Strychnine-sensitive stabilization of postsynaptic glycine receptor clusters

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SUMMARY

The cellular and molecular mechanisms underlying the postsynaptic aggregation of ionotropic receptors in the central nervous system are not understood. The glycine receptor (GlyR) and its cytoplasmic domain-associated protein, gephyrin, are clustered at the postsynaptic membrane and constitute a good model for addressing these questions. The glycine receptor is inhibited by strychnine. The effects of chronic strychnine treatment on the expression and cellular distribution of gephyrin and glycine receptor were therefore tested using primary cultures of spinal cord neurons. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis revealed that the glycine receptor α1, α2, β subunits and gephyrin mRNAs were expressed at comparable levels in strychnine-treated and untreated cultures. The number of immunoreactive cells and the subcellular distribution of gephyrin and GlyR subunits was determined with standard and confocal immunofluorescence. The proportion of gephyrin and glycine receptor-immunoreactive (IR) cells was unaffected by strychnine treatment. Confocal microscopy revealed that the glycine receptor was mainly localized intracellularly near the nucleus. This cytoplasmic glycine receptor was not associated with the Golgi apparatus nor with the rough endoplasmic reticulum and therefore is not likely to correspond to neosynthesized proteins. The number of GlyR clusters on the somatodendritic membrane was dramatically reduced on neurons displaying intracellular staining. In contrast, the subcellular distribution and the number of gephyrin clusters was not modified by the treatment. The fact that gephyrin postsynaptic localization was not modified by strychnine suggests that the aggregation of glycine receptor and gephyrin is governed by different mechanisms. The distribution of other cell surface molecules such as NCAM or GABA_A receptor β2/3 subunits was not modified by strychnine treatment. Chronic exposure of the cultures to tetrodotoxin did not affect gephyrin or glycine receptor cluster formation. Taken together, these results indicate that functional glycine receptor, but not electrical synaptic activity, is required for the formation of glycine receptor clusters.

Key words: Glycine receptor, Gephyrin, Strychnine, Intracellular distribution, Spinal cord, Cultured neuron

INTRODUCTION

At the neuromuscular junction (NMJ), postsynaptic nicotinic acetylcholine receptors (nAchR) are precisely localized in front of motor nerve terminals (see Hall and Sanes, 1993, and references therein). Similarly, in the central nervous system (CNS), high densities of neurotransmitter receptors are found on the postsynaptic side of synapses. Postsynaptic clustering has been reported in the mammalian brain for the GlyR (Triller et al., 1985, 1987; Van den Pol and Gorcs, 1988), the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) subtype of glutamate receptor (Petralia and Wenthold, 1992), and the α6 subunit of the γ-aminobutyric acid (GABA_A) receptor (Baude et al., 1992). These receptors are accumulated in front of terminal boutons releasing corresponding neurotransmitters. However, some GABA_A and glutamate receptor subtypes are also present at extrasynaptic plasma membrane sites (Baude et al., 1992, 1994, 1995; Sur et al., 1995). These observations raise questions about the mechanisms governing localization of ionotropic receptors at given postsynaptic membranes. At the NMJ, pre- and postsynaptic factors are involved in the formation of the postsynaptic microdomains. Molecules secreted by motor nerve endings regulate both nAchR gene expression and clustering (see Hall and Sanes, 1993, and references therein; Sanes, 1997). Within the postsynaptic muscle cell, the 43 kDa nAchR-associated protein rapsyn has been shown to be involved in nAchR clustering (see Cartaud and Changeux, 1993, and references therein). In the CNS, the discovery of several proteins colocalized and interacting with inhibitory or excitatory receptors has now advanced our understanding of postsynaptic receptor clustering: gephyrin anchors the GlyR in the postsynaptic membrane (Kirsch et al., 1993b), and PSD95/SAP90, GRIP, and Homer proteins interact with N-methyl-D-aspartate (NMDA)-, AMPA- and metabotropic-types of glutamate receptor, respectively (Brakeman et al., 1997; Dong et al., 1997; Kornau et al., 1995). The role of synaptic transmission in gene expression and distribution of neurotransmitter receptors (NT-R) at central synapses is not yet understood. In the CNS, one of the best systems for examining this question is the glycinergic synapse because: (i) GlyR is one
of the best characterized NT-R at the molecular and cellular level (see Vannier and Triller, 1997, and references therein); and (ii) activation of the GlyR associated channel can be selectively blocked by strychnine (see Cortés and Palacios, 1990, and references therein).

The GlyR is a ligand-gated chloride channel which mediates inhibition in the spinal cord, brain stem and other regions of the vertebrate CNS. It contains two types of transmembrane glycosylated subunits of 48 (α) and 58 (β) kDa associated in a pentamer (α3β2). Gephyrin, a peripheral membrane unglycosylated protein of 93 kDa, was copurified with the GlyR (see Betz et al., 1994, and references therein). As shown in rat spinal cord, both the GlyR and gephyrin are concentrated at postsynaptic membrane apposed to glycineric terminals (Triller et al., 1985, 1987; Todd et al., 1996). The anchoring function of gephyrin was demonstrated by antisense experiments leading to an inhibition of GlyR clustering (Kirsch et al., 1993b). It was later shown that gephyrin interacts with the GlyR β subunit (Kirsch et al., 1995). The gephyrin-binding region was then identified within the cytoplasmic loop between the M3-M4 segment of the β subunit (Meyer et al., 1995). Gephyrin also links the postsynaptic GlyR to the submembranous cytoskeleton by interacting with microfilaments and microtubules (Kirsch and Betz, 1995).

In the rat, three variants of the GlyR α subunit (α1-α3) have been isolated by screening cDNA and genomic libraries. They share sequence homologies but exhibit distinct functional properties and are differentially expressed during development. The so-called ‘neonatal’ GlyR is constituted by α2 subunit associated in homo-oligomers and replaced at later developmental stages by the α1/β heteromeric polypeptide (Becker et al., 1988; Hoch et al., 1989). Alternative splicing of both the α1 and α2 subunits lead to subunit diversity (Kuhse et al., 1990b, 1991; Malosio et al., 1991). The α3 subunit is predominantly expressed in the postnatal cerebellum (Kuhse et al., 1990a).

It has been shown in a previous study in the adult goldfish Mauthner cell that the stability of postsynaptic gephyrin clusters depends on the integrity of the presynaptic innervation (Seitanidou et al., 1992). To investigate whether postsynaptic GlyR-gephyrin complex cluster formation depends on glycineric transmission, we blocked the GlyR function of spinal cultured neurons by chronic treatment with strychnine. Strychnine, a convulsive alkaloid extracted from the Indian tree *Strychnox nux vomica*, selectively blocks spinal postsynaptic inhibition (see Dale et al., 1990, and references therein). It interacts with the N-terminal domain of the GlyR α subunit (Kd 1-10 nM) at a site distinct from the ligand-binding site (see Betz et al., 1994, and references therein). Strychnine also acts on non-glycinergic sensitive sites at a higher range of concentrations (Barron and Guth, 1987). Following strychnine treatment, we analyzed the mRNA expression and the subcellular localization of the GlyR αβ subunits and of gephyrin. We present here direct evidence that functional GlyR is required for GlyR but not gephyrin clustering.

**MATERIALS AND METHODS**

**Primary cultures**

Primary cultures of spinal cord neurons were prepared from embryos at day 14 of gestation of Sprague-Dawley rats as previously described (Béchade et al., 1996). Cells were plated at a density of 4×10⁵ cells/ml on sterilized glass coverslips (12 mm in diameter) in 4-well plates (Nunc) for immunofluorescence and in 35 mm dishes (Corning) for RT-PCR. Coverslips and Petri dishes were previously coated with 15 µg/ml poly D-L ornithine (Sigma). Culture supports were then incubated until plating, with medium containing 5% inactivated fetal calf serum (Gibco). Cells were plated in the serum-free supplement B27-Neurobasal medium combination (Gibco) which optimizes neuronal survival (Brewer et al., 1993). Cultures were kept at 37°C in 5% CO₂ for 11 days. The culture medium was renewed after 4 and 8 days in vitro (DIV). Starting the day after plating, strychnine (Sigma) was added to the culture medium every 24 hours to a final concentration of 0.1, 1, or 10 µM. Control cultures received a comparable volume of normal medium every 24 hours. On the third, seventh and eleventh DIV, cells were harvested for total RNA extraction or fixed for immunofluorescence.

**PCR analysis**

GlyR subunits and gephyrin mRNAs were identified in cultured spinal neurons by polymerase chain reaction (PCR) amplification. Total RNA was isolated using the RNAnow extraction procedure (Biogentex). After DNAase (20 units/reaction; Pharmacia) treatment, single-stranded cDNAs were synthesized by reverse transcription. Aliquots of cDNA equivalent to 200 ng of total RNA were amplified in a PCR thermocycler using the following conditions: denaturation, 95°C for 30 seconds; primer annealing, 56°C (for the α1 subunit), 60°C (for the α2 and β subunits), 58°C (for gephyrin and cyclophilin) for 30 seconds; and extension, 72°C for 1 minute. The number of cycles was: 25 for cyclophilin, 35 for the α1, α2 and β subunits, and 40 for gephyrin. Sequences of oligonucleotides covered for cyclophilin: the positions 490-510 (sense) and 622-642 (antisense) (Danielian et al., 1988), for α1; the positions 883-903 (sense) and 1,189-1,209 (antisense) (Grenningloeh et al., 1987), for α2; the positions 1,525-1,575 (sense) and 1,790-1,810 (antisense) (Kuhse et al., 1990b), for β; the positions 1,326-1,347 (sense) and 1,694-1,714 (antisense) (Grenningloeh et al., 1990), for gephyrin; the positions 326-346 (sense) and 804-824 (antisense) (Prior et al., 1992) of the corresponding rat cDNAs. PCR products were separated on a 2% agarose gel and visualized by ethidium bromide staining. Titrations were performed to ensure that PCR reactions were carried out in the linear range of amplification. For illustration, representative samples were assembled with Photoshop 3.0 (Adobe, Mountain View, CA) and the image was printed with a Personal laser Writer 320 printer.

**Antibodies**

Monoclonal antibodies (mAb) were used in this study: mAb7a which recognizes gephyrin (1/100, Pfeiffer et al., 1984; Boehringer); mAb4a which binds to all GlyR α and β subunit isoforms (1/200, Pfeiffer et al., 1984; gift from H. Betz), AP14 which detects MAP2A (1/20, Binder et al., 1984; gift from B. Riederer), mAb bd17 directed against the GABA₆ R β2/3 subunits (10 µg/ml, Ewert et al., 1990; Boehringer) and OB11 which recognizes the cytoplasmic domain of NCAM A and NCAM B (1/100, Neill and Barnstable, 1990; Sigma). As secondary antibodies, we used a carbamoyethyl indocyanine-3 (CY3)-coupled affinity-purified goat anti-mouse IgG (1/200; Jackson Immunoresearch Laboratories). Polyclonal antibodies (pAb) were also employed: a pAb directed against the rough endoplasmic reticulum (1/1,000, Louvard et al., 1982; gift from E. Courdier), a pAb which recognizes p210, a protein of the cis-Golgi network (1/1,000, Rios et al., 1994; gift from M. Bornens), a pAb anti-synapsin (1/1,000, De Camilli et al., 1983; gift from P. de Camilli). The pAb were recognized by a dichlorotriazynil-amino-fluorescein (DTAF)-conjugated affinity-purified goat anti-rabbit IgG (H+L) (1/200; Jackson Immunoresearch Laboratories).

**Immunocytochemistry**

For mAb7a, AP14, mAb bd17 and OB11 immunodetection, cells were
fixed with 4% (w/v) paraformaldehyde for 15 minutes. Cells were washed in phosphate buffer saline (PBS; 120 mM, pH 7.4), and permeabilized with 0.12% Triton X-100 in PBS with 0.12% (w/v) gelatin for 5 minutes. The permeabilized cells were incubated with primary antibodies for 1 hour, washed in PBS, and incubated in secondary fluorescent antibodies for 45 minutes. Each wash and antibody incubation step was performed at room temperature (RT). For mAb4a immunocytochemistry, a methanol/acetic acid (95/5) mixture was used as fixative (10 minutes at −20°C). Cells were rinsed in PBS and incubated successively in primary and secondary antibodies as above. For double-labeling experiments, primary and secondary antibodies were incubated sequentially. After extensive washes, cultures were mounted with Vectashield (Vector Lab.) and observed with a standard (IRMB-Leica) or a confocal (Molecular Dynamics) epifluorescence microscope. In all experiments, the specificity of immunolabeling and the absence of antibody cross-reaction in double-staining experiments were controlled by omission of the primary antibodies.

Quantitative analysis

The proportion of immunoreactive cells in the cultures was determined by visual inspection using a standard fluorescence microscope LEICA (objective ×63). For each experiment, 7 to 17 fields were analyzed. Each field corresponded to 9.06 ± 0.3 μm and 0.3 μm, respectively (objective ×63, numerical aperture 1.4), with images of 512×512 pixels. Digitized series of optical sections at different planes of focus were collected using a host computer (Indy, Silicon Graphics). The background noise was reduced and the contrast enhanced by applying a median (3x3x3) Gaussian filter. Maximum intensity projections were derived from these sections using Imagespace (Molecular Dynamics) software. The number and surface area of peripheral clusters were determined with NIH 1.52 software. The threshold intensity fluorescence was set manually for each cell to insure efficient detection and to avoid a coalescence of clusters. An image-object was computed if it comprised at least 4 pixels. For the determination of GlyR clusters per cell and of their surface area in strychnine-treated cultures, only cells with intracellular mAb4a staining were chosen. In this case, intracellular labeling was excluded from analysis by manual delineation of the peripheral neuronal region. For double-labeling experiments, both FITC and CY3 fluorescence were recorded simultaneously. The laser was set at 488 nm, a wavelength allowing a maximal excitation for FITC and an acceptable one for CY3. The corresponding emitted lights were separated by a beam splitter (565 nm) and recorded through separate photomultipliers. Pixel size was 0.1 μm (objective ×63, numerical aperture 1.4) with images of 512×512 pixels.

RESULTS

Primary cultures were prepared from 14-day embryonic rat spinal cords. With the serum-free culture conditions used here (Colin et al., 1996), neurons identified by neurofilament-staining (data not shown) represented 97% of the total cell population. As seen with phase contrast microscopy or following MAP2A detection, strychnine had no effect on the morphology of the neurons at any of the concentrations used (0.1; 1 or 10 μM; data not shown).

Absence of effect of strychnine treatment on GlyR and gephyrin mRNA levels and number of immunoreactive cells

After 3, 7 and 11 DIV, the total RNA was extracted from cultures exposed to different concentrations of strychnine and from untreated ones grown in parallel. RT-PCR amplification of gephyrin and GlyR α1, α2, and β subunits was performed using previously characterized oligonucleotide primers (see Materials and Methods). Different cycle numbers were performed to insure that PCR reactions were carried out in the linear range of amplification. The amplification of cyclophilin transcripts used as an internal control confirmed RNA integrity for each of these cDNA preparations. This approach allowed comparison of relative transcript levels in each set of experimental conditions. As exemplified here at 11 DIV (Fig. 1), gephyrin, GlyR α1, α2, and β subunits bands of correct size were of 0.21 ± 0.1 m and 0.3 m, respectively (objective ×63, numerical aperture 1.4), with images of 512×512 pixels. Digitized series of optical sections at different planes of focus were collected using a host computer (Indy, Silicon Graphics). The background noise was reduced and the contrast enhanced by applying a median (3x3x3) Gaussian filter. Maximum intensity projections were derived from these sections using Imagespace (Molecular Dynamics) software. The number and surface area of peripheral clusters were determined with NIH 1.52 software. The threshold intensity fluorescence was set manually for each cell to insure efficient detection and to avoid a coalescence of clusters. An image-object was computed if it comprised at least 4 pixels. For the determination of GlyR clusters per cell and of their surface area in strychnine-treated cultures, only cells with intracellular mAb4a staining were chosen. In this case, intracellular labeling was excluded from analysis by manual delineation of the peripheral neuronal region. For double-labeling experiments, both FITC and CY3 fluorescence were recorded simultaneously. The laser was set at 488 nm, a wavelength allowing a maximal excitation for FITC and an acceptable one for CY3. The corresponding emitted lights were separated by a beam splitter (565 nm) and recorded through separate photomultipliers. Pixel size was 0.1 μm (objective ×63, numerical aperture 1.4) with images of 512×512 pixels.

Strychnine (μM)

0 0.1 1 10

α1 ins

α1

α2

β

gephyrin

cyclophilin

Fig. 1. No effects of strychnine treatment on GlyR subunits and gephyrin mRNA levels. Total RNA was extracted from spinal neurons cultured for 11 DIV in the absence or presence of strychnine at the indicated concentrations. PCR was performed on corresponding cDNAs. The fragments amplified from GlyR α1, α1 ins, α2, β subunits and for gephyrin mRNAs were detected at comparable levels under all culture conditions. Cyclophilin amplification products indicate that PCR was performed on comparable levels of RNA. The last column corresponds to the RT-PCR control.
were amplified and the intensities of detection were comparable whatever the concentration of strychnine. Similar results were found at 3 and 7 DIV (data not shown). For the α1 subunit, two products were detected corresponding to the α1 and α1\textsuperscript{iso} mRNAs resulting from alternative splicing (Malosio et al., 1991). These data showed that chronic strychnine treatment did not alter the expression of all GlyR subunits and gephyrin mRNAs. In sister cultures, we determined after 3, 7 and 11 DIV, using the indicated concentrations of strychnine. Strychnine treatment at 0.1 and 1 μM produced no significant effects on the evolution of the proportion of gephyrin and GlyR-IR cells during neuronal maturation. At 10 μM, the percentage of cells displaying GlyR-IR was significantly reduced after 7 and 11 DIV. Data represent means (± s.d.) derived from three independent experiments. Differences with control values were tested using Student’s t-test (*P<0.01).

Subcellular distribution of GlyR and gephyrin under strychnine treatment

The subcellular distribution of gephyrin and GlyR was analyzed by confocal microscopy. The gephyrin-IR subcellular distribution was not modified by strychnine treatment. Gephyrin formed patches at the periphery of somata and neurites of untreated (Fig. 3A1, C1) and strychnine-exposed neurons (Fig. 3B1, D1). Between 7 and 11 DIV, the number of peripheral patches of gephyrin increased progressively on neuritic and somatic compartments independently of the concentration of strychnine (Fig. 3B1, D1). Under control conditions, GlyR clusters were present at the periphery of soma and neurites of neurons (Fig. 3A2) and their number increased from 7 DIV (Fig. 3A2) to 11 DIV (Fig. 3C2). Intracellular mAb4a IR was never observed in these cells. Following strychnine applications for 7 DIV, the GlyR-IR was detected intracellularly as visualized on confocal microscope optical sections (Fig. 3B2). The GlyR-IR formed large and round-shaped blobs (0.5-2 μm) near the nucleus. These intracellular fluorescent spherical elements were occasionally observed in the proximal portion of the neurites. In these neurons, only a few small GlyR clusters were visible at the surface of cell bodies. The effect on GlyR subcellular distribution was the same whatever the concentration of strychnine. The effects of strychnine on the cellular GlyR distribution varied between individual neurons on one coverslip. Some cells with intracellular GlyR-IR did not express mAb4a-positive clusters at their surface. Other cells were unlabeled for the GlyR. Other neurons did not show GlyR intracellular labeling; GlyR clusters could be observed over the somata and dendrites.

Fig. 2. Proportion of GlyR-IR and gephyrin-IR cells following strychnine treatment. The percentage of gephyrin- (A) and GlyR- (B) IR cells was determined after 3, 7 and 11 DIV, using the mAb7a. Strychnine treatment at 0.1 and 1 μM produced no significant effects on the evolution of the proportion of gephyrin and GlyR-IR cells during neuronal maturation. At 10 μM, the percentage of cells displaying GlyR-IR was significantly reduced after 7 and 11 DIV. Data represent means (± s.d.) derived from three independent experiments. Differences with control values were tested using Student’s t-test (*P<0.01).

The relationship of peripheral gephyrin and GlyR clusters to presynaptic endings was analyzed in double-labeling experiments with a polyclonal antibody against synapsin. We found that the vast majority of gephyrin clusters on dendrites and somata of strychnine-treated and untreated cells were apposed to synaptic boutons (data not shown). In untreated cultures, postsynaptic GlyR clusters were also observed along dendritic and somatic regions contacted by synapsin-positive axons (Fig. 4A1). The peripheral GlyR clusters which persisted on strychnine-treated neurons were also found in front of synapsin-stained profiles (Fig. 4A2). These data indicated that the synaptic connectivity within the culture is probably not modified by strychnine. As expected, the large and round-shaped blobs of intracellular GlyR-IR found in strychnine-treated cells were never colocalized with synapsin-stained endings. Moreover, when compared to untreated cells, GlyR clusters were more often absent in front of synaptic boutons. Double-labeling experiments of GlyR with a p210 pAb, a marker of the cis-Golgi apparatus (Fig. 4B1, B2) or with a pAb directed against the rough endoplasmic reticulum (RER) (Fig. 4C1, C2), were also performed on control and strychnine-treated cultures. We found that the GlyR-IR intracellular blobs were not colocalized with either of these two compartments.
suggesting that this intracellular IR did not correspond to neosynthesized GlyR.

The strychnine-induced GlyR cytoplasmic localization could also result from a global alteration of protein transport to the cell surface. Therefore, we have studied the effects of 10 \( \mu \text{M} \) strychnine treatment on the surface expression of two plasma membrane protein, NCAM and the GABA\(_A\) R \( \beta_2/3 \) subunits. The distribution pattern of NCAM was analyzed following strychnine treatment from DIV 0 to 11 and that of GABA\(_A\) R \( \beta_2/3 \) subunits after treatment from DIV 7 to 11. For the two antigens the IR was found on the soma and neurites and no redistribution could be observed in treated cells. NCAM-IR (Fig. 5A-B) was diffuse at the neuronal surface, and GABA\(_A\) R-IR (Fig. 5C-D) was patchy. Confocal sections showed that the staining was always located at the cell periphery independently from the treatment.

The observed redistribution of GlyR, induced by strychnine also raises the question as to whether other agents capable of blockades of other chloride-associated receptors could act on GlyR distribution. We have tested the effect of chronic exposure of neurons to: (1) 3 \( \mu \text{M} \) gabazine (Hussy et al., 1997); and (2) 50 \( \mu \text{M} \) bicuculine (Krishek et al., 1996). We found that this treatment (from 7 to 11 DIV) did not alter the subcellular distributions of both gephyrin and GlyR (not shown). This indirectly favors the notion that strychnine acts selectively on GlyR stability at the cell surface.

**Quantification of gephyrin and GlyR-IR**

The number of cells with gephyrin clusters and intracellular GlyR-IR and/or clusters was determined on control and strychnine-exposed cultures at different concentrations following 3, 7 and 11 days of treatment. The percentage of
neurons with gephyrin clusters followed a similar trend in the presence or absence of strychnine (see Fig. 2), indeed strychnine had no effect on the subcellular distribution of gephyrin clusters. The situation was different for the GlyR. After 7 (Fig. 6A1) or 11 (Fig. 6A2) days of strychnine treatment, the proportion of cells with intracellular GlyR-IR increased while the proportion of cells with peripheral GlyR clusters decreased. The changes were dose-dependent. As illustrated in Fig. 6B, in the case of a continuous exposure to 10 µM strychnine, the proportion of cells with internal GlyR-IR was already maximum at 7 DIV. The addition of 10 µM strychnine to the culture medium from DIV7 to DIV11 also induced an increase in the number of neurons with intracellular GlyR-IR (32%). It is worth noting that at 7 DIV, 50% of the neurons already expressed peripheral GlyR clusters. Interestingly, the effects of strychnine treatment were reversible (Fig. 6B). Strychnine withdrawal at DIV7 resulted in a reduction in the number of neurons with intracellular GlyR-IR to 4.6% after another 4 DIV. This was accompanied by the restoration of a peripheral GlyR localization and resulted in an increase in the proportion of cells with surface GlyR clusters to a value comparable to that seen in control cultures (54%).

The number of gephyrin and GlyR clusters per cell and the mean surface area of clusters were determined from series of confocal section. Only cells with intracellular GlyR-IR were considered for the quantification of GlyR clusters per cell in strychnine-treated cultures. In these cultures, neither the number nor the mean surface area of gephyrin-IR clusters was significantly different from the values found in a parallel control experiment. From 7 to 11 DIV, the number of gephyrin clusters per cell increased from 53±22 to 69±3 (Fig. 7A1). The mean surface area of gephyrin clusters was independent of the neuronal periphery (0.7±0.1 µm², respectively). The values found in the control experiments and their range between 7 DIV were consistent with what was found in previous experiments (Béchade et al., 1996; Kirsch et al., 1993b). In the presence of strychnine, the number of GlyR clusters at the neuronal periphery dropped dramatically compared to untreated cells: at 0.1 µM, only 7.2±1.9 and 11.1±4.2 GlyR clusters were detected at 7 and 11 DIV, respectively, compared with 22.4±8.6 and 49.2±9.9 in control experiments (Fig. 7B1). Significant effects were also observed for the size of GlyR clusters which dropped by 50% (Fig. 7B2).

Fig. 4. The relationships of strychnine-induced intracellular GlyR-IR with synapsin, cis-Golgi and rough endoplasmic reticulum-IR. Spinal neurons at day 7 in vitro cultured under control conditions (A1,B1,C1) or treated with 1 µM strychnine (A2,B2,C2). Cells double-labeled with mAb4a (red) and with polyclonal antibodies (green) directed against synapsin (A1,A2), cis-Golgi network (B1,B2), or rough endoplasmic reticulum (C1,C2). (A1) Under control conditions, many GlyR clusters were apposed to synapsin-IR (arrowheads, yellow) at the periphery of the cell soma. (A2) Strychnine-induced internalization of GlyR-IR (crossed arrows), most synapsin-IR (arrows) did not colocalize with GlyR-IR. A few GlyR-IR clusters still colocalized with synapsin-IR (arrowheads). Under control conditions, surface GlyR-IR clusters (arrowheads) were not associated with the Golgi apparatus (B1) or the RER (C1). In strychnine-treated neurons, intracellular GlyR-IR blobs (crossed arrows) are close to but do not colocalize with the Golgi apparatus (B2) or the RER (C2). In all images, n shows the position of the nucleus. Bars, 5 µM.
DISCUSSION

Cultured embryonic spinal cord neurons develop morphologically differentiated (Colin et al., 1996) and functional (Jackson et al., 1982) inhibitory synapses. We used this in vitro system to analyze the effects of a chronic blockade of GlyR channel activity by strychnine on: (i) GlyR subunit mRNA expression; and (ii) on receptor component subcellular distribution. The major finding of this study is that the strychnine treatment led to a dramatic diminution in the number of cell surface postsynaptic GlyR clusters. The GlyR-IR was then detected as blobs within the neuronal soma. Following strychnine treatment, the number of cells displaying intracellular labeling was dose-dependent and an increase was still observed between 1 and 10 μM. This result may be explained by the prevalent expression of low-strychnine sensitivity GlyR isotype in this type of neuronal culture (Hoch et al., 1989). In our cultures, only a low proportion of cells was labeled with GlyR α1-specific antibody after 11 DIV (data not shown) suggesting that the GlyR complex may be mostly constituted by the neonatal isoform. The GlyR ‘adult’ α1 and ‘neonatal’ α2 subunits differ in their functional properties and their developmental expression pattern with the latter being expressed at an earlier stage and displaying a lower strychnine-sensitivity than the former (Becker et al., 1988). Moreover, it has been shown that glycine-currents of cells cotransfected with α2 and gephyrin displayed a significantly 10-fold lower sensitivity to strychnine (Takagi et al., 1992). Therefore, the effects of strychnine are still specific in the range of concentrations used. Interestingly, the effect of strychnine on the internalization of GlyR was reversible. Furthermore, internalization of GlyR could be induced by 4 days of strychnine after 7 days without strychnine. Therefore, the plasma membrane peripheral GlyR clusters are stabilized by function.

Our experiments revealed that neither the neonatal GlyR α2 nor the adult GlyR α1 subunit mRNA levels was modified by strychnine treatment. It was shown in similar spinal cultured neurons (Béchade et al., 1996) that GlyR α1, α2 and gephyrin mRNAs levels increase markedly before synaptogenesis occurs. Therefore, functional glycinergic transmission is not directly responsible for the onset of GlyR subunit mRNA expression. This finding contrasts with data obtained at the NMJ (see references in Hall and Sanes, 1993) and in ciliary ganglia (Boyd et al., 1988; Levey et al., 1995) showing that AchR gene expression is regulated by either nerve-activity and/or soluble factors.

The cytoplasmic localization of the GlyR may result from: (1) an increased rate of its synthesis; (2) an alteration in its transport to the plasma membrane; or (3) a retrieval from the cell surface. These mechanisms are not mutually exclusive. During their synthesis and maturation, transmembrane GlyR
tested using Student’s *t*-test with control values. Differences with control values were tested with Student’s *t*-test (*P*<0.01).

Fig. 6. Dose-dependent strychnine effects on the proportion of cells with intracellular GlyR-IR. (A1,A2) Proportion of cells with peripheral GlyR clusters (hatched bars) and intracellular GlyR staining (closed bars) after 7 (A1) or 11 (A2) days with increasing concentrations of strychnine. The percentage of cells with peripheral GlyR clusters decreased progressively whereas the proportion of neurons with intracellular GlyR-IR increased with the concentration of strychnine. (B) Increasing proportion of cells with intracellular GlyR-IR between 3 and 7 DIV which remained stable between 7 and 11 DIV (continuous line). Strychnine withdrawal between 7 and 11 DIV resulted in a drop in the proportion of cells with intracellular GlyR-IR (small broken line). Strychnine exposure from DIV7 to DIV11 induced an increase in the proportion of cells with intracellular GlyR-IR (large broken line). Results are expressed as percentages and represent means (± s.d.) from three independent experiments. Differences with control values were tested using Student’s *t*-test (*P<0.01).

components necessarily move in the exocytic pathway sequentially through the RER and the Golgi complex before reaching the plasma membrane (Palade, 1975; Pfeffer and Rothman, 1987). In strychnine-treated cells, it seems unlikely that the GlyR remains blocked in an early compartment of the secretory pathway since cytoplasmic GlyR labeling was never found to be associated with the RER or the cis-Golgi cisternae. It cannot be excluded, however, that GlyR are stopped in a later compartment such as the trans-Golgi network or secretory vesicles. The size of the intracellular GlyR-IR blobs (0.5-2 μm) is not in favor of this hypothesis. Furthermore, the bulk exocytic pathway of membrane proteins to the cell surface is not modified by strychnine treatment: the subcellular localizations of NCAM and of GABA<sub>A</sub> receptors were not modified. Therefore, unless strychnine has a yet unknown specific target participating to GlyR traffic, the accumulation of intracellular receptor cannot be accounted for by a global retention of postsynaptic transport vesicles. Another possibility would be that postsynaptic GlyR blocked by strychnine would be removed from the dendritic and somatic surface via endocytosis. The subsequent internalization of GlyR could lead to an accumulation in an endocytotic compartment, followed or not by degradation in lysosomes. Internalization of receptors from the cell periphery has been documented in other systems. For instance, the nerve growth factor and the low-density lipoprotein receptors are internalized following interaction with their respective ligands (see Goldstein et al., 1985, and references therein; Greene and Shooter, 1980). Axonal and dendritic endocytosis has been demonstrated in cultured hippocampal neurons where endocytotic vesicles are transported from the peripheral dendritic or axonal regions to the cell body (Parton et al., 1992).

Fig. 7. Morphometrical analysis of GlyR and gephyrin clusters following strychnine treatment. Spinal neurons kept for 7 or 11 DIV under control conditions or treated with strychnine at the indicated concentrations were stained with mAb7a (A1,A2) or mAb4a (B1,B2). (A1-B1) Mean number of gephyrin (A1) and GlyR (B1) clusters/cell. (A2-B2) Mean surface area per cluster of gephyrin (A2) and GlyR (B2) clusters. Note the decrease in both the number of GlyR membranous clusters/cell and their surface area after 7 or 11 DIV of strychnine treatment whatever the concentration used. For each set of experimental conditions, quantification was performed on maximum-intensity confocal projections of 10 cells. The bars represent means (±s.d.) of three independent experiments. Differences with control values were tested with Student’s *t*-test (*P<0.01).
AchRs are present almost exclusively at the postsynaptic membranes of mature NMJs (see Hall and Sanes, 1993, and references therein). Surgical denervation (Loring and Salpeter, 1980), inhibition of quantal release of Ach from motoneurons with botulinum toxin or the block of AchRs with the α-bungarotoxin (Avila et al., 1989) accelerated the loss of stable junctional AchRs. Muscle paralysis induced by continuous application of TTX to the motor nerve also increased the rate of disappearance of AchRs in a similar way to denervation (Fumagalli et al., 1990). In our experiments, the few persisting GlyR peripheral clusters found on strychnine responsive neurons were significantly smaller than those seen at the surface of untreated neurons. These data suggest that GlyR clusters are first destabilized. This result is reminiscent of data obtained at the NMJ where large AchR clusters break up into several smaller clusters following denervation (Kuromi and Kidokoro, 1984; Slater, 1982). Similarly, denervation of adult frog cardiac ganglion cells lead to a reduction in the size of AchR patches (Sargent and Pang, 1988). At variance with the AChR, the stability of GlyR was not dependent upon evoked glycine release since we never observed intracellular GlyR-IR or a diminution in the size and number of GlyR clusters in cultured spinal cord neurons treated for up to 11 DIV with TTX (10 μM, data not shown). Indeed, glycine is present at high concentration in the culture medium, and this could mask the effect of the drug. However, neurons cultured in deprived glycine media displayed a comparable behaviour (data not shown). Similarly, TTX had no effect on either the overall levels of IR or the postsynaptic clustering of GluR1 and GABAA R β2/3 subunits in cultured hippocampal neurons (Craig et al., 1994). Further, spontaneous glycine quantal events occur at glycineric synapses in the presence of TTX (Korn and Faber, 1990), therefore glycine released by spontaneous exocytotic events may also be sufficient for the stabilization of GlyR clusters. Taken together, these results and ours suggest that expression and localization of central receptors are largely independent of evoked (i.e. synchronisation of quantal events) synaptic activity.

The key role of gephyrin in the postsynaptic localization of GlyR has been demonstrated in cultured embryonic neurons (Kirsch et al., 1993b). The lack of effect of strychnine on the subcellular distribution of gephyrin at the periphery of cultured neurons is striking. This could be due to interaction of gephyrin with other elements. Gephyrin clusters are stabilized by the subsynaptic cytoskeleton (Kirsch and Betz, 1995). Furthermore, gephyrin may interact with other transmembrane proteins. In the CNS, gephyrin is expressed in many regions with apparently no GlyR α isoforms (Kirsch and Betz, 1993; Kirsch et al., 1993a). The GABA R A is a good candidate for an interaction with gephyrin. Immunohistochemical studies have reported that glutamate decarboxylase (GAD)- or GABA-presynaptic terminals are asposed to gephyrin-IR synapses in the spinal cord (Bohllhalter et al., 1994; Triller et al., 1987). Both GABA R A β3-subunit and gephyrin are found in front of presynaptic axons enriched with both GABA- and glycine-like IR (Todd et al., 1996). In the rat retina, gephyrin and the GABAA R α1, α2 and α3 subunits are colocalized at synapses where the GlyR is absent (Sassoè-Pognetto et al., 1995). Alternatively, different mechanisms may control the subcellular organization of GlyR and gephyrin clusters: postsynaptic gephyrin clustering occurs before the formation of differentiated synapses while GlyR cluster formation is contemporaneous with the establishment of synaptic contacts (Béchade et al., 1996; Kirsch et al., 1993b). Furthermore, it has been shown in vivo in the goldfish Mauthner cell that chronic treatment with strychnine had no effect on the ultrastructural distribution of gephyrin (Seitanidou et al., 1992). This observation and our new data indicate that presynaptic neuron-derived factor(s) rather than synaptic transmission are involved in the control of the postsynaptic gephyrin localization.

In conclusion, this study shows that formation and/or stabilization of GlyR but not of gephyrin postsynaptic clusters depend upon functional GlyR. This indicates that different mechanisms are involved in the accumulation of these two molecules at the postsynaptic specialization. We suggest that the activation or conformational change of the GlyR are involved in the establishment and/or maintenance of the interaction between GlyR and gephyrin.

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