosolic aggregates instead (Kralic et al., 2006; Studer et al., 2006).

As discussed in previous chapters, some $GABA_A$ receptor subunits localize mainly extrasynaptically, such as the α4 and α5-subunits. These subunits were shown to form clusters devoid of gephyrin (Kralic et al., 2006; Serwanski et al., 2006), even though gephyrin immunoreactivity was identified at both synaptic and extrasynaptic sites (Danglot et al., 2003). The gephyrin-independent mechanism for α5-subunit clustering was identified as the actin-binding protein radixin (Loebrich et al., 2006). Radixin binds to the intracellular domain of the α5-subunit and directly links α5 to the actin cytoskeleton. The alternative mechanisms of gephyrin-independent $GABA_A$ receptor anchoring remain to be elucidated.

Apart from accumulating receptors in apposition to presynaptic release sites, clustering can also modulate the functional properties of receptors, especially the desensitization kinetics that play an important role in shaping synaptic currents (Mozrzymas et al., 2003b). When glycine receptor clustering was induced by co-expression of gephyrin in heterologous expression systems, the receptors rapidly entered into the desensitized state upon short agonist applications (Legendre et al., 2002). This acceleration in the desensitization kinetics, as compared to diffusely distributed glycine receptors, was proposed to rely on changes in the conformation of the intracellular loop of the glycine receptor subunits. On the other hand, declusterization of $GABA_A$ receptors by disrupting the microtubule cytoskeleton resulted in faster rise time and desensitization kinetics,

which was shown to affect both phasic and tonic GABAergic transmission (Petrini et al., 2003; Petrini et al., 2004). As receptors rapidly exchange between diffuse and confined states under basal conditions and during neuronal activity (Bannai et al., 2009), such modifications in receptor kinetics due to their level of confinement could provide a rapid mechanism for dynamic regulation of synaptic strength.

3.4 CELL ADHESION MOLECULES AT GABAERGIC SYNAPSES

Synapses are specialized intercellular junctions which ensure communication between neurons. According to a classical view, synaptic transmission is unidirectional: the arrival of the action potential in a presynaptic nerve terminal causes neurotransmitter molecules to be released and to bind to receptors localized on postsynaptic sites, leading to channel opening. However, to be highly efficient, synaptic transmission needs the coordinated activity of pre and postsynaptic elements. In particular, the postsynaptic cell should exert a backward control on presynaptic signaling. This may occur *via* retrograde messengers or specialized adhesion molecules such as neurexins and neuroligins which provide a direct link between the pre and the postsynaptic sites by bridging the synaptic cleft (Lisé and El-Husseini, 2006). Several lines of evidence support the idea that the binding between neuroligins and neurexins represents not only a structural but also a functional link between pre- and post synaptic elements.

Presynaptic neurexins were first identified as receptors for the black-widow spider venom α-latrotoxin, which induces a massive release of neurotransmitters (Ushkaryov et al., 1992). Neurexins are transmembrane proteins encoded by three vertebrate genes (*NRXN1-3*) with two independent promoters that lead to either α- or β-neurexins (Tabuchi and Südhof, 2002). Alternative splicing at the N-terminal extracellular site modulates neurexin binding to different neuroligin isoforms and synaptogenic activity. In particular, the splice site 4 (S4) was shown to affect β-neurexin binding to neuroligin1 (Boucard et al., 2005; Chih et al., 2006; Graf et al., 2006). Hippocampal neurons co-cultured with COS cells expressing different neurexin isoforms revealed that β-neurexin containing the S4 insert and α-neurexin could promote GABAergic postsynaptic specialization (Chih et al., 2006; Kang et al., 2008). Interestingly, deletion of α-neurexins

led to a severe reduction in neurotransmitter release, due to the impairment of N- and P/Q-type Ca^{2+} channels (Missler et al., 2003; Zhang et al., 2005). The precise mechanism of neurexin-Ca^{+2} channel regulation is unclear, but likely involves the extracellular domain of α-neurexins (Zhang et al., 2005). Interestingly, a direct interaction of postsynaptic neurexins with α1-subunit of $GABA_A$ receptors was recently discovered (Zhang et al., 2010). Overexpression of neurexins in cultured hippocampal neurons resulted in an impairment of GABAergic transmission and synapse maturation by a neuroligin-independent mechanism, suggesting a more diverse function for neurexins than previously envisioned (Zhang et al., 2010).

Neuroligins, on the other hand, are found at postsynaptic sites and contain a large extracellular domain homologous to acetylcholinesterase which mediates binding to neurexins and allows neuroligins to homo-multimerise (Südhof, 2008). There are four neuroligin genes in mammals (*NLGN1-4*), while humans also express *NLGN5* on the Y chromosome. Neuroligins are also subject to alternative splicing at two splice sites in the extracellular domain (named A and B), splice site B being specific for neuroligin 1 (Craig and Kang, 2007). Although some studies reported that the presence of splice site B (which is the dominant isoform in rat brain) restricts neuroligin 1 binding to β-neurexins lacking S4 (Boucard et al., 2005; Chih et al., 2006), neuroligin 1 containing the insert at splice site B was also shown to bind β-neurexin 1 containing S4 (Reissner et al., 2008). The role of alternative splicing in regulating neuroligin/neurexin interactions was further challenged in a recent study in which the binding affinities of all combinations of alternatively spliced β-neurexins and neuroligins 1-3 were measured (Koehnke et al., 2010). All neuroligins containing or lacking insert A or B could bind to all β-neurexin isoforms regardless of the S4 insertion. Furthermore, the crystal structure of β-neurexin 1 with or without the S4 insert were very similar, although conformational dynamics could contribute to β-neurexin specificity (Wei and Zhang, 2010).

Nevertheless, the importance of the neuroligin/neurexin complex for the formation or maintenance of synaptic connections was revealed by co-culture experiments in which neuroligins expressed in non-neuronal cells could induce neurons to form functional release sites onto these cells (Scheiffele et al., 2000). Similarly, neurexin expression in non-neuronal cells could cluster glutamatergic and GABAergic postsynaptic proteins on

the dendritic surface of co-cultured neurons (Graf et al., 2004; Nam and Chen, 2005). The synaptogenic activity of neuroligins and neurexins was mediated by their ability to cluster each other on the contacting neurons. Interestingly, neuroligin 1 was localized preferentially at glutamatergic synapses, while neuroligin 2 at GABAergic synapses. Neuroligin 3 was observed at both GABAergic and glutamatergic synapses (Budreck and Scheiffele, 2007; Graf et al., 2004; Song et al., 1999; Varoqueaux et al., 2004). This has led to the hypothesis that different neuroligin isoforms could mediate synaptic specificity (Figure 6). Indeed, HEK293 cells co-expressing neuroligin 1 and glutamate receptors or neuroligin 2 and $GABA_A$ receptors formed functional synapses with co-cultured neurons, while co-expression of neuroligin 1 and $GABA_A$ receptors did not produce any spontaneous IPSCs (Dong et al., 2007; Fu et al., 2003).

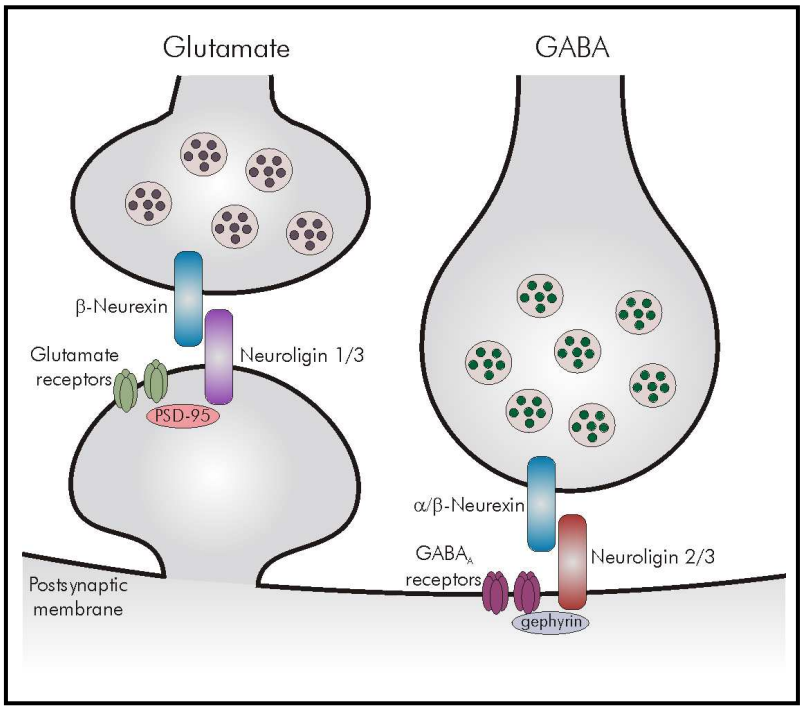


Figure 6. **Neuroligin/neurexin complex in synapse organization.** Postsynaptic neuroligins interact with presynaptic neurexins to stabilize synapses. Neuroligin 1 is localized preferentially at glutamatergic synapses, while neuroligin 2 is at GABAergic synapses. Neuroligin 3 is observed at both GABAergic and glutamatergic synapses. Neuroligins interact with postsynaptic scaffold proteins PSD-95 and gephyrin at glutamatergic and GABAergic synapses, respectively, which in turn mediate receptor clustering.

Overexpression and RNA interference studies revealed a more specific function for neuroligins in synaptic function *in vitro*, which consisted of regulating the balance between excitatory and inhibitory synapses (Chih et al., 2005; Chubykin et al., 2007). Even though overexpression of neuroligin 1-3 in cultured hippocampal neurons increased both excitatory and inhibitory terminals, neuroligin 2 had a much more pronounced effect on GABAergic terminals with respect to glutamatergic ones (Chih et al., 2005). Furthermore, knockdown of all three neuroligin isoforms with RNA interference selectively reduced miniature IPSCs with a mild effect on excitatory transmission, leading to an overall increase in the excitatory to inhibitory (E/I) balance (Chih et al., 2005). The E/I ratio is important for the overall cell excitability, and alterations of this parameter may underlie several pathological conditions including epilepsy and autism spectrum disorders (Fritschy, 2008; Rubenstein and Merzenich, 2003).

In vivo studies on mice knockout for neuroligin genes (*NLGNs 1-3*) revealed no abnormalities in synaptogenesis and synaptic morphology, but the animals died shortly after birth because of respiratory failure (Varoqueaux et al., 2006). In triple KO mice, the brainstem respiratory network was severely impaired due to an increase in excitatory versus inhibitory synaptic activity. Likewise, deletion of either neuroligin 1 or neuroligin 2 had no effect on the density of synapses in the hippocampus, but mice exhibited deficits in synaptic function, suggesting that neuroligins are dispensable for the initial synapse formation *in vivo* (Blundell et al., 2009; Blundell et al., 2010; Chubykin et al., 2007; Gibson et al., 2009).

Both *in vitro* and *in vivo* studies strongly implicate neuroligin 2 in the regulation of inhibitory synapses (Arancibia-Cárcamo and Kittler, 2009; Huang and Scheiffele, 2008). Neuroligin 2 selectively colocalizes with gephyrin and $GABA_A$ receptors, and overexpression of neuroligin 2 increases inhibitory synaptic contacts, leading to enhanced GABAergic transmission both *in vitro* and *in vivo* (Chubykin et al., 2007; Graf et al., 2004; Hines et al., 2008; Varoqueaux et al., 2004). In contrast, neuroligin 2 knockout animals display a reduced inhibitory synaptic transmission (Chubykin et al., 2007; Gibson et al., 2009). This leads to an increased cell excitability and impaired clustering of gephyrin and $GABA_A$ receptors (Jedlicka et al., 2010).

Despite the extensive evidence on the importance of neuroligin 2 in GABAergic transmission, how this molecule is selectively recruited to GABAergic synapses is still unclear. One of the prominent models for synapse-specific neuroligin distribution relies on the interaction of neuroligins with postsynaptic scaffold proteins (Figure 6). Intracellularly, neuroligins bind to PSD-95/Dlg/ZO-1 homology domains (PDZ) found in many scaffold proteins (such as PSD-95) in the glutamatergic postsynaptic densities. This interaction was shown to restrict neuroligin 1 localization to glutamatergic synapses (Irie et al., 1997; Prange et al., 2004). Interestingly, overexpression of PSD-95 relocalizes both neuroligin 1 and neuroligin 2 to excitatory synapses at the expense of inhibitory synapses (Graf et al., 2004; Levinson et al., 2005), while knockdown of PSD-95 increases neuroligin 1 localization at inhibitory synapses (Prange et al., 2004). Similarly, knockdown of gephyrin with RNA interference causes a shift of neuroligin 2 from inhibitory to excitatory synapses (Levinson et al., 2010). It is thus evident that at least *in vitro*, the balance between excitatory and inhibitory synapses could partly be regulated by neuroligin-scaffold protein interactions.

Yeast-2-hybrid screening recently led to the identification of a direct interaction between gephyrin and neuroligins, through a 15-residue gephyrin-binding motif (Poulopoulos et al., 2009). The gephyrin-binding motif was shared by all four neuroligin isoforms, so the specificity of neuroligin 2 for GABAergic synapses was proposed to come from its interaction with another postsynaptic protein, collybistin. According to the model proposed by the authors, initial neuroligin 2 localization at synapses would form nucleation sites for the recruitment for gephyrin-collybistin clusters, and collybistin activation by neuroligin 2 would further stabilize gephyrin scaffold formation and accumulate inhibitory receptors (Poulopoulos et al., 2009). A similar postsynaptic assembly model dependent on collybistin-activation was also proposed by Saiepour et al. (2010), whereby a direct interaction between the α2-subunit of $GABA_A$ receptors would activate collybistin and facilitate gephyrin targeting to synapses (Saiepour et al., 2010). The functional interplay between these two mechanisms for the assembly of GABAergic synapses remains to be elucidated.

4. HOMEOSTATIC SYNAPTIC PLASTICITY & E/I BALANCE

The properties of the neuronal network in the brain are constantly modified by activity-dependent plasticity processes. These provide feedback mechanisms to strengthen or weaken individual synaptic connections in response to different inputs. Changes in synaptic strength could drive the cells toward hyper-excitation or inhibition, and are thus counterbalanced by homeostatic plasticity mechanisms that allow neurons to adjust their properties according to their level of activity (Turrigiano, 2008). The current understanding of homeostatic plasticity mechanisms involves modifications in synaptic strength as well as in intrinsic excitability and in the number of neurons to maintain a 'set-point' of activity (Desai, 2003; Turrigiano and Nelson, 2000; Turrigiano and Nelson, 2004).

Changes in synaptic strength in response to perturbed network activity (i.e. synaptic scaling) was first described in cultured neocortical neurons, where blocking GABA-mediated inhibition initially raised firing rates, but over a 48-hour period, the amplitude of miniature EPSCs decreased and firing rates returned to control values (Turrigiano et al., 1998). The functional importance of synaptic scaling in network connectivity was further demonstrated *in vivo* in the rodent visual cortex, where quantal amplitude of excitatory currents could be scaled up or down as a function of development and sensory experience (Desai et al., 2002). Inhibitory synapses also undergo synaptic scaling following activity deprivation, albeit in the opposite direction from excitatory synapses. Upon tetrodotoxin treatment of hippocampal neurons in culture, the amplitude of miniature IPSCs is reduced, an effect associated with a decrease in postsynaptic $GABA_A$ receptors and presynaptic GAD65 immunoreactivity (Kilman et al., 2002). As such, neurons can tune the relative strengths of excitatory (E) and inhibitory (I) synapses and balance the E/I ratio in response to changes in activity.

Disruption of the E/I ratio was implicated in various neurological disorders, such as epilepsy, schizophrenia, and autism spectrum disorders. Some forms of epilepsy are associated with abnormal $GABA_A$ receptor function, due to an altered receptor trafficking and/or expression (Jacob et al., 2008). Multiple mutations in the $\gamma 2$, $\alpha 1$ and δ subunits of $GABA_A$ receptors have been identified in epileptic patients, although the precise

mechanisms by which these mutations contribute to seizures are unclear (Baulac et al., 2001; Cossette et al., 2002; Feng et al., 2006). Nevertheless, region-specific changes in $GABA_A$ receptor function and mutations affecting $GABA_A$ receptor assembly and trafficking contribute to altering the E/I balance and to the pathophysiology of epilepsy (Fritschy, 2008).

Deficits in GABAergic neurotransmission was also observed in schizophrenia, especially within the dorsolateral prefrontal cortex network associated with working memory (Lewis and Gonzalez-Burgos, 2006). Post-mortem studies revealed a reduction in the mRNA levels of GABA-synthesizing enzyme GAD67 and GABA membrane transporter GAT1 in a subpopulation of interneurons (Akbarian et al., 1995; Volk et al., 2001). A concomitant increase in the $GABA_A$ receptor α2-subunit immunoreactivity was observed on the axon initial segment of pyramidal neurons that receive input from these interneurons (Volk et al., 2002). Such alterations in the GABAergic transmission is thought to underlie deficits in the oscillatory activity in the prefrontal cortex network and lead to working memory dysfunction in schizophrenia (Lewis and Gonzalez-Burgos, 2006).

As discussed in the previous chapters, cell adhesion molecules such as neuroligins/neurexins, and postsynaptic scaffold proteins such as PSD-95 and gephyrin are crucial for the organization and maintenance of synapses. Disruptions in synaptic organization due to mutations in the genes encoding for neuroligins and neurexins are associated with autism spectrum disorders (Südhof, 2008). Autism spectrum disorders (ASDs) are a group of heterogeneous neurodevelopmental disorders characterized by impaired social interactions, communication deficits, stereotypic and repetitive behaviors. A small percentage of ASD patients carry missense and nonsense mutations in genes encoding neuroligins 3 and 4 (Jamain et al., 2003; Laumonnier et al., 2004), neurexin 1 (Kim et al., 2008) and Shank3, an intracellular binding partner for neuroligins (Durand et al., 2007). In line with the role of neuroligin/neurexin complex in maintaining the E/I balance, a model for ASDs has emerged in which an increased E/I ratio due to a combination of genetic and environmental factors leads to some forms of autism (Rubenstein and Merzenich, 2003).

The molecular mechanisms that establish and maintain synaptic connections and regulate the balance between excitatory and inhibitory inputs undoubtedly play a pivotal role in the normal functioning of the brain. Further understanding of these mechanisms will help unveil the pathophysiology of numerous cognitive disorders and lead to more effective treatment options for patients.

AIM OF THE WORK

The localization of $GABA_A$ receptors in precise apposition to release sites is a prerequisite for an efficient and reliable GABAergic transmission. Gephyrin is the backbone of the postsynaptic scaffold at inhibitory synapses, mediating receptor clustering and synaptic organization. The aim of this study was to examine the functional role of gephyrin in regulating GABAergic transmission in the hippocampus.

To study gephyrin function, different approaches have been used including gephyrin knockout mice and RNA interference. While these methods have some advantages they also have some shortcomings. Targeted deletion of gephyrin gene in knock-out mice leads to a lethal phenotype shortly after birth, limiting the use of these mice for post-natal studies. In addition, compensatory mechanisms might complicate the functional consequences of gephyrin gene deletion. On the other hand, RNA interference can be used to down regulate gephyrin gene expression, but this technique is temporally limited due to the short half-life of interfering RNAs. An efficient alternative to nucleic acid-based techniques may consist of knocking down protein function at the post-translational level.

I took advantage of the Intracellular Antibody Capture Technology to develop, in collaboration with A. Cattaneo (SISSA) and M. Visintin (Lay Line Genomics), single-chain antibody fragments against gephyrin (scFv-gephyrin), which had a loss-of-function effect on endogenous gephyrin clusters. When transfected into neurons, scFv-gephyrin harboring a nuclear localization signal could displace gephyrin away from its subcellular localization, leading to a loss of inhibitory receptor function.

In order to study the role of gephyrin in organizing GABAergic synapses, I investigated the distribution of $GABA_A$ receptors and gephyrin on hippocampal neurons in culture in the presence or absence of scFv-gephyrin. This study revealed a significant reduction in the density of gephyrin and synaptic γ2-subunit containing $GABA_A$ receptors, as well as a concomitant reduction in the density of the vesicular GABA transporter (VGAT), pointing towards pre- and postsynaptic effects of gephyrin ablation. Electrophysiological experiments showed that hampering gephyrin function leads to a severe impairment of

both phasic and tonic $GABA_A$ receptor-mediated inhibition, and alters the microscopic gating properties of $GABA_A$ receptors.

The presynaptic effect of gephyrin disruption was further examined by pair recordings from interconnected neurons, which showed that the probability of GABA release was reduced upon scFv-gephyrin transfection. A possible mechanism by which gephyrin can interfere with GABA release is through the neuroligin-neurexin complex. Therefore, in further experiments I explored whether hampering gephyrin function also affects the distribution of neuroligin 2, the neuroligin isoform enriched at GABAergic synapses. Upon scFv-gephyrin transfection, the density of neuroligin 2 clusters was significantly reduced, suggesting that gephyrin interacts with this protein. Furthermore, co-immunoprecipitation experiments from rat brain homogenates revealed that gephyrin can form a complex with endogenous neuroligin 2. Over-expressing neuroligin 2 with scFv-gephyrin was able to rescue the reduction in GABAergic innervation.

Neuroligins can localize at both GABAergic and glutamatergic synapses and modulate the excitatory/inhibitory balance at the network level. Unexpectedly, we found that knocking down gephyrin with scFv-gephyrin produced a severe impairment in glutamatergic innervation. Electrophysiological and immunocytochemical experiments revealed a significant reduction in the frequency of miniature excitatory postsynaptic currents, which was associated with a significant decrease in the vesicular glutamate transporter (VGLUT). However, over-expression of neuroligin 2 with scFv-gephyrin failed to modify changes in glutamatergic innervation, indicating that this effect was not due to a compensatory homeostatic mechanism. Additional co-immunoprecipitation experiments revealed that gephyrin interacts also with neuroligin 1 raising the possibility of a trans-synaptic effect of gephyrin at both inhibitory and excitatory synapses.

MATERIALS AND METHODS

Cell Cultures

Primary cell cultures were prepared as previously described (Andjus et al., 1997). Briefly, 2-4 days old (P2-P4) Wistar rats were decapitated after being anesthetized with an intraperitoneal injection of urethane (2 mg/kg). Hippocampi were dissected free, sliced, and digested with trypsin, mechanically triturated, centrifuged twice at 40 x *g*, plated in Petri dishes, and cultured for up to 14 days. Experiments were performed on cells cultured for at least 7 days. For paired recording experiments, neurons were plated at low density (~ 40000 cells/ml).

HEK-293 cells were maintained in DMEM supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 mg/ml) and transiently transfected with various plasmid constructs using standard calcium phosphate method. Cells were collected 24-48 hours after transfections.

scFv-gephyrin

The technique for isolating scFv-gephyrin has been described in the detail in Paper-1 (see Results). Briefly, a Single Pot Library of INTrabodies (SPLINT) was used to isolate antibodies against a gephyrin bait (aa 153–348). SPLINT contains genes encoding the heavy and light variable regions of the antibody producing cells. These are cloned in the Intracellular Antibody Capture Technology (IACT) format as antibody fragments (scFv). The library design includes the capability to rapidly isolate soluble and stable scFvs directly from gene sequences with no handling of proteins.

The selected intracellular antibodies were then expressed in mammalian cells, after subcloning of the scFv cassettes into the scFv-cyto-express plasmid. For intracellular detection, scFvs were equipped with the 11 amino acid long SV5 tag, preceded by an EGFP tag and a tandem of three repeats of nuclear localization signals (NLS) (Zacchi et al., 2008).

Construction of plasmid vectors

Complementary DNAs encoding full length FLAG-tagged gephyrin has been previously described (Zita et al., 2007). The N-terminal truncated gephyrin polypeptide (aa 2-188) fused to GFP is described in Maas et al. (2006). It acts as a dominant-negative protein due to its lack of dimerization motif, and is able to deplete endogenous gephyrin clusters in neurites within 24 h of expression. The murine HA-tagged neuroligin 1 (NLG1-HA) and HA-tagged neuroligin 2 (NLG2-HA) were constructed as reported elsewhere (Scheiffele et al., 2000). NLG2-GFP was constructed by using PCR-based mutagenesis. A PvuI restriction site was introduced ten amino acids downstream of the sequence encoding for the transmembrane domain of NLG2-HA. This restriction site was then used to clone the EGFP coding sequence amplified using oligonucleotides provided of the PvuI consensus sites. The last 94 amino acids of the cytoplasmic domains of both NLGs were inserted into pGEX4T1 vector for bacterial expressions as glutathione-S-transferase (GST)-NLs 94 aa fusion proteins. All PCR-amplified products were fully sequenced to exclude the possibility of second site mutations.

Immunofluorescence staining

Hippocampal neurons in culture were transfected with EGFP alone or cotransfected with EGFP and scFv-gephyrin using the calcium phosphate transfection method. For each Petri dish, 3 μg of DNA was transfected in total. Reliable cotransfection was ensured by routinely transfecting 0.9 μg of EGFP and 2.1 μg of scFv-gephyrin, and identified by the increased EGFP signal around the nucleus. For the rescue experiments, scFv-gephyrin and NLG2-HA were co-transfected at a ratio of 2:1.

Neurons were transfected at 7 DIV and used for immunostaining 48 hours later. All steps were carried out at room temperature. After fixation with 4% paraformaldehyde in PBS for 10 min, neurons were quenched in 0.1 M glycine in PBS for 5 min, and blocked in 10% FCS in PBS for 30 min. For the rescue experiments, cells were fixed with pre-cooled 4% PFA in PBS for 5 min at 4°C, then 5 min at room temperature. In order to label surface $GABA_A$ receptors, neurons were incubated with polyclonal antibodies against the amino-terminal of γ2 subunit for 1 hour. They were then permeabilized with 0.1% Triton X-100 in PBS for 2 min and blocked again for 15 min.

After incubation with primary antibodies for 1 hour, cells were incubated with AlexaFluorophore-conjugated secondary antibodies for 45 min. In the case of double-immunostaining, cells were incubated with biotinylated secondary antibodies (45 min) followed by Streptavidin-conjuaged fluorophores (30 min). The coverslips were washed in PBS, rinsed in water and mounted with VectaShield (Vector Labs).

The antibodies used were as follows: rabbit polyclonal anti-γ2 (Alomone Labs; 1:100), rabbit polyclonal anti-α5 (provided by W. Sieghart; 1:100), mouse monoclonal anti-VGAT (Synaptic Systems; 1:200), mouse monoclonal anti-gephyrin (mAb7a, Synaptic Systems; 1:400), mouse monoclonal anti-VGLUT1 (Synaptic Systems; 1:200), guinea pig polyclonal anti-VGLUT1 (Synaptic Systems; 1:2000), rabbit polyclonal anti-neuroligin 2 (Synaptic Systems; 1:200), rat monoclonal anti-HA 3F10 (Roche; 1:1000), biotinylated goat anti-rabbit IgG (Vector Labs; 1:200). All secondary antibodies were obtained from Invitrogen.

Confocal microscopy and image analysis

Fluorescence images were acquired on a TCS-SP confocal laser scanning microscope (Leica, Bensheim, Germany) with a 40X 1.4 NA oil immersion objective, additionally magnified 2 fold with the pinhole set at 1 Airy unit. Stacks of z-sections with an interval of 0.4 μm were sequentially scanned twice for each emission line to improve the signal/noise ratio. Cluster analysis was carried out using MetaMorph Imaging System (Universal Imaging, Westchester, PA, USA). First a binary mask was created using the EGFP staining to identify transfected neurons, and then cluster intensities in regions overlapping with the binary template were analyzed. Images were segmented to select immunofluorescent puncta over background labeling. Integrated Morphometry Analysis function of MetaMorph was used to quantify the number and size of clusters (4-5 cells from at least 4 different experiments). For the analysis of colocalization, a binary mask of the segmented image for each channel was created and overlapping regions with >3 pixels were defined as colocalized. For the rescue experiments, NLG2 staining was used to create the binary template for the NLG2-HA/scFv-gephyrin co-transfected cells. As excessive NLG2-HA expression masks the rescue effect and results in an overall increase in synaptic staining (similar to NLG2-HA overexpression alone), cells with moderate

amount of NLG2-HA expression (as identified by the unsaturated NLG2 flourescence signal) was selected for the analysis of the rescue effect. Representative figures were prepared using ImageJ software.

In vitro binding, immunoprecipitation and Western blot analysis

Transfections were performed with the calcium phosphate method. GST pull-down assays were performed as previously described (Zita et al., 2007). For NLGs and gephyrin co-immunoprecipitation, HEK 293 cells overexpressing NLG1-HA/NLG2-HA and gephyrin-FLAG were lysed in 50 mM Tris HCl pH 7.5, 100 mM NaCl, 0.1% Tween 20, 10% glycerol, 10 mM EDTA, 2 mM $MgCl_2$ and protease inhibitor cocktail, and immunoprecipitated by the anti-FLAG antibody. Analysis of NLG1/NLG2-gephyrin interactions were performed on postnuclear homogenates from neonatal rat brains using the following lysis buffer: 50 mM Tris HCl pH 7.5, 150mM NaCl, 0.5% CHAPS, 1mM EDTA, 10% glycerol and protease inhibitor cocktail**.** After 2 hours of incubation with monoclonal anti-gephyrin antibody, immunoprecipitation experiment was performed according to standard procedures. Primary antibodies were revealed by HRP-conjugated secondary antibodies (Sigma) followed by ECL (Amersham). The following primary antibodies were used: mouse monoclonal anti-FLAG M2 (Sigma); mouse monoclonal anti-gephyrin 3B11 (Synaptic Systems); high affinity rat monoclonal anti-HA 3F10 (Roche), rabbit polyclonal anti-NLG2 (Synaptic Systems); rabbit polyclonal anti-NLG1 (Synaptic Systems).

Electrophysiological recordings

Spontaneous excitatory and inhibitory postsynaptic currents (EPSCs and IPSCs) were recorded in hippocampal neurons in culture transfected either with scFv-gephyrin or EGFP at 22-24^{o}C using a Multiclamp 700A amplifier (Axon Instruments, Foster City, CA, USA). Patch electrodes pulled from borosilicate glass capillaries (Hilgenberg, Malsfeld, Germany) had a resistance of 4-6 MΩ when filled with an intracellular solution containing (in mM): CsCl 137, $CaCl_2$ 1, $MgCl_2$ 2, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) 11, ATP 2, and HEPES 10 (the pH was adjusted to 7.3-7.4 with CsOH). IPSCs were recorded at a holding potential of -70 mV, in the

presence of DNQX (20 μM) and D-AP5 (50 μM) to block AMPA and NMDA receptors, respectively whereas EPSCs in the presence of bicuculline (10 μM) and D-AP5 (50 μM) to block $GABA_A$ and NMDA receptors, respectively. Miniature PSCs were recorded in the presence of tetrodotoxin (TTX, 1 μM) to block sodium currents and propagated action potentials and the respective $GABA_A$ or AMPA/NMDA receptor antagonists.

For double-patch recordings, pairs of action potentials 50 ms interval, were evoked in non transfected presynaptic neurons (in current clamp mode) by injecting depolarizing current pulses at a frequency of 0.1 Hz. IPSCs were detected from postsynaptic transfected (scFv-gephyrin) and non transfected (controls) neurons in voltage clamp mode at a holding potential of 0 mV (near the reversal potential for glutamate). In this case, the intracellular solutions contained (in mM): $KMeSO_4$ 135, KCl 10, HEPES 10, $MgCl_2$ 1, Na_2ATP 2, and Na_2GTP 0.4 (the pH was adjusted to 7.3 with KOH). In all experiments, the cells were perfused with an external solution containing (in mM): NaCl 137, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 20, and HEPES 10, pH 7.4, with NaOH. Data were sampled at 10 kHz and low pass filtered at 3 kHz. The stability of the patch was checked by repetitively monitoring the input and series resistances during the experiments. Cells exhibiting 15-20% changes were excluded from the analysis. The series resistance was 10-15 MΩ. All drugs (except TTX that was purchased from Latoxan, Valence, France) were obtained from Tocris (Cookson Ltd, Bristol, UK). All drugs were dissolved in external solution, except DNQX, which was dissolved in dimethylsulphoxide (DMSO). The final concentration of DMSO in the bathing solution was 0.1%. At this concentration, DMSO alone did not modify the shape or the kinetics of synaptic currents.

GABA containing solution was applied to nucleated patches (held at -40 mV) using an ultrafast perfusion system based on piezoelectric driven theta-glass application pipette. The piezoelectric translator was from Physik Instrumente (Waldbronn, Germany), and the theta glass tubing was from Hilgenberg (Malsfeld, Germany). The open tip recordings of the liquid junction potentials revealed that the 10–90% exchange of the solution occurred within 40–80 μs. The speed of the solution exchange was also estimated around the nucleated patch by the 10–90% onset of the membrane depolarization induced by

application of high potassium saline (25 mM). In this case, the 10–90% rise time value (60–90 μs) was very close to that found for the open tip recordings.

The tonic $GABA_A$ receptor-mediated conductance was estimated by the outward shift of the baseline current after the application of the $GABA_A$ receptor antagonist bicuculline (10 μM) or picrotoxin (100 μM). Bicuculline and picrotoxin were delivered to the recorded neurons with a perfusion system consisting of glass barrels positioned close to the soma of the recorded cell (multibarrel RSC-200 perfusion system, Bio-Logic, Grenoble, France). Judging from the onset of the liquid junction potentials, a complete exchange of the solution around the open tip electrode occurred within 10–20 ms. Epochs of 300-500 ms each, were pooled together to calculate the baseline current amplitude and its standard deviation. The resulting all-point histograms were fitted with a Gaussian function. Only current recordings that exhibited a stable baseline were included in the analysis.

Data analysis

For the analysis requiring a high temporal resolution (e.g. rise time kinetics of mIPSCs and GABA-evoked currents) signals were sampled at 50-100 kHz and low pass filtered at 10 kHz with a Butterworth filter and stored on a computer hard disk. The analysis of spontaneous events was performed with Clampfit 10.1 software (Axon Instruments, Foster City, CA, USA). This program uses a detection algorithm based on a sliding template. The template did not induce any bias in the sampling of events because it was moved along the data trace one point at a time and was optimally scaled to fit the data at each position. The detection criterion was calculated from the template-scaling factor and from how closely the scaled template fitted the data. The threshold for detection was set at 3.5 times the SD of the baseline noise. Using the same program, the decay time constant of averaged mIPSCs was taken from the biexponential fit of the decay time. The rise time was estimated as the time needed for 10–90% increase of the peak current response.

The decaying phase of mIPSCs and GABA-evoked currents was fitted with exponential functions in the form:

$$y(t) = \sum_{i=1}^{n} A_i * \exp(- t / \tau_i),$$

where τ_i and A_i are the time constants and relative fractions of the respective components. In the case of analysis of normalized currents, the fractions of kinetic components fulfilled the normalization condition:

$$\sum_{i=1}^{n} A_i = 1.$$

Deactivation time courses of mIPSCs and GABA-evoked currents were fitted with the sum of two exponentials.

The mean time constant calculated as:

$$\tau_{mean} = \sum_{i=1}^{n} A_i * \tau_i,$$

was used to estimate the speed of the decaying process.

In the case of current responses elicited by long (250 ms) GABA pulses, the desensitization onset was described by:

$$y(t) = A_{fast} * \exp(- t / \tau_{fast)} + A_{slow} * \exp(- t / \tau_{slow}) + A_s$$

where A_{fast} and A_{slow} are the fractions of the fast and the slow component, respectively and τ_{fast} and τ_{slow} are the fast and the slow time constants. A_s is the steady state current. The goodness of the fit was assessed by minimizing the sum of the squared differences.

Brief (2 ms) paired pulses separated by a variable time interval were used to test whether or not the entrance of bound receptors into the desensitized state proceeded after the agonist removal. The parameter R was calculated according to the formula:

$$R = (I_2 - I_{end}) / (I_1 - I_{end})$$

where I_1 is the first peak amplitude, I_{end} is the current value immediately before the application of the second pulse, and I_2 is the second peak amplitude. During the 2 ms pulse the onset of the use-dependent desensitization is minimal. Thus, in the case of

continued entrance into the desensitized state after the first short agonist pulse, the peak of the second response (I_2) was smaller than the first one resulting in $R < 1$.

Variance analysis was used to estimate the single channel current (i) from the mean current (I_{mean}) and current variance (σ^2). Variance (σ^2) was calculated according to the formula:

$$\sigma^2 = \sum_{n=1}^{n} (AC_i - AC_m)^2/(n-1)$$

where n is the number of samples per record, AC_i is the current mediated by $GABA_A$ receptors at sample i and AC_m is the mean AC current. Assuming N independent and identical channels, $I_{mean} = NP_oi$, where *i* is a single-channel current, P_o – single channel open probability and I_{mean} – the mean measured current. In these conditions, the plot σ^2 – I_{mean} follows a parabolic relationship in the form:

$$\sigma^2 = i \cdot I_{mean} - I_{mean}^{\ 2}/N$$

Single channel conductance (γ) was estimated according to the equation:

$$\gamma = i/(V_H - V_R)$$

where V_H is the holding potential and V_R is the reversal potential for chloride that in conditions of nearly symmetric chloride used in our experiments was very close to zero and in calculations using the formula (8) V_R was assumed equal to zero.

For evoked IPSCs, transmission failures were identified visually. Mean IPSCs amplitude was obtained by averaging successes and failures. The paired-pulse ratio (PPR), known to be inversely correlated to the initial release probability (Dobrunz and Stevens, 1997), was calculated as the ratio between the mean amplitudes of IPSC2 over IPSC1. The coefficient of variation (CV^{-2}) was calculated as the square root of the ratio between the standard deviation of IPSC1 and the mean amplitude of IPSC1. Values are given as mean ± S.E.M. Although otherwise stated, significance of differences was assessed by Student's *t*-test. The differences were considered significant when $P<0.05$.

RESULTS

The results are presented in the following papers:

Paper 1:

Zacchi, P., Dreosti, E, Visintin, M, Moretto-Zita, M, Marchionni, I, Cannistraci, I, **Kasap, Z**, Betz, H, Cattaneo, A, Cherubini, E (2008) Gephyrin selective intrabodies as a new strategy for studying inhibitory receptor clustering. *J Mol Neurosci* **34**:141-148.

Paper 2:

Marchionni, I.*, **Kasap, Z***, Mozrzymas, J.W, Sieghart, W, Cherubini, E, Zacchi, P (2009) New insights on the role of gephyrin in regulating both phasic and tonic GABAergic inhibition in rat hippocampal neurons in culture. *Neuroscience* **164**:552-562.

Paper 3:

Kasap Varley, Z.*, Pizzarelli, R*, Antonelli, R, Stancheva, S.H, Kneussel, M, Cherubini, E, Zacchi, P (2010) Gephyrin regulates GABAergic and glutamatergic synaptic transmission in hippocampal cell cultures. *(Submitted)*.

-Paper 1-

Gephyrin selective intrabodies as a new strategy for studying inhibitory receptor clustering.

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Gephyrin Selective Intrabodies as a New Strategy for Studying Inhibitory Receptor Clustering

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Abstract The microtubule-binding protein gephyrin is known to play a pivotal role in targeting and clustering postsynaptic inhibitory receptors. Here, the Intracellular Antibodies Capture Technology (IATC) was used to select two single-chain antibody fragments or intrabodies, which, fused to nuclear localization signals (NLS), were able to efficiently and selectively remove gephyrin from glycine receptor (GlyR) clusters. Co-transfection of NLS-tagged individual intrabodies with gephyrin-enhanced green fluorescent protein (EGFP) in HEK 293 cells revealed a partial relocalization of gephyrin aggregates onto the nucleus or in the perinuclear area. When expressed in cultured neurons, these intrabodies caused a significant reduction in the number of immunoreactive GlyR clusters, which was associated with a decrease in the peak amplitude of glycine-evoked whole cell currents as assessed with electrophysiological experiments. Hampering protein function at a posttranslational level may represent an attractive alternative for interfering with gephyrin function in a more spatially localized manner.

Keywords Gephyrin · Glycine receptor clusters · Intracellular antibodies · Hippocampal neurons · IATC

Introduction

At inhibitory synapses, the microtubule-binding protein gephyrin is known to play a pivotal role in targeting/clustering postsynaptic inhibitory glycine receptors (GlyRs) (Feng et al. 1998) and γ2-subunit containing gamma-aminobutyric acid A ($GABA_A$) receptors (Kneussel et al. 1999). Gephyrin binds with high affinity to the β subunit of the GlyR (Meyer et al. 1995), whereas it only functionally associates with $GABA_A$ receptor subtypes (Kirsch and Betz 1995; Meyer et al. 1995). Receptor anchoring is based on the interaction of gephyrin with the actin- and microtubule-based cytoskeleton (Kirsch 2006). GlyR clustering has been proposed to rely on the ability of gephyrin to multimerize into a submembraneous hexagonal protein lattice (Sola et al. 2004). The critical contribution of gephyrin to the postsynaptic localization of GlyRs in the spinal cord has been elegantly demonstrated by gene ablation experiments (Feng et al. 1998). Gephyrin knockout (KO) mice display a severe neuromotor phenotype, which is responsible for their death shortly after birth. The motor impairment observed in gephyrin-deficient mice has been mainly attributed to a

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severe deficit in glycinergic neurotransmission in the spinal cord (Feng et al. 1998). Interestingly, the complex motor disorder and neurologic symptoms observed in gephyrin-deficient mice exhibit some phenotypic features of hyperexplexia in humans, suggesting the possible involvement of gephyrin in this disease.

Gephyrin KO mice have been extremely useful for unveiling several aspects of gephyrin-mediated receptor localization at central synapses (Feng et al. 1998). However, the lethality exhibited by these mice soon after birth represents an important limitation to their use as animal models for postnatal studies. To circumvent this problem, we have developed a more versatile and powerful molecular tool to achieve functional ablation of gephyrin based on intracellular expression of anti-gephyrin single-chain antibody fragments (scFv) or intrabodies (Biocca et al. 1990). Unlike nucleic acid-based technologies, such as antisense (Kramer and Cohen 2004), zinc-finger proteins (Beerli and Barbas 2002), targeted gene disruption, or (ribonucleic acid) RNA interference (Tian et al. 2004), intrabodies operate at the posttranslational level, offering new experimental opportunities to analyze the function of a given molecule (Manikandan et al. 2007). Intrabodies can sterically prevent the interaction of their targets with other protein partners, or stabilize/destabilize them, thus preventing/facilitating their turnover and/or degradation. Finally, intrabodies fused to specific cellular localization sequences can target their antigens to specific subcellular compartments (Visintin et al. 2004a).

In the present study, a nuclear localization signal (NLS)-targeted intrabody for the removal of endogenous gephyrin from inhibitory receptor clusters in cultured hippocampal neurons has been developed. As not all antibodies isolated by standard technologies fold well under conditions of intracellular expression (Visintin et al. 2004a), we exploited a yeast two-hybrid based intrabody selection technology, named Intracellular Antibody Capture Technique (IACT) (Visintin et al. 1999), to select anti-gephyrin intrabodies.

Material and Methods

SPLINT Selection

A Single Pot Library of INTrabodies (SPLINT) was used to isolate antibodies against ghephyrin (aa 153–348) bait. SPLINT contains genes encoding the heavy and light variable regions of the antibody producing cells (Visintin et al. 2004b). These are cloned in the IACT format as antibody fragments (scFv) (Visintin et al. 1999). The library design includes the capability to rapidly isolate soluble and stable scFvs directly from gene sequences with no handling of proteins.

A functional domain of the gephyrin protein (GDL aa 153–348) was cloned in the pMIC-BD1 vector and the expression of the fusion protein was assayed after the protein was extracted from the transformed yeast strain L40. The antigen was found to be expressed in optimum quantities in the yeast cells and did not transactivate the reporter genes (HIS3 and lacZ).

SPLINT library was then transformed into L40 yeast strain by using a rearranged lithium acetate transformation protocol. SPLINT and the ghephyrin bait were cotransformed into yeast cells as described (Visintin et al. 2002). Colonies were isolated on histidine-deficient plates and screened for β-gal activity on filters. Individual blue colonies were isolated by restreaking and again reassayed for β-gal activity. To isolate different anti-gephyrin scFvs, a colony-polymerase chain reaction (PCR) and BstNI fingerprinting analysis were performed and the isolated plasmids encoding anti-gephyrin scFvs were individually retested in a secondary IACT screening confirmed that true positives were identified that interact specifically with the original bait, but not with other lexA fusions (lexA-lamin).

Cell Culture

Primary cell cultures were prepared as previously described (Andjus et al. 1997). Briefly, 2- to 4-day-old (P2-P4) Wistar rats were decapitated after being anesthetized with an intraperitoneal injection of urethane (2 mg/kg). This procedure is in accordance with the regulation of the Italian Animal Welfare Act and was approved by the local authority veterinary service. Hippocampus was dissected free, sliced, and digested with trypsin, mechanically triturated, centrifuged twice at 40×*g*. Dissociated neurons were plated in Petri dishes, and cultured for up to 14 days. Experiments were performed on cells cultured for at least 7 days. All the other cell lines used were routinely cultured at 37°C in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) Penicillin (100 U/ml) and Streptomycin (100 μg/ml).

Immunofluorescence Staining

HEK 293 cells were fixed with 4% (*w*/*v*) paraformaldehyde/4% sucrose for 15 min, permeabilized with 0.1% (*v*/*v*) NP-40 for 5 min, and then blocked with 10% (*w*/*v*) fetal bovine serum in phosphate-buffered saline (PBS) for 30 min. Hippocampal neurons were fixed for 10 min with methanol at −20°C or for 15 minutes, and processed as described above. Antibody staining was performed by incubation for 1 hour with primary antibodies and 45 min for secondary antibodies in blocking buffer. Enhanced green fluorescent protein (EGFP) was visualized by auto-

fluorescence. Cluster density and brightness were analyzed with the Meta-Morph Imaging System (Universal Imaging, Westchester, PA). Twenty neurons were analyzed for each experiment (3–4 dendritic regions for each cell).

Immunoprecipitation and Western Blot Analysis

Transfections were performed with the standard calcium phosphate method. For gephyrin-EGFP and scFvGeph-2/scFvGeph-9 coimmunoprecipitation HEK 293 cells overexpressing gephyrin-EGFP and scFvGeph-2/scFvGeph-9 were lysed in a lysis buffer containing 50 mM Tris 7.5, 150 mM NaCl, 0.5% NP40, 2 mM ethylenediaminetetraacetic acid (EDTA) and 10% glycerol and immunoprecipitated by the anti-SV5 antibody. Western blotting was performed according to standard procedures. Primary antibodies were revealed by HRPO-conjugated secondary antibodies (SIGMA), followed by enhanced chemiluminescence (Amersham).

Electrophysiological Recordings

Glycine- and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)-evoked currents were recorded at room temperature (22–24°C) in the whole-cell configuration of the patch-clamp technique using a Multiclamp 700A (Axon Instruments, Foster City, CA, USA). Patch electrodes, formed from thin borosilicate glass (Hilgenberg, Malsfeld, Germany) had a resistance of 4–8 MΩ when filled with an intracellular solution containing: CsCl 137 mM, 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES) 10 mM, BAPTA 11 mM, $MgCl_2$ 2 mM, Mg ATP 2 mM, $CaCl_2$ 1 mM. The composition of the external solution was: NaCl 137 mM, KCl 5 mM, $MgCl_2$ 1 mM, $CaCl_2$ 2 mM, glucose 20 mM, hemisodium HEPES 10 mM (the pH was adjusted to 7.3–7.4 with NaOH).

The stability of the patch was checked by repetitively monitoring the input and series resistance during the experiments. Cells exhibiting more than 15% changes were excluded from the analysis. Glycine- and AMPA-evoked currents were recorded at a holding potential of −40 mV. Glycine and AMPA were applied through a multibarrel RSC-200 perfusion system (Bio-logic, Grenoble, France). With this system, a complete exchange of the solution around the cell was obtained in less than 30 ms. In the case of glycine-evoked current, recordings were routinely performed in the presence of the $GABA_A$ receptor antagonist picrotoxin (50 μM), the $GABA_B$ receptor antagonist CGP 55845 (1 μM) and the broad spectrum ionotropic glutamate receptor antagonist kynurenic acid (1 mM).

Drugs used were kynurenic acid, glycine, AMPA, strychnine all purchased from Sigma (Milan, Italy). Picrotoxin and CGP 55845 were purchased from Tocris Cookson, Ltd. (Bristol, UK). Drugs were dissolved in water except CGP 55845 and picrotoxin that were dissolved in dimethylsulphoxide (DMSO) and ethanol, respectively. The final concentration of DMSO in the bathing solution was 0.1%. At this concentration, DMSO alone did not modify the shape or the kinetics of the evoked currents. Currents were sampled at 20–100 kHz and filtered with a cut-off frequency of 1 kHz. Data are expressed as mean ± SEM. Statistical comparison was made using the unpaired *t*-test. $P<0.05$ was taken as significant.

Results

Selection of Two Gephyrin-Specific Intrabodies Using the IAC Technology

To select intracellular competent intrabodies by the two-hybrid system, the linker region of gephryin (Fig. 1a) was cloned to the 3' of LexA (LexA-GLD; aa 153–348) and used to challenge a mouse SPLINT (Single Pot Library of INTracellular Antibodies) library of intrabodies (Visintin et al. 2004b). From the selection procedure a total of 90 colonies able to grow in the absence of histidine and showing activation of β-Galactosidase were obtained. The scFv-VP16 plasmids were isolated and sorted by their restriction patterns and sequences. The specificity of scFvs with different DNA fingerprints were reanalyzed using yeast strains expressing LexA-GLD and LexA-lamin, as nonrelevant antigen. Two different anti-gephyrin scFvs, designated scFvGeph-2 and scFvGeph-9, were thus identified. Analysis of the V region nucleotide sequences of the two selected anti-gephyrin intrabodies revealed that they were derived from germline V region genes with very few somatic mutations (data not shown). Both scFv shared the same VH region V11 that belongs to the subgroup VH-VIII, whereas VL regions belong to the kappa subgroup (kk4 and 19–32 for scFv2 and 9, respectively).

The selected intracellular antibodies were then expressed in mammalian cells, after subcloning of the scFv cassettes into the scFv-cyto-express plasmid (Persic et al. 1997). For intracellular detection, scFvs were equipped with the 11 amino acid long SV5 tag, preceded, in the nuclear tagged forms, by a tandem of three repeats of nuclear localization signals (NLS). Extracts from HEK 293 cells transiently transfected with scFvGeph-2 and scFvGeph-9 for different times demonstrated that the highest cytoplasmic expression was reached ~24–30 hours posttransfection. ScFvGeph-2 appeared to be more stable than scFvGeph-9, yielding higher expression levels in mammalian cells as compared to scFvGeph-9 (Fig. 1b). Similar results were obtained for the intrabodies provided of the NLS (data not shown).

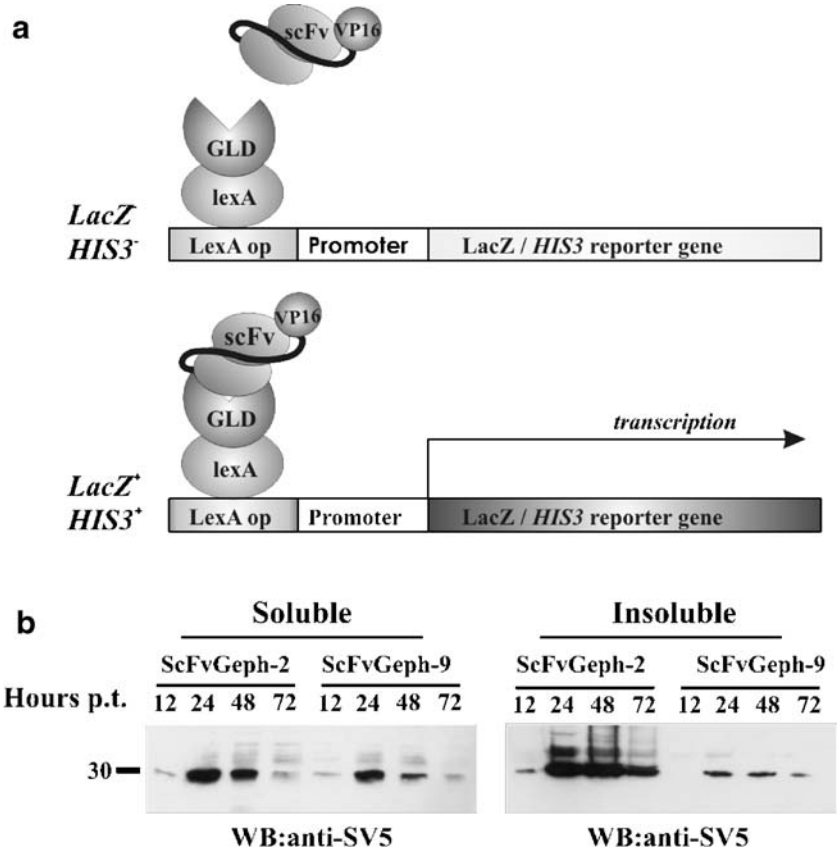

Figure 1 Isolation and characterization of scFv selected by IACT-SPLINT technology. **a.** The scFv library is screened in yeast with the Gephyrin Linker Domain of (GLD) as a bait. Only those scFvs that retain the specific binding ability *in vivo* can activate the reporter genes, *His3* and *LacZ*. **b.** Western immunoblot using mAb anti-SV5 of cellular extracts of HEK 293 cells, transiently transfected with the indicated scFvs tagged with SV5. On the left, soluble fraction of scFvGeph-2 and scFvGeph-9 collected at different times after transfection, as indicated (*Hours*). On the right, insoluble fractions analyzed as in the right panel

Gephyrin-Specific Intrabodies Recognize Gephyrin in Mammalian Cells

The ability of the selected intrabodies to recognize gephyrin in mammalian cells was assessed in immunocytochemical experiments performed on HEK 293 cells co-transfected with scFvGeph-2/scFvGeph-9 and gephyrin fused to EGFP. It is well-known that ectopically expressed gephyrin forms large intracytoplasmic aggregates characterized by their ability to actively sequester gephyrin interacting proteins (Kins et al. 2000; Meyer et al. 1995). Intrabodies expressed as leaderless cytoplasmic proteins showed a diffuse intracellular staining, typical of soluble cytoplasmic proteins (Fig. 2a). When gephyrin-EGFP and individual intrabodies were co-transfected, a massive fraction of scFvGeph-2/scFvGeph-9 was relocalized to gephyrin intracytoplasmic aggregates, thus resulting in colocalization of the two proteins (Fig. 2a). When the same experiments were performed using overexpression of the NLS-tagged intrabodies, a dramatic change in gephyrin distribution was observed (Fig. 2b). The efficient translocation of intrabodies into the nucleus because of the presence of the NLS was associated with a partial reduction in the size of gephyrin cytoplasmic aggregates, most of them concentrated in the perinuclear area of the cell. The ability of scFvs to interact specifically with gephyrin was further analyzed by co-immunoprecipitation from extracts of HEK 293 cells co-expressing the two proteins. As shown in Fig. 2c, scFvGeph-2/scFvGeph-9 antibodies were able to co-immunoprecipitate gephyrin, suggesting that they interacted with gephyrin intracellularly.

Gephyrin-Specific Intrabodies Alter Endogenous Glycine Receptor Function

The ability of the anti-gephyrin intrabodies to bind and remove endogenous gephyrin from glycine receptor clusters was functionally assessed on cultured hippocampal neurons, known to highly express GlyRs (Danglot et al. 2004; Ito and Cherubini 1991). To this aim, NLS-tagged scFvGeph-2/ scFvGeph-9 were additionally equipped with EGFP tags to easily follow their fate within the transfected neurons. Twenty-four hours after transfection, immunocytochemical experiments revealed that not only gephyrin was

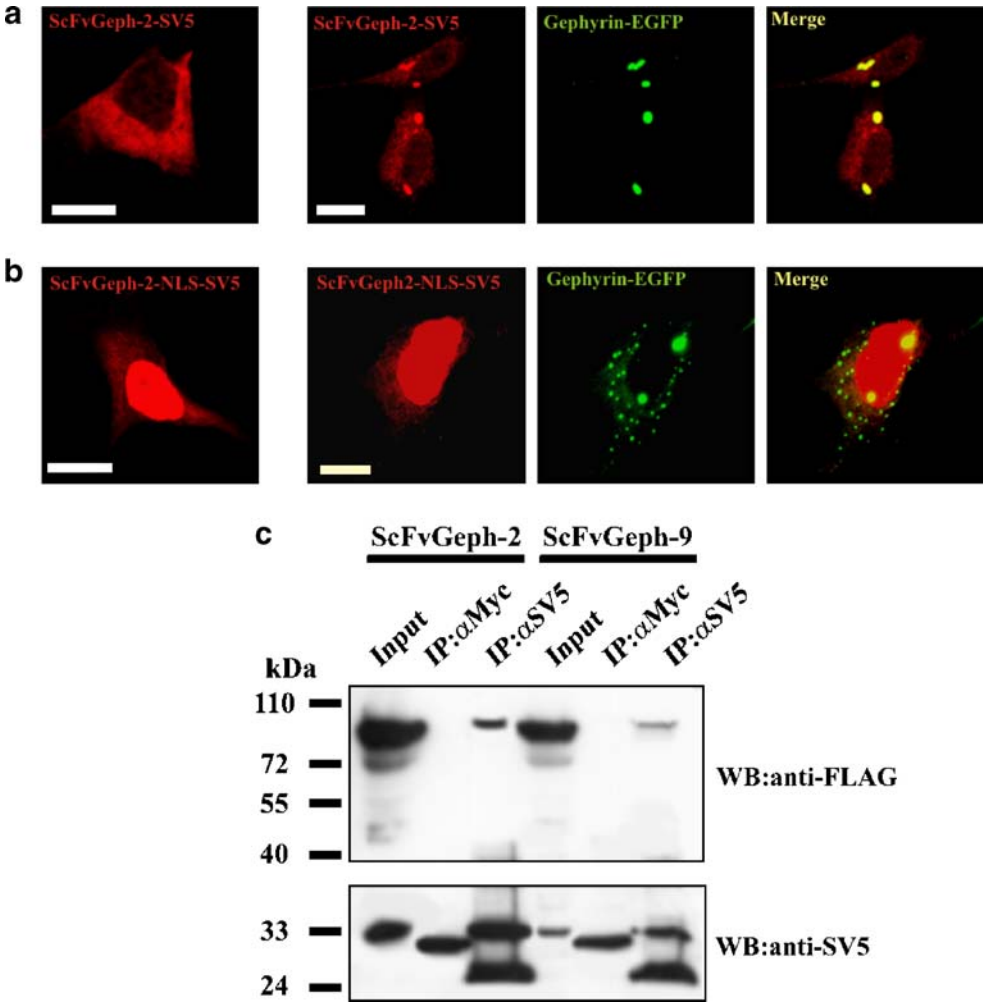

Figure 2 Gephyrin-specific intrabodies interact with gephyrin in mammalian cells. **a.** Immunofluorescence assay of the subcellular distribution of SV5-tagged anti-gephyrin intrabody (scFv-Gephyrin) ectopically expressed in HEK 293 cells in single transfection experiment (left panel) and in co-transfection with gephyrin-EGFP (right panels). ScFv-Gephyrin distribution was revealed with the anti-SV5 monoclonal antibody followed by anti-mouse TRITC-conjugated secondary antibody. Gephyrin distribution was revealed by the intrinsic green fluorescence of EGFP. **b.** Single (left panel) and double (right panels) transfection of the nuclear target NLS anti-Gephyrin intrabody (scFv-Gephyrin-NLS) was visualized as described in A. (Scale bar, 10 μm). **c.** Lysates of HEK 293 cells co-transfected with gephyrin-FLAG and scFv-Geph-2 or scFvGeph-9 were immunoprecipitated with monoclonal antibodies anti-SV5 or anti-Myc as negative control. Immunoprecipitates were analyzed by western blotting using anti-FLAG and anti-SV5 antibodies, as indicated

efficiently removed from most subsynaptic sites (Fig. 3a), but also that the number of GlyR clusters were dramatically reduced (Fig. 3b) compared to neurons transfected with EGFP alone. A quantitative analysis of immunoreactive gephyrin puncta in scFv-gephyrin-NLS transfected neurons revealed that in comparison to cells transfected only with EGFP, cluster fluorescence was significantly reduced. Fluorescence intensity values per square micron (μm^2) of dendritic surface were 2,857±500 and 1,205±134 in controls and scFv-gephyrin-NLS transfected cells, respectively (20 cells, detected in four different experiments, Fig. 3c). Similar results were found for GlyRs (fluorescence intensity values per square micron of dendritic surface were 2,211±378 and 1,152±72 in controls and in scFv-gephyrin-NLS transfected cells, respectively; n=20 in both cases; Fig. 3c).

Glycine receptor responses in NLS-EGFP-tagged scFvGeph-2 transfected neurons were analyzed in electrophysiological experiments, using the whole-cell configuration of the patch clamp technique. Recordings were routinely performed in the presence of the GABAA receptor antagonist picrotoxin (50 μM), the $GABA_B$ receptor antagonist CGP 55845 (1 μM), and the broad spectrum ionotropic glutamate receptor antagonist kynurenic acid (1 mM). All cells tested (n=99) responded to glycine application with inward currents of variable amplitude. These were readily blocked by the selective glycine receptor antagonist strychnine (0.5 μM), indicating that they were mediated by glycine receptors (data not shown). As shown in Fig. 4a, glycine-evoked currents from transfected cells were significantly reduced in amplitude as compared to controls (nontransfected cells from the same dish) or to hippocampal neurons transfected only with EGFP. Glycine (0.6 mM) induced current responses whose peak amplitude was 0.75±0.11 nA (n=11), 1.52±0.12 nA (n=9), and 1.6±0.16 nA (n=9) in NLS-scFvGeph-2, EGFP and nontransfected cells, respectively. The value obtained in transfected cells was significantly different from that detected in EGFP positive cells (p<0.01) or in nontransfected neurons (p<0.01). In comparison with nontransfected or EGFP-transfected cells, NLS-scFvGeph-2-transfected neurons exhibited a concentration–response curve for glycine that was compressed downward and to the right (Fig. 4b). EC_{50} values were 254 μM and 125 μM for NLS-scFvGeph-2- and

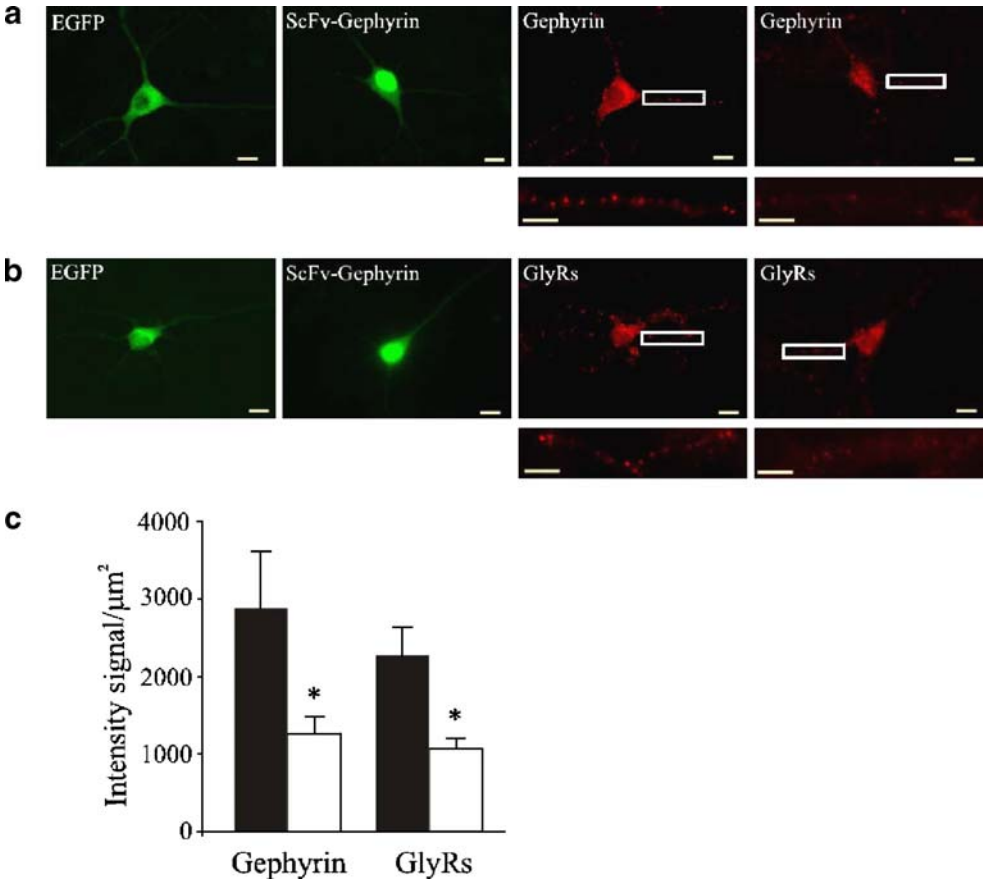

Figure 3 ScFv-Geph2-EGFP-NLS transfected in hippocampal neurons displaces gephyrin from its subsynaptic sites. **A**, **B**. Hippocampal neurons transfected with EGFP as negative control (*EGFP*) and with scFv-Geph-2-EGFP-NLS (*scFv-Geohyrin*). Endogenous gephyrin (**a**) and GlyR (**b**) distributions were revealed using the monoclonal antibodies mAb7a (for gephyrin) and mAb4a (for GlyR) followed by anti-mouse TRITC-conjugated secondary antibody (Invitrogen). Note that the nuclear accumulation of scFv-Gephyrin-NLS was associated with a marked reduction of both gephyrin (scFv + Gephyrin in A) and glycine receptor clusters (scFv + GlyRs in B). Bottom panels represent enlargements of the boxed areas. Scale bars: 10 μm. **c.** Quantitative analysis of cluster fluorescence on hippocampal neurons transfected with EGFP and scFv-Gephyrin-NLS. Each column represents the intensity signal normalized to the unit area (in μm^2) of dendritic surface (20 cells in each column) in control conditions (black) and in the presence of scFv-Gephyrin-NLS (white); * $p<0.05$

EGFP-transfected cells, respectively. In contrast, current responses elicited in the same neurons by α-amino-3-hydroxy-5-methylisoxazole-4 propionic acid (AMPA 50 μM, in the presence of tetrodotoxin [TTX] 1 μM) were similar in amplitude (0.20±0.03 nA, $n=11$ and 0.21± 0.02 nA, $n=11$, $p>0.5$; Fig. 4a) in both NLS-scFvGeph-2- and EGFP-transfected cells, respectively, indicating that gephyrin intrabodies selectively reduce surface glycine receptor numbers.

Discussion

The main goal of the present study was to select competent cytoplasmic intrabodies against the scaffolding molecule gephyrin to use as a molecular tool to knock out gephyrin function *in vivo*. This strategy is of particular relevance as disrupting gephyrin gene in knockout mice leads to a lethal phenotype, thus hindering the study of gephyrin functions at later stages of postnatal development. To exploit the intrabodies-based retargeting scheme for depleting endogenous gephyrin from inhibitory synapses, the linker or intervening region of gephyrin molecule was used for the two-hybrid selection method. This domain was chosen as it is not engaged in gephyrin oligomerization, a process regarded as the molecular mechanism responsible for inhibitory receptors accumulation at postsynaptic sites (Sola et al. 2004). As a consequence, the selected intrabodies should recognize gephyrin upon its self-assembly into the submembraneous protein lattice underlying inhibitory receptor clusters. Several lines of evidence support that the selected intrabodies are fully functional inside the cell. First, both intrabodies immunoprecipitate gephyrin upon their co-expression in HEK 293 cells. Second, they are specifically relocalized to gephyrin cytoplasmic aggregates. Interestingly, in the NLS-tagged format to divert intrabodies localization, they were able to promote a partial relocalization of gephyrin aggregates into the nucleus. We believe

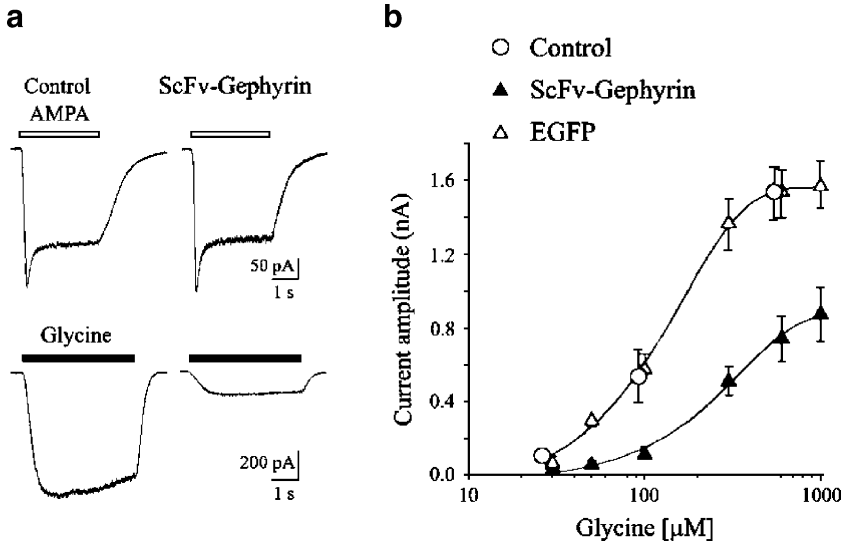

Figure 4 AMPA and glycine evoked currents on hippocampal neurons transfected with ScFv-Geph2-EGFP-NLS **a.** Upper traces: current responses evoked at −40 mV by application of AMPA (50 μM; open bars) in nontransfected (left) and scFv-Gephyrin-NLS transfected neurons present in the same dish (right). Lower traces: current responses evoked at −40 mV by application of glycine (100 μM; closed bars) in nontransfected (left) and scFv-Gephyrin-NLS transfected neurons present in the same dish (right). **b.** Dose–response curves for glycine-evoked currents obtained in hippocampal neurons transfected with EGFP (open triangles) or with scFv-Gephyrin-NLS (scFv-Gephyrin, closed triangles). Open circles refer to current responses obtained from nontransfected cells present with scFv-Gephyrin-NLS transfected neurons on the same dish. Each point is the average of 9–20 individual responses

that the lack of a massive translocation of gephyrin aggregates into the nucleus is probably caused by their size—too big to allow nuclear pore crossing. Finally, and most importantly, when ectopically expressed in cultured hippocampal neurons, both selected intrabodies were able to remove endogenous gephyrin from GlyRs clusters, leading to a decreased number of GlyR immunoreactive punctae associated with a severe reduction in the mean amplitude of glycine-evoked currents.

Certainly, alternative strategies can be used to target and inactivate intracellular proteins. One of the most currently used is the RNA interference (RNAi), which acts at the level of mRNA degradation and subsequent inhibition of protein synthesis. However, major technical challenges of this technique are the specificity of sequence match necessary to achieve gene silencing and the nonspecific effects of small interfering RNAs (siRNA). Concerning the latter, microarray analyses have demonstrated that siRNA with only partial complementarity to mRNAs can cause a reduction in the RNA levels of a large number of transcripts (Jackson et al. 2003). Many of these mRNA changes are caused by the interaction of the siRNA with partially complementary sites and thus are considered "off-target" effects (Birmingham et al. 2006; Saxena et al. 2003). Another major deficiency of utilizing siRNAs is their relatively short active half-life, which would limit their effects on the cell, unless these are expressed via transfected recombinant DNA. By contrast, intrabodies possess a much longer active half-life compared to siRNA, and are also much more specific to their target molecules (Visintin et al. 2004a). Therefore, it would be particularly advantageous to utilize an intrabody instead of iRNA when the active half-life of the target molecule is long, as in the case of neuronal gephyrin. In addition, in the case of proteins with multifunctional domains, intrabodies could be designed to block selectively a particular binding interaction domain therefore leading to the loss of a certain function while sparing others.

Hampering protein function at the posttranslational level may have distinctive advantages, especially when dealing with cells with complex and spatially extended morphology, such as nerve cells, where functional knock-down with intrabodies could be achieved in a more spatially localized manner, or where one could target subcellular pools of a given target protein. The recombinant antibody we selected could be exploited for developing conditional transgenic mice where scFv expression is under the control of a neuronal cell type specific (tissue-specific) promoter. Alternatively, viral expression vectors may be used to transduce gephyrin-specific intrabodies in nondividing neuronal populations in specific areas of the CNS, giving the possibility to follow the consequences of gephyrin expression and its functional ablation within the same biological preparation. In particular, the occurrence in the same neuronal tissue of cells phenotypically knocked out for gephyrin with WT ones may allow studying the mechanisms regulating neurotransmitter receptor movements on the cell surface and the contribution of receptor clustering to the cross talk between pre and postsynaptic membranes.

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-Paper 2-

New insights on the role of gephyrin in regulating both phasic and tonic GABAergic inhibition in rat hippocampal neurons in culture

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NEW INSIGHTS ON THE ROLE OF GEPHYRIN IN REGULATING BOTH PHASIC AND TONIC GABAergic INHIBITION IN RAT HIPPOCAMPAL NEURONS IN CULTURE

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Abstract—Gephyrin is a tubulin-binding protein that acts as a scaffold for clustering glycine and $GABA_A$ receptors at postsynaptic sites. In this study, the role of gephyrin on $GABA_A$ receptor function was assessed at the post-translational level, using gephyrin-specific single chain antibody fragments (scFv-gephyrin). When expressed in cultured rat hippocampal neurons as a fusion protein containing a nuclear localization signal, scFv-gephyrin were able to remove endogenous gephyrin from $GABA_A$ receptor clusters. Immunocytochemical experiments revealed a significant reduction in the number of synaptic γ2-subunit containing $GABA_A$ receptors and a significant decrease in the density of the GABAergic presynaptic marker vesicular GABA transporter (VGAT). These effects were associated with a slow down of the onset kinetics, a reduction in the amplitude and in the frequency of miniature inhibitory postsynaptic currents (mIPSCs). The quantitative analysis of current responses to ultrafast application of GABA suggested that changes in onset kinetics resulted from modifications in the microscopic gating of $GABA_A$ receptors and in particular from a reduced entry into the desensitized state. In addition, hampering gephyrin function with scFv-gephyrin induced a significant reduction in $GABA_A$ receptor-mediated tonic conductance. This effect was probably dependent on the decrease in GABAergic innervation and in GABA release from presynaptic nerve terminals. These results indicate that gephyrin is essential not only for maintaining synaptic $GABA_A$ receptor clusters in the right position but also for regulating both phasic and tonic inhibition.

Key words: recombinant antibodies, synaptic and extrasynaptic $GABA_A$ receptors, $GABA_A$ receptors gating, GABAergic innervation, VGAT, immunocytochemistry.

In the CNS, the efficiency of GABAergic signaling relies on the temporally and spatially regulated expression of $GABA_A$ receptors, which are located at both synaptic and extrasynaptic sites. While synaptic receptors regulate phasic inhibition responsible for point-to-point communication between neurons, extrasynaptic receptors mediate tonic inhibition, involved in the control of neuronal gain and cell excitability (Semyanov et al., 2004; Cavelier et al., 2005; Farrant and Nusser, 2005). Clustering of inhibitory receptors at synaptic and extrasynaptic sites is dynamically controlled by highly regulated events, which require the precise interplay of various proteins and active transport processes along the cytoskeleton (Kneussel and Loebrich, 2007). The tubulin-binding protein gephyrin, originally purified in association with glycine receptors (Meyer et al., 1995) has been proposed to serve as a scaffold for anchoring glycine and $GABA_A$ receptors to synaptic membranes (Kneussel and Loebrich, 2007). Gephyrin has been shown to bind with high affinity to the β subunits of glycine receptors (Meyer et al., 1995) and a recent study has provided convincing evidence that this protein can directly interact with the α2 subunit of $GABA_A$ receptors (Tretter et al., 2008). To elucidate the functional role of gephyrin on GABAergic signaling, a variety of different approaches has been used including, antisense oligonucleotides (Essrich et al., 1998), gephyrin −/− mice (Kneussel et al., 1999; Levi et al., 2004), and RNA interference (iRNA, Jacob et al., 2008; Yu et al., 2007). These studies have revealed that gephyrin is a key player in the synaptic aggregation of $GABA_A$ receptors. Here, using immunocytochemical and electrophysiological techniques, we have investigated in more detail the functional role of gephyrin on phasic and tonic inhibition in hippocampal neurons in culture.

To hamper gephyrin function we have used a method recently developed in our laboratory based on single-chain antibody fragments against gephyrin (scFv-gephyrin) which, unlike nucleic acid-based technologies, operate at the post-translational level (Zacchi et al., 2008). ScFv-gephyrin were linked to a nuclear localization signal (NLS) that sequestered endogenous gephyrin in the nucleus. We found that the reduction of gephyrin and synaptic γ2-subunit containing $GABA_A$ receptor clusters was accompanied with a decrease density of vesicular GABA transporter

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Abbreviations: BSA, bovine serum albumin; DMSO, dimethylsulfoxide; DNQX, 6,7-dinitroquinoxaline-2,3-dione; EGFP, enhanced green fluorescent protein; FCS, fetal calf serum; GABA, γ-aminobutyric acid; mIPSCs, miniature inhibitory postsynaptic currents; NLS, nuclear localization signal; PBS, phosphate-buffered saline; scFv, single chain antibody fragments; TTX, tetrodotoxin; VGAT, vesicular GABA transporter.

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(VGAT). Electrophysiological experiments revealed a severe alteration of both phasic and tonic $GABA_A$ receptor-mediated inhibition.

EXPERIMENTAL PROCEDURES

Cell culture

All experiments were carried out in accordance with the European Community Council Directive of November 24, 1986 (86/609EEC) and were approved by the local authority veterinary service. Experiments were designed to minimize the number of animals used and their suffering. Primary cell cultures were prepared as previously described (Andjus et al., 1997). Briefly, 2–4 days old (P2–P4) Wistar rats were decapitated after being anesthetized with an i.p. injection of urethane (2 mg/kg). Hippocampi were dissected free, sliced, and digested with trypsin, mechanically triturated, centrifuged twice at $40\times g$, plated in Petri dishes, and cultured for up to 14 days. Experiments were performed on cells cultured for at least 7 days.

ScFv-gephyrin

The technique for isolating scFv-gephyrin has already been reported (Zacchi et al., 2008). The Intracellular Antibodies Capture Technology (Visintin et al., 2002) was used to select a single-chain antibody fragment or intrabody against the linker domain (aa 153–348) of gephyrin. This intrabody, fused to NLS, was able to efficiently and selectively remove gephyrin from glycine and $GABA_A$ receptors.

Immunofluorescence staining

Hippocampal neurons in culture were transfected with enhanced green fluorescent protein (EGFP) alone or cotransfected with EGFP and scFv-gephyrin using the magnetofection protocol (Buerli et al., 2007) as described by the manufacturer (OZ Biosciences, Marseille). Briefly, NeuroMag beads were coated with DNA and added to the culture medium. Following 15 min of incubation on a magnetic plate, the cells were left to grow at 37 °C.

Neurons were transfected at seven DIV and used for immunostaining 48 h later. All steps were carried out at room temperature. After fixation with 4% paraformaldehyde/4% sucrose in phosphate-buffered saline (PBS) for 10 min, neurons were quenched in 0.1 M glycine in PBS for 5 min, and blocked in 0.2% bovine serum albumin (BSA)/1% fetal calf serum (FCS) in PBS for 30 min. In order to label surface $GABA_A$ receptors, neurons were incubated with polyclonal antibodies against the amino-terminal of γ2 (1:100) or α5 (1:100) subunits for 1 h. They were then permeabilized with 0.1% Triton X-100 in PBS for 2 min and blocked again in 0.2% BSA/1% FCS in PBS for 15 min. After incubation with monoclonal antibodies against VGAT (1:200) or gephyrin (1:500) for 1 h, they were incubated with a mixture of secondary antibodies (AlexaFluor 594 goat anti-rabbit, 1:500 and biotinylated goat anti-rabbit, 1:100) for 45 min, followed by incubation with Streptavidin-conjugated Marina Blue or Streptavidin-conjugated AlexaFluor 405 (1:100) for 30 min. The coverslips were washed in PBS and mounted with VectaShield (Vector Laboratories). The fixation protocol did not permeabilize the neurons as revealed by the lack of immunostaining when an antibody against an intracellular antigen (MAP2) on non-permeabilized neurons was used.

The antibodies used were as follows: rabbit polyclonal anti-γ2 (Alomone Laboratories), rabbit polyclonal anti-α5 (provided by W. Sieghart), mouse monoclonal anti-VGAT (Synaptic Systems), mouse monoclonal anti-gephyrin (mAb7a, Synaptic Systems), biotinylated goat anti-rabbit IgG (Vector Laboratories). All secondary antibodies were obtained from Invitrogen.

Confocal microscopy and image analysis

Fluorescence images were acquired on a TCS–SP confocal laser scanning microscope (Leica, Bensheim, Germany) with a 63×1.4 NA oil immersion objective, additionally magnified two fold with the pinhole set at one Airy Unit. Stacks of z-sections with an interval of 0.4 μm were sequentially scanned twice for each emission line to improve the signal/noise ratio. Cluster analysis was carried out using MetaMorph Imaging System (Universal Imaging, Westchester, PA, USA). First a binary mask was created using the EGFP staining to identify transfected neurons, then $GABA_A$ receptor and VGAT or gephyrin intensities in regions overlapping with the binary mask were analyzed. Images were segmented to select immunofluorescent puncta over background labeling, and clusters were defined as >5 pixels (0.067 μm^2) as determined by visual inspection. Integrated Morphometry Analysis function of MetaMorph was used to quantify the number and size of clusters. For the analysis of colocalization, a binary mask of the segmented image for each channel was created and overlapping regions with >3 pixels were defined as colocalized. Five to six–cells from at least four different experiments were quantified. Representative figures were prepared using Adobe Photoshop CS3.

Electrophysiological recordings

Spontaneous miniature $GABA_A$-mediated synaptic currents (mIPSCs) and GABA-evoked currents were recorded in hippocampal neurons in culture transfected either with scFv-gephyrin or EGFP at 22–24 °C using a Multiclamp 700 A amplifier (Axon Instruments, Foster City, CA, USA). Patch electrodes were pulled from borosilicate glass capillaries (Hilgenberg, Malsfeld, Germany). They had a resistance of 4–6 MΩ when filled with an intracellular solution containing (in mM): CsCl 137, $CaCl_2$ 1, $MgCl_2$ 2, 1,2-bis(2-aminophenoxy)ethane-N,N,N′N′-tetraacetic acid (BAPTA) 11, ATP 2, and HEPES 10 (the pH was adjusted to 7.3–7.4 with CsOH). The composition of the external solution was (in mM): NaCl 137, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 20, and HEPES 10, pH 7.4, with NaOH. mIPSCs were recorded, at a holding potential of -70 mV, in the presence of tetrodotoxin (TTX, 1 μM), 6,7-dinitroquinoxaline-2,3-dione (DNQX, 20 μM) and CGP55845 (1 μM) to block sodium currents and propagated action potentials, AMPA and $GABA_B$ receptors respectively. The stability of the patch was checked by repetitively monitoring the input and series resistance during the experiments. Cells exhibiting 15–20% changes were excluded from the analysis. The series resistance was 5–7 MΩ, and it was compensated by 70–80%.

GABA containing solution was applied to nucleated patches (held at -40 mV) using an ultrafast perfusion system based on piezoelectric driven theta-glass application pipette (Mozrzymas et al., 1999; Barberis et al., 2000; Petrini et al., 2003, 2004). The piezoelectric translator was from Physik Instrumente (Waldbronn, Germany), and the theta glass tubing was from Hilgenberg (Malsfeld, Germany). The open tip recordings of the liquid junction potentials revealed that the 10–90% exchange of the solution occurred within 40–80 μs. The speed of the solution exchange was also estimated around the nucleated patch by the 10–90% onset of the membrane depolarization induced by application of high potassium saline (25 mM). In this case, the 10–90% rise time value (60–90 μs) was very close to that found for the open tip recordings.

The tonic $GABA_A$ receptor-mediated conductance was estimated by the outward shift of the baseline current after the application of the $GABA_A$ receptor antagonist bicuculline (10 μM) or picrotoxin (100 μM). Epochs of 300–500 ms each, were pooled together to calculate the baseline current amplitude and its standard deviation. The resulting all-point histograms were fitted with a Gaussian function. Only current recordings that exhibited a stable baseline were included in the analysis.

Drugs used were TTX, DNQX, CGP55845, bicuculline methiodide, picrotoxin. All drugs (except TTX that was purchased from Latoxan, Valence, France) were obtained from Tocris (Cookson Ltd., Bristol, UK). All drugs were dissolved in ACSF, except DNQX, and picrotoxin that were dissolved in DMSO and ethanol, respectively. The final concentration of DMSO in the bathing solution was 0.1%. At this concentration, DMSO alone did not modify the shape or the kinetics of synaptic currents. Bicuculline and picrotoxin were delivered to the recorded neurons with a perfusion system consisting of glass barrels positioned close to the soma of the recorded cell (multibarrel RSC-200 perfusion system, Bio-Logic, Grenoble, France). Judging from the onset of the liquid junction potentials, a complete exchange of the solution around the open tip electrode occurred within 10–20 ms.

Data analysis

For the analysis requiring a high temporal resolution (e.g. rise time kinetics of mIPSCs and GABA-evoked currents) signals were sampled at 50–100 kHz and low pass filtered at 10 kHz with a Butterworth filter and stored on a computer hard disk. The analysis of minis was performed with Clampfit 9 software (Axon Instruments, Foster City, CA, USA). This program uses a detection algorithm based on a sliding template. The template did not induce any bias in the sampling of events because it was moved along the data trace one point at a time and was optimally scaled to fit the data at each position. The detection criterion was calculated from the template-scaling factor and from how closely the scaled template fitted the data. The threshold for detection was set at 3.5 times the SD of the baseline noise. Using the same program, the decay time constant of averaged mIPSCs was taken from the biexponential fit of the decay time. The rise time was estimated as the time needed for 10–90% increase of the peak current response.

The decaying phase of mIPSCs and GABA-evoked currents was fitted with exponential functions in the form:

$$y(t)=\sum_{i=1}^{n} A_i\times\exp(-t/\tau_i), \quad (1)$$

where τ_i and A_i are the time constants and relative fractions of the respective components.

In the case of analysis of normalized currents, the fractions of kinetic components fulfilled the normalization condition:

$$\sum_{i=1}^{n} A_i=1. \quad (2)$$

Deactivation time courses of mIPSCs and GABA-evoked currents were fitted with the sum of two exponentials.

The mean time constant calculated as:

$$\tau_{mean}=\sum_{i=1}^{n} A_i\times\tau_i, \quad (3)$$

Was used to estimate the speed of the decaying process.

In the case of current responses elicited by long (250 ms) GABA pulses, the desensitization onset was described by:

$$y(t)=A_{fast}\times\exp(-t/\tau_{fast})+A_{slow}\times\exp(-t/\tau_{slow})+A_s \quad (4)$$

where A_{fast} and A_{slow} are the fractions of the fast and the slow component, respectively and τ_{fast} and τ_{slow} are the fast and the slow time constants. A_s is the steady state current. The goodness of the fit was assessed by minimizing the sum of the squared differences.

Brief (2 ms) paired pulses separated by a variable time interval were used to test whether or not the entrance of bound receptors into the desensitized state proceeded after the agonist removal. The parameter R was calculated according to the formula:

$$R=(I_2-I_{end})/(I_1-I_{end}) \quad (5)$$

where I_1 is the first peak amplitude, I_{end} is the current value immediately before the application of the second pulse, and I_2 is the second peak amplitude. During the 2 ms pulse the onset of the use-dependent desensitization is minimal. Thus, in the case of continued entrance into the desensitized state after the first short agonist pulse, the peak of the second response (I_2) was smaller than the first one resulting in $R<1$.

Variance analysis was used to estimate the single channel current (i) from the mean current (I_{mean}) and current variance (σ^2). Variance (σ^2) was calculated according to the formula:

$$\sigma^2=\sum_{n=1}^{n} (AC_i-AC_m)^2/(n-1) \quad (6)$$

where n is the number of samples per record, AC_i is the current mediated by $GABA_A$ receptors at sample i and AC_m is the mean AC current. Assuming N independent and identical channels, $I_{mean}=NP_oi$, where i is a single-channel current, P_o—single channel open probability and I_{mean}—the mean measured current. In these conditions, the plot σ^2-I_{mean} follows a parabolic relationship in the form:

$$\sigma^2=i\times I_{mean}-I_{mean}^2/N \quad (7)$$

Single channel conductance (γ) was estimated according to the equation:

$$\gamma=i/(VH-VR) \quad (8)$$

where V_H is the holding potential and V_R is the reversal potential for chloride that in conditions of nearly symmetric chloride used in our experiments was very close to zero and in calculations using the formula (8) V_R was assumed equal to zero.

Unless otherwise stated, data are expressed as mean±SEM, and all the values included in the statistics represent recordings from separate cells. Statistical comparisons were made with the use of unpaired t-test. $P<0.05$ was taken as significant.

RESULTS

ScFv-gephyrin reduced the number of gephyrin clusters and γ2 subunit containing synaptic $GABA_A$ receptors

Double-immunostaining was performed on neurons transfected with EGFP alone as control or cotransfected with EGFP and scFv-gephyrin. Cotransfection was necessary to visualize the dendritic processes as scFv-gephyrin alone resulted in strong EGFP signal only around and within the nucleus (due to NLS). Cultured hippocampal neurons were stained for surface $GABA_A$ receptors containing γ2 subunit, then permeabilized and stained for gephyrin. As shown in Fig. 1A, a significant reduction in the number of gephyrin clusters was observed in scFv-gephyrin transfected neurons as compared to controls (0.097± 0.015 *vs* 0.159±0.014 clusters/μm^2, $P<0.01$; $n=22$). ScFv-gephyrin also caused a decrease in the total number of γ2 subunit immunoreactive puncta although this did not reach a significant level. γ2 subunit-containing $GABA_A$

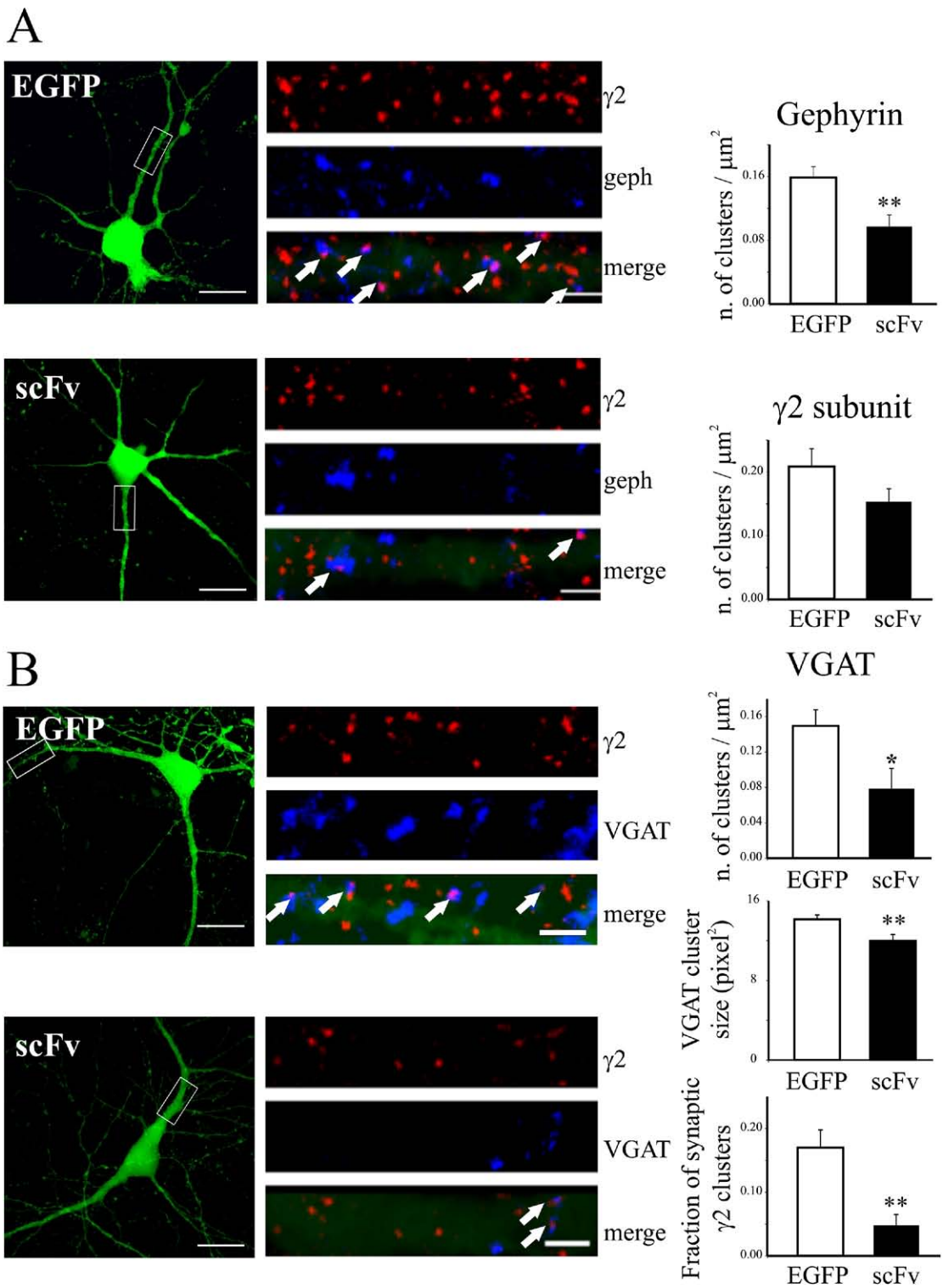

Fig. 1. scFv-gephyrin reduce the number of gephyrin clusters and alters GABAergic synapses. (A) On the left: neurons transfected (green) with EGFP (upper panel) or EGFP and scFv (lower panel). Right panels are magnifications of the white boxes marked on the left. Neurons were double immunostained for surface γ2 (red) and intracellular gephyrin (blue). Colocalizing puncta (pink) are indicated by arrows on the merge window. Quantifications of cluster densities for gephyrin and γ2 subunits are shown on the right. (B) Neurons were treated as in (A) but double immunostained for surface γ2 (red) and VGAT (blue). Note the significant reduction in the density and size of VGAT clusters, and fraction of γ2 clusters co-localizing with VGAT (merged window). Scale bars: 20 μm and 2 μm. * $P<0.05$; ** $P<0.01$.

receptors can localize at both synaptic and extrasynaptic sites (Danglot et al., 2003). In order to distinguish between these two receptor populations, neurons were stained for surface γ2 subunits and for VGAT (the vesicular GABA transporter) considered a GABAergic presynaptic marker (Dumoulin et al., 2000). Interestingly, a significant decrease in VGAT immunoreactivity was found in scFv-gephyrin transfected cells (n=11; Fig. 1B). VGAT clusters were reduced in number (from 0.149±0.018 to 0.078±0.023 clusters/μm^2, P<0.05 and size (from 14.1±0.4 to 11.9±0.6 pixel,2 P<0.01). The fraction of γ2 subunit clusters co-localizing with VGAT was reduced from 17.0±2.8% to 4.8±1.7%, P<0.01.

It should be stressed that in spite of a significant reduction in the number of synaptic γ2 subunits containing $GABA_A$ receptor clusters, in scFv-gephyrin transfected cells the fluorescence intensity of individual clusters (calculated by normalizing cluster fluorescence intensity to cluster area and expressed in arbitrary units) was only slightly reduced without reaching a significant level (154.2±3.2 and 146.2±3.4 in EGFP and scFv-gephyrin, respectively; n=20; P>0.05; data not shown). We cannot exclude that, minor changes in receptor density within clusters, not detectable in our experimental conditions, could influence the kinetic properties of the receptors.

Overall, these results demonstrate that scFv-gephyrin have a pronounced effect on synaptic γ2 subunit containing $GABA_A$ receptors co-localizing with VGAT, suggesting that the impairment of gephyrin function alters GABAergic innervation.

Immunocytochemical investigation of α5 subunit containing $GABA_A$ receptors, which in the hippocampus are mainly extrasynaptic (Fritschy et al., 1998; Brünig et al., 2002; but see Serwanski et al., 2006) did not reveal any significant difference between the number of immunoreactive puncta in scFv-gephyrin- and EGFP-transfected neurons (0.29±0.03 and 0.29±0.05 clusters/100 μm^2 in control, n=18, and scFv-gephyrin, n=14, respectively; data not shown) suggesting that scFv-gephyrin did not affect α5 containing $GABA_A$ receptors.

ScFv-gephyrin slowed down the onset kinetics and reduced the amplitude and frequency of mIPSCs

Miniature IPSCs were recorded from cultured hippocampal neurons in the whole-cell configuration of the patch-clamp technique at a holding potential of −70 mV in the presence of TTX (1 μM), DNQX (20 μM), and CGP55845 (1 μM). Miniature events were reversibly blocked by bicuculline (10 μM) indicating that they were $GABA_A$ receptor-mediated (n=6; data not shown). mIPSCs were recorded from EGFP and scFv-gephyrin transfected neurons. Miniature events were recorded also from neighboring non-transfected cells in the same dishes containing scFv-gephyrin transfected neurons. No differences in amplitude, frequency and kinetics were observed between EGFP and non-transfected cells and therefore data were pooled together and considered as controls. As shown in the representative example of Fig. 2A, B, in comparison to controls, mIPSCs recorded from scFv-gephyrin transfected neurons exhibited a significant reduction in amplitude and frequency associated with a slow-down of their onset kinetics. On average, the mean 10–90% rise time of mIPSCs was 1.1±0.1 and 1.6±0.1 ms in controls (n=23) and scFv-gephyrin transfected neurons (n=18) respectively (Fig. 2C). This effect was associated with a significant (P<0.01) reduction in the peak amplitude of mIPSCs (from 42.6±3.0 to 29.8±1.7 pA) and with a shift to left of the cumulative amplitude distribution. ScFv-gephyrin also affected mIPSCs frequency: on average this was 0.28±0.04 and 0.18±0.03 Hz, in control and scFv-gephyrin, respectively (Fig. 2C).

ScFv-gephyrin did not alter the deactivation kinetics of mIPSCs. mIPSCs decay was fitted with a biexponential function: τ_{fast} was 11.3±0.6 and 10.5±1.1 ms and τ_{slow} was 38.6±2.6 and 44.1±4.2 ms in control and scFv-gephyrin, respectively (P>0.05; data not shown). Altogether, these results indicate that removal of gephyrin from the subsynaptic membrane severely affects $GABA_A$ receptor-mediated phasic inhibition.

ScFv-gephyrin slowed down the onset kinetics of currents evoked by ultrafast application of saturating concentrations of GABA

In order to investigate the mechanisms underlying changes in mIPSCs kinetics induced by scFv-gephyrin, we examined the currents evoked by ultrafast applications of GABA (Jones and Westbrook, 1995; Mozrzymas et al., 1999; Barberis et al., 2000; Petrini et al., 2003). Using this approach it is possible to determine the microscopic gating of $GABA_A$ receptors with a time resolution similar to that of synaptic events. For this purpose we studied current responses evoked in nucleated patches obtained from controls and scFv-gephyrin transfected cells. Similarly to mIPSCs, in scFv-gephyrin transfected neurons, a significant (P<0.01) slow down of the onset kinetics of currents evoked by brief (2 ms) pulses of a saturating concentration of GABA (10 mM) was observed (Fig. 3A). The mean 10–90% rise time value was 0.42±0.01 ms (n=17) and 0.48±0.02 ms, in control and scFv-gephyrin transfected neurons, respectively. According to the kinetic model proposed by Jones and Westbrook (1995), the activation of $GABA_A$ receptors results from two kinetically separated steps: the binding of the agonist to the receptor and the conformational change from the bound–closed to the bound-open state. The binding step strictly depends on GABA concentration since the effective rate of binding is proportional to k_{on}×(GABA). Thus, in the presence of low concentrations of GABA, the binding step becomes much slower than the conformational change and represents the rate-limiting step of the whole process. Thus, changes in binding rate should affect the onset kinetics of GABA-evoked responses. Fast application of low concentrations of GABA (100 μM) produced current responses with similar onset kinetics in controls and in scFv-gephyrin transfected cells (Fig. 3A). On average, the 10–90% rise time values of responses evoked by 100 μM GABA were 4.36±0.23, and 4.71±0.27 ms, in controls and scFv-gephyrin transfected cells, respectively (n=17 for each group; P>0.05). As shown in Fig. 3B, the rise time values

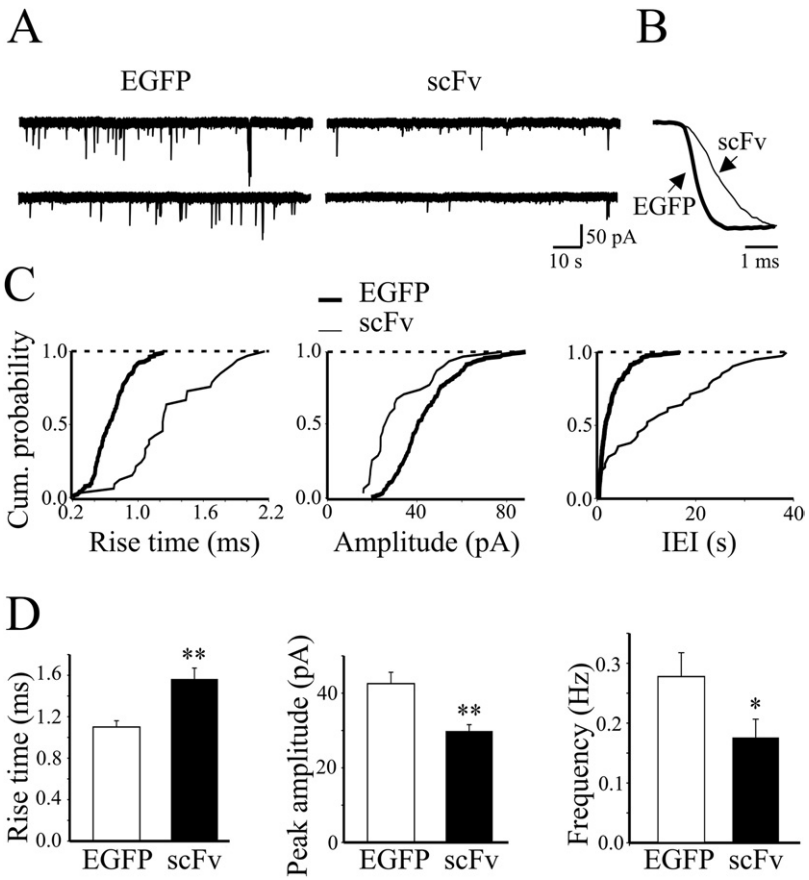

Fig. 2. scFv-gephyrin reduce the peak amplitude, frequency and slows down the onset kinetics of mIPSCs. (A) mIPSCs recorded from controls and scFv-gephyrin transfected neurons. (B) Average of individual mIPSCs from the traces shown in (A) are normalized and superimposed to emphasize differences in the onset kinetics between controls and scFv-gephyrin transfected cells (C) Cumulative 10–90% rise time, amplitude and interevent interval distribution of mIPSCs from the EGFP- (thick line) and the scFv-gephyrin transfected neurons (thin line) shown on (A). (D) Each column represents the mean 10%–90% rise time, peak amplitude and frequency values of mIPSCs recorded from EGFP (white) and scFv-gephyrin (black) transfected cells. * $P<0.05$; ** $P<0.01$.

of current responses obtained from scFv-gephyrin transfected cells, normalized to controls (EGFP), were significantly higher only for saturating concentrations of GABA, indicating that the impairment of $GABA_A$ receptor clusters by scFv-gephyrin does not affect the agonist binding process.

The peak amplitude and the decay of currents evoked by saturating concentrations of GABA (10 mM for 2 ms; data not shown) were not affected by scFv-gephyrin transfection. On average, the peak amplitudes of GABA-evoked currents were 2062.2±275.8 and 2116.5±463.7 pA while the decay time constants (τ_{mean}) were 69.1±3.4

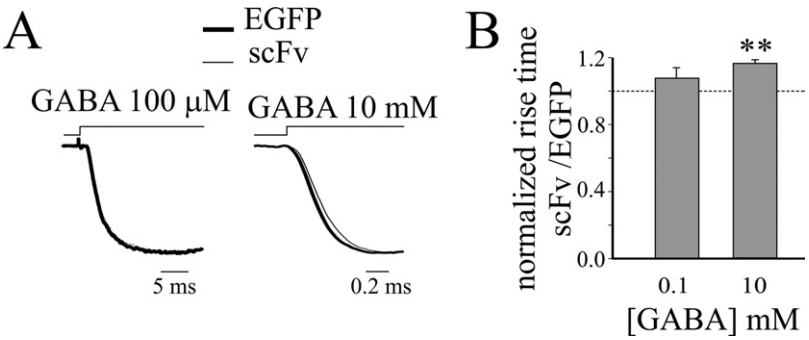

Fig. 3. scFv-gephyrin reduce the onset of GABA-evoked currents, only at saturating agonist concentration. (A) Normalized and superimposed onset of current responses evoked by ultrafast application of a nonsaturating (100 μM, left) or a saturating (10 mM, right) concentration of GABA for 2 ms to neurons transfected with EGFP (thick lines) or with scFv-gephyrin (thin lines). Note that with GABA 100 μM the two traces overlap. Each trace is the average of 5 responses. (B) Each column represents the mean 10–90% rise time value of currents evoked by 100 μM and 10 mM of GABA, respectively, in scFv-gephyrin transfected neurons normalized to the corresponding values obtained from EGFP transfected cells ($n=17$). ** $P<0.01$.

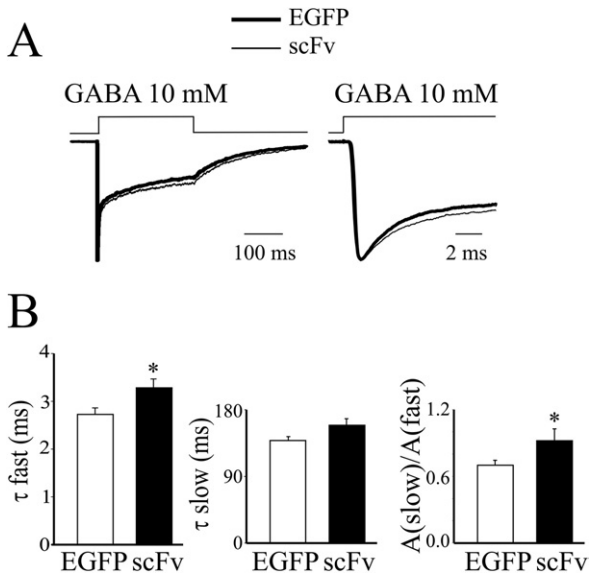

Fig. 4. scFv-gephyrin reduce the desensitization kinetics of GABA-evoked currents. (A) Currents evoked by long (250 ms) pulses of GABA (10 mM) in control (thick line) and scFv-gephyrin transfected cells (thin line). On the right the two normalized and superimposed traces are shown on an expanded time scale. (B) Mean fast (τ_{fast}) and slow (τ_{slow}) time constants of current responses obtained from control (white; $n=23$) and from scFv-gephyrin (black; $n=17$) transfected neurons. Note that scFv-gephyrin affected only τ_{fast} * $P<0.05$.

and 72.8±4.7 ms in controls ($n=23$) and scFv-gephyrin transfected cells ($n=14$), respectively ($P>0.05$; data not shown).

ScFv-gephyrin reduced the rate and extent of desensitization kinetics of GABA-evoked currents

$GABA_A$ receptors undergo rapid desensitization during activation, a process known to contribute to shape synaptic currents (Jones and Westbrook, 1995; Morzrymas et al., 2003a, 2004). To see whether in cells transfected with scFv-gephyrin $GABA_A$ receptor desensitization was altered, we applied (*via* the ultrafast agonist application system) long pulses (250 ms) of a saturating concentration of GABA (10 mM). We found that, in comparison with controls, cells expressing scFv-gephyrin exhibited a significant reduction in current desensitization (Fig. 4A).

The desensitization time course could be fitted with a biexponential function. In controls ($n=23$), τ_{fast} and τ_{slow} were 2.7±0.1 and 139.6±5.1 ms, respectively (the corresponding A_{fast} and A_{slow} values were 0.43±0.04 and 0.29±0.04; Fig. 5B). In scFv-gephyrin ($n=17$) τ_{fast} and τ_{slow} were 3.3±0.2 and 158.4±8.3 ms, respectively (the corresponding A_{fast} and A_{slow} were 0.33±0.03 and 0.27±0.01; Fig. 4B). Values of τ_{fast} (but not τ_{slow}) measured in controls and in scFv-gephyrin transfected cells were significantly different ($P<0.05$).

ScFv-gephyrin did not affect the recovery process of GABA responses in the paired pulse protocols

The paired pulse protocol was used to analyze whether scFv-gephyrin may affect the recovery process assessed as the R parameter (see Experimental procedures). The peak current value elicited by the second pulse is clearly smaller than that evoked by the first one and the difference between these amplitudes reflects the number of receptors that fell into the desensitized state. The current peak elicited by the second pulse is proportional to the number of receptors that have dissociated the agonist and are ready to be activated again. Paired pulses (2 ms duration each) of saturating GABA (10 mM) were applied at different time intervals (ranging between 5 and 1000 ms; see example of Fig. 5A). As shown in the summary graph of Fig. 5B, data points obtained in controls and in scFv-gephyrin overlapped, indicating that the impairment of gephyrin function did not affect the recovery parameter.

However, it should be taken into account that the recovery process is a complex phenomenon involving multiple re-entries into the open and desensitized states due to the functional coupling between desensitization-resensitization, opening-closing and unbinding, therefore it cannot be ascribed solely to the kinetics of entry or exit from the desensitized state of $GABA_A$ receptors (Jones and Westbrook, 1995; Barberis et al., 2000; Petrini et al., 2003).

ScFv-gephyrin reduced the tonic $GABA_A$ receptor mediated conductance

In the attempt to investigate whether the impairment of GABAergic function by scFv-gephyrin involves also the tonic $GABA_A$ receptor mediated conductance, we measured the baseline shift induced by bath application of the $GABA_A$ receptor antagonists bicuculline or picrotoxin. Application of bicuculline (100 μM) caused a shift in the baseline current of 12.3±2.2 and 5.9±1.1 pA in controls ($n=15$) and in scFv-gephyrin transfected cells ($n=14$), respectively (Fig. 6A–C). These values were significantly different ($P<0.05$). As shown in the representative example of Fig. 6B, the all-point histogram of base-line current obtained in scFv-gephyrin transfected neurons (black) was shifted to the right with respect to control (gray). Similar results were obtained with picrotoxin (100 μM; $n=5$; data not shown).

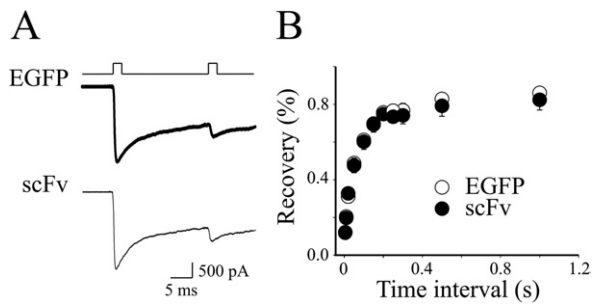

Fig. 5. In paired pulse protocols, scFv-gephyrin do not affect the recovery of the second peak of GABA-evoked currents. (A) Paired pulses of GABA (2 ms, 10 mM) elicited at 20 ms interval in control (thick line) and in scFv-gephyrin transfected cells (thin line). (B) Normalized recovery of the second peak evoked in EGFP (open circles) and in scFv-gephyrin (closed circles) transfected cells. Each point represents the mean of 7–11 experiments. The error bars are often within the symbols.

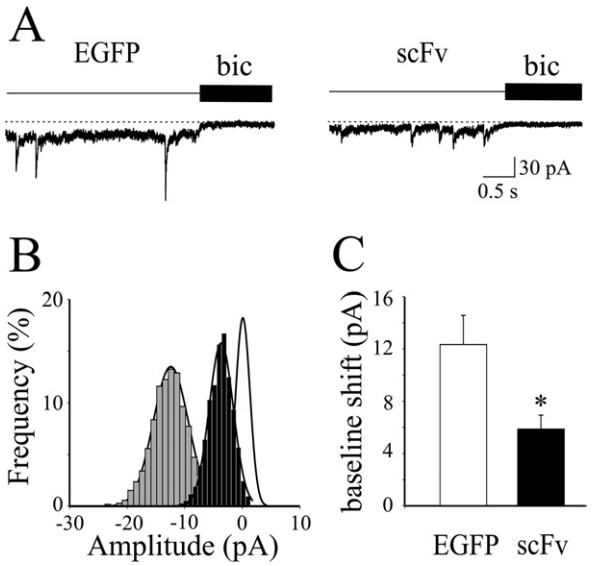

Fig. 6. scFv-gephyrin reduce the amplitude of the tonic conductance mediated by ambient GABA. (A) Currents recorded in the absence (thin lines) and in the presence of bicuculline (bic 10 μM; thick lines) from control and scFv-gephyrin transfected cells. Holding potential −60 mV. The dotted lines represent the holding current in the presence of bicuculline. (B) All-point histogram of 500 ms traces recorded from the neurons shown in A. Note that, in comparison to control (gray) in scFv-gephyrin (black), the all point histogram is shifted to the right. In white is the distribution observed in the two cells in the presence of bicuculline. The thin black lines represent the gaussian fit of the distributions. (C) Summary data of the mean tonic current amplitude (base-line shift) obtained from EGFP (white, $n=15$) and scFv-gephyrin (black, $n=14$) transfected cells. * $P<0.05$.

These results suggest that scFv-gephyrin affect not only phasic but also tonic inhibition.

ScFv-gephyrin did not alter $GABA_A$ receptor mediated single channel conductance

In a series of experiments we have examined whether changes of $GABA_A$ receptor function observed in scFv-gephyrin transfected cells could be attributed to alterations in $GABA_A$ receptor properties. To this aim, single channel conductance was estimated by analyzing the noise variance of current responses evoked by applications of GABA (100 μM or 10 mM) in controls ($n=38$) or in scFv-gephyrin transfected cells ($n=25$). In both experimental conditions, the relationship between variance and mean current amplitude was obtained and the fit of the parabolic equation (eq. 7) yielded the single channel current (i) and the number of channels (N) in the considered patches (Fig. 7). Assuming a reversal potential for chloride equal to zero, the single channel conductance was calculated using eqn. 8. The mean single channel conductances determined in this way were 28.58±1.96 and 28.32±1.69 pS, in controls and in scFv-transfected cells, respectively (Fig. 7C; $P>0.05$).

The number of channels (N) within patches, determined for controls and scFv-gephyrin transfected cells did not show any significant difference (2036±245 and 2169±574 for controls and scFv-gephyrin, respectively, $P>0.05$). There was a slight trend of increase in the maximum open channel probability (P_{max}, calculated as P_o at current peak) following gephyrin impairment (0.86±0.032 and 0.9±0.057 for control and scFv-gephyrin, respectively) but this difference did not reach statistical significance ($P>0.05$). These results indicate that scFv-gephyrin did not modify either the mean single channel conductance or the number of channels within patches.

DISCUSSION

The effects of gephyrin on GABAergic function has been extensively studied (Fritschy et al., 2008). In the present study, we have confirmed and extended previous findings using a novel tool (scFv-gephyrin), which interferes with gephyrin at post-translational level (Zacchi et al., 2008). In particular, we found that the reduction in number of gephyrin and synaptic γ2 subunit containing $GABA_A$ receptor clusters was associated with a severe impairment of both phasic and tonic $GABA_A$ receptor-mediated inhibition. It is

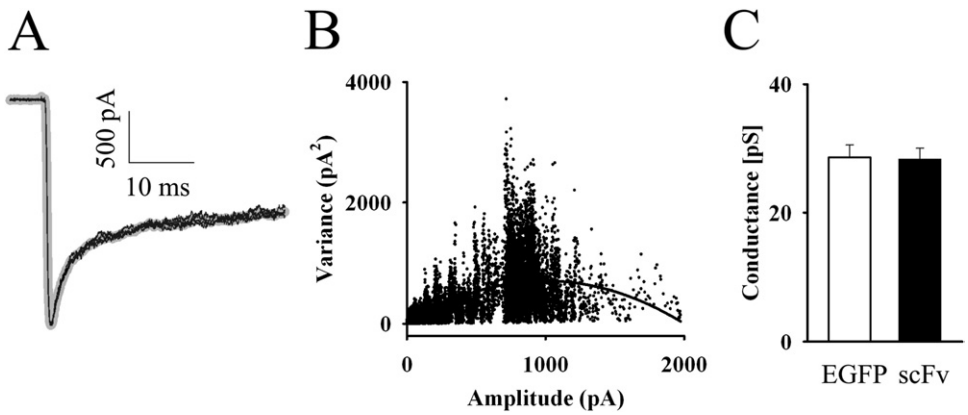

Fig. 7. Single channel conductance determined from the non-stationary noise analysis is not affected by scFv-gephyrin. (A) Typical traces elicited by 10 mM GABA. Averaged trace, to make it visible, is marked with thick grey line while 4 individual traces are plotted with thin black lines. (B) Typical variance vs. amplitude plot with a parabolic fitting according to Eqn 7. (C) Statistics of single channel conductance calculated with Eqn 8. The single channel currents were obtained from fitting of Eqn 7 to the variance vs. amplitude for each individual recording.

therefore likely that gephyrin exerts a crucial role not only in keeping synaptic $GABA_A$ receptors in place but also for ensuring a correct communication between the pre and the postsynaptic element of the synapses.

Although the exact mechanisms by which scFv-gephyrin reduce the number of gephyrin and synaptic $\gamma 2$ subunit containing $GABA_A$ receptor clusters is presently unknown, we cannot exclude the possibility that the recombinant antibodies could also affect $GABA_A$ receptor trafficking directly or indirectly. However, if this effect is present it seems to be minor since, at the cell surface, the total number of $\gamma 2$-containing $GABA_A$ receptor clusters was only slightly reduced. In addition, it is noteworthy that, similar to our results, a reduction in amplitude and frequency of spontaneous IPSCs was found when gephyrin was deleted by iRNA or by over expressing gephyrin EGFP (Yu et al., 2007). Interestingly, a reduction in the number of $GABA_A$ receptor clusters and GABAergic innervation was also found when $GABA_A$ receptors trafficking was prevented by knocking down the palmitoyl acyltransferase GODZ with GODZ iRNA (Fang et al., 2006). The crosstalk between the post- and presynaptic elements of the synapses may occur through specialized adhesion molecules such as neuroligins, known to trans-synaptically interact with neurexins to form and maintain both GABAergic and glutamatergic synapses (Sudhof, 2008). However, whether gephyrin interacts with these molecules remains to be elucidated.

In our case, changes in amplitude and frequency of mIPSCs were accompanied by a slow down of their onset kinetics. Interestingly, similar kinetic changes were recently observed in collybistin $-/-$mice. Collybistin is a guanine nucleotide exchange factor required for gephyrin-dependent $GABA_A$ receptor clustering at inhibitory synapses (Papadopoulos et al., 2007). In the present experiments, the analysis of currents evoked in nucleated patches by ultrafast application of GABA allowed us to give a mechanistic interpretation of the results considering the kinetic properties of $GABA_A$ receptors under non-equilibrium conditions similar to those presumably occurring at synapses (Mozrzymas et al., 1999; Barberis et al., 2000). Like mIPSCs, currents evoked in scFv-gephyrin transfected cells by saturating concentrations of GABA, exhibited a slower rise time. The lack of this effect at non-saturating agonist concentrations (100 μM) suggests that impairing gephyrin function does not affect the agonist binding process (Jones and Westbrook, 1995; Petrini et al., 2003, 2004). Interestingly, slower onset of currents evoked by saturating (GABA) was associated with a reduction in the rate and extent of desensitization. Although it is not immediately apparent, both effects can be explained with a reduction of the desensitization rate. Indeed, as for AMPA receptors (Clements et al., 1998), it has been shown that the amplitude and the rising phase of GABA-evoked currents strictly depend on receptors desensitization (Mozrzymas et al., 2003a; Mozrzymas, 2004). Thus, in accordance with Jones and Westbrook's model, at saturating (GABA), binding is completed very quickly and current onset time constant is roughly described by the reciprocal of sum of the opening and desensitization rates ($\tau = 1/(\beta + d)$, where β is opening and d, the desensitization rate). Thus, consistent with our results, if desensitization rate decreases the current onset is expected to become slower. Contrary to the present data, a reduced entry into the desensitized state should be associated with an increase in current amplitude (Mozrzymas et al., 2003a,b). The reduced amplitude of mIPSCs detected here could be attributed to the reduction in GABAergic innervation (and possibly GABA release) which may have counterbalanced the expected modification in current amplitude due to receptor gating. VGAT immunostaining data and the reduction in mIPSCs frequency indicate a loss of functional synaptic sites. However, no changes in amplitude of GABA-evoked currents were detected in nucleated patches from scFv-gephyrin transfected cells. This discrepancy could be explained by the fact that current responses to rapid agonist application, although commonly used to mimic mIPSCs, differ from the synaptic ones in the targeted receptors (a mixture of synaptic and extrasynaptic ones) and in a more severe loss of intracellular soluble modulators (nucleated patches vs. whole-cell). The possibility that scFv-gephyrin could have reduced single channel conductance, thus obscuring the increase in current amplitude, is unlikely as the non-stationary noise analysis indicated that gephyrin impairment did not significantly affect this parameter. In contrast to the present data, the observed reduction in desensitization rate is expected to accelerate the deactivation kinetics (Jones and Westbrook, 1995; Mozrzymas et al., 2003a, 2007). The reason for this discrepancy in presently unclear: probably deactivation kinetic is a more complex phenomenon difficult to fit into a simplified model such that of Jones and Westbrook (1995). This may involve several additional desensitized as well as open and closed states.

The precise mechanism by which hampering gephyrin function affects the gating properties of $GABA_A$ receptors is still unknown. One possibility is that, to confine receptors into clusters may enhance their allosteric interaction possibly *via* protein domains present on neighboring receptors. In the case of glycine receptors it has been hypothesized that an allosteric interaction may occur between the large intracellular TM3–TM4 loop, containing consensus sequences for receptor phosphorylation (Cherubini and Conti, 2001) and the intracellular TM1-TM2 domain. This would lead to conformational changes and enhanced desensitization, particularly in case of high receptor density (Nikolic et al., 1998; Legendre et al., 2002; Muller et al., 2008). In support of this hypothesis is the observation that, increasing the density of glycine receptors in HEK cells co-transfected with gephyrin, enhanced fast desensitization of glycine-evoked currents, in the absence of apparent changes in receptor affinity (Legendre et al., 2002). Whether this could be extrapolated to $GABA_A$ receptors remains to be demonstrated.

In the present experiments, interfering with gephyrin function altered not only phasic but also tonic inhibition mediated by extrasynaptic $GABA_A$ receptors (Semyanov et al., 2004). It is well known that after being assembled

from their component subunits in the endoplasmic reticulum, $GABA_A$ receptors are targeted to their appropriate synaptic and extrasynaptic sites on the plasma membrane (Jacob et al., 2008). Like most neurotransmitter receptors, also $GABA_A$ receptors are subject to exchanges between synaptic and extrasynaptic domains by lateral diffusion (Triller and Choquet, 2005; Thomas et al., 2005; Renner et al., 2008; Jacob et al., 2008). Interestingly, the lateral mobility of $GABA_A$ receptors is regulated by gephyrin which facilitates their accumulation and stabilization at inhibitory synapses (Jacob et al., 2005). Therefore, it is likely that hampering gephyrin function with scFv-gephyrin affects lateral diffusion and synaptic and extrasynaptic $GABA_A$ receptors dynamics.

CONCLUSION

The new experimental approach described here based on scFv-gephyrin has unveiled new insights into the dynamic mechanisms by which this protein interacts with $GABA_A$ receptors to regulate synaptic activity at both pre and postsynaptic sites.

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-Paper 3-

Gephyrin regulates GABAergic and glutamatergic synaptic transmission in hippocampal cell cultures

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Gephyrin regulates GABAergic and glutamatergic synaptic transmission in hippocampal cell cultures

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Running title: gephyrin and synaptic transmission

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Abstract

Gephyrin is a scaffold protein essential for stabilizing glycine and $GABA_A$ receptors at inhibitory synapses. Here, recombinant intrabodies against gephyrin (scFv-gephyrin) were used to assess whether this protein exerts a transynaptic action on GABA and glutamate release. Pair recordings from interconnected hippocampal cells in culture, revealed a reduced probability of GABA release in scFv-gephyrin transfected neurons as compared to controls. This effect was associated with a significant decrease in VGAT, the vesicular GABA transporter and in neuroligin 2 (NLG2), a protein which, interacting with neurexins, ensures the cross talk between the post and the presynaptic site. Interestingly, hampering gephyrin function also produced a significant reduction in VGLUT, the vesicular glutamate transporter, an effect accompanied with a significant decrease in frequency of miniature excitatory post-synaptic currents. The loss of GABAergic but not glutamatergic innervation could be rescued by over-expressing NLG2 in gephyrin-deprived neurons suggesting that indeed NLG2 was involved in down regulation of GABA release. Pull-down experiments demonstrated that gephyrin interacts not only with NLG2 but also with NLG1, the isoform enriched at excitatory synapses. These results suggest a key role of gephyrin in regulating transynaptic signalling at both inhibitory and excitatory synapses.

Introduction

Speed and reliability of synaptic transmission are essential for information coding and require the presence of clustered neurotransmitter receptors at the plasma membrane in precise apposition to presynaptic releasing sites. The postsynaptic organization comprises a large number of proteins that ensure the correct targeting, clustering and stabilization of neurotransmitter receptors. Among them, the tubulin-binding protein gephyrin plays a crucial role in the functional organization of inhibitory synapses (Fritschy *et al,* 2008). Through its self-oligomerizing properties, gephyrin can form a hexagonal lattice that trap glycine (Sola *et al,* 2004) and $GABA_A$ receptors in the right place at postsynaptic sites (Kneussel *et al,* 1999; Tretter *et al,* 2008) by linking them to the cytoskeleton. Disruption of endogenous gephyrin leads to reduced $GABA_A$ receptor clusters (Kneussel *et al,* 1999), an effect that has been shown to be accompanied by a loss of GABAergic innervation (Yu *et al,* 2007; Marchionni *et al,* 2009). This observation suggests the existence of a cross talk between the post and presynaptic sites. The retrograde control of presynaptic signaling may occur via neuroligins (NLGs), postsynaptic cell adhesion molecules known to transynaptically interact with presynaptic neurexins (Sudhof, 2008). NLG1 is enriched at glutamaterigic synapses (Song *et al,* 1999; Chih *et al,* 2005), while NLG2 is preferentially associated with GABAergic connections (Varoqueaux *et al,* 2004). Over-expression of NLGs has been shown to increase the number of GABAergic and glutamatergic synaptic contacts (Levinson *et al,* 2005). Interestingly, increasing the expression level of PSD-95, the scaffolding molecule which directly binds NLG1, caused an enhancement of the glutamatergic innervation at the expense of the GABAergic one. This effect was accompanied by the recruitment of NLG2 to glutamatergic synapses (Gerrow *et al,* 2006, Levinson *et al,* 2005; Prange *et al,* 2004). Moreover, the recent demonstration of a direct interaction between NLG2 and gephyrin (Poulopoulos *et al,* 2009) suggests a role for this protein in regulating transynaptic signaling at inhibitory connections. Altogether, these findings have led to the hypothesis that scaffolding molecules can establish and maintain the proper excitatory (E)/inhibitory (I) balance, necessary for the correct functioning of neuronal networks, by modulating neuroligin localization and function at particular synapses (Craig and Kang, 2007; Dalva *et al,* 2007; Gerrow and El-Husseini, 2006). Understanding the molecular

mechanisms involved in the maintenance of a proper E/I balance is challenging since an alteration of this parameter underlies several devastating forms of neurological diseases including autism spectrum disorders (Rubenstein and Merzenich, 2003).

Previous studies on cultured hippocampal neurons have demonstrated that removal of gephyrin with single chain antibody fragments (scFv-gephyrin; Zacchi *et al*, 2008) produce changes in the gating properties of $GABA_A$ receptors associated with a decrease in GABAergic innervation (Marchionni *et al*, 2009).

In the present study, scFv-gephyrin were used to further characterize the transynaptic contribution of gephyrin in maintaining and stabilizing GABAergic synapses.

Double patch experiments from monosynaptically connected cells revealed a reduction in the probability of GABA release in scFv-gephyrin transfected cells. Moreover, transfection with scFv-gephyrin affected not only GABA but also glutamate release as demonstrated by the reduction in frequency of spontaneous and miniature glutamatergic synaptic events. Immunocytochemical data revealed a significant reduction in the number of NLG2 clusters together with a decrease of VGAT and VGLUT, the vesicular GABA and glutamate transporter, respectively. Finally biochemical experiments demonstrated that gephyrin can form a complex with both NLG2 and NLG1 in the brain, suggesting a role of this scaffold protein in regulating both excitatory and inhibitory synaptic transmission.

Materials and Methods

Neuronal and cell cultures

All experiments were carried out in accordance with the European Community Council Directive of 24 November 1986 (86/609 EEC) and were approved by the local authority veterinary service. Primary cell cultures were prepared as previously described (Andjus *et al*, 1997). Briefly, 2-4 days old (P2-P4) Wistar rats were decapitated after being anesthetized with an intraperitoneal injection of urethane (2 mg/kg). Hippocampi were dissected free, sliced, and digested with trypsin, mechanically triturated, centrifuged twice at 40 x *g*, plated in Petri dishes, and cultured for up to 14 days. Experiments were performed on cells cultured for at least 7 days. For paired recording experiments, neurons were plated at low density (~ 40000 cells/ml).

HEK-293 cells were maintained in DMEM supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 mg/ml) and transiently transfected with various plasmid constructs using standard calcium phosphate method. Cells were collected 24-48 hours after transfections.

Construction of plasmid vectors, scFv-gephyrin

Complementary DNAs encoding full length FLAG-tagged gephyrin has been previously described (Moretto-Zita *et al*, 2007). The N-terminal truncated gephyrin polypeptide (aa 2-188) fused to GFP is described in Maas *et al*, (2006). It acts as a dominant-negative protein due to its lack of dimerization motif, and is able to deplete endogenous gephyrin clusters in neurites within 24 h of expression. The murine HA-tagged NLG1 and HA-tagged NLG2 were constructed as reported elsewhere (Scheiffele *et al*, 2000; Chih *et al*, 2006). NLG2-GFP was constructed by using PCR-based mutagenesis. A PvuI restriction site was introduced ten amino acids downstream of the sequence encoding for the trans-membrane domain of NLG2-HA. This restriction site was then used to clone the EGFP coding sequence amplified using oligonucleotides containing PvuI consensus sites.

The last 94 amino acids of the cytoplasmic domains of both NLGs were inserted into pGEX4T1 vector for bacterial expressions as glutathione-S-transferase (GST)-NLs 94 aa

fusion proteins. All PCR-amplified products were fully sequenced to exclude the possibility of second site mutations.

The technique for isolating scFv-gephyrin has already been reported (Zacchi *et al*, 2008).

Neuronal transfection and Immunocytochemistry

Hippocampal neurons in culture were transfected with EGFP alone or cotransfected with EGFP and scFv-gephyrin using the calcium phosphate transfection method. For each Petri dish, 3 μg of DNA was transfected in total. Reliable cotransfection was ensured by routinely transfecting 0.9 μg of EGFP and 2.1 μg of scFv-gephyrin, and identified by the increased EGFP signal around the nucleus. For the rescue experiments, scFv-gephyrin and full length HA-tagged NLG2 (NLG2-HA) were co-transfected at a ratio of 2:1.

Neurons were transfected at 7 DIV and used for immunostaining 48 hours later. All steps were carried out at room temperature. After fixation with 4% paraformaldehyde (PFA) in PBS for 10 min, neurons were quenched in 0.1M glycine in PBS for 5 min, and permeabilized with 0.1% Triton X-100 in PBS for 2 min. For the rescue experiments, cells were fixed with pre-cooled 4% PFA in PBS for 5 min at 4°C, then 5 min at room temperature. They were then blocked in 0.2% BSA/1% FCS or 10% FCS in PBS for 30 min. After incubation with primary antibodies for 1 hour, cells were incubated with AlexaFluorophore-conjugated secondary antibodies (1:400) for 45 min. In the case of double-immunostaining, cells were incubated with biotinylated secondary antibodies (1:100, 45 min) followed by Streptavidin-conjuaged fluorophores (1:100, 30 min). The coverslips were washed in PBS, rinsed in water and mounted with VectaShield (Vector Labs).

The antibodies used were as follows: mouse monoclonal anti-VGAT (1:200, Synaptic Systems), mouse monoclonal anti-VGLUT1 (1:200, Synaptic Systems), rabbit polyclonal anti-Neuroligin 2 (1:200, Synaptic Systems), biotinylated goat anti-mouse IgG (Vector Labs). All secondary antibodies were obtained from Invitrogen.

In vitro binding, immunoprecipitation and Western blot analysis

Transfections were performed with the calcium phosphate method. GST pull-down assays were performed as previously described (Moretto-Zita *et al*, 2007). For NLGs and

gephyrin co-immunoprecipitation, HEK 293 cells overexpressing NLG1-HA/NLG2-HA and gephyrin-FLAG were lysed in 50 mM Tris HCl pH 7.5, 100 mM NaCl, 0.1% Tween 20, 10% glycerol, 10 mM EDTA, 2 mM $MgCl_2$ and protease inhibitor cocktail, and immunoprecipitated by the anti-FLAG antibody. Analysis of NLG1/NLG2-gephyrin interactions were performed on postnuclear homogenates from neonatal rat brains using the following lysis buffer: 50 mM Tris HCl pH 7.5, 150mM NaCl, 0.5% CHAPS, 1mM EDTA, 10% glycerol and protease inhibitor cocktail**.** After 2 hours incubation with monoclonal anti-gephyrin antibody, immunoprecipitation experiment was performed according to standard procedures. Primary antibodies were revealed by HRP-conjugated secondary antibodies (Sigma) followed by ECL (Amersham). The following primary antibodies were used: mouse monoclonal anti-FLAG M2 (Sigma); mouse monoclonal anti-gephyrin 3B11 (Synaptic Systems); high affinity rat monoclonal anti-HA 3F10 (Roche), rabbit polyclonal anti-NLG2 (Synaptic Systems); rabbit polyclonal anti-NLG1 (Synaptic Systems).

Confocal microscopy and image analysis

Fluorescence images were acquired on a TCS-SP confocal laser scanning microscope (Leica, Bensheim, Germany) with a 40X 1.4 NA oil immersion objective, additionally magnified 2 fold with the pinhole set at 1 Airy unit. Stacks of z-sections with an interval of 0.4 μm were sequentially scanned twice for each emission line to improve the signal/noise ratio. Cluster analysis was carried out using MetaMorph Imaging System (Universal Imaging, Westchester, PA, USA). First a binary template was created using the EGFP staining to identify transfected neurons, then cluster intensities in regions overlapping with the binary template were analyzed. Images were segmented to select immunofluorescent puncta over background labeling, and clusters were defined as >3 pixels as determined by visual inspection. Integrated Morphometry Analysis function of MetaMorph was used to quantify the number and size of clusters (4-5 cells from at least 4 different experiments). For the rescue experiments, NLG2 staining was used to create the binary template for the NLG2-HA/scFv-gephyrin co-transfected cells. As excessive NLG2-HA expression masks the rescuing effect and results in an overall increase in synaptic staining (similar to NLG2-HA over-expression alone), cells with moderate

amount of NLG2-HA expression (as identified by the unsaturated NLG2 fluorescence signal) was selected for the analysis of the rescue effect. Representative figures were prepared using ImageJ software.

Electrophysiological recordings

Spontaneous excitatory and inhibitory postsynaptic currents (EPSCs and IPSCs) were recorded from cultured hippocampal neurons transfected either with scFv-gephyrin or EGFP at 22-24^{o}C using a Multiclamp 700A amplifier (Axon Instruments, Foster City, CA, USA). Patch electrodes pulled from borosilicate glass capillaries (Hilgenberg, Malsfeld, Germany) had a resistance of 3-4 MΩ when filled with an intracellular solution containing (in mM): CsCl 137, $CaCl_2$ 1, $MgCl_2$ 2, BAPTA 11, ATP 2, and HEPES 10 (the pH was adjusted to 7.3-7.4 with CsOH). IPSCs were recorded at a holding potential of -70 mV, in the presence of DNQX (20 μM) and D-AP5 (50 μM) to block AMPA and NMDA receptors, respectively whereas EPSCs in the presence of bicuculline (10 μM) and D-AP5 (50 μM) to block $GABA_A$ and NMDA receptors, respectively. Miniature PSCs were recorded in the presence of tetrodotoxin (TTX, 1 μM) to block sodium currents and propagated action potentials and the respective $GABA_A$ or AMPA/NMDA receptor antagonists.

For double-patch recordings, pairs of action potentials (at 50 ms interval), were evoked in non transfected presynaptic neurons (in current clamp mode) by injecting depolarizing current pulses at a frequency of 0.1 Hz. IPSCs were detected from postsynaptic transfected (scFv-gephyrin) and non transfected (controls) neurons in voltage clamp mode at a holding potential of 0 mV (near the reversal potential for glutamate). In this case, the intracellular solutions contained (in mM): $KMeSO_4$ 135, KCl 10, HEPES 10, $MgCl_2$ 1, Na_2ATP 2, and Na_2GTP 0.4 (the pH was adjusted to 7.3 with KOH). In all experiments, the cells were perfused with an external solution containing (in mM): NaCl 137, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 20, and HEPES 10, pH 7.4, with NaOH. Data were sampled at 10 kHz and low pass filtered at 3 kHz. The stability of the patch was checked by repetitively monitoring the input and series resistances during the experiments. Cells exhibiting 15-20% changes were excluded from the analysis. The series resistance was 10-15 MΩ. All drugs (except TTX that was purchased from

Latoxan, Valence, France) were obtained from Tocris (Cookson Ltd, Bristol, UK). All drugs were dissolved in external solution, except DNQX, which was dissolved in dimethylsulphoxide (DMSO). The final concentration of DMSO in the bathing solution was 0.1%. At this concentration, DMSO alone did not modify the shape or the kinetics of synaptic currents.

Data Analysis

The analysis of spontaneous events was performed with Clampfit 10.1 software (Axon Instruments, Foster City, CA, USA). This program uses a detection algorithm based on a sliding template. The template did not induce any bias in the sampling of events because it was moved along the data trace one point at a time and was optimally scaled to fit the data at each position. The detection criterion was calculated from the template-scaling factor and from how closely the scaled template fitted the data.

For evoked IPSCs, transmission failures were identified visually. Mean IPSCs amplitude was obtained by averaging successes and failures. The paired-pulse ratio (PPR), known to be inversely correlated to the initial release probability (Dobrunz and Stevens, 1997), was calculated as the ratio between the mean amplitudes of IPSC2 over IPSC1. The coefficient of variation (CV^{-2}) was calculated as the square root of the ratio between the standard deviation of IPSC1 and the mean amplitude of IPSC1 (Korn and Faber, 1991).

Values are given as mean ± S.E.M. Unless otherwise stated, significance of differences was assessed by Student's *t*- test. The differences were considered significant when $P<0.05$.

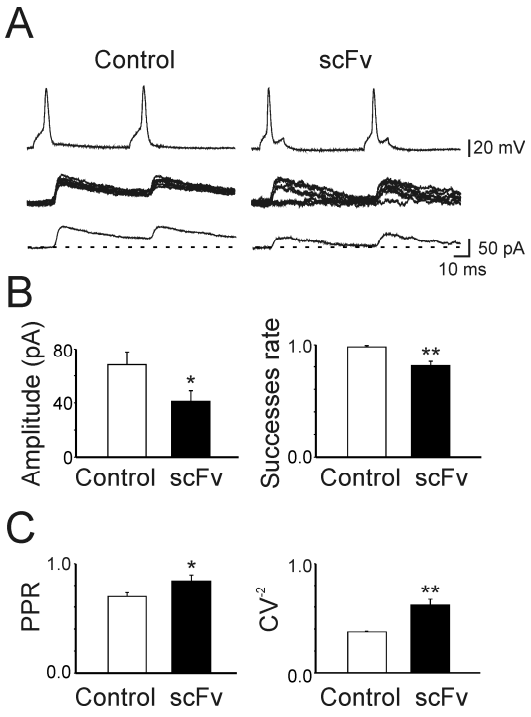
Results

Impairing gephyrin function with scFv-gephyrin reduces the probability of GABA release

As recently reported (Marchionni *et al*, 2009), transfecting cultured hippocampal neurons with scFv-gephyrin reduced the number of gephyrin and synaptic γ2 subunit containing $GABA_A$ receptor clusters. These effects were associated with a severe impairment of both phasic and tonic $GABA_A$ receptor-mediated inhibition. The mechanisms underlying these effects relied on changes in GABAergic innervation as suggested by the concomitant reduction in the number and size of presynaptic VGAT (the vesicular GABA transporter) clusters.

According to the quantal theory, the synaptic efficacy *E*, the mean amplitude of unitary IPSCs, can be defined as $E=mQ$, where *m* is the quantal content or mean number of quanta released per presynaptic action potential and *Q* is the quantal size or amplitude of the unitary IPSC (Katz, 1969). Whereas *Q* depends on both pre and postsynaptic mechanisms, m depends on presynaptic factors, namely the number of release sites *N* and the probability of release (*P*). To see whether a decrease in quantal content could account for the observed effects, simultaneous recordings were obtained from pairs of interconnected neurons (the postsynaptic one expressing or not expressing scFv-gephyrin; see Methods). As shown in Figure 1, IPSCs evoked in non transfected cells by pairs of presynaptic action potentials (50 ms apart, delivered at a frequency of 0.1 Hz, Control) were highly reliable and usually did not exhibit synaptic failures. In contrast, with respect to control, IPSCs from scFv-gephyrin transfected cells (n=6) exhibited a significant reduction in amplitude (from 68.2 ± 9.7 pA to 41.1 ± 7.8 pA; $p<0.05$, Mann-Whitney Rank test) and in successes rate (from. 0.98 ± 0.01 to 0.80 ± 0.03; $p<0.01$, Mann-Whitney Rank test; Figure 1A and B). These effects were associated with a significant increase in the PPR (from 0.69 ± 0.03 to 0.84 ± 0.05; $p<0.05$; Figure 1C) which is considered an index of presynaptic release probability (Zucker, 1989; Dobrunz and Stevens, 1997). Furthermore, the coefficient of variation (CV^{-2}) was significantly increased (from 0.6 ± 0.05 to 0.8 ± 0.03; $p<0.01$; Figure 1C), indicating changes in quantal content (Korn and Faber, 1991).

Figure 1



Hampering gephyrin function with scFv-gephyrin reduces the probability of GABA release
A. Pair recordings obtained from two interconnected neurons. The postsynaptic cell was transfected with scFv-gephyrin (scFv; right). As control a neighboring non transfected cell was used (Control; left). Upper traces are pairs of action potentials evoked in presynaptic cells at 50 ms interval by depolarizing current steps of variable amplitude every 10 s. Middle traces are monosynaptic IPSCs (successes and failures) evoked at 0 mV (E_{GABA} -70 mV) by presynaptic action potentials. Lower traces are averaged responses. *B.* Mean amplitude and successes rate obtained in monosynaptically connected cells in control (white columns; n=7) and in scFv-transfected neurons (black columns). *C.* Paired-pulse ratio and CV^{-2} of monosynaptically connected neurons (n=6) recorded from control and scFv-transfected cells. * $p<0.05$; ** $p<0.01$.

Overall these data strongly suggest that hampering gephyrin function with scFv-gephyrin reduces the probability of GABA release.

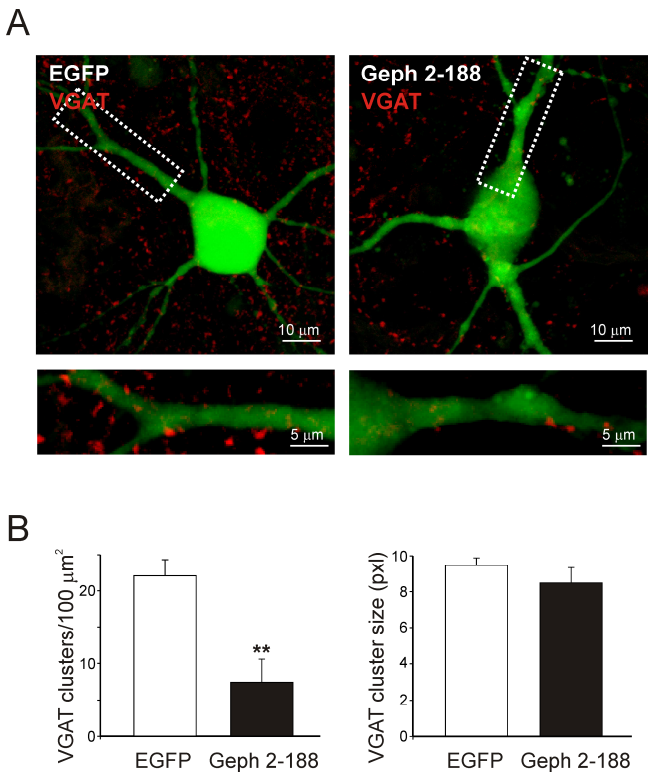
Gephyrin 2-188, a dominant-negative form of gephyrin, mimics the effect of scFv-gephyrin on GABAergic function

To validate the results obtained with scFv-gephyrin, a truncated gephyrin polypeptide comprising the N-terminal (amino acids 2-188) of gephyrin fused with EGFP, known to

act as a dominant-negative protein, was used (Maas *et al*, 2006). Due to the lack of dimerization motif, this polypeptide interferes with the endogenous gephyrin lattice formation and depletes gephyrin clusters in neurites within 24 hours of expression on cultured neurons.

Immunocytochemical experiments on hippocampal neurons transfected with gephyrin 2-188 revealed a significant reduction in the number of VGAT clusters (without effects on their size), indicating an effect on GABAergic innervation similar to that observed for scFv-gephyrin (Supplementary Figure 1).

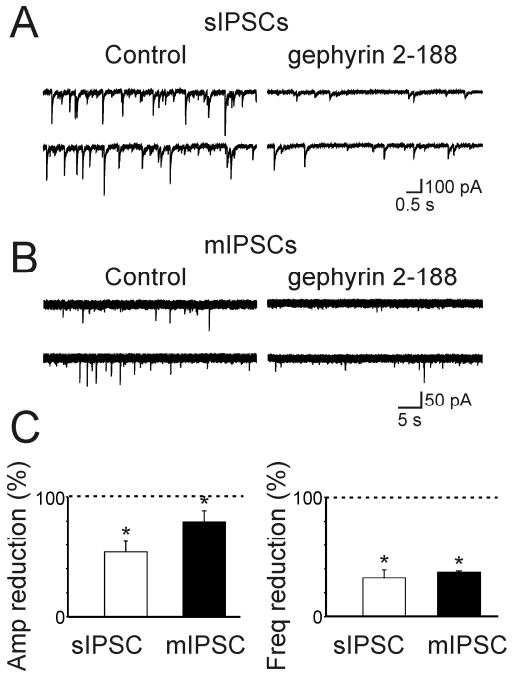
Suppl. Figure 1



Gephyrin 2-188 reduces the density of GABAergic synapses. *A.* Neurons were transfected (green) with EGFP (left) or a dominant-negative construct fused to GFP (Geph 2-188; right) and immunostained for VGAT (red). Bottom panels are magnifications of the white boxes marked on top. *B.* Quantification of VGAT cluster density (left) and cluster size (right). Note the significant reduction in the density of VGAT clusters. **p<0.01 (for comparison with scFv-gephyrin see Figure 2 in Marchionni *et al*, 2009).

As for scFv-gephyrin, this effect was accompanied by a significant reduction in amplitude and frequency of spontaneous and miniature IPSCs (in cell transfected with gephyrin 2-188 the reduction in amplitude of sIPSCs and mIPSCs was 54 ± 9 % and 79 ± 9 % of controls, respectively; the reduction in frequency of sIPSCs and mIPSCs the 32 ± 7 % and 37 ± 1 % of controls, respectively; Supplementary Figure 2).

Suppl. Figure 2



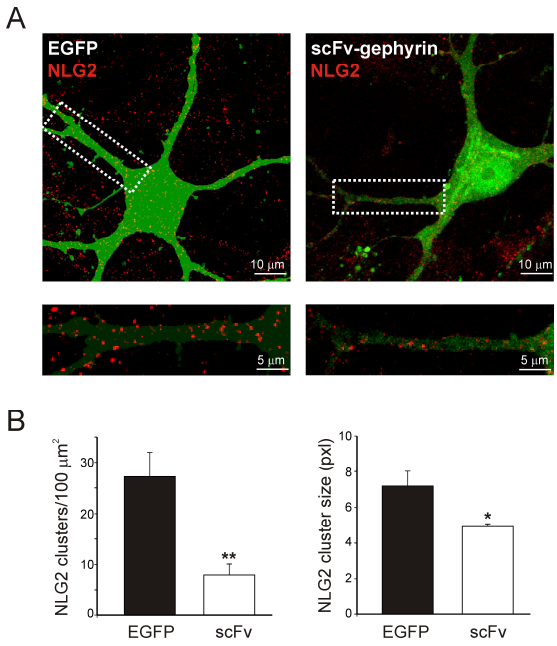
Gephyrin 2-188 reduces the peak amplitude and frequency of sIPSCs and mIPSCs. *A.* Samples of spontaneous IPSCs recorded from controls and gephyrin 2-188 transfected neurons at a holding potential of -70 mV in the presence of D-AP5 (50 μM) and DNQX (20 μM) *B.* Samples of miniature IPSCs recorded from controls and gephyrin 2-188 transfected neurons in the presence of tetrodotoxin (1 μM). *C.* Each column represents the reduction in amplitude (left) and in frequency (right) of sIPSC (white; n=9) and mIPSCs (black; n=7) obtained from gephyrin 2-188 transfected neurons and expressed as percentage of controls (dashed lines). * $p<0.05$.

These data further support the hypothesis that gephyrin not only regulates postsynaptic organization of synaptic $GABA_A$ receptors but also GABAergic innervation.

Gephyrin removal reduces the density and size of Neuroligin 2 clusters

How can gephyrin interfere with GABA release? One possibility is that this protein interacts with cell adhesion molecules such as neuroligins which, by binding neurexins, ensure the crosstalk between the pre- and post-synaptic sites (Sudhof, 2008). Of particular interest is neuroligin 2 (NLG2), since this protein is known to play a pivotal role in the organization of GABAergic synapses (Poulopoulos *et al*, 2009). To verify whether disrupting gephyrin affects NLG2 distribution, transfected hippocampal neurons with scFv-gephyrin were immunostained for NLG2.

Figure 2



scFv-gephyrin reduces the total number and size of NLG2 clusters. *A.* Neurons were transfected (green) with EGFP (left) or EGFP and scFv-gephyrin (right) and immunostained for NLG2 (red). Bottom panels are magnifications of the white boxes marked on top. *B.* Quantification of NLG 2 clusters density (left) and cluster size (right). Note the significant reduction in the density and size of NLG2 clusters. * $p<0.05$; ** $p<0.01$.

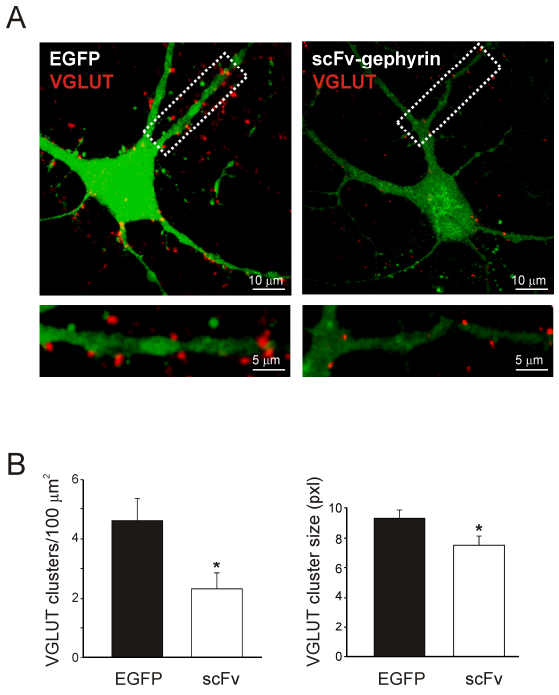
As shown in Figure 2, scFv-gephyrin transfected neurons exhibited a significant reduction in the density of NLG2-positive clusters as compared to EGFP transfected controls (7.9 ± 2.1 clusters/100 µm^2 for scFv-gephyrin *vs* 27.2 ± 4.8 clusters/100 µm^2 for

EGFP; p<0.01; n=9). In addition, the average size of these clusters was smaller for scFv-gephyrin- than for EGFP-transfected neurons (4.9 ± 0.2 μm^2 for scFv-gephyrin *vs* 7.2 ± 0.8 μm^2 for EGFP; p<0.05; n=9). NLG2 did not re-localize to glutamatergic synapses since the synaptic fraction co-localized with VGLUT was merely detectable (4.1 ± 0.01 % in control and 5.3 ± 0.02 % in scFv-gephyrin transfected cells, respectively; these values were not significantly different; p>0.05; data not shown).

Impairing gephyrin function with scFv-gephyrin reduces glutamatergic innervation

The interaction of NLGs with scaffolding proteins is crucial for ensuring the correct excitatory/inhibitory balance, critical for the proper functioning of neuronal networks. Therefore, the following experiments were performed to assess whether disrupting gephyrin function with scFv-gephyrin can affect not only GABAergic but also glutamatergic transmission.

Figure 3

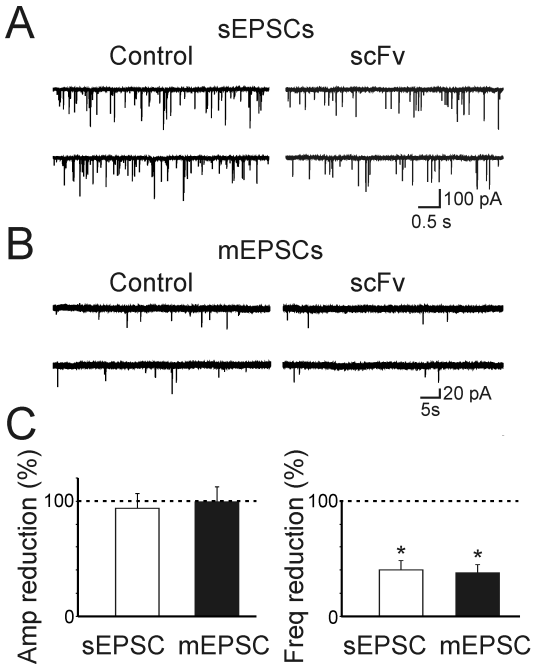


scFv-gephyrin reduces the number and size of glutamatergic synapses. *A.* Neurons were transfected (green) with EGFP (left) or EGFP and scFv-gephyrin (right) and immunostained for the presynaptic glutamatergic marker VGLUT (red). Bottom panels are magnifications of the white boxes marked on top. *B.* Quantification of VGLUT clusters density (left) and clusters size (right). Note the significant reduction in the density and size of VGLUT clusters. * p<0.05; ** p<0.01.

To this aim, cultured hippocampal neurons transfected with scFv-gephyrin were immunostained for the vesicular glutamate transporter VGLUT, a widely used marker for presynaptic glutamatergic terminals (Yu and De Blas, 2008). As compared to controls (EGFP-transfected cells) in scFv-gephyrin transfected cells VGLUT immunopositive clusters were significantly reduced in density and size (Figure 3). In particular, the density of VGLUT clusters was reduced from 4.6 ± 0.8 clusters/100 μm^2 in EGFP to 2.3 ± 0.5 clusters/100 μm^2 in scFv-gephryin ($p<0.05$; n=12). The size of these clusters was reduced from 9.3 ± 0.5 μm^2 to 7.5 ± 0.6 μm^2 ($p<0.05$).

Furthermore, whole cell voltage clamp recordings performed in the presence of bicuculline (10 μM) and D-AP5 (50 μM), to block $GABA_A$ and NMDA receptors, respectively, revealed a significant reduction in frequency (but not in amplitude) of spontaneous EPSCs (the frequency reached 40 ± 8 %; $p<0.05$; n=12; the amplitude 95 ± 13 %; $p>0.05$; n=12) recorded from scFv-gephyrin transfected neurons as compared to controls (Figure 4A and C).

Figure 4



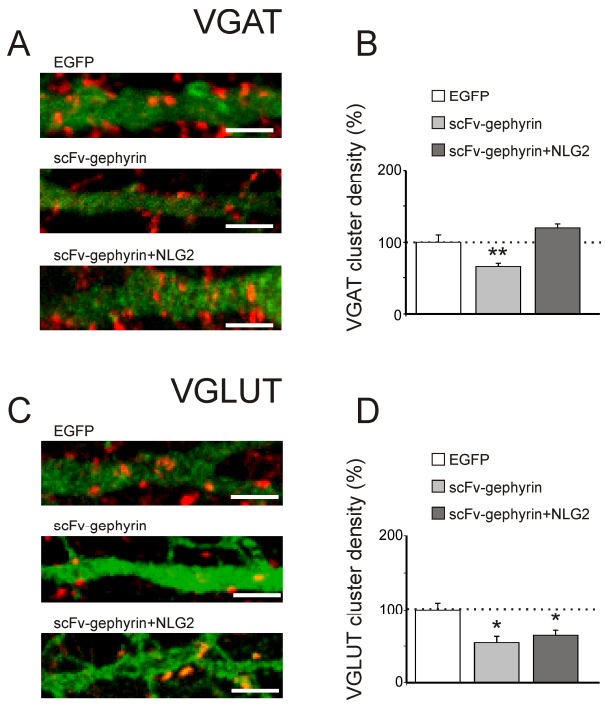
scFv-gephyrin reduces the frequency but not the amplitude of sEPSCs and mEPSCs. *A.* Samples of spontaneous EPSCs recorded from controls and scFv-gephyrin transfected neurons at a holding potential of -70 mV in the presence of bicuculline (10 μM) and D-AP5 (50 μM). *B.* Samples of miniature EPSCs recorded from controls and scFv-gephyrin transfected neurons at a holding potential of -70 mV in the presence of tetrodotoxin (1 μM). *C.* Each column represents the reduction in amplitude (left) and in frequency (right) of sIPSC (white) and mIPSCs (black) obtained from scFv-gephyrin transfected neurons (n= 12) and expressed as percentage of controls (n= 12; dashed lines). * $p<0.05$.

Similarly, in scFv-gephyrin transfected cells, the frequency of miniature EPSCs recorded in the presence of TTX was significantly reduced with respect to controls (to 37 ± 7 %; $p<0.05$; from 0.78 ± 0.14 Hz to 0.32 ± 0.05 Hz; n= 12) while the amplitude was unchanged (to 100 ± 13 %; $p>0.05$; from 34 ± 6 pA to 34 ± 5 pA; n=7; Figure 4B and C). Altogether, these results strongly support the involvement of gephyrin in regulating not only GABAergic but also glutamatergic synaptic transmission.

The loss of GABAergic but not glutamatergic innervation in gephyrin-deprived neurons can be rescued by over expressing NLG2

To further assess the possibility that the reduced GABAergic innervation in scFv-gephyrin transfected cells is mediated by NLG2 which may convey information in a retrograde way from post to presynaptic sites, NLG2 was co-expressed with scFv-gephyrin. In immunocytochemical experiments, co-expression of NLG2 with scFv-gephyrin induced a significant increase in the density of VGAT-positive clusters as compared to cells transfected with scFv-gephyrin alone (180 ± 8 %; from 10.6 ± 0.7 clusters / 100 μm^2 to 19.1 ± 0.9 clusters / 100 μm^2; $p<0.01$; n=11 and 8 for scFv and scFv/NLG2, respectively), restoring VGAT cluster density to control levels (Figure 5A and B). In line with previous studies (Chih *et al*, 2005), over expression of NLG2 alone led to a two-fold increase in the density of VGAT clusters as compared to EGFP transfected controls (data not shown).

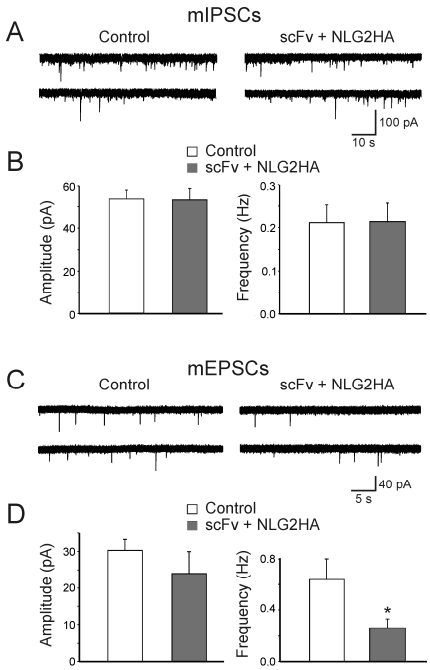
Figure 5



Co-expression of NLG2 with scFv-gephyrin restores the loss of GABAergic but not glutamatergic innervation. *A.* Representative images of neurons transfected with EGFP (top), scFv-gephyrin (middle) or co-transfected with scFv-gephyrin and NLG2-HA (bottom). Dendrites were visualized by EGFP signal or NLG2 staining (green). Neurons were immunostained for VGAT (red). Scale bar, 5 µm. *B.* Quantification of VGAT cluster densities relative to the mean value obtained from EGFP-transfected neurons (dashed line). **p<0.01. *C* and *D* as in *A* and *B* but for neurons immunostained for VGLUT (red). Scale bar, 5 µm.

Parallel electrophysiological experiments from cultured neurons revealed no changes in amplitude and frequency of spontaneous mIPSCs between cells co-transfected with scFv-gephyrin and NLG2 and controls (neighboring non-transfected cells). On average, the frequency of mIPSCs was 0.211 ± 0.040 Hz and 0.213 ± 0.044 Hz (p=0.97) while the amplitude was -55 ± 4 pA and -53 ± 6 pA (p=0.7) in control (n=7) and in co-transfected neurons (n=8), respectively (Figure 6A and B). Altogether these experiments indicate that overexpression of NLG2 is able to rescue the loss of GABAergic innervation induced by scFv-gephyrin.

Figure 6



Co-expression of NLG2 with scFv-gephyrin rescues GABAergic but not glutamatergic synaptic transmission. *A.* Samples of spontaneous mIPSCs recorded from cells co-transfected with scFv-gephyrin plus NLG2-HA and from neighbouring non transfected cells (Control) at a holding potential of -70 mV in the presence of TTX (1 μM), DNQX (20 μM) and D-AP5 (50 μM) *B.* Each column represents the mean amplitude (left) and frequency (right) of mIPSC from control (white; n=7) and from scFv-gephyrin transfected cells (black; n=8). *C* and *D* as in *A* and *B* but for mEPSCs recorded in the presence of TTX (1 μM) and bicuculline (10 μM) from cells co-transfected with scFv-gephyrin plus NLG2-HA (n=9) and from neighboring non transfected cells (Control, n=8).

It is possible that the observed reduction in glutamatergic innervation following gephyrin depletion with scFv-gephyrin represents a homeostatic compensatory mechanism to prevent hyperexcitability and to maintain the right E/I balance within the neuronal network (Turrigiano and Nelson, 2004). If this is the case, rescuing GABAergic innervation should lead to a concomitant change in glutamatergic transmission. However, this was not the case because over expressing NLG2 in gephyrin depleted neurons failed to restore VGLUT immonoreactive puncta (0.5 ± 0.1 and 0.6 ± 0.1 clusters / 100 μm^2 for scFv and scFv/NLG2, respectively; $p>0.5$; Figure 5C and D) as well as the frequency of

mEPSCs to control levels. The frequency of mEPSCs was 0.64 ± 0.16 Hz and 0.26 ± 0.07 Hz, in the absence or presence of NLG2 over expression; $p<0.05$; the amplitude of mEPSCs was 29 ± 3 pA. and 25 ± 7 pA, in the absence (n= 8) or in the presence (n=9) of NLG2 over expression ($p>0.05$; Figure 6C and D).

Gephyrin directly interacts with NLG2 and NLG1

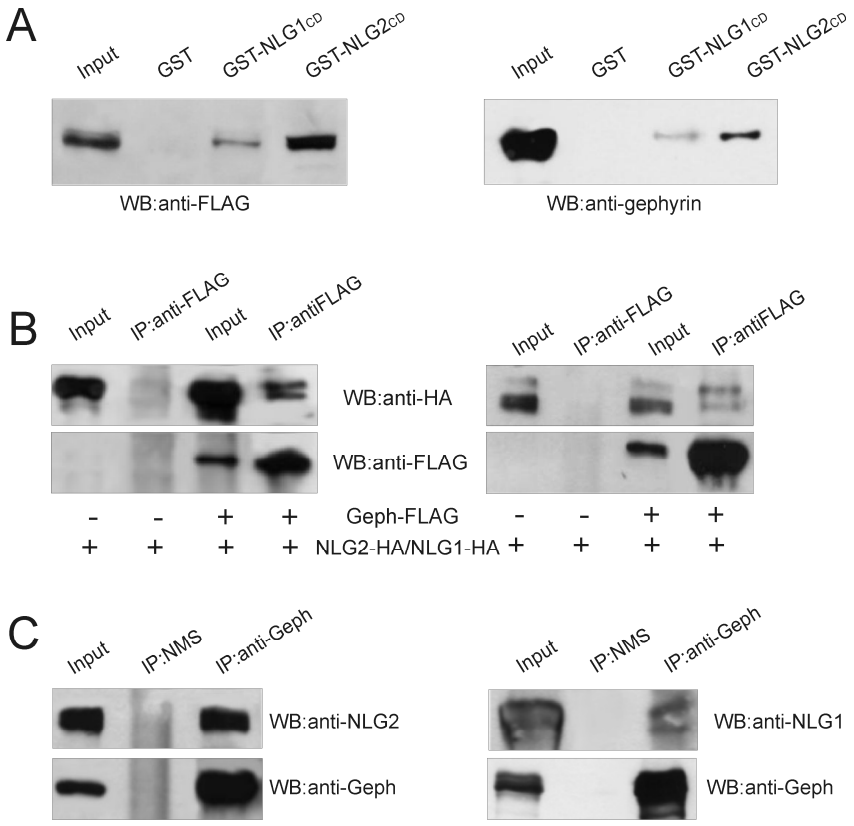
It has been recently reported that at inhibitory synaptic contacts gephyrin directly binds NLG2 (Poulopoulos *et al,* 2009). The amino-acid sequence identified as gephyrin-binding motif on NLG2 is highly conserved in all NLGs, and indeed gephyrin binds to all four NLGs in yeast two-hybrid assays (Poulopoulos *et al,* 2009). To test whether gephyrin can form a complex with NLG1 in mammalian cells, lysates of HEK 293 cells transfected with gephyrin-FLAG were subjected to pull-down assay with beads loaded with GST-NLG1 cytoplasmic domain ($NLG1_{CD}$), GST-$NLG2_{CD}$ or with GST alone as negative control. In agreement with previous observations (Poulopoulos *et al,* 2009), $NLG2_{CD}$ was able to precipitate a consistent amount of gephyrin-FLAG (Figure 7A, left panel). Interestingly a small but significant fraction of gephyrin-FLAG was also found in complex with GST-$NLG1_{CD}$ (Figure 7). Similar pull-down experiments were then performed to assay the ability of endogenous gephyrin present on neonatal rat brain homogenates to interact with NLG1 and NLG2. Also in this case gephyrin was not only associated with GST-$NLG2_{CD}$ fusion protein but also with GST-$NLG1_{CD}$ (Figure 7A, right panel). In this case immunoblot analysis was performed using a monoclonal antibody raised against the C-terminal domain of gephyrin protein.

We then performed immunoprecipitation experiments to investigate the presence of NLG1-HA/gephyrin-FLAG complexes *in vitro*. HEK 293 cells were co-transfected with plasmids encoding for NLG1/2-HA and gephyrin-FLAG, or NLG1/2-HA alone, and cell lysates were immunoprecipitated with the anti-FLAG monoclonal antibody. The bound protein complexes were analysed by Western blotting using anti-HA and anti-FLAG for NLG1 and gephyrin detection, respectively. As shown in Figure 7B (right panel), NLG1-HA was immunoprecipitated only from cells co-expressing gephyrin-FLAG. The same experimental conditions were also applied to detect the expected presence of NLG2-HA/gephyrin-FLAG complexes in mammalian cells. Indeed we found that a lower

amount of gephyrin-FLAG was able to precipitate a higher amount of NLG2-HA as compared to NLG1-HA, thus supporting previous *in vitro* observations.

Finally endogenous NLG2, and interestingly also NLG1, were found in native complexes with gephyrin upon co-immunoprecipitation from mouse brain homogenates (Figure 7C). These data suggest that gephyrin, by directly interacting with NLG2 and to a lesser extent with NLG1, may affect not only GABAergic but also glutamatergic synaptic transmission.

Figure 7



Gephyrin interacts with NLG2 and NLG1. A. GST-NLG1/2$_{CD}$ pull-down assay using lysates of HEK 293 cells transfected with gephyrin-FLAG (left) and rat brain lysates (right). *B.* Lysates of HEK 293 cells transfected with either NLG2-HA (left panel) or NLG1-HA (right panel) in the presence of gephyrin FLAG or with the vector alone (as a negative control) were immunoprecipitated with monoclonal anti-FLAG antibodies. Immunoprecipitates were analyzed by Western blotting using anti-HA and anti-FLAG monoclonal antibodies. *C.* Co-immunoprecipitation experiments on rat brain lysates using a monoclonal anti-gephyrin antibody and NMS as negative control. Immunoprecipitates were analyzed by Western blotting using a monoclonal anti-gephyrin antibody and a polyclonal antibody against NLG2 and NLG1.

Discussion

The tubulin-binding protein gephyrin is a core protein of inhibitory postsynaptic densities which interacts with the cytoskeleton to stabilize inhibitory receptors in precise apposition to presynaptic active zones (Fritschy *et al,* 2008). In a previous study, we have demonstrated that disrupting endogenous gephyrin with selective scFv-gephyrin altered the gating properties of $GABA_A$ receptors, an effect that was found to be associated with modifications of GABAergic innervation (Marchionni *et al,* 2009). In the present study we hypothesized that hampering gephyrin function affects not only the number of release sites (as suggested by the reduction in VGAT clusters) but also the probability of GABA release. In support of this view, in double patch experiments from interconnected neurons, we found that, with respect to controls, scFv-gephyrin expressing cells exhibited a significant decrease in amplitude of individual synaptic currents accompanied by a clear increase in the number of transmitter failures and a reduction in the PPR. Changes in transmitter failures and in PPR are consistent with an increase in release probability (Katz, 1969; Zucker 1989).

The role of gephyrin in ensuring a correct communication between pre and postsynaptic elements of the synapses was further validated by the experiments in which a truncated form of gephyrin (gephyrin 2-188; Maas *et al,* 2006) was used. This gephyrin mutant lacks the dimerization motif but it can still interact with endogenous gephyrin molecules, producing dominant-negative effects on postsynaptic gephyrin clusters. Similar to scFv-gephyrin, over expression of gephyrin 2-188 caused a reduction in GABAergic innervation and a decrease in frequency of spontaneous and miniature IPSCs, further confirming a key role of gephyrin in maintaining the stability of GABAergic connections within the neuronal network. The ability of gephyrin to influence presynaptic innervation was already suggested by Yu *et al,* (2007) even though no mechanistic interpretation was provided.

The presynaptic action of gephyrin on GABA release implies the coordinated activity of other signaling molecules that interact directly or indirectly with gephyrin to ensure the corrected cross-talk between the post and presynaptic elements of the synapse. Possible candidates are NLGs, specialized cell adhesion molecules that functionally couple the postsynaptic densities with the transmitter release machinery by forming transynaptic

complexes with their presynaptic binding partners, neurexins (Sudhof, 2008). The role of NLG-neurexin complex as a coordinator between postsynaptic and presynaptic sites has been investigated at excitatory CA3-CA1 synapses in the hippocampus. This study has revealed a retrograde modulation of neurotransmitter release by PSD-95-NLG complex (Futai *et al,* 2008). The authors found that over expression of the glutamatergic scaffold protein PSD-95 enhanced release probability *via* a mechanism involving the NLG-neurexin complex.

The reduction in the probability of GABA release after scFv-gephyrin transfection likely involves a similar mechanism, as NLG2 is preferentially concentrated at inhibitory synapses (Varoqueaux *et al,* 2004) and directly binds gephyrin through a conserved cytoplasmatic domain (Poulopoulos *et al,* 2009 and present data). Consistent with our experiments, it has been recently shown that NLG2-/- mice exhibit a reduction in quantal content associated with a decrease in quantal size of unitary responses between fast spiking GABAergic interneurons and principal cells in the neocortex (Gibson *et al,* 2009). Gephyrin has been shown to be selectively recruited by NLG2 *via* collybistin (Poulopoulos *et al,* 2009). Interestingly, similarly to our experiments knock out mice for collybistin exhibited a reduction in the frequency of mIPSCs (Papadopoulos et al. 2007) suggesting the possible involvement of collybistin in gephyrin-dependent transynaptic signaling.

Our findings, while providing additional evidence that native gephyrin-NLG2 complexes are present in the brain (even in the absence of any cross-linking agent), support the role of gephyrin in maintaining NLG2 at GABAergic synapses. Indeed, immunocytochemical experiments showed a significant decrease in the total number and size of NLG2 clusters upon scFv-induced gephyrin removal. The possibility to restore GABAergic innervation in gephyrin-deprived neurons by over expressing NLG2 further supports the involvement of gephyrin-NLG2 complex in transynaptic signaling.

A recent study (Levinson *et al,* 2010) has shown that knocking down gephyrin with siRNA led to a shift of endogenous NLG2 from inhibitory to excitatory synapses, in the absence of any change in the density of NLG2 clusters. In the present experiments instead we have observed a clear reduction in the density of NLG2 clusters without a detectable re-localization of this protein to glutamatergic synapses. Since scFv-mediated

removal of gephyrin is associated with a significant reduction of synaptic γ2 containing $GABA_A$ receptors (Marchionni *et al,* 2009) and evidence has been provided for the reciprocal stabilization of NLG2 by $GABA_A$ receptors (Dong *et al,* 2007), the reduction of NLG2 staining could be a consequence of the loss of gephyrin-dependent $GABA_A$ receptors clustering. We can not exclude the possibility that scFv-gephyrin may affect the function of additional gephyrin-bound factors important for the efficient localization of NLG2 to and/or from GABAergic terminals. Conventional kinesin (KIF5) and the dynein motor complex have been shown to be involved in microtubule-dependent transport of gephyrin, thus contributing to postsynaptic remodeling (Maas *et al,* 2006; Maas *et al,* 2009). Since microtubule motors transport and remodel a variety of transmembrane and submembrane postsynaptic proteins (Hirokawa and Takemura, 2005; Kneussel, 2005), similar mechanisms may account for NLG2 transport.

Unexpectedly, hampering gephyrin function with scFv-gephyrin produced a significant reduction not only of GABAergic but also of glutamatergic innervation as assessed by the significant decrease in density of VGLUT positive puncta associated with a significant reduction in frequency, but not in amplitude, of spontaneous and miniature glutamatergic events. This effect was not due to a sort of homeostatic plasticity because, overexpressing NLG2 in gephyrin depleted neurons failed to re-establish glutamatergic innervation.

Co-immunoprecipitation experiments have revealed the existence of native complexes not only between gephyrin and NLG2 but also with NLG1, which is primarily localized at excitatory synapses (Song *et al,* 1999). This confirms and extends previous data obtained with the yeast two hybrid system (Poulopoulos *et al,* 2009).

Gephyrin might interact with both NLG2 and NLG1 to regulate both excitatory and inhibitory inputs converging on the same neuron thus controlling the E/I balance at the network level. While the present experiments clearly demonstrate that gephyrin *via* NLG2 controls GABA release, the precise mechanism by which this scaffolding molecule affects glutamate release remains to be elucidated.

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CONCLUSIONS AND FUTURE PERSPECTIVES

In this thesis I have analyzed the role of gephyrin in the organization and function of GABAergic synapses. To this aim I took a loss-of-function approach to knock down endogenous gephyrin using intracellular single chain antibody fragments against gephyrin (scFv-gephyrin). Deletion of gephyrin by scFv-gephyrin induced: (1) impairment of both phasic and tonic GABAergic inhibition. (2) reduction in GABAergic innervation and in the probability of GABA release. (3) reduction in VGAT and neuroligin 2 immunoreactivity. The trans-synaptic effect of scFv-gephyrin on GABAergic innervation probably involved the neuroligin 2/neurexin complex since it could be rescued by over-expressing neuroligin 2 in gephyrin-depleted neurons. In addition, hampering gephyrin function caused a reduction in glutamatergic innervation. Although this effect is particularly interesting, the underlying mechanism remains to be elucidated.

As outlined in Zacchi *et al.* (2008), scFv-gephyrin were selected using the Intracellular Antibody Capture Technique, which is a yeast two-hybrid based system that allows the selection of antibody fragments suitable for intracellular expression. The C-domain linker region of gephyrin was used as bait to select gephyrin-specific scFv, as this domain is not involved in gephyrin oligomerization. In this way, scFv-gephyrin tagged with a nuclear localization signal could recognize gephyrin within the submembranous lattice scaffold and relocate it towards the nucleus.

The main advantages of using scFv-gephyrin over gephyrin knockout and RNA interference approaches is that it bypasses possible compensatory effects following targeted gene deletion and has a longer half-life compared to interfering RNAs. Acting at the post-translational level, scFv-gephyrin provides an alternative approach to study the role of gephyrin in synaptic organization. Upon transfection into cultured hippocampal neurons, scFv-gephyrin disrupted endogenous gephyrin clusters and impaired glycine receptor function (Zacchi et al., 2008).

As already mentioned, disrupting gephyrin function with scFv-gephyrin resulted in a significant reduction in the density of gephyrin and synaptic γ2-subunit containing $GABA_A$ receptors. This effect was associated with a reduction in the amplitude and

frequency of miniature IPSCs as well as changes in the gating properties of $GABA_A$ receptors. Using an ultrafast agonist application system, I was able to demonstrate that hampering gephyrin function resulted in a slow-down of the onset kinetics and fast entry into the desensitized state of $GABA_A$ receptors. Overall, this led to a severe impairment in phasic inhibition. Unexpectedly, I also observed a significant reduction in tonic inhibition upon scFv-gephyrin transfection. As tonic inhibition is mediated by extrasynaptic $GABA_A$ receptors, gephyrin appears to effect not only synaptic but also extrasynaptic receptors. This could be *via* changes in the lateral mobility of receptors on the neuronal membrane. Gephyrin was shown to play an important to role in cell surface dynamics of $GABA_A$ receptors (Jacob et al., 2005). An overall increase in the receptor diffusion rate could thus be reflected in a modification of both phasic and tonic inhibition, as receptors with altered kinetics move in and out of synapses. In future experiments, in collaboration with A. Barberis and E. Petrini (IIT, Genova), single particle tracking experiments using fluorophore-tagged $GABA_A$ receptors would be a good approach to study changes in the mobility of synaptic (for instance $\alpha 2$) and extrasynaptic (for instance $\alpha 5$) $GABA_A$ receptors following scFv-gephyrin transfection. Especially with the use of photostable quantum dots to label endogenous membrane proteins, it would be possible to follow the movement of receptors without photobleaching the fluorophore (Triller and Choquet, 2008). RNA interference against gephyrin was shown to reduce gephyrin clusters and to produce a three fold increase in the mobility of $\gamma 2$ and $\beta 3$ subunit-containing $GABA_A$ receptors (Jacob et al., 2005). In addition, it was recently found that interfering with gephyrin oligomerization induces an increase in lateral mobility of extrasynaptic but not synaptic glycine receptors in spinal cord neurons in culture (Calamai et al., 2009).

A still unsolved problem is the mechanism underlying the reduction of glutamatergic innervation following transfection with scFv-gephyrin. Using the yeast 2-hybrid screening gephyrin was found to bind not only to neuroligin 2, but to all four neuroligin isoforms (Poulopoulos et al., 2009). Similarly, we observed that gephyrin could co-immunoprecipitate neuroligin 1, albeit at a lower level than neuroligin 2, from rat brain homogenates. Among the different neuroligin isoforms, neuroligin 1 is preferentially associated with glutamatergic synapses, while neuroligin 2 is found mostly at

GABAergic synapses (Graf et al., 2004). Although the functional significance of the gephyrin-neuroligin 1 interaction is still unclear, it points towards a possible mechanism for gephyrin to regulate both excitatory and inhibitory synapses, exerting a homeostatic control on the E/I balance at the network level. We are planning to perform rescue experiments to see whether in gephyrin-depleted neurons, over-expression of neuroligin 1, can reverse the effect of scFv-gephyrin on glutamatergic innervation. This will be assessed with electrophysiological recordings of mEPSCs as well as with immunocytochemical experiments using VGLUT specific antibodies on hippocampal neurons in culture. These experiments will allow establishing the possible role of neuroligin 1 in trans-synaptic regulation of glutamate release by endogenous gephyrin.

Among different neuroligin isoforms, neuroligin 3 was found to be localized at both glutamatergic and GABAergic synapses (Budreck and Scheiffele, 2007). Therefore, the possibility that gephyrin modulates both types of synapses through neuroligin 3 can not be excluded. A recent study demonstrated that the localization of neuroligin 3 at GABAergic or glutamatergic synapses can be modulated by the relative level of postsynaptic scaffold proteins gephyrin and PSD-95 (Levinson et al., 2010). The fate of neuroligin 3 after scFv-gephyrin transfection would further elucidate the role of gephyrin in the localization of this neuroligin isoform.

Alterations in the E/I balance are thought to play a crucial role in autism spectrum disorders (ASD). Interestingly, a small percentage of ASD patients carry missense and nonsense mutations in genes encoding neuroligins 3 and 4 (Jamain et al., 2003; Laumonnier et al., 2004; Yan et al., 2005). We intend to investigate the role of neuroligin 3 in GABAergic signaling, using a mouse model that was recently shown to exhibit a phenotype reminiscent of that present in human patients with ASD. These knock-in mice carry a mutation in the *NLGN3* gene which alters a conserved arginine residue to a cysteine (R451C) within the extracellular esterase-homology domain (Tabuchi et al., 2007). Functional characterization of the R451C mice has revealed a ~90% loss in neuroligin 3 in the forebrain. However the mutation led to a gain-of-function effect, with an increase in GABAergic inhibition and an increase in the level of VGAT and gephyrin, associated with enhanced spatial learning (Tabuchi et al., 2007). Knock-in mice carrying the R451C mutation are currently under study in our lab, with the aim of elucidating the

role of neuroligin 3 in GABAergic transmission and in the overall network activity, as well as the mechanism by which the R451C mutation leads to enhanced inhibitory transmission. In particular, we are interested to see whether correlated network activity such as giant depolarizing potentials (GDPs) in the hippocampus develop normally and whether GABA shifts from the depolarizing to the hyperpolarizing direction at the right time (during the second postnatal week). Changes in GABAergic signaling have been proposed to contribute to many developmental disorders and evidence has been provided that ASD symptoms in children can drastically improve after treatment with bumetanide (Lemonnier and Ben-Ari, 2010), which blocks the chloride importer NKCC1, shifting GABA reversal from the depolarizing to the hyperpolarizing direction.

During the course of experiments aimed at characterizing the full length neuroligin 2 expression on human embryonic kidney cells (HEK 293), I observed that the cells over-expressing neuroligin 2 exhibited a large number of filopodia-like protrusions. These were characterized by an extension of the actin cytoskeleton, as revealed by the F-actin staining with phalloidin (Figure 7).

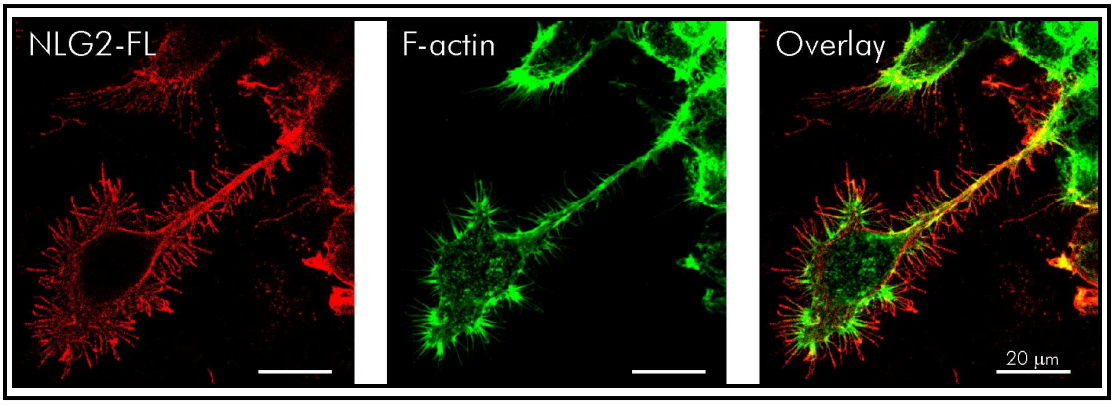

Figure 7. **Neuroligin 2 enhances filopodial extensions on HEK 293 cells.** HEK 293 cells were transfected with the full length neuroligin 2 protein (NLG2-FL) and stained for neuroligin 2 (red; left) and F-actin (green; middle). The overlay of the two channels shows the filopodial protrusions exploring the surrounding area.

Neuroligin 2 staining was observed on the cell membrane, along the actin protrusions. In order to study these morphological changes in more detail, we are planning to perform live cell imaging experiments to measure the mobility of the cells in the presence or absence of neuroligin 2 over-expression. For this purpose I inserted an EGFP tag to the full length neuroligin 2 construct. HEK 293 cells transfected with EGFP-tagged neuroligin 2 will be visualized under an epifluorescent microscope.

In collaboration with the group of V. Torre (SISSA), we are also planning to measure the force exerted by the protrusions on a silica bead trapped with optical tweezers in the absence or presence of neuroligin 2 over-expression. These experiments are aimed at better understanding the morphological changes exerted by neuroligin 2 expression alone, in the absence of the neuroligin/neurexin interaction. As filopodial extensions are the initial steps in the formation of synapses, a possible effect of neuroligin 2 on the properties of these extensions have implications for the synaptogenesis.

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