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A crosstalk between β 1 and β 3 integrins controls glycine receptor and gephyrin trafficking at inhibitory synapses

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Abstract

The regulation of glycine receptor (GlyR) number at synapses is fundamental for the efficacy of inhibition and the control of neuronal excitability in the spinal cord. GlyR accumulation at synapses depends on the scaffolding molecule gephyrin and is linked to GlyR synaptic dwell time. However, the mechanisms that tune GlyR synaptic exchanges depending on the neuronal environment are unknown. Integrins are cell adhesion molecules and signaling receptors. Using single quantum dot and FRAP imaging, we demonstrate in rats that β 1 and β 3 integrins regulate the synaptic dwell time of both GlyRs and gephyrin to adjust synaptic strength. β 1 and β 3 integrins crosstalk via calcium/calmodulin-dependent protein kinase II and adapt GlyR lateral diffusion and gephyrin-dependent trapping at synapses. This provides a mechanism to maintain or adjust the steady state of postsynaptic molecule exchanges and the level of glycinergic inhibition in response to neuron- and glia-derived signals or extracellular matrix remodeling.

Introduction

While synapses are relatively stable structures, their molecular components are exchanged within short time scales¹. Postsynaptic scaffolding molecules are renewed in tens of minutes both *in vitro* and *in vivo* (e.g. ²⁻⁴). In the neuronal membrane, recent advances in single particle imaging techniques have enabled the visualization of neurotransmitter receptor lateral diffusion into and out of synapses^{1, 5}. As receptors are mostly inserted in and removed from the plasma membrane at non-synaptic sites¹, lateral diffusion and trapping at synapses are fundamental mechanisms for the sorting and accumulation of receptors at excitatory or inhibitory synapses. Synaptic strength is closely related to the dwell time of the postsynaptic molecules, and both can be adjusted during synapse maturation and plasticity^{1, 3}. However, how changes in the neuronal environment are translated into changes in the dwell times of synaptic molecules remains unknown.

Integrins mediate adhesion between the extracellular matrix (ECM) and the cytoskeleton and transduce bidirectional signaling cascades^{6, 7}. They are transmembrane heterodimers composed of one α and one β subunit, which are receptors for ECM molecules, soluble factors and counter-receptors. At least ten integrin subunits are expressed in the central nervous system⁸ and some are localized at synapses (e.g.⁸⁻¹¹). Integrins are central elements in neuron-neuron and neuron-glia interactions during synapse maturation, synapse plasticity and after injury^{10, 12, 13}. Recent studies have highlighted the involvement of the $\beta 1$ and $\beta 3$ integrins at excitatory synapses. In hippocampal neurons, $\beta 1$ integrins are required for long-term potentiation and spatial memory^{9, 14-16}. $\beta 3$ integrins coordinate the maturation of the pre- and post-synaptic compartments and mediate activity-dependent regulation of excitatory synaptic strength^{10, 11}. The function of integrins at inhibitory synapses remains to be determined.

In this study, we have investigated the function of $\beta 1$ and $\beta 3$ integrins at glycinergic synapses in spinal cord neurons. Glycine receptors (GlyRs) play a major role in controlling motor neuron excitability and in processing sensory signals¹⁷. We have focused on GlyRs containing the $\alpha 1$ subunit, which predominate in the adult spinal cord¹⁷. These receptors are $\alpha 1/\beta$ hetero-pentamers and are stabilized at synapses by direct interaction between the β subunit and gephyrin, the main inhibitory scaffolding protein⁴. We find that $\beta 1$ and $\beta 3$ integrins adjust glycinergic

synaptic strength with opposing actions: while $\beta 1$ integrins increase GlyR numbers at synapses, $\beta 3$ integrins reduce GlyR numbers and glycinergic synaptic strength. Using single molecule and bulk approaches, we demonstrate that these effects result from a regulation of both gephyrin exchange and GlyR dwell time at synapses. The opposing actions of $\beta 1$ and $\beta 3$ integrins rely on calcium/calmodulin-dependent protein kinase II (CaMKII), which regulates GlyR trapping and accumulation at synapses. We also find that $\beta 1$ and $\beta 3$ integrins can be further activated by extracellular factors such as thrombospondin I (TSP1) and fibrinogen that are released after injury. Thus, the crosstalk between $\beta 1$ and $\beta 3$ integrins provides a mechanism for the regulation of glycinergic synaptic strength integrating signals from neurons and glia.

Results

Opposite actions of $\beta 1$ and $\beta 3$ integrins on synaptic GlyRs

To test the possible involvement of integrins at glycinergic synapses, we blocked integrin function in cultured spinal cord neurons using echistatin, a peptide from viper venom containing an arginine-glycine-aspartate (RGD) motif, which specifically inhibits integrins containing the $\beta 1$ and $\beta 3$ subunits¹⁸. We analyzed the effect of 1-hr treatment with echistatin (100 nM) on GlyR synaptic clusters by immunocytochemistry, using synapsin I as a presynaptic marker. After echistatin treatment, GlyR clusters were brighter than in control conditions (**Fig. 1a-b**). GlyR immunoreactivity (IR) at synapses increased to $177 \pm 11\%$ of the control ($n_{\text{Ctr}} = 52$, $n_{\text{Echis}} = 51$, $p < 0.001$, **Fig. 1c**). Similar results were obtained with a synthetic RGD peptide ($n_{\text{GRGESP}} = 54$, $n_{\text{GRGDSP}} = 54$, $p < 0.001$, **Fig. 1c**). Together, this indicates that integrins control GlyR accumulation at synapses.

To discriminate between the effects of $\beta 1$ and $\beta 3$ integrins on GlyR clusters, we used monoclonal antibodies that specifically block $\beta 1$ or $\beta 3$ integrins. One-hour treatment with $\beta 3$ -blocking antibodies ($\alpha\beta 3$, 25 $\mu\text{g/ml}$) increased GlyR cluster-IR to $198 \pm 11\%$ of the control ($n_{\text{Ctr}} = 60$, $n_{\alpha\beta 3} = 60$, $p < 0.001$, **Fig. 2a-b**). By contrast, treatment with $\beta 1$ -blocking antibodies ($\alpha\beta 1$, 25 $\mu\text{g/ml}$) reduced GlyR cluster-IR to $57 \pm 2\%$ of the control ($n_{\text{Ctr}} = 60$, $n_{\alpha\beta 1} = 60$, $p < 0.001$). The difference between the effect of RGD peptides and that of $\alpha\beta 1$ can be explained by the fact that, depending on the

α subunit, some $\alpha\beta 1$ heterodimers are not receptors for RGD containing molecules⁶ and are therefore not inhibited by these peptides. One-hour treatment with $\alpha\beta 1$ or $\alpha\beta 3$ had no effect on synapse density or on synapse size (**Supplementary Fig. 1**). In line with this, the robust changes in GlyR cluster-IR after $\alpha\beta 1$ and $\alpha\beta 3$ treatments were not associated with similar modifications of GlyR cluster size (**Supplementary Fig. 1**). This is consistent with previous reports on synapse number or morphology^{11, 14, 15, 19}.

To further substantiate the opposite effects of the $\beta 1$ and $\beta 3$ integrin blocking antibodies, we overexpressed truncated forms of $\beta 1$ and $\beta 3$ integrins, in which the extracellular domain was replaced by EGFP (CT $\beta 1$ and CT $\beta 3$, respectively¹¹). The low transfection efficiency allowed us to analyze the effects of postsynaptic integrin blockade, by examining transfected postsynaptic neurons that were not surrounded by transfected axons. After 24 hrs, overexpression of CT $\beta 3$ resulted in a robust increase in receptor cluster-associated fluorescence ($206 \pm 23\%$ of the control) whereas overexpression of CT $\beta 1$ had the opposite effect ($55 \pm 4\%$ of the control) ($n_{EGFP} = 37$; $n_{CT\beta 1} = 40$; $n_{CT\beta 3} = 38$, $p < 0.001$, **Supplementary Fig. 2**). The direction and the amplitude of these changes were similar to those observed after 1-hr treatment with the blocking antibodies. Together, these results demonstrate that $\beta 1$ and $\beta 3$ integrins regulate GlyR numbers at synapses in opposing directions.

The functional consequences of the regulation of GlyR numbers at synapses were tested with whole-cell patch-clamp recordings. We compared the miniature glycinergic postsynaptic currents (mIPSCs) on the same cells before and after antibody application. This showed that mIPSC amplitudes were increased after $\beta 3$ integrin inhibition, but reduced after $\beta 1$ integrin inhibition (**Fig. 2c-d**), consistent with the effects observed by immunocytochemistry. Twenty minutes after $\alpha\beta 3$ application, mIPSC amplitudes were increased to $132 \pm 4\%$ of the amplitudes before treatment, whereas they were decreased to $76 \pm 4\%$ after $\alpha\beta 1$ application ($n = 5$ for each condition, $p < 0.001$, **Fig. 2e**). The coefficient of variation of the mIPSC amplitudes was not modified after $\alpha\beta 1$ or $\alpha\beta 3$ treatment (**Supplementary Fig. 3**), indicating that $\beta 1$ and $\beta 3$ integrins uniformly scale the synaptic strength of all glycinergic synapses. Moreover, the kinetics of the mIPSCs remained unchanged (**Supplementary Fig. 3**). The frequency of the mIPSCs varied between cells from 1.0 ± 0.4 Hz to 7.5 ± 0.3 Hz,

but was not modified by $\alpha\beta 1$ and $\alpha\beta 3$ treatments (statistical test: non significant (NS); **Fig. 2f**). The effects of $\alpha\beta 3$ and $\alpha\beta 1$ treatments were not observed with denatured antibodies (data not shown). These data indicate that $\beta 1$ and $\beta 3$ integrins scale inhibitory synaptic strength in opposite directions by modifying GlyR number at synapses.

Integrins control GlyR lateral diffusion

We have previously shown that GlyR diffusion-trapping is a major mechanism for the regulation of GlyR number at synapses^{5, 20, 21}. To test the effects of integrin inhibition on GlyR diffusion properties in the neuronal membrane, we stained active presynaptic boutons with FM4-64 and monitored the surface mobility of endogenous GlyRs labeled with quantum dots (GlyR-QDs) in single particle tracking (SPT) experiments. The SPT method provides a high spatial resolution (≈ 10 nm)⁵. Consistent with previous observations^{5, 20}, some GlyR-QDs remained at synapses or diffused in the extrasynaptic membrane, while others entered or escaped the synaptic area during the recording session (**Fig. 3a**). After $\alpha\beta 3$ treatment, GlyR-QDs generally explored larger surfaces of the extrasynaptic membrane, but smaller surfaces at synapses. After $\alpha\beta 1$ treatment, GlyR-QDs were very mobile within and outside synapses and frequently exchanged between the synaptic and extrasynaptic compartments.

Quantification of these experiments indicated that, in the extrasynaptic membrane, GlyR-QD diffusion coefficients were about twice higher than control values after both $\alpha\beta 3$ and $\alpha\beta 1$ treatments ($n_{\text{Ctr}} = 1112$, $n_{\alpha\beta 3} = 626$, $n_{\alpha\beta 1} = 682$, $p < 0.001$, **Fig. 3b,d**). At synapses however, $\beta 3$ and $\beta 1$ integrin blockade had opposite effects: GlyR-QD diffusion coefficients were decreased after $\alpha\beta 3$ treatment ($n_{\text{Ctr}} = 288$, $n_{\alpha\beta 3} = 152$, $p < 0.01$), whereas they were increased after $\alpha\beta 1$ treatment ($n_{\alpha\beta 1} = 176$; $p < 0.001$, **Fig. 3c**). The diffusion coefficients were decreased to 75% of the control after $\beta 3$ integrin inhibition and increased to 170% of the control after $\beta 1$ integrin inhibition (**Fig. 3d**). Therefore, the effects of $\beta 1$ and $\beta 3$ integrin inhibition are comparable at the extrasynaptic membrane but opposite at synapses, indicating distinct regulations in the two compartments.

Integrins control GlyR trapping and dwell time at synapses

Given the effects on GlyR diffusion at synapses, we hypothesized that integrins regulate the level of confinement of synaptic receptors. Therefore, we analyzed the trajectories of GlyR-QDs in more detail (**Fig. 4a**). As shown by plots of the mean square displacement (MSD), GlyRs were more confined after $\beta 3$ integrin inhibition, but less confined after $\beta 1$ integrin inhibition (**Fig. 4b**). In control conditions, the average diameter of the confinement domain at synapses was 175 ± 9 nm ($n = 228$ trajectories), consistent with previous data²⁰. The size of the confinement domain was reduced to $71 \pm 5\%$ of the control after $\beta 3$ integrin inhibition whereas it was enlarged to $150 \pm 10\%$ of the control after $\beta 1$ integrin inhibition ($n_{a\beta 3} = 115$, $n_{a\beta 1} = 124$, $p < 0.001$, **Fig. 4c**). This indicates that $\beta 1$ integrin inhibition induces a relaxation of the constraints that stabilize the GlyRs at synapses, and therefore facilitates GlyR exit from synapses, whereas $\beta 3$ integrin inhibition has the opposing action.

Next, we tested whether the changes in GlyR confinement affected the time spent by GlyRs at synapses when they diffuse in the plasma membrane. Receptor dwell time in a synapse may vary between seconds and tens of minutes⁵, thus exceeding the duration of our recordings (38.4 s). Therefore, we used a dwell time index to estimate the mean fraction of time spent in a synapse (see **Online Methods**). The cumulative distribution of this index highlighted two populations of receptors: a 'stable' population corresponding to receptors always detected at synapses during the recording session (dwell time index = 1), and a 'mobile' population corresponding to receptors that exchange between the synaptic and extrasynaptic compartments (dwell time index < 1) (**Fig. 4d**). $\beta 1$ integrin inhibition reduced GlyR-QD dwell time index from 0.51 ± 0.02 in control condition to 0.34 ± 0.03 , whereas $\beta 3$ integrin inhibition increased it to 0.60 ± 0.03 ($n_{\text{Ctr}} = 377$, $n_{a\beta 3} = 205$, $n_{a\beta 1} = 253$; $a\beta 3$: $p < 0.05$, $a\beta 1$: $p < 0.001$; **Fig. 4e**). Thus, GlyRs spent more time at synapses after $\beta 3$ integrin inhibition and more time in the extrasynaptic membrane after $\beta 1$ integrin inhibition. Furthermore, we found that integrin-mediated regulation of GlyRs did not depend on GlyR endocytosis, exocytosis, protein synthesis or protein degradation (**Supplementary Fig. 4**), which contrasts with what has been observed for GluR2-containing AMPA receptors¹¹ (**Supplementary Fig. 4**). Together, these data demonstrate that $\beta 1$ and $\beta 3$ integrins set the equilibrium between the pools of synaptic and extrasynaptic GlyRs and thereby GlyR number at synapses.

Integrins regulate gephyrin stabilization at synapses

At synapses, GlyRs are transiently stabilized by the gephyrin-based postsynaptic scaffold⁴. Therefore, we tested whether changes in gephyrin assembly were associated with the regulation of GlyR trapping at synapses. In immunocytochemistry, gephyrin cluster-IR increased to $161 \pm 5\%$ of the control value after $\alpha\beta 3$ treatment ($n_{\text{Ctr}} = 60$, $n_{\alpha\beta 3} = 60$, $p < 0.001$), whereas it decreased to $59 \pm 3\%$ after $\alpha\beta 1$ treatment ($n_{\alpha\beta 1} = 60$, $p < 0.001$, **Fig. 5a-b**), indicating that integrins control the level of gephyrin at synapses. These effects were not associated with modification of gephyrin expression and did not depend on protein synthesis or protein degradation (**Supplementary Fig. 5**), indicating that integrins control the exchange between synaptic and non-synaptic gephyrin molecules.

To examine the effects of $\beta 1$ and $\beta 3$ integrin blockade on the synaptic dynamics of gephyrin, we transfected neurons with a gephyrin::Venus construct (VeGe²²) and performed fluorescence recovery after photobleaching (FRAP) of individual synaptic VeGe clusters (**Fig. 5c-d**). Over a 20 minute time scale, the recovery curves exhibited two main phases: a 'fast' component corresponding to a population of molecules exchanged in a few seconds with non-synaptic non-bleached molecules, and a 'slow' component corresponding to a population of molecules that could reside in a cluster for tens of minutes (**Fig. 5e**). Integrin inhibition had a noticeable effect on the fast phase of the recovery, which was more rapid and reached a higher plateau after $\beta 1$ integrin inhibition but was reduced after $\beta 3$ integrin inhibition. In contrast, the slow phase was not dramatically altered. Ten minutes after photobleaching, the fluorescence recovery was $50 \pm 2\%$ of the initial value in control condition ($n = 26$, **Fig. 5f**), which is similar to the recovery obtained with endogenous mRFP-gephyrin clusters from a knock-in mouse². It was increased to $58 \pm 2\%$ after $\beta 1$ integrin inhibition ($n = 21$, $p < 0.01$) and reduced to $33 \pm 1\%$ after $\beta 3$ integrin inhibition ($n = 21$, $p < 0.001$). Therefore, integrins determine the steady state of gephyrin synaptic exchange. Altogether, our data indicate that the modulation of gephyrin synaptic turnover by $\beta 1$ and $\beta 3$ integrins controls the dwell time and the number of GlyRs at synapses.

Integrins control GlyR synaptic trapping via CaMKII

In contrast to other adhesion molecules such as cadherins and neuroligins, there is no known direct interaction between integrins and postsynaptic proteins. Nonetheless, integrins interact with a number of cytoskeleton-related molecules and signaling proteins⁷ that might regulate the interactions in the postsynaptic scaffold. We have previously shown that F-actin disruption increases GlyR lateral diffusion, and reduces the amount of both GlyRs and gephyrin at synapses²⁰. We therefore investigated the involvement of the actin cytoskeleton in integrin-mediated regulations of GlyR lateral diffusion. We found that F-actin disruption with latrunculin (Lat, 3 μ M, 25 min) abolished the effect of β 1 and β 3 integrin inhibition in the extrasynaptic membrane ($n_{\text{Lat}} = 484$, $n_{\text{Lat}+\alpha\beta 3} = 621$, $n_{\text{Lat}+\alpha\beta 1} = 386$, statistical tests: NS, **Fig. 6a**). At synapses, however, latrunculin did not prevent the $\alpha\beta$ 1-induced increase or the $\alpha\beta$ 3-induced decrease in GlyR diffusion coefficients ($n_{\text{Lat}} = 103$, $n_{\text{Lat}+\alpha\beta 3} = 185$, $n_{\text{Lat}+\alpha\beta 1} = 73$, $p < 0.001$ and $p < 0.05$, respectively). The effects on GlyR clusters were also maintained after latrunculin treatment: β 3 integrin blockade increased GlyR cluster-IR to $167 \pm 9\%$, whereas β 1 integrin blockade reduced it to $77 \pm 4\%$ ($n_{\text{Lat}} = 40$, $n_{\text{Lat}+\alpha\beta 3} = 40$, $n_{\text{Lat}+\alpha\beta 1} = 40$; $p < 0.001$ and $p < 0.01$, respectively, **Fig. 6b**). This indicates that the lateral mobility of GlyRs depends on actin in the extrasynaptic membrane, whereas integrins regulate GlyR trapping at synapses via a distinct pathway.

We then examined several kinases involved in integrin signaling. Inhibition of Src kinases did not block the effect of $\alpha\beta$ 1 or $\alpha\beta$ 3 on GlyR cluster-IR (**Supplementary Fig. 6**), suggesting that Src kinases do not contribute to these regulations. PKC inhibition with GF109203X (GFX, 50 nM, 25 min) alone did not significantly modify GlyR lateral diffusion or GlyR accumulation at synapses (**Supplementary Fig. 7**). However, PKC inhibition prevented the effects of integrin inhibition on the lateral diffusion of GlyRs in the extrasynaptic membrane ($n_{\text{GFX}} = 675$, $n_{\text{GFX}+\alpha\beta 3} = 321$, $n_{\text{GFX}+\alpha\beta 1} = 353$, statistical tests: NS, **Fig. 6c**) and at synapses ($n_{\text{GFX}} = 230$, $n_{\text{GFX}+\alpha\beta 3} = 168$, $n_{\text{GFX}+\alpha\beta 1} = 218$, statistical tests: NS). PKC inhibition also abolished the $\alpha\beta$ 3-induced increase and the $\alpha\beta$ 1-induced decrease in the number of GlyRs at synapses (GFX: $100 \pm 6\%$, $n = 40$; GFX + $\alpha\beta$ 3: $94 \pm 4\%$, $n = 40$; GFX + $\alpha\beta$ 1: $98 \pm 5\%$, $n = 40$, statistical tests: NS, **Fig. 6d**). Since integrins regulate GlyR mobility in and out of synapses by different pathways (see above), these results indicate that

PKC is necessary for integrin activation but is not responsible for the synapse-specific antagonism between $\beta 1$ and $\beta 3$ integrins.

Next, we tested the involvement of CaMKII. In the extrasynaptic membrane, inhibition of CaMKII with KN-93 (KN93, 10 μ M, 1 hr) had no effect on GlyR mobility ($n_{\text{Ctr}} = 279$, $n_{\text{KN93}} = 505$, statistical tests: NS, **Fig 6e**) and did not alter $\alpha\beta 1$ - and $\alpha\beta 3$ -induced increase of GlyR diffusion coefficients ($n_{\text{KN93}+\alpha\beta 3} = 489$, $n_{\text{KN93}+\alpha\beta 1} = 450$, $p < 0.001$). At synapses however, CaMKII inhibition increased GlyR diffusion coefficients ($n_{\text{Ctr}} = 90$, $n_{\text{KN93}} = 133$, $p < 0.001$) and decreased their synaptic accumulation (Ctr: $100 \pm 6\%$, $n = 40$; KN93: $59 \pm 3\%$, $n = 40$, $p < 0.001$, **Fig. 6f**). Furthermore, CaMKII inhibition completely abolished the effects of $\alpha\beta 1$ and $\alpha\beta 3$ on GlyR mobility at synapses ($n_{\text{KN93}+\alpha\beta 3} = 158$, $n_{\text{KN93}+\alpha\beta 1} = 131$, statistical tests: NS, **Fig. 6e**) and on GlyR cluster-IR (KN93: $100 \pm 4\%$, $n = 40$; KN93 + $\alpha\beta 3$: $99 \pm 4\%$, $n = 40$; KN93 + $\alpha\beta 1$: $101 \pm 4\%$, $n = 40$, statistical tests: NS, **Fig. 6f**). The effects of $\alpha\beta 1$ and $\alpha\beta 3$ on gephyrin clusters were also occluded by the CaMKII inhibitor (KN93: $100 \pm 4\%$, $n = 40$; KN93 + $\alpha\beta 3$: $100 \pm 5\%$, $n = 40$; KN93 + $\alpha\beta 1$: $98 \pm 4\%$, $n = 40$, statistical tests: NS). These experiments reveal the involvement of CaMKII at glycinergic synapses. They indicate that both $\beta 1$ and $\beta 3$ integrins signal via CaMKII, which is responsible for their opposing actions at synapses. Furthermore, they demonstrate that controlling GlyR mobility in the synapse is crucial to adjust the number of synaptic GlyRs.

TSP1 and fibrinogen have opposite effects at synapses

Little is known about the ligands of integrins involved in the regulation of synapse function. We examined the effects of TSP1 and fibrinogen, two known $\beta 1$ and $\beta 3$ integrin ligands released after injury²³⁻²⁶. In our experiments, the $\beta 1$ integrin ligand TSP1 (2 μ g/ml, 1 hr) slowed down GlyR lateral diffusion in the extrasynaptic membrane ($n_{\text{Ctr}} = 487$, $n_{\text{TSP1}} = 355$, $p < 0.001$) and stabilized GlyRs at synapses ($n_{\text{Ctr}} = 159$, $n_{\text{TSP1}} = 130$, $p < 0.001$, **Fig. 7a-b**). The changes in diffusion properties were associated with an increased accumulation of GlyRs (Ctr: $100 \pm 4\%$, $n = 60$; TSP1: $147 \pm 5\%$, $n = 60$; $p < 0.001$) and gephyrin at synapses (Ctr: $100 \pm 4\%$, $n = 60$; TSP1: $150 \pm 6\%$, $n = 60$; $p < 0.001$, **Fig. 7c**). The effects of TSP1 were opposite to those observed after inhibition of $\beta 1$ integrins and were abolished by prior incubation with $\alpha\beta 1$ (**Supplementary Fig. 8**). This shows that TSP1 increases GlyR trapping through $\beta 1$ integrins.

The $\beta 3$ integrin ligand fibrinogen (Fib, 1.5 mg/ml, 1 hr) slowed GlyR lateral mobility in the extrasynaptic membrane and destabilized GlyRs at synapses (extrasynaptic: $n_{\text{Vehicle}} = 614$, $n_{\text{Fib}} = 413$, $p < 0.001$; synaptic: $n_{\text{Vehicle}} = 218$, $n_{\text{Fib}} = 152$, $p < 0.001$; **Fig. 7d-e**). Consistent with this, fibrinogen decreased GlyR and gephyrin synaptic levels to $67 \pm 2\%$ and $73 \pm 2\%$ of the control values, respectively (GlyR and gephyrin: $n_{\text{Vehicle}} = 60$, $n_{\text{Fib}} = 60$, $p < 0.001$, **Fig. 7f**). The effects of fibrinogen were opposite to those observed after inhibition of $\beta 3$ integrins and were abolished by prior incubation with $\alpha\beta 3$ (**Supplementary Fig. 8**), showing that fibrinogen decreases GlyR trapping via $\beta 3$ integrins. Taken together, these results strongly suggest that GlyR diffusion-trapping and numbers at synapses can be regulated by extracellular factors released after injury.

Discussion

In this study, we have shown that integrins control glycinergic synaptic strength by regulating the number of GlyRs at synapses. Using SPT with blocking antibodies and known integrin ligands, we demonstrate that $\beta 1$ integrins decrease GlyR diffusion coefficients and increase GlyR confinement at synapses, whereas $\beta 3$ integrins have opposing actions. $\beta 1$ and $\beta 3$ integrins adjust GlyR dwell time at synapses and shift the equilibrium between the pools of synaptic and extrasynaptic receptors toward an increased and decreased synaptic localization, respectively. Importantly, blocking the effects of integrins on GlyR diffusive properties at synapses abolished the regulation of GlyR postsynaptic accumulation. Furthermore, the effects of integrins were not associated with modification of GlyR surface expression and did not depend on endocytosis, protein synthesis or protein degradation. Together, these data reveal that $\beta 1$ and $\beta 3$ integrins control GlyR synaptic trapping and residence time, which determine the number of GlyRs at synapses in spinal cord neurons.

It is now well accepted that GlyRs may be associated with gephyrin along the secretion pathway²⁷ and that a large proportion of GlyRs diffuse into and out of synapses in association with gephyrin^{2, 28}. This implies that GlyRs can be trapped at synapses by GlyR/gephyrin or gephyrin-gephyrin interactions. Consequently, modulating gephyrin oligomerization is expected to affect the stabilization of both GlyRs and gephyrin molecules at synapses. In line with this, it has been shown that overexpression of gephyrin variants with altered oligomerization properties affects

GlyR lateral diffusion and postsynaptic accumulation^{2, 29}. Alternatively, the modulation of GlyR/gephyrin interaction may affect GlyR synaptic trapping. It has been shown that gephyrin interaction with its partner Pin1 regulates GlyR/gephyrin binding³⁰. Modifications in the gephyrin-binding sequence of the GlyR β subunit have also been proposed to affect GlyR synaptic localization during activity-dependent homeostatic regulation of glycinergic synapses²¹. So far, the modulation of GlyR/gephyrin interaction has not been associated with changes in gephyrin postsynaptic accumulation^{21, 30}. Our immunocytochemistry and FRAP experiments showed that β 1 and β 3 integrin inhibition modified both the amount of synaptic gephyrin and the exchange between synaptic and non-synaptic gephyrin molecules. This indicates that β 1 and β 3 integrins modulate gephyrin oligomerization properties. The regulation of gephyrin oligomerization alone can account for the effects on GlyR and gephyrin dynamics because GlyRs and gephyrin may be co-trafficked and because gephyrin provides the binding sites for GlyRs at synapses. We conclude that gephyrin exchange at synapses is tightly linked to GlyR stabilization. This provides important insight into the dynamics of synaptic multimolecular assemblies in the control of synaptic strength.

Strikingly, β 1 and β 3 integrins affected inhibitory synaptic strength in opposite directions. β 1 and β 3 integrins have been shown to regulate other cell functions differentially. For example, L-type calcium channels are up-regulated by β 1 integrins and down-regulated by β 3 integrins in arteriolar smooth muscles for vasomodulation³¹. In macrophages, β 1-mediated phagocytosis and migration (but not adhesion) are inhibited by β 3 integrins³². This suggests that the functional counteraction between β 1 and β 3 integrins may be a general mechanism to adapt cellular responses to the extracellular environment. We performed experiments aimed at identifying the molecular basis of the opposing actions of β 1 and β 3 integrins at glycinergic synapses. We found that PKC was necessary for integrin effects in and outside synapses, consistent with the requirement of PKC for integrin activation⁷. We also found that CaMKII was responsible for β 1 and β 3 integrin actions specifically at synapses. CaMKII inhibition abolished the effects of integrin blockade at synapses but not in the extrasynaptic membrane. Furthermore, CaMKII inhibition increased GlyR mobility at synapses and strongly decreased GlyR synaptic localization. These effects were similar to those observed after β 1 integrin inhibition

and after $\beta 3$ integrin activation with fibrinogen. Consistent with this, $\beta 1$ and $\beta 3$ integrins have been shown to activate and inhibit CaMKII, respectively³². Together, these data indicate that $\beta 1$ and $\beta 3$ integrins crosstalk via CaMKII to adjust GlyR trapping at synapses depending on the neuronal environment (**Supplementary Fig. 9**).

Our results reveal that CaMKII stabilizes GlyRs at inhibitory synapses. By contrast, CaMKII has been shown to destabilize PSD-95 at excitatory synapses³³ and to inhibit high order complex formation between Homer and Shank *in vitro*³⁴. This suggests that CaMKII regulates the dynamics of excitatory and inhibitory postsynaptic membranes in opposite directions to control neuronal activity. An important issue will be to identify the molecular targets of CaMKII for the regulation of inhibitory postsynaptic assembly dynamics. One possibility is that CaMKII directly targets gephyrin. Indeed, using *in vitro* phosphorylation tests and mass spectrometry, we found that CaMKII can directly phosphorylate gephyrin on at least six serine residues (**Supplementary Fig. 10**). Three of these sites have already been found to be phosphorylated *in vivo* (S296³⁵, S318³⁵, S337³⁶), suggesting that they are of physiological relevance. The sites we identified here are mainly located at the junction between the linker region and the C-terminal domain of gephyrin, which dimerizes and is essential for the formation of higher order oligomers (see^{29, 37}, **Supplementary Fig. 10**). Further studies are required to determine whether $\beta 1$ and $\beta 3$ integrins modulate the phosphorylation state of these sites in living spinal cord neurons and how the multiple phosphorylation of gephyrin modulates its oligomerization properties.

Our results showed that $\beta 1$ and $\beta 3$ integrins modulate inhibitory synapses in response to TSP1 or fibrinogen, two molecules released after injury²⁴⁻²⁶. TSP1 activates $\beta 1$ integrins²³ and mediates neurite outgrowth and synaptogenesis for functional recovery³⁸. By contrast, fibrinogen, a blood protein massively deposited in the spinal cord after injury, inhibits neurite outgrowth via $\beta 3$ integrins²⁶. Therefore, in addition to their role in maintaining inhibitory synaptic strength under basal conditions, $\beta 1$ and $\beta 3$ integrins could modulate glycinergic inhibition in pathological and regenerative situations. These regulations may be crucial *in vivo* for the modulation of pain pathways^{39, 40} as well as spinal locomotor networks, which mainly rely on the proper equilibrium between glycinergic inhibition and glutamatergic

excitation⁴¹. Interestingly, $\beta 3$ integrins have been shown to regulate the numbers of AMPA receptors at excitatory synapses and are required for synaptic scaling in response to TNF- α ¹¹. This glia-dependent form of plasticity helps adapt the activity of neuronal networks following chronic activity blockade or sensory deprivation^{42, 43}. Altogether, these data and our own suggest that integrins orchestrate inhibitory and excitatory neurotransmission to adjust neuronal excitability in normal and pathological conditions.

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Author contributions

C.C. performed and analyzed all the experiments except *in vitro* phosphorylation assays and mass spectrometry and wrote the manuscript with the help of the authors. P.M. performed *in vitro* phosphorylation assays. R.Y.T-C. performed mass spectrometry and analyzed data. I.M.M. supervised phosphorylation analyses with mass spectrometry. A.T. supervised the project.

Figure Legends

Figure 1 RGD-peptides increase GlyR numbers at synapses.

(a) Double immunodetection of $\alpha 1$ -GlyR and synapsin I in spinal cord neurons (12 days *in vitro*) in control or after treatment with echistatin (1 hr, 100 nM). Scale bar: 20 μm . (b) Cumulative probability plot of synaptic GlyR cluster-associated fluorescence in control conditions (black) and after 1-hr echistatin treatment (gray) ($n_{\text{Ctr}} = 2740$; $n_{\text{Echis}} = 2549$; from 21 cells by condition; $p < 0.001$, *MW-test*). (c) Increased GlyR-associated fluorescence intensity at synapses after treatment with echistatin or GRGDSP peptide (RGD, gray) compared to control or GRGESP treatment (RGE, black). Mean \pm SEM, *** $p < 0.001$, *t-test*; a.u.: arbitrary unit.

Figure 2 $\beta 1$ and $\beta 3$ integrin blocking antibodies have opposite effects on GlyR numbers at synapses.

(a) Immunodetection of $\alpha 1$ -GlyR on non-permeabilized cells in control conditions (Ctr) or after 1-hr treatment with $\beta 3$ (a $\beta 3$) or $\beta 1$ (a $\beta 1$) function-blocking antibodies. Scale bar: 20 μm . (b) Normalized fluorescence intensity associated with GlyR clusters (mean \pm SEM, *** $p < 0.001$, *t-test*). (c) Examples of glycinergic mIPSCs and cumulative probability plot of their amplitudes from 2-min recording periods from the same cells before and 20 min after a $\beta 3$ or a $\beta 1$ treatment. Scale bars: 100 pA, 1s. (d) Average mIPSC amplitude before and after a $\beta 1$ or a $\beta 3$ treatment or after a 20-min time lag (Ctr) for each cell. (Ctr: NS; a $\beta 1$ and a $\beta 3$: $p < 0.01$, *MW-test*). (e) Relative effects on mIPSC amplitudes. Mean amplitudes after treatment are plotted relative to the mean amplitudes before treatment (*** $p < 0.001$, ANOVA). (f) Mean effects on mIPSC frequency (NS, ANOVA). Error bars indicate SEM.

Figure 3 $\beta 1$ and $\beta 3$ integrins control GlyR lateral diffusion.

(a) Maximum intensity projections of 512 frames recorded at 13 Hz during 38.4 s. The GlyR-QD traces and FM4-64-stained synapses are green and red, respectively; yellow denotes their overlap. The GlyR-QD explored area increased after a $\beta 3$ or a $\beta 1$ treatment in the extrasynaptic membrane (arrowheads). At synapses, a $\beta 3$ and a $\beta 1$ reduced and increased GlyR-QD mobility (arrows), respectively. Scale bar: 5 μm . (b) Cumulative probability plot of GlyR-QD diffusion coefficients in the extrasynaptic membrane (ES, $p < 0.001$, *MW-test*). (c) Cumulative probability plot of GlyR-QD diffusion coefficients at synapses (S, a $\beta 3$: $p < 0.01$, a $\beta 1$: $p < 0.001$, *MW-test*). (d)

Normalized effects of $\alpha\beta 1$ and $\alpha\beta 3$ on GlyR-QD diffusion coefficients (mean \pm SEM; *** $p < 0.001$, ANOVA). Control values indicate the relative fluctuation between two control distributions of diffusion coefficients (ES: $n_{\text{Ctra}} = 300$, $n_{\text{Ctrb}} = 524$; S: $n_{\text{Ctra}} = 300$, $n_{\text{Ctrb}} = 524$). Note that $\beta 1$ and $\beta 3$ integrin inhibitions have comparable effects in the extrasynaptic membrane but opposite effects at synapses.

Figure 4 $\beta 1$ and $\beta 3$ integrins modulate GlyR confinement and dwell time at synapses.

(a) Examples of GlyR-QD trajectories (black) over FM4-64 stained synapses (red) from control (Ctr), $\alpha\beta 3$ - and $\alpha\beta 1$ -treated neurons. Trajectories analyzed in **b** are marked with *. Scale bar: 1 μm . (b-c) Increased and decreased confinement of GlyR-QD after $\alpha\beta 3$ (green) and $\alpha\beta 1$ (red), respectively. MSD versus time plot (b) for GlyR-QD trajectories indicated in **a**. Normalized size of the confinement domain at synapses (c). Note that the smaller the confinement domain, the greater the confinement (mean \pm SEM, ** $p < 0.01$, *** $p < 0.001$, MW-test). (d-e) Dwell time of GlyR-QDs at synapses. Cumulative probability plot (d) and mean dwell time index (e). Error bars indicate SEM; * $p < 0.05$; *** $p < 0.001$, MW-test.

Figure 5 $\beta 1$ and $\beta 3$ integrins control gephyrin amount and exchanges at synapses.

(a) Immunodetection of gephyrin in control conditions (Ctr) or after $\alpha\beta 3$ or $\alpha\beta 1$ treatment. Scale bar: 20 μm . (b) Normalized fluorescence intensity associated with endogenous gephyrin clusters (mean \pm SEM; *** $p < 0.001$, t-test). (c) Representative example of transfected neurons expressing a Venus-tagged gephyrin (VeGe, green). VeGe forms clusters in front of active presynaptic boutons stained with FM4-64 (red). The white square outlines the region shown in **d**. Scale bar: 1 μm . (d) Photobleaching of an individual synaptic VeGe cluster (arrow) and time-lapse recording of the fluorescence recovery during 20 min. The color look-up indicates the level of fluorescence. (e) Fluorescence recovery curves (mean \pm SEM). (f) Mean fluorescence recovery 10 min after the photobleaching (error bars: SEM, ** $p < 0.01$; *** $p < 0.001$, MW-test). F_0 : initial fluorescence.

Figure 6 Actin, PKC and CaMKII mediate integrin-dependent regulation of GlyR lateral dynamics.

(a, c, e) Cumulative distributions of GlyR-QD diffusion coefficients. (b, d, f) Histograms of normalized fluorescence intensity associated with GlyR clusters (mean \pm SEM). (a-b) Effects of $\alpha\beta 1$ (red) and $\alpha\beta 3$ (green) treatments after F-actin disruption with latrunculin (Lat, 3 μ M, black). Latrunculin abolishes extrasynaptic but not synaptic effects on GlyR diffusion and cluster-IR. (c-d) Effects of $\alpha\beta 1$ (red) and $\alpha\beta 3$ (green) treatments after PKC inhibition (GFX, 50 nM, black). GFX abolishes extrasynaptic and synaptic effects on GlyR diffusion and cluster-IR. (e-f) Effects of CaMKII inhibition (KN93, 10 μ M, black) compared with the control (Ctr, blue) and effects of $\alpha\beta 1$ (red) and $\alpha\beta 3$ (green) treatments after CaMKII inhibition. (e) In the extrasynaptic membrane, KN93 has no effect on GlyR mobility. At synapses, KN93 increases GlyR mobility and prevents $\alpha\beta 1$ and $\alpha\beta 3$ effects. (f) At synapses, CaMKII inhibition decreases GlyR cluster-IR (blue scale, left) and prevents $\alpha\beta 1$ and $\alpha\beta 3$ effects on GlyR cluster-IR (black scale, right). *** $p < 0.001$, ** $p < 0.01$; for complete statistics, see text.

Figure 7 Thrombospondin 1 and fibrinogen have opposite effects at inhibitory synapses.

(a) Typical behavior of GlyR-QDs after 1-hr treatment with thrombospondin 1 (TSP1, 2 μ g/ml). Maximum intensity projections of 512 frames recorded at 13 Hz. GlyR-QD explored area: green; FM4-64-stained synapses: red. GlyR-QDs have a reduced mobility in (arrows) and out (arrowheads) of synapses. Scale bar: 1 μ m. (b) Distributions of GlyR-QD diffusion coefficients at extrasynaptic and synaptic locations in control condition (Ctr, black) or after TSP1 treatment (red) ($p < 0.001$, *MW-test*). (c) Normalized fluorescence intensity associated with GlyR and gephyrin clusters at synapses (mean \pm SEM; *** $p < 0.001$, *t-test*). (d) Typical behavior of GlyR-QD after 1-hr treatment with fibrinogen (Fib, 1.5 mg/ml). Same legends as for panel a. GlyR-QDs are very mobile at synapses but not in the extrasynaptic membrane. (e) Distributions of GlyR-QD diffusion coefficients at extrasynaptic and synaptic locations after treatment with fibrinogen (green) or with its vehicle (veh, black) ($p < 0.001$, *MW-test*). (f) Same legend as for panel c (mean \pm SEM; *** $p < 0.001$, *t-test*). Note that TSP1 increases the stabilization and the number of GlyRs at synapses whereas fibrinogen has opposite effects.

Online methods

Primary neuronal culture

Primary cultures of spinal cord neurons were prepared from embryonic Sprague Dawley rats at day 14 as previously described⁴⁴ with few modifications. Cells were plated at a density of 5×10^4 cells/cm². They were maintained in neurobasal medium supplemented with B27 (1X) and L-glutamine (2 mM) at 37°C in 5% CO₂ for 11–14 days. The medium was changed every 4–5 days.

Constructs and transfection

Transfections were performed using the Lipofectamine2000 (Invitrogen) method. We used plasmids encoding gephyrin as a fusion protein with Venus yellow fluorescent protein (Vege²²), EGFP, CTβ1, and CTβ3 (gift from Dr Yukiko Goda, described in¹¹), and *c-myc-α1βgb*⁴⁵. Cells were transfected 9 or 10 days after plating and imaged 48 hrs (Vege) or 24 hrs (other constructs) later.

Cell treatment

Monoclonal function-blocking antibodies against integrins were hamster anti-rat β1 monoclonal antibody (αβ1, clone Ha2/5) and mouse anti-rat β3 monoclonal antibody (αβ3, clone F11). They were from BD Pharmingen and used at 25 μg/ml unless otherwise noted. Blocking antibodies stabilize the inactive conformation of integrins⁶. Echistatin (100 nM), latrunculin A (3 μM), cycloheximide (100 μM) and leupeptin (100 μg/ml) were from Sigma. GRGDSP and GRGESP were from Bachem and used at 200 μM. Purified human platelet TSP1 was from either Sigma or Haematologic Technologies. TSP1 was used at a concentration of 2 μg/ml. Purified human plasma fibrinogen (Calbiochem) was used at 1.5 mg/ml in 1.2 mM sodium citrate buffer. GF109203X/Bisindolylmaleimide I (Calbiochem) was used at 50 nM. KN-93 (10 μM), dynasore (80 μM), MG 132 (50 μM) and PP2 (10 μM) were from Tocris. Unless otherwise noted, cells were treated with αβ1, αβ3, echistatin, TSP1 or fibrinogen for 1 hr before fixation or imaging. Except cycloheximide (2 hrs), inhibitors were added 25

min before integrin blocking antibodies. In live cell imaging experiments, reagents were present in the recording medium.

Antibodies for immunodetection

α 1-GlyRs were immunodetected using either a mouse anti- α 1-GlyR (mab2b, 0.7 to 1.25 μ g/ml, Synaptic System) or a homemade rabbit anti- α 1-GlyR antibody raised against the same extracellular epitope (1:800 to 1:1500). No difference in GlyR lateral dynamics was observed with the mouse and rabbit primary antibodies²¹. Other primary antibodies were mouse anti-gephyrin (mAb7a, 1.25 μ g/ml, Synaptic System), rabbit anti-gephyrin (homemade, 1:1000), 9E10 (Millipore) and rabbit anti-synapsin I (1.7 μ g/ml, Synaptic System). Secondary antibodies used in immunocytochemistry were Cy3-conjugated goat anti-mouse (1.25 μ g/ml), Cy3-conjugated goat anti-rabbit (2.5 μ g/ml), Alexa 488-conjugated goat anti-rabbit (2.5 μ g/ml). Secondary antibodies used in SPT were biotinylated Fab fragments (goat anti-mouse: 1 μ g/ml, goat anti-rabbit: 2 μ g/ml). All secondary antibodies were from Jackson ImmunoResearch, except for the Alexa 488-conjugated antibody, which was from Invitrogen.

Immunocytochemistry, image acquisition and quantitative analysis

Immunocytochemistry, image acquisition and fluorescence quantification were performed as previously described²⁰. Images shown in Supplementary Fig. 2 were obtained using a Leica DM5000B microscope equipped with Yokogawa CSU 10 spinning disk. Sets of neurons compared with quantification were fixed, labeled and imaged in parallel. Analyses were performed using MetaMorph (MetaImaging). GlyR and gephyrin synaptic amounts were quantified in double-labeling experiments by measuring the fluorescence intensity associated with GlyR or gephyrin clusters apposed to presynaptic terminals labeled with synapsin I. In our dissociated spinal cord neurons, 90% of GlyR and gephyrin clusters are apposed to presynaptic terminals⁴⁶. Therefore, all GlyR and gephyrin clusters were analyzed when cells were treated with $\alpha\beta$ 1 and $\alpha\beta$ 3. $\alpha\beta$ 1 and $\alpha\beta$ 3 effects on GlyR cluster-IR were examined on non-permeabilized neurons. A procedure based on wavelet decomposition was used to quantify synapse density and synapse size (see²²).

Single particle imaging

Cells were labeled and imaged in MEM recording medium consisting of MEM (Invitrogen) supplemented with 10 mM HEPES, 33 mM glucose, 2 mM glutamine, 1 mM sodium pyruvate and B27 at 37°C. Cells were incubated for 5 min with primary antibodies, washed, incubated for 5 min with biotinylated secondary Fab and washed. Cells were then incubated for 1 min with streptavidin-coated quantum dots emitting at 605 nm (1 nM, Invitrogen) in borate buffer (50 mM). After extensive washing, active presynaptic terminals were stained for 40 s with FM4-64 (3 μM, Invitrogen) in the presence of 40 mM KCl. Cells were imaged in an open chamber mounted on a inverted microscope (IX71, 60X objective, NA=1.45, Olympus). QDs and FM4-64 were detected using a Hg+ lamp and appropriate filters (QD: D455/70x, HQ605/20m; FM4-64: D535/50x, E590lpv2; Chroma Technology). QDs were recorded during 512 consecutive frames at 13 Hz with a CCD camera (Cascade 512BFT; Roper Scientific) and MetaView (Metalmaging). Cells were imaged within 30 min after the labeling.

Single particle tracking and quantitative analysis of lateral diffusion

Analyses were restricted to single QDs identified by the intermittency of their fluorescence. Synapses were identified from FM4-64 images using a procedure based on wavelet decomposition (see²²).

- Single particle tracking

SPT was performed using homemade software written with MATLAB (The MathWorks)²⁸. QD localization was determined with a spatial accuracy of about 10 nm by cross correlating the image with a Gaussian model of the point spread function and GlyR-QD trajectories were reconstructed as previously detailed²⁸.

- Diffusion coefficients

Diffusion coefficients were calculated from the longest fragment of the trajectory spent in the synaptic and extrasynaptic compartment, determined by comparison of the trajectories with the FM image. Only fragments longer than 30 consecutive frames were taken into account. The mean square displacement (MSD) was calculated using $MSD(ndt) = (N - n)^{-1} \sum_{i=1}^{N-n} ((x_{i+n} - x_i)^2 + (y_{i+n} - y_i)^2).dt$, where x_i, y_i are the coordinates of an object on frame i , N is the total number of steps in the trajectory, dt is the time interval between two successive frames and ndt is the time interval over which displacement is averaged⁴⁷. Diffusion coefficients (D) were

calculated with a fit between data points 2 and 5 of the MSD curves versus time (t) as previously described²⁸. Distributions rather than mean values were compared because of the large dispersal of the values (over four orders of magnitude). Diffusion parameters could vary from one culture another, imposing comparisons with internal controls. Diffusion coefficients at synapses were compared in the 25%–75% inter-quartile range. To evaluate the effects of $\alpha\beta 1$ and $\alpha\beta 3$ treatments, the distributions were divided into 10 classes with an equal number of values, and the mean diffusion coefficient was calculated for each class. Ratios of the means in $\alpha\beta 1$ and $\alpha\beta 3$ conditions over the means in control were used as an indicator of $\alpha\beta 1$ and $\alpha\beta 3$ effects.

- Confinement

Confined trajectories were determined according to the relative deviation of the experimental MSD with the one expected in the case of Brownian diffusion (RD, adapted from⁴⁸). The size of the confinement domain for trajectories showing restricted motion was calculated as previously described²⁸.

- Dwell time at synapses

The time spent by single GlyR-QDs in the synaptic and extrasynaptic compartments and the number of transitions between the two compartments were determined as previously described²⁰. A dwell time index was defined as follows: for GlyR-QDs transiting between the synaptic and extrasynaptic compartments, dwell time index = time spent at synapses / (total time detection \times n exits from synapses); for GlyR-QDs always detected at synapses, dwell time index = 1. Only GlyR-QDs with a dwell time index > 0.03 were taken into account.

Fluorescence recovery after photobleaching

Experiments were performed at 37°C in MEM recording medium. Active synapses were stained with FM 4-64 as described above. Cells were observed on an inverted microscope (Eclipse TE2000-E, 100X objective, NA=1.4, Nikon) equipped with a DG-4 illumination system (Sutter Instruments) and appropriate filter sets (Semrock, Optoprim), a CCD camera (QuantEM 512SC, Roper Scientific) and a FRAP-3D™ system (Roper Scientific). The procedure was controlled with MetaMorph. Fluorescence was photobleached using a 488 nm laser at 65 mW for 80 ms in three to four regions of interest (diameter: 1–1.2 μm) centered on individual VeGe synaptic

clusters. Photobleaching in the 3–4 regions of interest was quasi-simultaneous. Fluorescence was monitored in time-lapse. Three images were acquired before the photobleaching within a period of 1 min. The recovery after photobleaching was monitored as follows: 1 image every 10 s for 5 min, 1 image every 20 s for 5 min and 1 image every 60 s for 10 min. At each time point, three images were acquired in z-stack with a step size of 0.4 μm . Data were analyzed from maximum intensity projection using MetaMorph. Fluorescence recovery was measured after background subtraction and correction for the ongoing photobleaching as follows: $F_{\text{corr}_t} = (F_t/F_0)/(F_{\text{nb}_t}/F_{\text{nb}_0})$, where F_t is the fluorescence at time t , F_0 is the mean fluorescence before bleaching, F_{nb_t} is the average fluorescence intensity of three non-bleached spots at time t , and F_{nb_0} is the average fluorescence intensity of the same non-bleached spots before bleaching.

Electrophysiology

Whole-cell patch-clamp recordings were performed using a multiclamp 700B controlled by Clampex 10 (Axon Instruments). Patch-pipettes had a tip resistance of 3.5–5 $\text{M}\Omega$ when filled with an intracellular solution containing 68 mM K₂Glu, 68 mM KCl, 0.2 mM EGTA, 2 mM MgSO₄, 20 mM HEPES buffer, 3 mM Na₂ATP, and 0.2 mM Na₃GTP. The external solution contained 136 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 1.3 mM MgCl₂, 10 mM HEPES, and 10 mM D-glucose. It was supplemented with 1 μM TTX, 50 μM D-AP5, 2 μM NBQX and 5 μM gabazine (SR95531) to isolate glycinergic mIPSC. All the supplements were from Tocris. About 10 min after mIPSC isolation, $\alpha\beta 1$ or $\alpha\beta 3$ (20 $\mu\text{g}/\text{ml}$) was added to the external solution for 5 min (flow \approx 1 ml/min) to allow the immunoreaction. mIPSCs were recorded at a holding potential of -60 mV at 30°C. Recordings were filtered at 4 kHz and sampled at 20 kHz using a Digidata 1440A (Axon Instruments). Throughout the experiment, the access resistance was tested periodically and cells were discarded if the access resistance changed by more than 15% or was more than 20 $\text{M}\Omega$. mIPSCs were detected using the template procedure of the Clampfit 10 program (Axon Instruments) and analyzed over 2-min periods. mIPSCs recorded 15 min after treatment with antibodies were compared with mIPSCs before application. In control cells, mIPSCs from 2-min periods separated by 20 min were compared. All cells with mean mIPSC frequency higher than 1 Hz were taken into account.

Cell surface biotinylation, quantification of GlyR and gephyrin levels

After 1 hr treatment with $\alpha\beta 1$ or $\alpha\beta 3$, cells were washed 3 times with ice-cold Dulbecco's PBS (DPBS) supplemented with 0.8 mM CaCl_2 and 0.5 mM MgCl_2 , pH 7.4 (DPBS⁺⁺). Then, they were incubated twice with biotinylation reagent (1 mg/ml NHS-SS-biotin, Pierce) for 12 min with gentle shaking on ice. Cells were then washed and unbound biotin was quenched with 50 mM glycine in DPBS⁺⁺/BSA for 15 min. Cell extracts were collected by scraping the cells in a Tris buffer (TB) consisting of 50 mM Tris-HCl (pH=8), 100 mM NaCl, 1 mM PMSF (Sigma) and a protease inhibitor cocktail (Roche). Neurons were lysed with 3% Triton X100 in TB supplemented with 2 mM EDTA for 1 hr on ice. Cleared Triton extracts were collected after centrifugation for 15 min at 10,000 g. The blocking antibodies were removed by incubation with protein G immobilized on sepharose (Sigma) in TB supplemented with 1% Triton X100 and 2 mM EDTA (Binding buffer, BB) for 1 hr at 4°C. The blocking antibodies were not detected with western blot following this procedure. 20% of the sample was collected to constitute the *total* receptor fraction. The remaining 80% was mixed with neutravidin beads (Pierce) for 2 hrs at 4°C to purify the biotinylated proteins (*surface* proteins). Proteins were separated and analyzed by SDS-PAGE followed by immunoblotting with mAb4a antibody (Synaptic System, 1:600), rabbit anti-GluR2 antibody (Millipore, 1:200); rabbit anti-GAPDH antibody (Abcam, 1:2,000) and rabbit anti-gephyrin antibody (Santa Cruz, 1:1,500). Proteins were visualized with enhanced chemiluminescence (ECL+, Amersham). The level of *surface* receptor was normalized to the corresponding level of *total* receptor detected. The level of *total* receptor or gephyrin was normalized to the corresponding level of GAPDH.

In vitro phosphorylation assay

The Ge(4') gephyrin isoform was used to address gephyrin phosphorylation by CaMKII *in vitro*. This gephyrin isoform was cloned from the rat spinal cord⁴⁹. It is specifically expressed in the central nervous system⁵⁰ and it binds to the GlyR. The C4' cassette of gephyrin does not interfere with GlyR/gephyrin binding *in vitro*⁴⁹ but it contains a consensus site for phosphorylation by CaMKII. Recombinant Ge(4') has been described previously (Ge(2,4') in reference²⁹). Gephyrin was expressed in transformed *E. coli* and purified under native conditions by nickel-nitrilotriacetic acid

(Ni-NTA) affinity chromatography as described²⁹. Recombinant CaMKII α (Cell Signaling Technology) and GST-CaMKII β fusion protein (Sigma) were preincubated for 10 min at 30°C in kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 2 mM DTT, 0.1 mM Na₂EDTA, 200 μ M ATP and 10 mM NaF, 2 mM Na₃VO₄, 5 mM Na₄P₂O₇.H₂O) supplemented with 2 mM CaCl₂ and 1.2 μ M calmodulin. In control experiments, CaMKII was preincubated in the absence of CaCl₂ and calmodulin (inactive CaMKII). Recombinant gephyrin Ge(4') was incubated in kinase buffer with activated or inactive CaMKII for 30 min at 30°C. The reaction was stopped by heating at 65°C for 20 min. CaMKII activity was verified using the highly selective CaMKII substrate autocalmitide-2 (Sigma).

Mass Spectrometry

- On-bead digestion of gephyrin

Gephyrin (2 μ g) bound to Ni-NTA agarose beads were reduced with 2 mM dithiothreitol for 60 min and alkylated with 4.2 mM iodoacetamide for 1 hr at 21°C. 100 μ l of 100 mM ammonium bicarbonate pH 7.8 and 13 μ l MeOH was added and pH adjusted to 7.8. Samples were heated for 15 min at 60°C prior to the addition of trypsin (1:150 enzyme/substrate). Samples were digested for 3 hr at 37°C and the reaction stopped by the addition of TFA then lyophilised to remove MeOH. Autocalmitide-2 samples were treated in the same manner.

- Enrichment of gephyrin phosphopeptides

Titanium oxide and immobilised metal affinity chromatography pipettes were used for enriching phosphopeptides from the digests. MonoTip TiO pipettes were prepared as per manufacturer's instructions (GL Sciences). Phosphopeptides were bound and washed as previously described³⁶ and then eluted twice with 150 μ l 0.3 M ammonium hydroxide solution. Unbound peptides were lyophilised and resuspended in 100 μ l of binding solution (10% Acetonitrile, 0.1% acetic acid). ZipTip 10 μ l Metal Chelator pipette tips (Waters) were prepared and the residual phosphopeptides bound and washed as per manufacturer's instructions (Waters). All phosphopeptide-enriched samples were quickly lowered to pH=3 using TFA and then cleaned with ZipTip C18 tips (Waters) prior to MS analysis.

- MS analysis

Samples were analysed on a calibrated hybrid LTQ Orbitrap XL ETD mass spectrometer (Thermo Scientific). Peptides were resuspended in 3% ACN and 0.2% formic acid and loaded on a 10 cm fused silica column packed with 3 μ m 200 Å pore size C18 resin. Peptides were eluted via an ACN gradient of 5–30% ACN over 35 min and 30–80% ACN over the subsequent 13 min in a buffer containing of 0.2% formic acid at flow rate of 200 nl/min. One scan cycle comprised of a full scan MS survey spectrum from m/z 300–2000 acquired in the FT-Orbitrap, followed by up to 6 sequential CID and ETD MS/MS scans with fragment ion detection in the linear ion trap. CID was performed with a target value of 1e4 in the linear trap, collision energy at 35%, Q value at 0.25 and activation time at 30 ms. AGC target values were 5e5 for full FTMS scans and 1e4 for ion trap MSn scans. The ETD anion target value was set at 1e6 and activation time at 100 ms. Supplementary activation was employed to enhance the fragmentation efficiency for 2+ precursors and charge state dependent ETD time enabled. A Data dependent decision tree was used to control ETD dissociation based on charge and m/z. The ETD reaction time was 120 ms and isolation width was 2 m/z. For all experiments, dynamic exclusion was used with 1 repeat count, 30 s repeat duration, and 10 s exclusion duration. Samples were acquired using internal lock mass calibration set on m/z 429.088735 and 445.120025.

- Database analysis

MS and MS/MS data were processed with Mascot distiller and searched using Mascot version 2.2. Monoisotopic masses of 2+ or greater charged peptides were searched with a peptide tolerance of 8 ppm and an MS/MS tolerance of 0.6 Da for fragment ions using a rat protein database downloaded from the European Bioinformatic Institute. Modifications used for searches included carbamidomethyl (C, variable) and phosphorylation (STY, variable). Only strictly tryptic peptides with a maximum of 4 missed cleavage sites were allowed. Positive identification of phosphorylated peptides was performed using a variety of strict criteria. Only bold-red, rank 1 peptides with Mascot expect values of less than 0.05 were considered and then manually evaluated for precise site assignment as previously described³⁶.

Statistics

Data were obtained from at least three independent experiments, except when a β 1 and a β 3 effects were tested after another treatment (2 independent experiments).

Statistical analyses were performed with StatView (Abacus Concept) or R. Differences in diffusion coefficients, confinement domain size, dwell time index, FRAP and mIPSCs were assessed using the Mann-Whitney test (*MW-test*). Data obtained with immunocytochemistry were compared using the unpaired two-tailed Student's t test (*t-test*). Multiple comparisons of mean effects on diffusion coefficients, mIPSC amplitudes and mIPSC frequency were performed using analysis of variance (*ANOVA*) followed by the least significant difference post-hoc test. Normality of the distributions was determined using the one-sample Kolmogorov-Smirnov test. A test was considered significant when $p < 0.05$. Data are expressed as mean \pm SEM.

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Supplementary Information Titles

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Journal: Nature Neuroscience

Article Title:	A crosstalk between $\beta 1$ and $\beta 3$ integrins controls glycine receptor and gephyrin trafficking at inhibitory synapses
Corresponding Author:	Antoine Triller

Supplementary Item & Number (add rows as necessary)	Title or Caption
Supplementary Figure 1	Effects of $\alpha\beta 1$ and $\alpha\beta 3$ treatments on synapse density and synapse size and comparison between the effects on GlyR cluster size and fluorescence.
Supplementary Figure 2	Effects of postsynaptic blockade of $\beta 1$ and $\beta 3$ integrins on GlyR clusters by overexpression of dominant-negative constructs.
Supplementary Figure 3	Effects of $\beta 1$ and $\beta 3$ integrin blocking antibodies on the coefficient of variation of mIPSC amplitudes and on mIPSC kinetics.
Supplementary Figure 4	$\alpha\beta 1$ and $\alpha\beta 3$ effects on GlyRs do not depend on endocytosis, exocytosis, protein synthesis or protein degradation.
Supplementary Figure 5	$\alpha\beta 1$ and $\alpha\beta 3$ effects on gephyrin do not depend on protein synthesis or protein degradation.
Supplementary Figure 6	Inhibition of Src kinases with PP2 does not prevent the effects of $\alpha\beta 1$ and $\alpha\beta 3$ on GlyR clusters.
Supplementary Figure 7	PKC inhibition with GF109203X does not affect GlyR lateral dynamics.
Supplementary Figure 8	TSP1 affects GlyR lateral dynamics via $\beta 1$ integrins whereas fibrinogen activates $\beta 3$ integrins.
Supplementary Figure 9	Model for the regulation of GlyR lateral dynamics by integrins.

Supplementary Figure 10

CaMKII directly phosphorylates gephyrin *in vitro*.

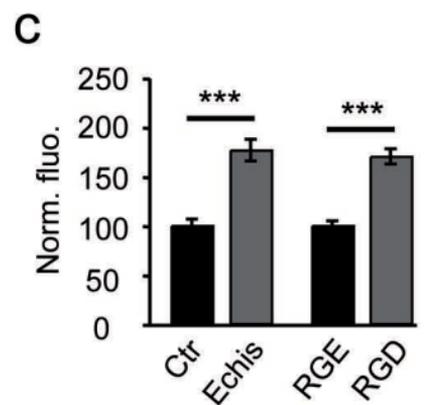
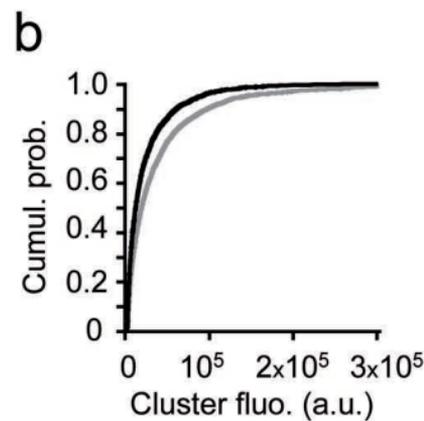
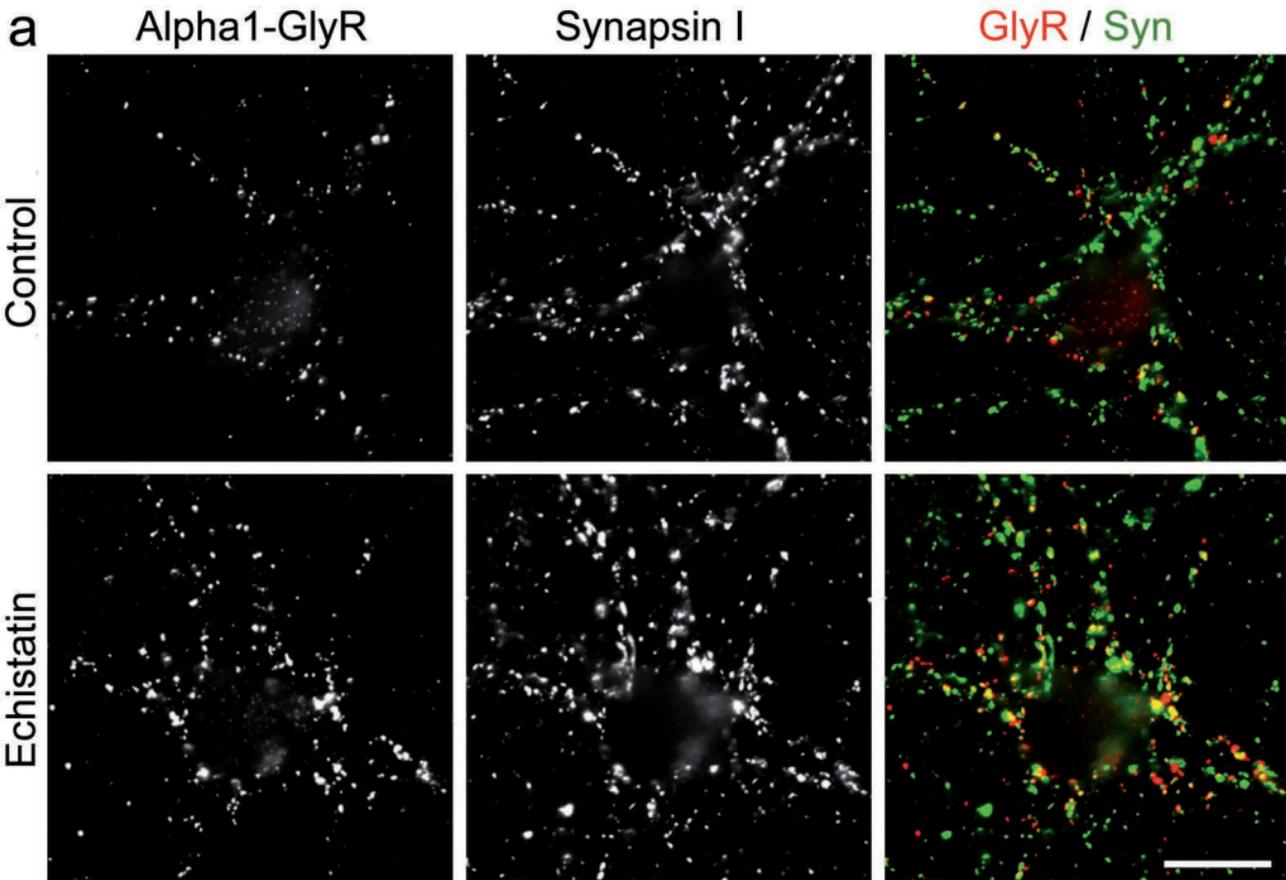


Figure-1 (Triller)

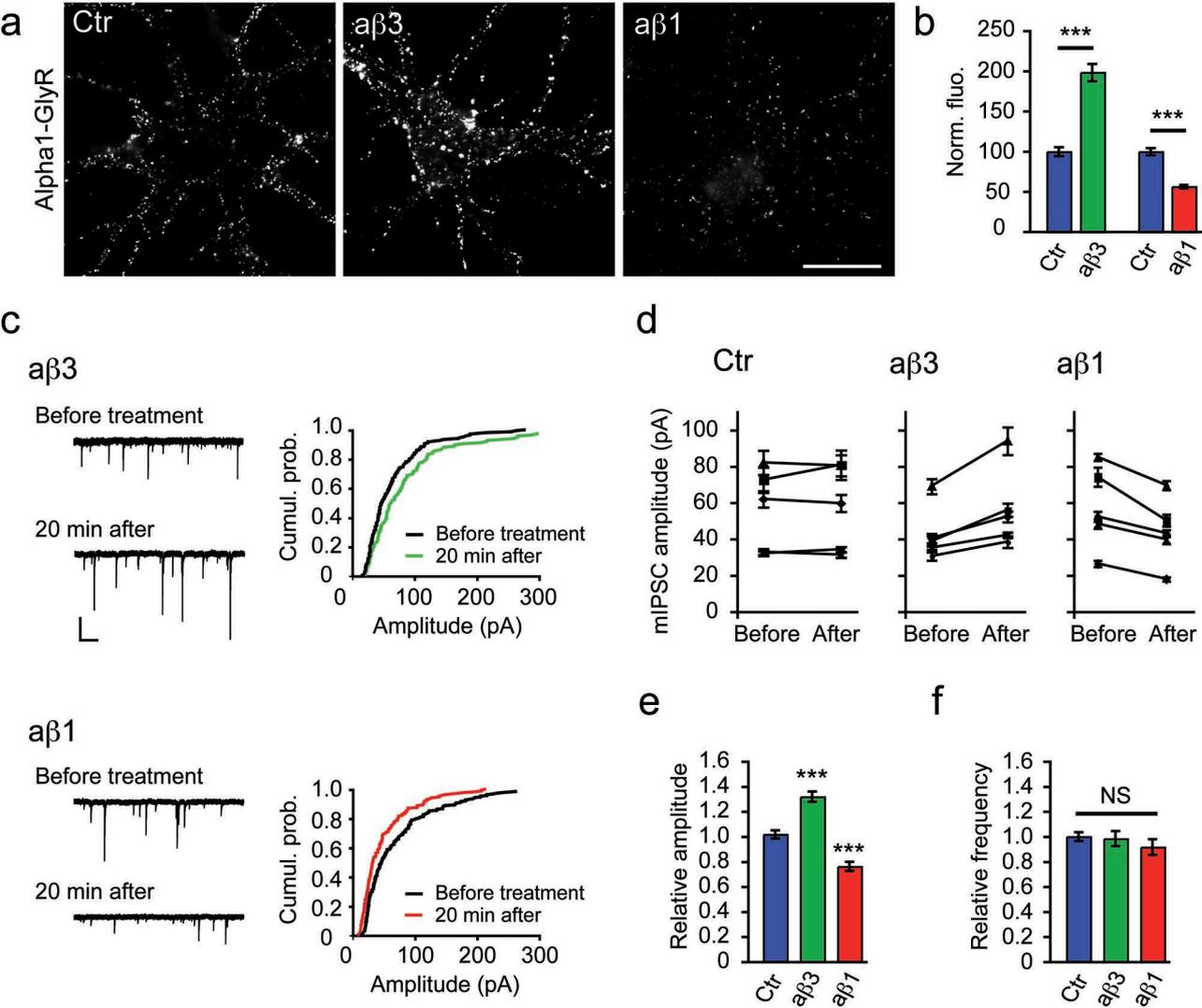


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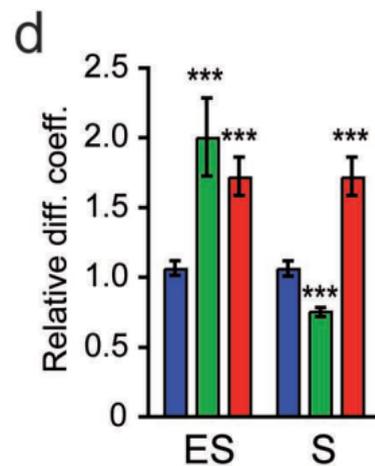
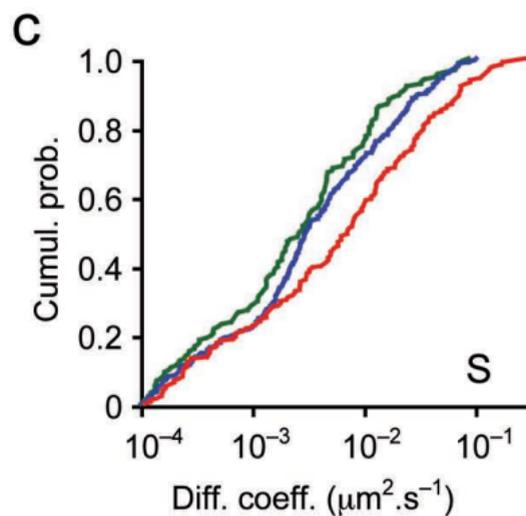
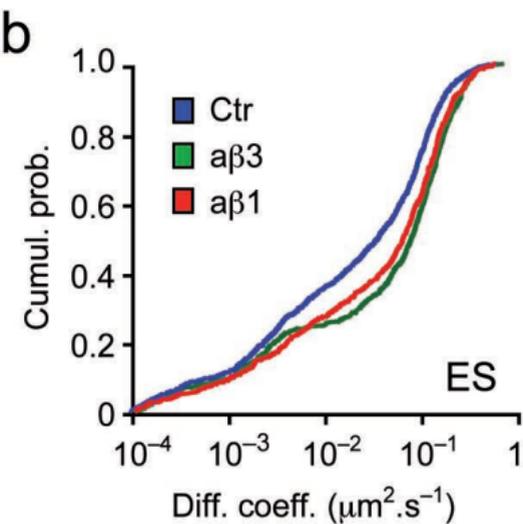
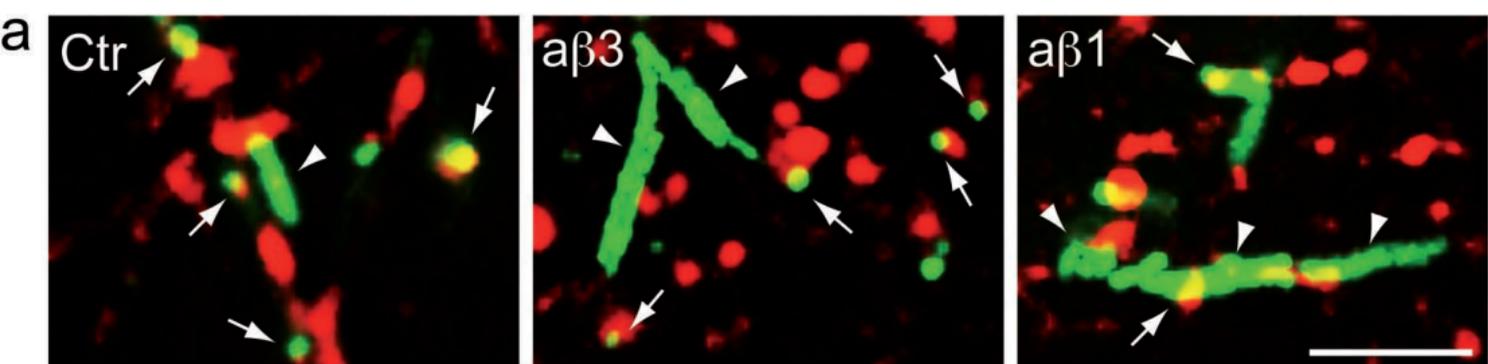


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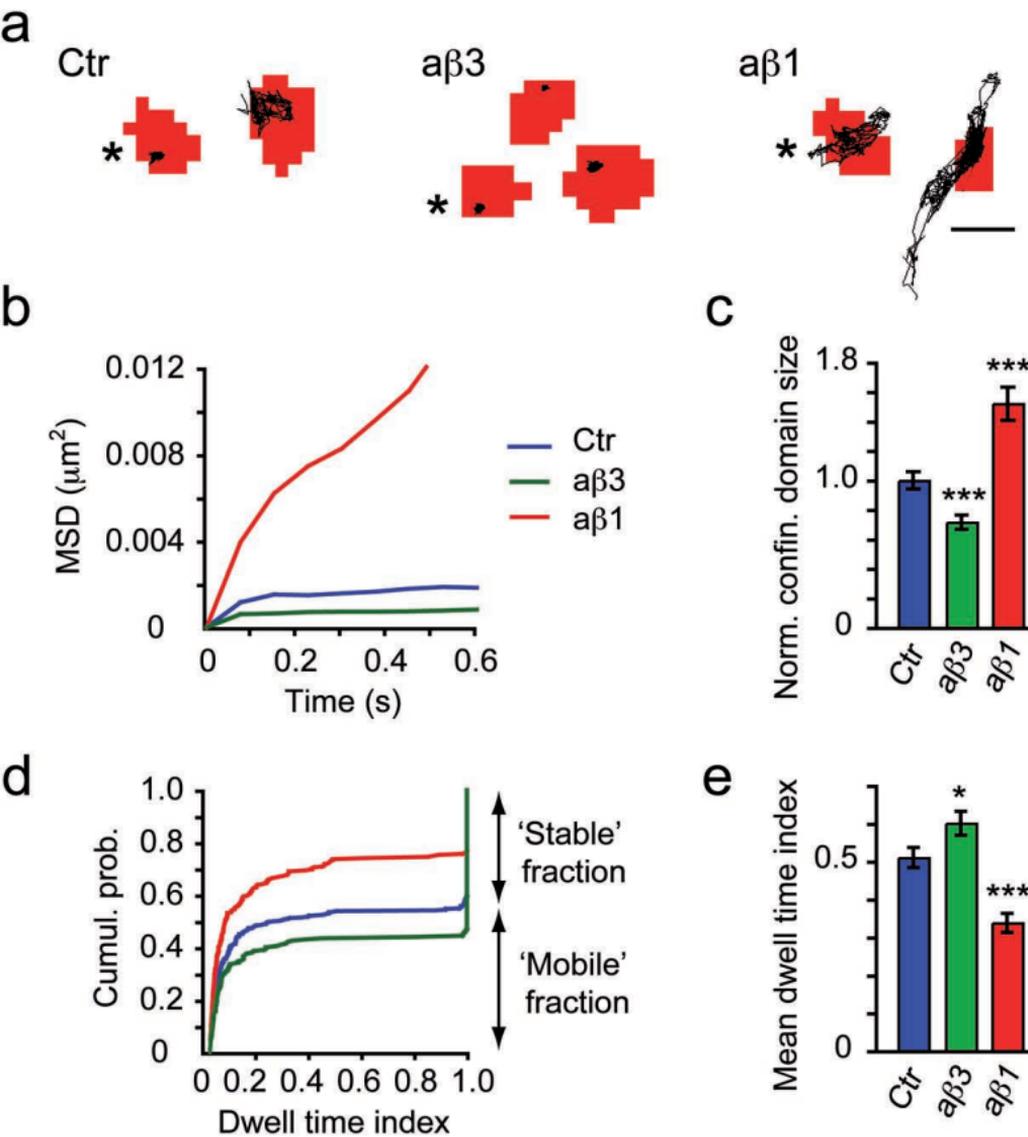


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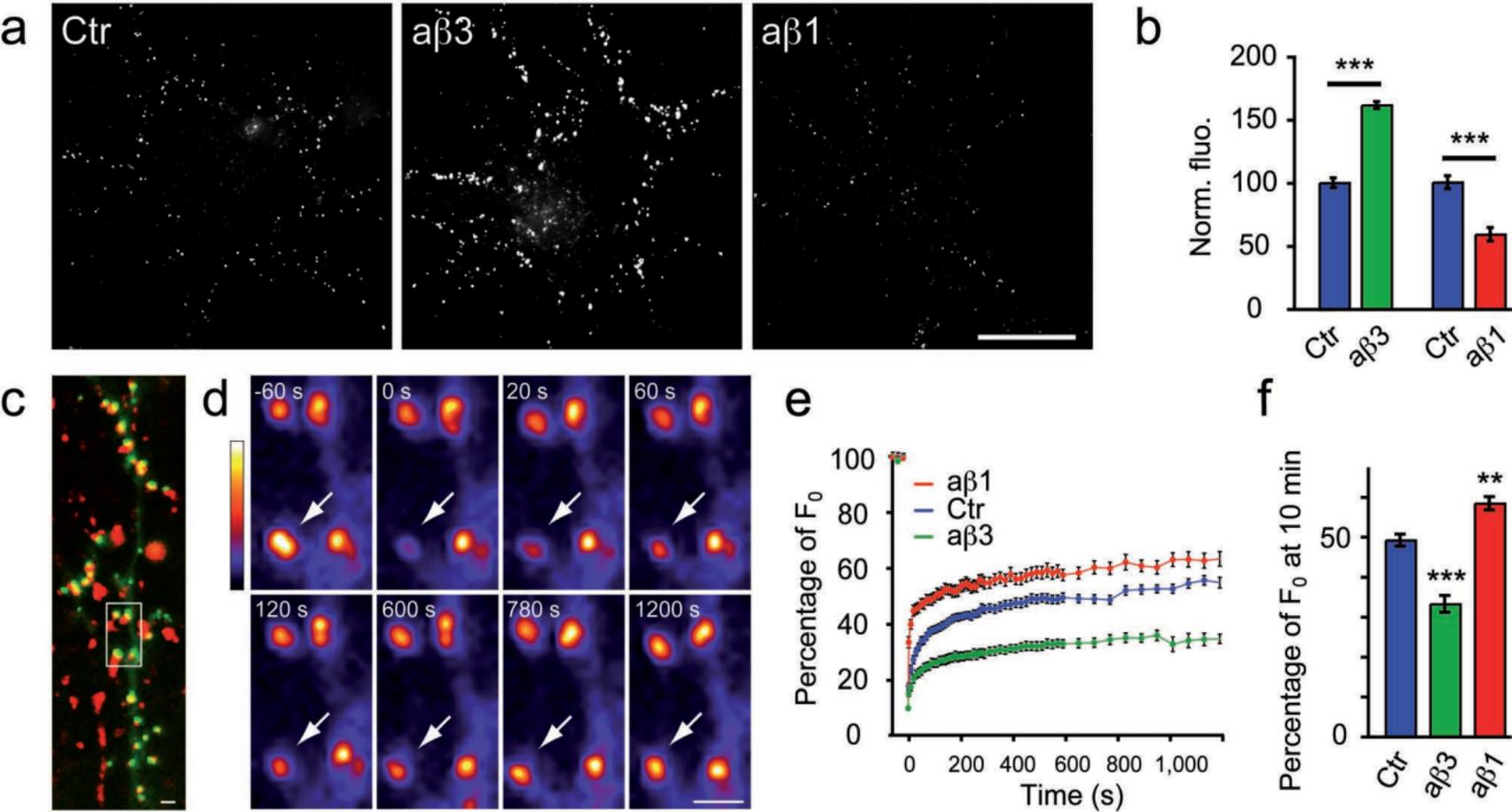


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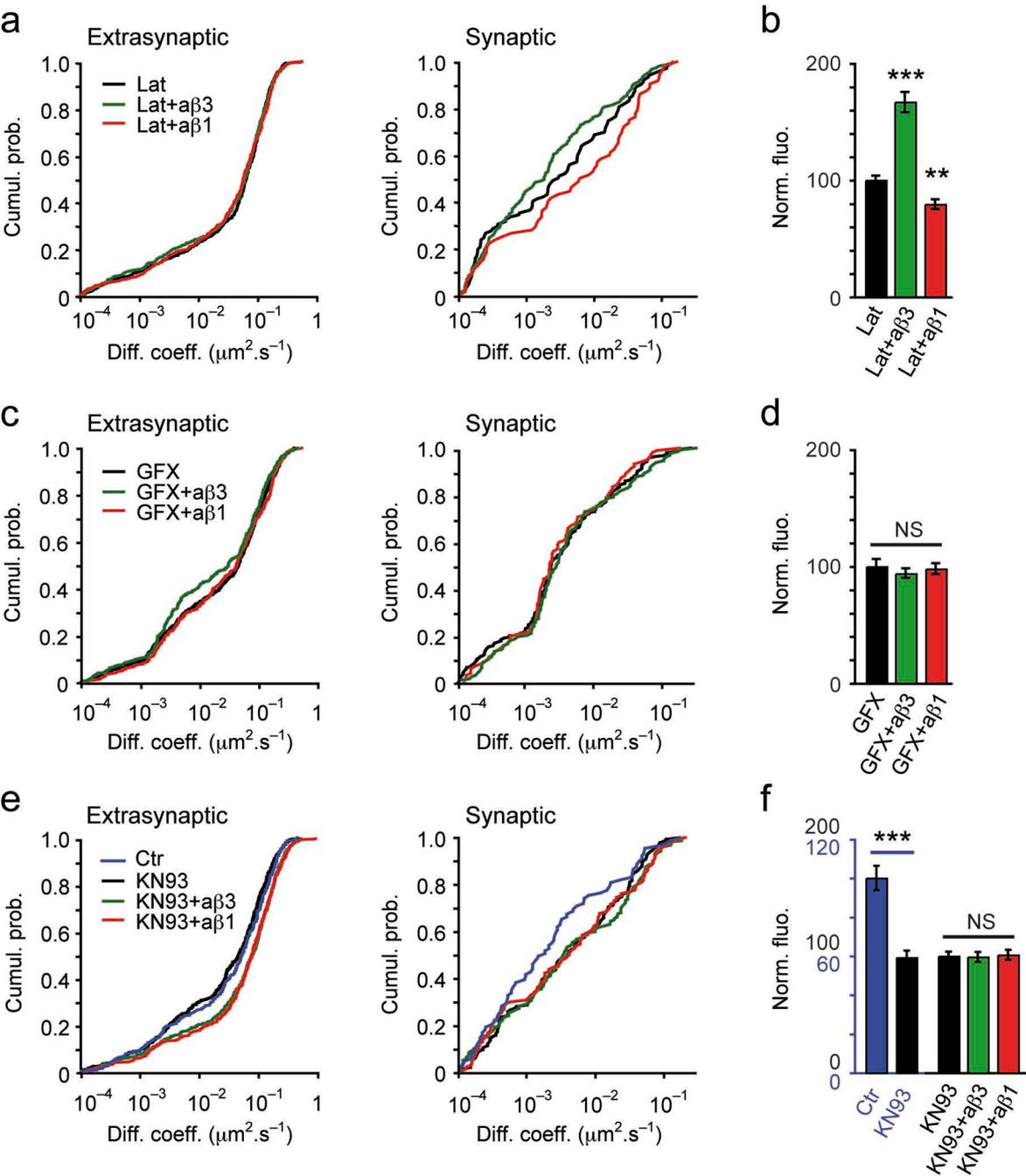


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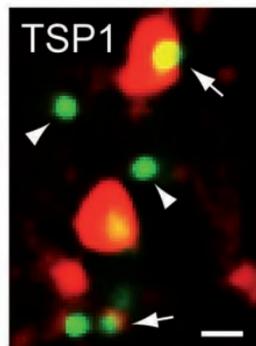
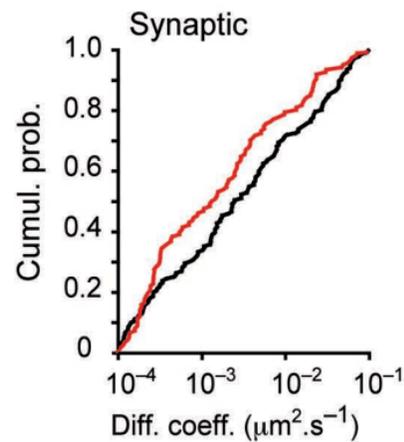
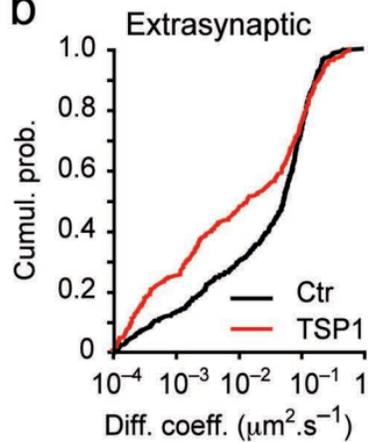
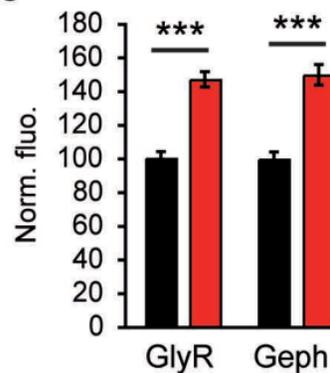
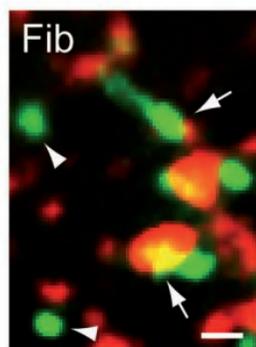
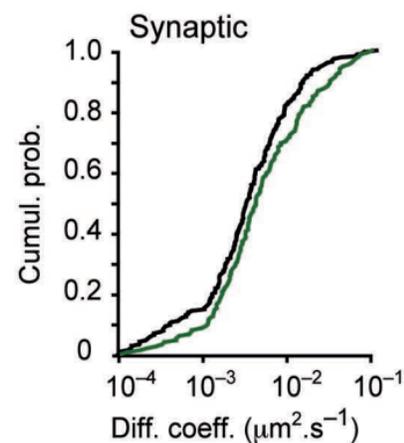
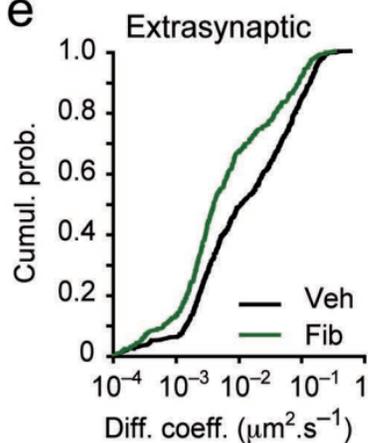
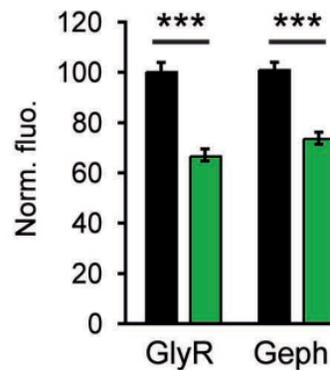
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Figure-7 (Triller)