Developmental-Dependent Action of Microtubule Depolymerization on the Function and Structure of Synaptic Glycine Receptor Clusters in Spinal Neurons

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INTRODUCTION

Postsynaptic densities (PSD) harbor neurotransmitter receptors that are anchored in the membrane at precise locations opposite to presynaptic active zones releasing neurotransmitters. Cytoskeletal components are believed to stabilize these molecular complexes. As such, both tubulin and actin are major components of the PSD (Cotman 1978; Matsus and Taff-Jones 1978), and several bridging proteins have been proposed to link membrane macromolecules to the underlying subsynaptic cytoskeleton within the PSD (reviewed in Sheng and Pak 2000).

Gephyrin, the first protein identified as a synaptic bridging component, is a key constituent of the PSD at inhibitory synapses (Triller et al. 1985, 1987). Gephyrin antisense and knock-out experiments indicated that gephyrin is associated with glycine receptors (GlyRs) and GABA A receptors (GABA A Rs) in postsynaptic clusters (Essrich et al. 1999; Feng et al. 1998; Kisch et al. 1993; Kneussel et al. 1999). While it is known that gephyrin strongly binds GlyRs through a peptide domain within the M3-M4 cytoplasmic loop of the GlyR β subunit (Meyer et al. 1995), the mechanism of interaction with GABA A Rs is not understood. In addition, in vitro binding studies suggested possible interactions of gephyrin with the cytoskeleton. Gephyrin binds tubulin and microtubules directly with high affinity and significant cooperativity (Kirsch et al. 1991) and can interact with microfilaments through intermediate actin-binding proteins such as profilin (Mamamoto et al. 1998) or regulators of actin filament dynamics like callybistin (Kins et al. 2000). Hence, a commonly proposed model for the structure of glycinerergic synapses hypothesizes that gephyrin anchors GlyRs to subsynaptic cytoskeleton (Kirsch 1999; Kneussel and Betz 2000). Tubulin depolymerization would then be expected to disrupt synaptic GlyR clusters.

Morphological studies performed in spinal cord cultures showed that microtubule depolymerization affected the number, structure, and intensity of gephyrin/GlyR postsynaptic clusters (Kirsch and Betz 1995). However, microtubule disruption in hippocampal cultures failed to alter gephyrin/GABA A clusters (Allison et al. 2000) and led to the conclusion that neither microtubules nor cytoplasmic tubulin-dimers play a significant role in the organization of gephyrin-containing inhibitory synapses. The reasons for these conflicting results are unknown.

Using the whole cell patch-clamp technique, we previously reported that the function of synaptic GlyRs, but not extrasynaptic receptors, was changed in cultured spinal cord neurons dialyzed with the microtubule depolymerization agent colchicine (van Zundert et al. 2002). In contrast, the function of...
glycinergic synapses, which contain the neonatal α2β receptor. After synaptic maturation, however, mainly the adult α1β GlyR remained in the postsynaptic membrane and colchicine was not effective. GABA_ARs postsynaptic clusters were unaltered at any developmental stage. Preliminary results were presented in abstract form (van Zundert et al. 2001).

METHODS

Cell culture

Mouse (C57BL/6) spinal cord neurons obtained from five to six embryos (13–14 days) were plated at 300,000 cells/ml into 35-mm tissue culture dishes coated with poly-L-lysine (MW > 350 kDa, Sigma Chemical, St. Louis, MO). The neuronal feeding medium consisted of 90% minimal essential medium (GIBCO, Grand Island, NY), 5% heat-inactivated horse serum (GIBCO), 5% fetal bovine serum (GIBCO), and a mixture of nutrient supplements (Aguayo and Pancetti 1994). Fresh media was replaced every 3 days. Experiments were performed on 5–17 DIV neurons. The microtubule interacting molecules colchicine, γ-lumicolchicine, nocodazole, and taxol (20 μM) were added to the culture media for 3 h or dialyzed into the neurons via the recording patch pipette as previously reported (Kirsch and Betz 1995; Rosenmund and Westbrook 1993; van Zundert et al. 2002).

Electrophysiology

For voltage- and current-clamp recordings in the whole cell configuration, patch electrodes were filled with (in mM) 140 KCl, 10 BAPTA, 10 HEPES (pH 7.4), 4 MgCl_2, and 2 ATP-Na_2. The external solution contained (in mM) 150 NaCl, 10 KCl 2.0 CaCl_2, 1.0 MgCl_2, 10 HEPES (pH 7.4), and 10 glucose. After the whole cell configuration was established, the capacitance of the cell and the series resistance were compensated using the amplifier (>80%). Spontaneous glycinergic miniature postsynaptic currents (mIPSCs) were isolated by the addition of CNQX (2 μM, RBI), bicuculline (2 μM, RBI), TTX (0.1 μM, Sigma), and MgCl_2 (2 mM) to the external solution (van Zundert et al. 2002). They were confirmed as glycinergic mIPSCs by a complete blockade with 750 nM strychnine. At 1 and 25 min after establishing the whole cell configuration, the spontaneous events occurring during a 3-min interval were analyzed. The properties of the mean mIPSC amplitude and frequency at 1 min were used as an internal control (van Zundert et al. 2002). By presenting the data as normalized (25 min/1 min), the results obtained with different drug treatments were pooled and analyzed in terms of their statistical differences. The noise was not significantly changed when neurons were dialyzed with any of the cytoskeletal disrupters. Synaptic currents were recorded using Axotape 7.0 software and analyzed off-line (Axon Instruments, Union City, CA). Every identified synaptic event collected during the test intervals displaying an amplitude above the background noise (12–15 pA) was analyzed using MiniAnalysis 5.0 software (Synaptosoft). Data were excluded if the uncompensated series resistance (<8 MΩ) increased by >15% during the recordings. Unless otherwise noted, all reagents were purchased from Sigma. Analysis of mIPSC properties included peak amplitude, inter-event interval, rise-time, and decay-time constant. The decay-phase of mIPSCs was best fitted with a single exponential curve. To avoid analysis of data points with excess noise at the peak and baseline, the rise and decay-phase were fitted between 10 and 90% of its amplitude.

Immuncytochemistry

Neurons were fixed for 5 (GlyR) or 15 min (GABA_A Rs) with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Fixing time was increased to 30 min for other antibodies. Cultures were permeabilized with a pretreatment of 0.25% Triton X-100 (15 min) to allow better access to intracellular epitopes and then blocked with 10% normal horse serum (NHS) for 30 min to reduce nonspecific staining. Cells were incubated for 1 h with pair combinations of the following primary antibodies: mouse monoclonal antibody (MmAb) against gephyrin (mAb7a; 1:100; Boehringer-Mannheim, Indianapolis, IN), rabbit polyclonal antibody (RpAb) against the α1 and α2 subunit of the GlyR (1:10; Calbiochem, La Jolla, CA), guinea pig polyclonal antibody (GpAb) against the γ2 subunit of the GABA_A R (1:1,000; kindly provided by Dr. J. M. Fritsche, University of Zurich), MmAb against synaptophysin (1:100; Oncogene, San Diego, CA), RpAb against synaptophysin (1:500; Zymed Laboratories Inc., San Francisco, CA), MmAb against SV2 (1:200; kindly provided by K. M. Buckley, Harvard Medical School, Boston, MA), and RpAb against synapsin I (1:1,000; Calbiochem). We tested the following combinations of antibodies: gephyrin/synapsin I, synaptophysin/SV2, synaptophysin/synapsin I, GlyR/gephyrin, GABA_A Rγ2/gephyrin, GlyR/SV2, GABA_A Rγ2/SV2, and GABA_A Rγ2/synapsin I. Immunoreactive (IR) sites were visualized after incubation for 1 h with appropriate secondary antibodies raised in donkey and conjugated to FITC or Cy3 (1:50; Jackson ImmunoResearch Laboratories, West Grove, PA). With every combination, we used a MmAb mixed with a RpAb or GpAb. Presynaptic markers were usually labeled with FITC-conjugated secondary antibodies and postsynaptic gephyrin and GABA_A Rγ2 antibodies with Cy3-conjugated antibodies. For the triple immunolocalization, together with the normal GABA_A Rγ2/gephyrin double immunolabeling, synapsin I was labeled with a biotin-SI-conjugated RpAb (1:50) and stained with Cy5-conjugated streptavidin (1:250; Jackson ImmunoResearch Laboratories). The cells were coverslipped using Vectashield (Vector Laboratories, Burlingame, CA).

Image visualization and sampling

For quantitative analysis of immuncytochemical data, samples of spinal neurons (10–15 cells from 2 separate experiments of paired control and colchicine-treated cells) were chosen randomly for imaging using confocal microscopy (Olympus Fluvioview; 100× oil immersion objective, N.A. 1.35, digitally zoomed 3 times, pixel size = 0.07 μm). Stacks of optical sections separated by 0.5 μm in the z axis were acquired throughout whole cells. Dual color immunofluorescent images were captured in simultaneous two-channel mode. Antibody dilutions were chosen for adequate immunofluorescent intensity to minimize cross-talk between the channels. If even minimal cross-talk between FITC and Cy3 fluorescence was observed (FITC usually labeled the presynaptic marker and resulted in brighter fluorescence), this was abolished by collecting Cy3 fluorescence above 610 nm. The number of synaptic gephyrin and GlyR clusters was determined as the number of clusters apposed/co-localized to punctate synapsin I or SV2 immunoreactivity. Co-localization was studied by superimposing both color channels. For triple immunolocalizations, neurons were imaged using a Leica TCS confocal microscope using multitracking imaging of each channel independently and optical and confocal conditions similar to those previously used and described above.

Analysis of cluster size

In contrast to previous confocal quantitative studies on gephyrin cluster sizes done in tissue sections (Geiman et al. 2000; Lim et al. 1999; Oleskevich et al. 1999; Triller et al. 1990), the majority of synapses in our cultures were positioned on the lateral sides of the cells, and relatively few crossed over the top of neurons. Therefore
there were only few examples of gephyrin clusters viewed “en face” where surface areas could be resolved accurately. Hence, we measured cluster lengths. Frequently, individual clusters were imaged in more than one serial optical section. The optical section that contained the largest and brightest immunofluorescence for each individual cluster was selected for measurement. For cluster size measurements, the stacks of confocal optical sections were analyzed using ImagePro software (ver. 4.1; Media Cybernetics, Silver Spring, MD). Individual clusters were detected in single optical sections by thresholding the image from the maximum arbitrary gray level (AGL) pixel intensity (4095) to 25% of this value. These image segmentation parameters allowed us to detect even the fainter clusters while outlining intensely fluorescent clusters inside their diffraction halo. Using this method, individual clusters were automatically segmented and outlined, and their maximum length was measured. For each experiment, imaging conditions were kept constant, and no postcapture modifications were done in images used for quantitative analysis. The data were compiled in Microsoft Excel, analyzed in Statview, and plotted using Origin. Importantly, our measurements of gephyrin cluster size gave similar results to previous studies in spinal cord cultures (Meier et al. 2000; Rosenberg et al. 2001) and spinal cord sections visualized with either confocal microscopy (Oleskevich et al. 1999), conventional fluorescence microscopy, or three-dimensional reconstruction with electron microscopy (Alvarez et al. 1997). For illustration, the neuron was reconstructed from the stack of optical sections, and further figure composition and labeling was done in CorelDraw 3.0, CorelDraw 8.0, or SigmaPlot 4.0.

RT-PCR: Total RNA extraction and cDNA synthesis

To extract total RNA from the spinal cord cultures, cells were lysed with 1 ml Trizol (5 min, 20°C, GIBCO), and 0.2 ml of chloroform was added to each sample. After vigorous shaking, the mixture was centrifuged (12,000g, 15 min, 4°C), and RNA was precipitated by mixing the aqueous phase with isopropyl alcohol (0.5 ml, 10 min, room temperature) and ethanol (75%) and centrifuged at 12,000g. After removal of the supernatant, the RNA was dried under the hood (5 min, 20°C) and centrifuged at 12,000g. The RNA was prepared according to their stage of development as immature (5–7 DIV), intermediate (10–12 DIV), and mature (15–17 DIV) neurons. It was found that the mean amplitude of glycinergic mIPSCs increased from 33 ± 4 pA in immature neurons to 56 ± 8 pA in mature neurons (Fig. 1C1). At the same time, the frequency increased from 0.84 ± 0.22 Hz in immature neurons to 1.64 ± 0.56 Hz in mature neurons (Fig. 1D1). Interestingly, we found that colchicine dialysis reduced both the amplitude (37 ± 5% reduction, P < 0.001) and frequency (72 ± 15% reduction, P < 0.01) in immature neurons (n = 5), while having no effect in mature neurons (n = 8; Fig. 1, C2 and D2). Smaller colchicine effects on both the amplitude (18 ± 3% reduction; P > 0.01) and the frequency (61 ± 7% reduction; P < 0.01) were found in neurons of intermediate stage of development (n = 7).

Colchicine-induced reductions of glycinergic mIPSCs activity are due to postsynaptic microtubule depolymerization

A summary of the effects produced by different agents acting on microtubules on the normalized mean mIPSC amplitude is shown in Fig. 2A. The amplitude of glycinergic mIPSCs remained highly stable (1.07 ± 0.07 presented as the amplitude ratio 25 min/1 min, from 34.7 ± 7 pA at 1 min) when the neurons were dialyzed with normal internal solution.
for 25 min ($n = 7$). On the other hand, dialysis of sister neurons with the microtubule disrupter colchicine (20 $\mu$M, $n = 10$) or nocodazole (20 $\mu$M, $n = 5$) reduced the normalized mIPSC amplitude to 0.71 ± 0.04 ($P < 0.001$) and 0.88 ± 0.01 ($P < 0.05$), respectively. When dialyzed with the inactive analog of colchicine, lumiocolchicine (20 $\mu$M), a cytotoxin that increases the stabilization of tubulin dimers (Nogales 2000), was unable to change the amplitude of the glycinergic transmission (0.98 ± 0.001). Taxol (20 $\mu$M, $n = 4$), a cytotoxin that increases the stabilization of tubulin dimers (Nogales 2000), was unable to affect the glycinergic transmission, and extracellular application of this alkaloid (20 $\mu$M; 3 h to the media) did not alter the number and morphology of gephyrin clusters (data not shown; Kirsch and Betz 1995), suggesting that stabilization of actin filaments and actin dynamics did not affect the integrity of postsynaptic gephyrin/GlyR clusters.

It was previously shown in Xenopus oocytes that extracellular colchicine inhibited overexpressed $\alpha_2$ and $\alpha_1$ GlyR subunits with IC$_{50}$s of approximately 64 and 324 $\mu$M, respectively (Machu 1998). We obtained four lines of experimental evidence indicating that our results with 20 $\mu$M intracellular colchicine cannot be explained by this competitive inhibition. First, analysis with extracellular colchicine (1-1,000 $\mu$M) showed that the whole cell glycine-activated current ($C_{50}$) was inhibited with very similar affinities in immature and mature neurons (IC$_{50}$ of 143 ± 28 and 187 ± 13 $\mu$M, respectively; $P > 0.05$, Fig. 2C). Second, internal nocodazole, which was reported to be devoid of antagonistic properties on the GlyR when applied extracellularly (Machu 1998), was still able to reduce the mIPSC amplitude (Fig. 2A). Third, the application of 20 $\mu$M external colchicine for 20 min did not affect the average mIPSC amplitude (0.93 ± 0.03, $n = 8$, $P > 0.05$, Fig. 2A) or the cumulative probability amplitude distributions ($P > 0.05$, Fig. 2B). Finally, intracellular GTP (500 $\mu$M), a guanine nucleotide with low membrane permeability and known for its capacity to stabilize microtubules (Nogales 2000), blocked the colchicine-induced reduction of glycinergic
mIPSCs amplitude (1.0 ± 0.08, n = 4, Fig. 2A). Control experiments showed that GTP had no effect when added alone to the internal solution (0.98 ± 0.04, n = 4, Fig. 2A).

**Effect of colchicine cannot be explained by a presynaptic site of action**

We also analyzed the number of mIPSC events and found that the frequency was decreased when the neurons were dialyzed with either colchicine (0.31 ± 0.08 presented as the frequency ratio 25 min/1 min, \( P < 0.001 \)) or nocodazole (0.49 ± 0.11, \( P < 0.05 \)). On the other hand, normal internal solution (0.84 ± 0.15, \( P > 0.05 \)) or addition of γ-lumicolchicine (0.85 ± 0.17, \( P > 0.05 \)) to the patch pipette was unable to significantly alter mIPSC frequency. A decrease in mIPSC frequency can be interpreted as a reduction in the probability of neurotransmitter release (Walmsley et al. 1998). Intracellular dialysis of membrane permeable colchicine could therefore have altered presynaptic cytoskeleton structures associated with neurotransmitter release after diffusion into the presynaptic terminal. However, our results with extracellular colchicine and GTP do not support this idea.

Alternatively, functional autaptic synapses could form under our culture conditions (Bekkers and Stevens 1991), and colchicine action could be partly due to alterations in autaptic transmission. To investigate this possibility, we examined whether the neurons used in our studies showed evidence of glycineergic autaptic activity. Under the low Cl⁻ gradient used, GlyR activation should be associated with a negative sign current. Figure 2D illustrates the effect of applying an 80-mV
depolarizing voltage step into a neuron held at –80 mV. This depolarizing pulse was able to activate a large somatic “unclamped” Na+ inward current (approximately 2 nA) but no autaptic glycinergic postsynaptic current was detected (Fig. 2D). In addition, the frequency of spontaneous glycinergic mIPSCs in the same neuron was unchanged even after a sustained depolarization elicited by a high-frequency (5 Hz) train of depolarizing pulses (Fig. 2D, inset). Additional experiments using current-clamp recordings showed that soma-elicited action potentials were not able to induce strychnine-sensitive autaptic synaptic potentials (Fig. 2E). From the 11 neurons examined, only 1 displayed an autaptic response, suggesting that it is unlikely that the colchicine effect is produced by alterations in autaptic transmission.

The above experiments ruled out the possibility that a presynaptic mechanism was associated to the action of colchicine on the glycinergic mIPSC frequency. Therefore we can argue that colchicine decreased the frequency of mIPSCs by reducing the current amplitude in such a way that a number of synaptic events fall under the threshold of detection (approximately 12 pA). To determine whether a change in mIPSC peak amplitude might account for the decrease in frequency, the mIPSC amplitude was reduced 27 ± 2% (37 ± 4 to 27 ± 2 pA, n = 5) by lowering the Cl– driving force. This closely simulates the colchicine-induced reduction in mIPSC amplitude (shown in Fig. 2A). As found with colchicine, the decrease in mIPSC quantal size was accompanied by a large (54 ± 5%) reduction in frequency, supporting the idea that the reduction in frequency after 25 min of colchicine treatment is best explained by an alteration in current amplitude.

Colchicine treatment decreased the size and intensity of gephyrin/GlyR clusters in immature neurons but not in mature neurons

We next investigated whether the developmentally regulated action of colchicine might be correlated with morphological changes in postsynaptic gephyrin clusters on microtubule disruption (Kirsch and Betz 1995). Immature, intermediate, and mature neurons were treated for 3 h with 20 µM colchicine (Kirsch and Betz 1995; van Zundert et al. 2002). After fixation, gephyrin clusters were immunolabeled with monoclonal antibody 7a, and the size (maximal length), density (luminosity), and numbers of synaptic and extrasynaptic gephyrin clusters were analyzed. Synaptic gephyrin clusters were detected by combining gephyrin-IR with a presynaptic marker (see METHODS). We considered gephyrin clusters synaptic when they were opposite to immunolabeled boutons and extrasynaptic if they were not. We compared synapsin I to other presynaptic markers like synaptophysin or SV2 and found that these three

FIG. 3. Colchicine altered the properties of gephyrin clusters in immature neurons. A1–D1: confocal images of synapsin I-IR terminals (green) in the soma and processes of spinal interneurons. A2–D2: gephyrin clusters (red) in the same cells from A1–D1. A3–D3: superimposed images of gephyrin and synapsin I immunofluorescence. Synaptic gephyrin clusters are found apposed to or co-localized (yellow) with synapsin I-IR. A: images obtained from an immature control neuron. Note that only approximately 60% of the gephyrin clusters are apposed/co-localized with synapsin I, suggesting that many nonsynaptic gephyrin clusters are present in immature neurons. B: application of colchicine (20 µM for 3 h) caused a reduction in both size and intensity of synaptic gephyrin clusters in immature neurons (B3). C: mature neurons show more synaptic terminals and gephyrin clusters compared with immature neurons. Size of the gephyrin clusters is also increased, and the majority (>95%) are localized at synapses. D: colchicine does not affect morphology of gephyrin clusters in mature neurons. Images were reconstructed from a stack of optical sections. Insets: magnified views of boxed areas showing examples of synaptic and nonsynaptic gephyrin clusters. Scale bar: 10 µm.
presynaptic markers labeled a similar population of boutons. Synaptophysin and synapsin I showed 78 ± 9% co-localization (n = 4 neurons), whereas synaptophysin and SV2 were co-localized in >90% of the boutons (n = 4 neurons). We found that synapsin I was the brighter marker and labeled a few more varicosities and thus was chosen for further studies. Analysis of synapsin I-IR showed that colchicine treatment was unable to significantly alter the bouton size in both immature (0.93 ± 0.04 μm in control vs. 0.89 ± 0.05 μm in treated) and mature neurons (0.80 ± 0.03 μm in control vs. 0.77 ± 0.04 μm in treated).

Immature control neurons showed gephyrin-IR clusters at the periphery of the neuronal soma and along proximal neurites (Fig. 3A). Not all gephyrin clusters were apposed or co-localized (Fig. 3A3), indicating the existence of extrasynaptic gephyrin clusters at this developmental stage. Synapsin I immunoreactivity was very robust in all stages of development in spinal cord cultures (Colin et al. 1996). In addition, the proportion of extrasynaptic to synaptic gephyrin clusters in immature neurons from 0.39 ± 0.02 μm in control to 0.30 ± 0.02 μm after treatment (P < 0.01; data not shown), indicating that large clusters were more sensitive to the colchicine treatment than smaller clusters. On colchicine application, the immunofluorescent intensity was also reduced from 916 ± 37 to 746 ± 21 arbitrary gray level units (Fig. 4B, P < 0.001). At intermediate developmental stages, a smaller but significant decrease in gephyrin cluster size was detected after colchicine treatment (85 ± 5% of control; P < 0.05; from 0.39 ± 0.01 μm in control to 0.33 ± 0.02 μm after treatment), but the immunofluorescent intensity was unchanged (P > 0.05).

During in vitro development, more synaptic interactions among the neurons are formed as indicated by an increased number of synapsin I contacts in mature neurons (Fig. 3C1). Correspondingly, mature neurons expressed approximately three times more gephyrin clusters, and these were always juxtaposed to synapsin I (Fig. 3C3). S psychotic gephyrin clusters grew bigger in size to a mean length of 0.48 ± 0.01 μm (Fig. 3C2). Extrasynaptic clusters were of similar size (0.33 ± 0.01 μm) compared with the previous developmental stages (0.31 ± 0.02 μm in both immature and intermediate neurons). Consistent with our electrophysiological data, colchicine did not alter synaptic or extrasynaptic gephyrin cluster number, size, or immunofluorescent intensity in mature neurons (Figs. 3D and 4, A and B).

![Graphs and images](http://jn.physiology.org/)

**FIG. 4.** Size and luminosity of gephyrin clusters were affected in a maturation-dependent manner by colchicine. Immature, intermediate, and mature spinal neurons were treated with colchicine and stained with antibodies against gephyrin and synapsin I to identify synaptic gephyrin clusters. A: colchicine decreased the mean synaptic gephyrin cluster size of immature and intermediate neurons. Size of gephyrin clusters in mature neurons was not significantly affected by colchicine. Note the increase in cluster size with time of development in vitro. B: luminosity of synaptic gephyrin clusters expressed in arbitrary gray levels (AGL) is decreased following colchicine treatment in immature neurons but not in intermediate or mature neurons. Each symbol represents means ± SE obtained from 14 neurons. Asterisks indicate a statistically significant action of colchicine compared with control. *P < 0.05, **P < 0.01, ***P < 0.001. C and D: mean gephyrin cluster size plotted as a function of mean glycinegic mIPSC amplitude at the 3 developmental stages in control (open symbols) and colchicine-treated (closed symbols) neurons. High correlations were found with intracellular (C) and extracellular colchicine treatments (D).
These results, as well as previous studies (Lim et al. 1999; Oleskevich et al. 1999), suggest a positive correlation between gephyrin/GlyR cluster size and glycinergic mIPSC amplitude. Next, we performed a correlative analysis between the average mean peak amplitude of glycinergic mIPSCs and gephyrin cluster size in control and after colchicine application either by dialysis into the neuron via the patch pipette or to the media for 3 h, similar to the protocol used to obtain the structural data (see Fig. 3). As shown in Fig. 4, C and D, both colchicine treatments gave a high positive correlation which ranged from 0.92 to 0.97. Similar results were found when the quantal amplitude was correlated with the gephyrin cluster intensity \((r = 0.88)\). The results support the conclusion that colchicine action on glycinergic activity is produced by a modification in postsynaptic receptor clusters.

The data in Fig. 3 was obtained using an antibody against gephyrin. Gephyrin immunolabeling constitutes a good method for performing high-resolution morphological analysis of postsynaptic cluster size and structure due to its favorable fixation tolerance and epitope display characteristics (Alvarez et al. 1997; Kirsch and Betz 1995; Triller et al. 1985, 1990). Additional experiments were performed with an antibody that targets GlyR \(\alpha1/\alpha2\) subunits, which co-localized with \(75 \pm 2\%\) of gephyrin clusters (Fig. 5A). Similar to the action on gephyrin clusters, colchicine (3 h to the media) decreased the size of synaptic GlyR clusters in immature neurons (71 \(\pm 6\%\) of control, \(P < 0.01\); Fig. 5, C and D), and the effect became less evident in intermediate neurons (90 \(\pm 7\%\) of control, \(P > 0.05\)). Mature neurons were not analyzed because colchicine had no action on either glycinergic mIPSCs or gephyrin cluster size/intensity.

Previous studies have indicated that gephyrin is also associated to GABA\(_A\)Rs (Essrich et al. 1999; Kneussel et al. 1999), and we found that 48 \(\pm 4\%\) of \(\gamma2\)-containing GABA\(_A\)Rs clusters co-localized with gephyrin in immature spinal neurons (Fig. 5B). Therefore it is possible that colchicine treatment

**FIG. 5.** Colchicine affects the morphology of synaptic gephyrin/glycine receptor (GlyR) clusters, but not synaptic GABA\(_A\)R\(\gamma2\) clusters. A: images show that GlyR (green) and gephyrin (red) clusters are highly (80%) co-localized (yellow, arrows). Few isolated gephyrin and GlyR clusters (double arrowheads) are found in these neurons. B: GABA\(_A\)R\(\gamma2\) (red) and gephyrin clusters (green) are found to co-localize at approximately 50% (yellow, arrows). Isolated GABA\(_A\)R\(\gamma2\) (double arrowheads) and gephyrin clusters (arrow heads) are frequently found in these neurons. C: images of GlyR clusters alone (green, C1) and superimposed with SV2 (red, C2) indicate the existence of synaptic GlyR clusters (yellow, arrows). Note that not all GlyR clusters are apposed/co-localized with SV2 (arrowheads). D: application of colchicine (20 \(\mu\)M for 3 h) reduced the size of synaptic GlyR clusters. E: similar colchicine treatment did not alter the morphology of GABA\(_A\)R\(\gamma2\) clusters. F: triple immunolocalization shows that GABA\(_A\)R\(\gamma2\) (red) gephyrin (green) clusters are apposed (blue) and co-localized with synapsin I (white), suggesting the synaptic localization of gephyrin/GABA\(_A\)R\(\gamma2\) cluster complexes. Images are reconstructed from a stack of optical sections. All confocal images were obtained from immature spinal interneurons. Insets: magnified views of boxed areas demonstrating examples of synaptic and non-synaptic GlyR clusters. Scale bar: 10 \(\mu\)m.
might also affect the properties of synaptic GABA\textsubscript{A}R clusters. Contrary to this effect, our results showed that colchicine did not alter the size of synaptic GABA\textsubscript{A}R2 clusters in immature (108 ± 3% of control) and intermediate (106 ± 3% of control) neurons (Fig. 5E). This lack of effect is in agreement with a previous study from our laboratory that showed that colchicine was unable to alter GABA\textsubscript{A}ergic synaptic currents in these spinal neurons (van Zundert et al. 2002).

**Immature cultured spinal cord neurons express α2β and α1β GlyRs in their synapses**

During the course of our study, we noted that colchicine treatment not only caused a reduction in the amplitude and frequency of glycine\textsubscript{ergic} mIPSCs, but it also accelerated its kinetics in immature neurons (Fig. 6A). Interestingly, the decay-phase of mIPSCs became approximately three times faster with neuronal maturation, and colchicine was no longer able to accelerate these rapid currents (Fig. 6, B and C). For instance, while glycine\textsubscript{ergic} mIPSCs in control immature neurons displayed a mean decay-time constant of 13.1 ± 1.8 ms (n = 6 cells), this value was 8.3 ± 1.1 ms in colchicine-treated neurons (n = 5 cells, P < 0.05). In intermediate (7.9 ± 1.4 ms, n = 5) and mature neurons (4.3 ± 0.4 ms, n = 5 cells), the glycine\textsubscript{ergic} currents displayed a faster decay-time constant and colchicine did not significantly modify these values (Fig. 6, B and C). The mean rise-time of mIPSCs, on the other hand, slightly decreased during maturation (15% from 2.6 ± 0.3, P > 0.05) and was unaffected by colchicine. The properties of developing mIPSCs in spinal neurons are in agreement with those reported in developing brain stem motoneurons (Singer et al. 1998).

Previous reports have proposed that acceleration of mIPSC kinetics with synaptic development results from a switch between neonate α2β GlyRs (slow) to adult α1β GlyRs (fast) (Ali et al. 2000; Krupp et al. 1994; Singer et al. 1998; Takahashi et al. 1992). Two independent experiments support the idea that these changes are recapitulated in cultured mouse spinal cord neurons. First, using RT-PCR, we found that α1 and β-subunit mRNA levels were high at all three developmental stages, while the α2-subunit expression was strongly down-regulated in mature neurons (Fig. 7B). No mRNA for the α3-subunit was detected during any of the three developmental stages. Similar expression patterns were previously described during the development of cultured rat spinal neurons (Bechade et al. 1996) and brain stem motoneurons (Singer et al. 1998). Second, we found that 100 μM picrotoxin, a toxin known to differentially affect α2β (IC\textsubscript{50} = 0.3 mM) and α1β (IC\textsubscript{50} = 1 mM) GlyRs (Pribilla et al. 1992), significantly decreased the amplitude and decay-time of glycine\textsubscript{ergic} mIPSCs in immature neurons (Fig. 7C), but not in mature neurons (Fig. 7D). Supporting the idea that the β-subunit is required to cluster the GlyR in the postsynaptic membrane (Meyer et al. 1995), we found that continuous application of 10 μM picrotoxin, a concentration known to selectively inhibit α homomeric (IC\textsubscript{50} = 7 μM; Pribilla et al. 1992), was unable to affect glycine\textsubscript{ergic} mIPSCs (data not shown). Taken together, these data suggest the presence of both α2β and α1β GlyRs in the postsynaptic membrane of immature neurons and predominantly α1β receptor in mature synapses.

**Colchicine and picrotoxin target the same population of postsynaptic GlyRs**

The previous results suggest that colchicine and 100 μM picrotoxin could affect the same population of slow immature α2β GlyRs. Therefore, in the next series of experiments, we analyzed whether glycine\textsubscript{ergic} activity of colchicine treated neurons (3 h in the media; 20 μM) displayed a different sensitivity to picrotoxin. We reasoned that if colchicine provoked the reduction of α2β GlyRs, this would reduce picrotoxin sensitivity of the synaptic currents. The open circles in the graph of Fig. 8A summarize data from five immature control neurons. In the absence of picrotoxin, glycine\textsubscript{ergic} mIPSC amplitudes ranged from 15 to 150 pA (mean, 42 ± 7 pA), and the decay-time constant varied from 2 to 35 ms (mean, 12.5 ± 2.4 ms). Picrotoxin caused a strong reduction in glycine\textsubscript{ergic} events with a large amplitude and slow decay-phase (Fig. 8A, ○). After treatment of sister cultures with colchicine, we found that the average amplitude and decay-time constant of mIPSCs was reduced to 32 ± 4 pA and 7.7 ± 1.4 ms, respectively (Fig. 8B, ○). Interestingly, most glycine\textsubscript{ergic} mIPSCs in these colchicine-treated neurons (n = 4) were resistant to picrotoxin application (Fig. 8B, ○).

A correlation (r = 0.65) between the decay-time constant and the amplitude of mIPSCs was found in control and treated neurons (Fig. 8, A and B, lines). This correlation is unlikely to be due to dendritic cable properties because no correlation was detected between decay-time constant and rise-time (r = 0.35; Fig. 8, C and D, lines). The mean rise-time under control conditions was 2.9 ± 0.4 ms and remained similar after treatment with colchicine and picrotoxin (P > 0.05, data not shown).

In summary, these results suggest that glycine\textsubscript{ergic} mIPSCs...
largely unmodified (van Zundert et al. 2002). In cultured spinal missions mediated by GABA\(_\text{A}\) Rs and AMPARs remained reduced by intracellular dialysis of colchicine, the synaptic transmission. For instance, although glycinergic transmission was reduced by intracellular dialysis of colchicine in the postsynaptic neurons, GlyRs and GABA\(_\text{A}\) Rs frequently co-localize in postsynaptic gephyrin-containing clusters opposite to presynaptic boutons containing inhibitory amino acids (Dumoulin et al. 2000). Hence, it is not likely that alterations in neurotransmitter release would affect glycinergic and leave GABA\(_\text{A}\)-ergic transmission unaffected. In addition, application of extracellular colchicine (20 min) did not reduce the frequency or amplitude of glycinergic mIPSCs. Further evidence supporting a postsynaptic action of colchicine was obtained during this study. First, intracellular application of the microtubule stabilizer GTP completely blocked the colchicine-induced reduction in glycinergic activity. Second, the rare occurrence of autapses in these cultured spinal neurons indicate that the effect of colchicine cannot be explained by depolymerization of vesicle-associated microtubules in presynaptic autaptic terminals. Finally, we found that the reduction in event frequency with colchicine could be well reproduced by changing the Cl\(^-\) driving force to obtain mIPSCs with smaller amplitudes.

Colchicine has been shown to competitively inhibit \(\alpha_2\) and \(\alpha_1\) GlyR subunits in Xenopus oocytes with IC\(_{50}\)s of approximately 64 and 324 \(\mu\)M, respectively (Machu 1998). Analysis of whole cell glycine-activated current during in vitro development of spinal cord cultures demonstrated that immature and mature neurons display a similar sensitivity to colchicine with IC\(_{50}\)s of 143 ± 28 and 187 ± 13 \(\mu\)M, respectively. In addition, extracellular application of 20 \(\mu\)M colchicine had no effect on glycinergic mIPSCs. Taken together, the effects observed with...
internal dialysis of 20 μM colchicine in immature and mature neurons are best explained by a postsynaptic mechanism consequent to microtubule disruption (see van Zundert et al. 2002).

In addition, our results provide good evidence supporting the idea that microtubules are important to maintain gephyrin/GlyRs clustered in the postsynaptic membrane of immature neurons, since we found that colchicine efficiently reduced the size and luminosity of postsynaptic gephyrin/GlyR clusters. Previous studies (Lim et al. 1999; Oleskevich et al. 1999), in addition to our present data, suggest that the amplitude of glycine-mediated mIPSCs and the size of gephyrin clusters are positively correlated. Colchicine reduced both parameters in immature, but not in mature neurons. In conclusion, the reduction in the amplitude of the glycineric mIPSCs after microtubule disruption can be best explained by a structural alteration of the postsynaptic density that results in decreased numbers of functional GlyRs in the synapse.

Microtubule disruption preferentially affects immature α2β-GlyRs

While the amplitude of the mIPSC appears to reflect the number of postsynaptic receptors, its decay is dictated by the kinetic properties of single channels. For example, in overexpression studies, α1 and α2 subunits assemble homomeric GlyRs with mean open times of 2 and 174 ms, respectively (Takahashi et al. 1992). In parallel with the known subunits switching from α2 to α1 subunits during postnatal development (Becker et al. 1988), patch-clamp analysis in spinal cord slices demonstrated that the open time of native GlyR channels decreased from 40 ms at E20 to 6 ms at P22 (Takahashi et al. 1992). Therefore it is believed that the differences in the decay-phase of glycineric mIPSCs during development depend on a transition between slow α2 and fast α1 subunits (Krupp et al. 1994; Legendre 2001; Singer et al. 1998; Takahashi et al. 1992). We also found that the time-course of the glycineric mIPSCs markedly accelerated with maturation of spinal neurons in culture. Analysis of mIPSCs properties showed that immature slow events were more sensitive to picrotoxin than mature faster events. However, our data suggest that immature glycineric currents are primarily associated to α2β subunits since these slow decay mIPSCs were affected by higher picrotoxin concentrations than those used to inhibit (IC50 = 7 μM) homomeric α receptors (Legendre 1997; Pribilla et al. 1992; Tapia and Aguayo 1998; Ye 2000). This conclusion is in agreement with other previous studies in immature spinal neurons showing the presence of a 48 pS channel, typical of heteromeric α2β receptors, and not a >85 pS associated to homomeric receptor (Bormann et al. 1987, 1993, Takahashi and Momiyama 1991; Twyman and MacDonald 1991). Similar to results obtained in newborn brain motoneurons (Singer et al. 1998), our results indicate that immature neurons express GlyRs that are formed by α2 and β subunits. During maturation, the mRNA level for the α2-subunit decreased, shifting the balance of mRNA expression toward α1 and β-subunits. In addition, analysis of the decay-time constant and the sensitivity to picrotoxin suggests that mature neurons express primarily α1β GlyRs. This finding is in agreement with previous studies (Krupp et al. 1994; Singer et al. 1998; Takahashi et al. 1992).

These data suggest that mature and immature GlyRs are composed of β-subunits and that they are linked to underlying postsynaptic microtubules. However, disruption of microtubules in immature neurons selectively affected a population of glycineric currents displaying slow decay-times, thus leaving faster events unaffected. These results indicate that microtubules regulate the immature α2β GlyR, but not the adult α1β receptor. Two lines of evidence support this idea. First, the picrotoxin-sensitive population of mIPSCs was no longer evident after disruption of microtubules with colchicine, suggesting they represent the same population of receptors. Second, mature neurons with fast α1β GlyRs were insensitive to colchicine.

Based on the current understanding regarding functional and structural properties of synaptic GlyRs, we postulate a model in which immature neurons express both α2β and α1β GlyRs at the postsynaptic membrane, while mature neurons contain mainly α1β GlyRs at the synapse (Fig. 9). Microtubules, via gephyrin, anchor both types of GlyRs in the postsynaptic membrane. On microtubule depolymerization, the molecular complex made up by gephyrin and α2β GlyRs is liberated and diffuses to the extrasynaptic membrane, reducing glycineric mIPSCs and gephyrin/GlyR cluster size and density. In contrast, colchicine treatment does not affect α1β GlyR/gephyrin clusters in immature and mature neurons (Fig. 9). This can be explained by the linkage of GlyR/gephyrin complexes to microtubules that are stabilized by post translational modifications, such as acetylation and/or the binding of molecules such as MAPs (Nogales 2000). In addition, the interaction of GlyRs with subsynaptic microtubules could depend on the type of α subunit present in the receptor rather than to differences in cytoskeleton properties. Interestingly, more than 10 potential
gephyrin isoforms can be generated by alternative splicing, allowing α1β- and α2β-GlyRs to associate with gephyrin variants that have distinct affinities for cytoskeletal elements. Thus the developmental dependent change of the GlyR molecular composition could affect the manner in which the receptor interacts with the gephyrin scaffold and subsynaptic microtubules.

Another possibility is that, after enough α1β GlyR/gephyrin complexes have accumulated in the synapse during maturation, gephyrin forms stable hexagonal scaffolds in the PSD, which maintain α1β GlyRs anchored at the postsynaptic membrane and independent of microtubules (Fig. 9; see also Kneussel and Betz 2000; Liu et al. 2000). Thus as proposed for actin filaments in excitatory hippocampal synapses (Allison et al. 2000; Zhang and Benson 2001), the state of microtubules could play a role in the development and maintenance of predominantly immature glycineergic synapses, which need to be highly plastic to shape efficient synaptic transmission. In agreement with this idea, electron microscopy studies have not consistently found microtubules within the 20- to 30-nm region beneath adult symmetric (inhibitory) or gephyrin-containing synapses (Alvarez et al. 1997; Peters et al. 1991; Triller et al. 1985, 1987). It is possible that postsynaptic microtubules are not well preserved in routine or immunocytochemical electron microscopy preparations as previously shown for presynaptic microtubules (Gray 1975). Accordingly, some microtubules were shown in close relationship to immature and mature postsynaptic densities (not necessarily inhibitory) in tissue sections or cultures pretreated with taxol, cryosubstitution techniques, or other procedures aimed to stabilize cytoskeletal components (Bird 1989; Ichimura and Hashimoto 1988; Le-Beux and Willemot 1975; Westrum and Gray 1977). However, the most convincing ultrastructural evidence available on microtubule presence in the PSD was obtained in neonatal synapses (Westrum and Gray 1977). To our knowledge, no systematic ultrastructural analysis of the spatial relationships between microtubules and the postsynaptic density of inhibitory synapses is available.

In conclusion, this study shows that disruption of microtubules by colchicine reduced both postsynaptic glycineergic currents and the size and density of synaptic gephyrin clusters in immature spinal neurons. Thus this study expands those on regulation of n-methyl-d-aspartic acid receptors (NMDARs), AMPARs, and nicotinic acetylcholine receptors (nAChRs) by the state of the cytoskeleton (Allison et al. 1998, 2000; Sattler et al. 2000; Shoop et al. 2000). Moreover, our data suggest that microtubule disruption affects immature synapses rather than mature synapses. This property could explain previous controversial findings in which disruption of microtubules altered the number and morphology of gephyrin clusters in 10–12 DIV spinal neurons (Kirsch and Betz 1995), while gephyrin distribution in >21 DIV hippocampal neurons was not affected by microtubule depolymerization (Allison et al. 2000).

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