Glycinergic Miniature Synaptic Currents and Receptor Cluster Sizes Differ Between Spinal Cord Interneurons

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Glycinergic miniature synaptic currents and receptor cluster sizes differ between spinal cord interneurons. J. Neurophysiol. 82: 312–319, 1999. The structural features of a synaptic connection between central neurons play an important role in determining the strength of the connection. In the present study, we have examined the relationship between the structural and functional properties of glycinergic synapses in the rat spinal cord. We have analyzed the structure of glycine receptor clusters on rat ventral horn interneurons using antibodies against the glycine receptor clustering protein, gephyrin. We have examined the properties of quantal glycinergic currents generated at these synapses using whole cell patch-clamp recordings of miniature postsynaptic inhibitory currents (mIPSCs) in rat spinal cord slices in vitro. Our immunolabeling results demonstrate that there is a considerable variability in the size of glycine receptor clusters within individual neurons. Furthermore there are large differences in the mean cluster size between neurons. These observations are paralleled closely by recordings of glycinergic mIPSCs. The mIPSC amplitude varies significantly within and between neurons. Results obtained using combined immunolabeling and electrophysiological recording on the same neurons show that cells with small glycine receptor clusters concurrently exhibit small mIPSCs. Our results suggest that the differences in the size of glycine receptor clusters may constitute an important factor contributing to the observed differences in mIPSC amplitude among spinal cord interneurons.

INTRODUCTION

Glycine is a major inhibitory neurotransmitter in the mammalian spinal cord (Aprison 1990). One of the major roles of spinal glycinergic neurotransmission is the shaping of motoneuron output via a number of well-characterized glycinergic interneurons in the ventral horn of the spinal cord (largely located in lamina VII) (Gao and Ziskind-Conhaim 1995; Jankowska 1992). A recent immunolabeling study in the ventral horn of the adult cat spinal cord revealed striking differences in the size and morphology of postsynaptic glycine receptor clusters over the soma and dendrites of various kinds of motoneurons and interneurons (Alvarez et al. 1997). One interneuron class, the Renshaw cell (which provides recurrent inhibitory control over the motoneuron output) was found to exhibit particularly large and complex clusters, whereas motoneurons and other interneurons displayed smaller and less complex clusters, on average. Furthermore most cell types (with the notable exception of Renshaw cells) exhibited an increase in the size of glycine receptor clusters at dendritic locations distal from the cell body (Alvarez et al. 1997). These observations naturally raise questions about the functional significance of such differences in postsynaptic receptor cluster size and morphology between cells (Walmsley et al. 1998). In the present study, we have investigated this issue by combining immunolabeling of glycine receptor clusters and electrophysiological recordings of glycinergic synaptic currents, using an in vitro slice preparation of rat spinal cord (Gao et al. 1998).

First, we have examined the morphology of glycine receptor clusters on rat ventral horn neurons in rats aged 10–15 days postnatal. We used antibodies against the glycine receptor clustering protein, gephyrin, to reveal the size and structure of the postsynaptic receptor clusters. Gephyrin is involved in directing the clustering and anchoring of glycine receptors at postsynaptic sites by cross-bridging glycine receptor subunits and the underlying cytoskeleton (Kirsch et al. 1991, 1993a,b; Meyer et al. 1995). In the ventral horn of the spinal cord, gephyrin has been shown to colocalize in a 1:1 relationship with the α transmembrane subunit of the glycine receptor and to map accurately the localization and structural organization of the postsynaptic receptor cluster (Alvarez et al. 1997; Colin et al. 1998; Todd et al. 1995, 1996; Triller et al. 1985, 1990a,b; Walmsley et al. 1998). In spinal cord cells in culture, gephyrin expression has been shown to precede the insertion of glycine receptors in the plasma membrane (Bechade et al. 1996; Kirsch et al. 1993a,b). In rat spinal cord, gephyrin immunoreactive (gephyrin-ir) clusters without glycine receptor subunits frequently are observed during the first week of postnatal development (Colin et al. 1998). However, the close relationship between gephyrin and glycine receptor clusters is established firmly at day 10 postnatal (Colin et al. 1998). This may not be the case in other regions of the CNS, particularly in the forebrain (Kirsch and Betz 1993) and retina (Sassoe-Poggetto et al. 1995). Gephyrin immunolabeling therefore was used as a marker of postsynaptic glycine receptor clusters in spinal cord slices from animals >10 days of age. Gephyrin immunolabeling constitutes a better method for performing high-resolution morphological analysis of cluster size and structure than immunofluorescence against transmembrane subunits of the receptor due to its favorable fixation tolerance and epitope display characteristics (Alvarez et al. 1997; Kirsch and Betz 1993; Triller et al. 1985, 1990b).

Second, as a measure of glycine synaptic function in rat ventral horn neurons, we have recorded miniature inhibitory postsynaptic currents (mIPSCs) in spinal cord slices. The mIPSCs represent the postsynaptic response to the spontaneous
release of transmitter from vesicles at individual release sites and thus provide a functional measure of the population of glycinergic synapses over the cell surface. Finally, we have combined electrophysiological recordings of glycine mIPSCs with gephyrin immunolabeling of receptor clusters in the same cells using intracellular injection of Lucifer yellow or Neurobiotin to identify each cell. Our results provide evidence on the role of site to site differences in receptor cluster size and electrotonic attenuation of synaptic currents in determining the variability in mIPSC amplitude within the same cell and in differences between cells in rat ventral horn interneurons.

METHODS

Electrophysiology

Transverse slices (150–300 μm) were made of the lumbar spinal cord of 10–15 day old Wistar rats after anesthesia (sodium pentobarbitone, 35 mg/kg ip) and decapitation. Whole cell patch electrode recordings were made at room temperature (22–25°C) from ventral horn neurons visualized in the slices using infrared differential interference contrast (DIC) optics. All recordings were made in sections of lumbar 4 or 5 spinal cord segments. In this region, lamina VII extends ventrally to contact the ventral funiculus of the white matter separating a lateral and a medial motoneuron pool (lamina IX). Most recordings were obtained from interneurons located in this intermediate region that lacks motoneuron cell bodies and corresponds with the classical ‘Renshaw cell area’ defined by previous authors in the lower lumbar segments of the cat (Fyffe 1990). The recorded neurons were classified as interneurons based on their location outside lamina IX and their small size. Slices were superfused with a Ringer solution containing (in mM): 130 NaCl, 3.0 KCl, 1.3 MgSO₄, 2.0 CaCl₂, 1.25 NaH₂PO₄, 26.2 NaHCO₃, and 10 glucose, equilibrated with 95% O₂-5% CO₂. Patch electrodes (3–6 MΩ resistance) contained (in mM): 120 CsCl, 4 NaCl, 4 MgCl₂, 0.001 CaCl₂, 10 HEPES, 2 Mg-ATP, 0.2 GTP-Tris, and 0.2–10 EGTA (pH 7.2). A cesium-chloride-based internal solution was used to improve recordings by blocking potassium conductances and to provide a large driving potential for the chloride-mediated glycinergic synaptic currents at a holding potential of −60 mV. For intracellular labeling, 0.2–2.0 mg/ml Lucifer yellow or Neurobiotin was added to the internal solution. Series resistance, which was <10 MΩ, was compensated by >80%. Synaptic currents were recorded and filtered at 10 kHz with an Axopatch 200B amplifier (Axon Instruments) before being digitized at 20 kHz. Data also were recorded on videotape with a VCR (Vetter) and digitized off-line. Data acquisition and analysis were performed using Axograph (Axon Instruments). The amplitudes of spontaneous IPSCs were measured using semi-automated detection procedures (Axograph 4.0). A sliding template was used to detect all spontaneous events with amplitudes >4 standard deviations of the background noise (δ-n was typically 1.5 pA). The detection technique uses a template with the time course of a typical synaptic event and slides this template along the current trace. The template is scaled optimally to fit the trace at each position. The event detection criterion is proportional to the scaling factor and inversely proportional to the goodness-of-fit between the template and the current trace at each position. An event is detected when this criterion exceeds a specified threshold level (Clements and Bekkers 1997). The rise time of the spontaneous IPSCs was calculated from 20 to 80% of the peak amplitude.

Immunohistochemistry

Slices were immersion fixed for 5–30 min using 2–4% paraformaldehyde in 0.1 M phosphate buffered (pH 7.4), dehydrated, and rehydrated in alcohols to enhance penetration of antibodies and incubated for 3–4 days with mouse monoclonal antibodies against gephyrin (mAb7a; Boehringer-Mannheim) or α1 subunits (mAb2b; gift from Drs. J. Kirsch and H. Betz, Max-Plank Institute, Frankfurt) both diluted 1:100 in 0.01 M phosphate-buffered saline (pH 7.2) with 0.3% Triton-X-100. Immunoreactive sites were visualized using secondary antibodies coupled to Cy3 (dilution 1:25; Jackson Labs). Neurobiotin was revealed with FITC-coupled avidin-D (dilution 1:10; Vector Labs). Sections with Neurobiotin-labeled cells were processed only with gephyrin antibodies. This combination allowed for longer fixation times and better structural analysis. The FITC-neurobiotin intracellular label and the Cy3-gephyrin immunofluorescence were imaged using a Leica confocal microscope. Pixel size was 0.1 μm (60× oil objective N. A. 1.4). Optical sections of 0.5 μm in thickness were acquired through the whole cell body and dendritic arbor of the recorded cells (20–40 sections per cell). Full penetration of the immunostaining through the section was confirmed under the confocal microscope. Gephyrin-immunoreactive clusters were analyzed using NIH Image. Morphological analysis was performed on the stack of raw optical sections obtained from the confocal microscope without any image processing. To avoid errors due to inaccuracies in the z axis, only clusters that were visualized “en face” were measured. Hence, only clusters at the top and bottom surfaces of the reconstructed neuron were analyzed. These represent a sizable sample of the whole cluster population of the cell. When clusters are visualized en face they appear in optimal focus in just one optical section. The stack of images was moved up or down to obtain the best focus for each cluster which was outlined using NIH image. Moving the stack up or down also allowed us to identify clusters that extend into multiple optical sections due to local changes in the convexity or orientation of the membrane (as at the point of emergence of a primary dendrite). Top and bottom surfaces were identified as the optical planes at the extremes of the confocal z-axis image series. Top and bottom surfaces contained many en face clusters. Regions in middle planes of the z-axis series displayed tangential clusters restricted exclusively to the perimeter cell membrane. Cell contours (outlined by gephyrin-ir clusters) were smooth in all x-yz axes (see Figs. 1 and 6). Our measurements of cluster size were similar to previous estimates obtained from thinner sections and visualized with confocal microscopy (Triller et al. 1990b), standard epifluorescence microscopy or three-dimensional reconstruction of cluster regions using electron microscopy (Alvarez et al. 1997). Small inaccuracies in area measurements due to unaccounted small amounts of xy shrinkage or ambiguities in border definition due to tissue diffraction and fluorescent halo typically occur in every histological preparation and have been discussed previously (Alvarez et al. 1997). Such inaccuracies would affect measurements of clusters on all cells and not significantly influence the major conclusions of this comparative study. Measurements were made of area, immunostaining density, and the lengths of major and minor axes for each immunofluorescent cluster. Care was taken to subtract the area occupied by inside holes in complex clusters that exhibited perforations in the postsynaptic region. For illustration, the top or bottom surfaces of half-cells were reconstructed from the stack of optical sections using Image-Pro Plus 3.0 software (Media Cybernetics), a Gaussian filter was applied to increase image contrast, and composite figures were assembled and labeled using Adobe Photoshop 4.0.

Tetrodotoxin (TTX; 1 μM; Sigma), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM; Tocris), bicuculline methochloride (10 μM; Tocris), strychnine hydrochloride (1 μM; Sigma), and d-tubocurarine chloride (30 μM; Sigma) were added, as indicated, to the Ringer solution and applied by bath perfusion. Results are expressed as means ± SD, and statistical tests of significance were determined with an unpaired t-test or a nonparametric test for correlation (Kendall rank; Statview). The terms cluster size and cluster area are used interchangeably.
RESULTS

Immunolabeling of glycine receptor clusters

Gephyrin immunoreactivity was examined over the surface of neurons of the ventral horn in lumbar spinal cord sections (150–300 μm), in rats aged 10–15 days postnatal. At all ages examined, punctate gephyrin-ir clusters were found over the soma and dendrites of both motoneurons and interneurons. The present study was focused on a population of spinal cord interneurons located in ventral lamina VII, which comprise different interneurons including Renshaw cells. Most cells exhibited relatively small, simple and dot-like gephyrin-ir clusters in their somatic and proximal dendritic surface (Fig. 1A). A more infrequent type of interneuron was observed with a substantial complement of large, intensely fluorescent clusters on the soma and proximal dendrites (Fig. 1B). [Lamina VII interneurons displaying this distinctive complement of gephyrin-ir clusters have been characterized as Renshaw cells in previous studies based on electrophysiological criteria (Alvarez et al. 1997), calbindin content (Carr et al. 1998), and synaptic complement (Alvarez et al. 1999). In the present study, such cells have been identified putatively as Renshaw cells, based on the appearance of their gephyrin-ir clusters.]

Cluster sizes were measured on six interneurons displaying characteristic Renshaw cell-like gephyrin-ir clusters and six interneurons with small clusters, sampled from the same slices in which we performed electrophysiological experiments. Figure 1, A and B, illustrates examples of gephyrin-ir clusters on two ventral horn interneurons from the same 13-day-old animal. In these and in all cells examined, there was a large variability in the size of gephyrin-ir clusters within individual neurons. This variability was demonstrated in the corresponding histograms of cluster size and the measures of the coefficient of variation (CV = SD/mean; Fig. 1, C and D). In addition to this large variability, histograms of cluster size exhibited a pronounced skew. A striking feature of the gephyrin-ir labeling was the difference in cluster size and cluster morphology between cells, as exemplified by the two cells of Fig. 1, A and B. One of these cells (Fig. 1A) exhibited mostly small gephyrin-ir clusters, whereas the other cell, most likely a Renshaw cell (Fig. 1B), exhibited many large clusters. This difference between cells was obvious in the histograms of cluster size and the measures of mean cluster size (Fig. 1, C and D).

Measurements of cluster areas were performed for 12 cells, all from the spinal cords of 13-day-old animals (Fig. 2). Figure 2A illustrates a histogram of cluster areas (summed) on six cell somas exhibiting small clusters (mean area = 0.25 μm²) and Fig. 2B shows the histogram for six cell somas with distinctively large clusters (mean area = 0.59 μm²). The mean CV was 0.5 for cells exhibiting small clusters and 0.7 for cells with large clusters. Figure 2C illustrates the linear relationship between the mean gephyrin-ir cluster area and the variability (standard deviation) for all cells (r = 0.99; P < 0.001). It is obvious from this plot that there was a wide range in the mean gephyrin-ir cluster size of all cells (range = 0.18–0.73 μm², n = 12). The SD of cluster area was larger for the cells exhibiting a larger mean area, reflecting the observation that these cells exhibit a very large spread in cluster areas rather than a narrow distribution around a larger mean cluster size.

The distinction between neurons with small clusters and those with large clusters appears to be regulated developmentally. In parallel with the present study, we examined the developmental aspects of gephyrin-ir clustering on rat ventral horn neurons in animals from 10 to 15 days postnatal. Neurons with small gephyrin-ir clusters were found at all ages between 10 and 15 days. However, in the Renshaw cell region of ventral lamina VII, the cluster size shows a developmental maturation such that the clusters become significantly larger from 13 days onward. Measurements were made of gephyrin-ir cluster areas...
found on the soma and first 10–15 μm of proximal dendrites of 23 cells over the age range 10–15 days postnatal (5–6 cells for each day). The mean cluster area was 0.37 ± 0.29 μm² at 10 days, 0.37 ± 0.28 μm² at 12 days, 0.63 ± 0.48 μm² at 13 days, and 0.58 ± 0.37 μm² at 15 days. Because the difference in mean cluster area between cells was more obvious for animals >12 days postnatal, we performed the measures of cluster size on 13-day-old animals and the majority of electrophysiological recordings in animals >13 days (n = 17 of 26).

**Glycine mIPSCs**

Whole cell patch recordings were made from ventral horn interneurons (n = 26) in 150 to 300 μm-thick spinal cord slices. Cells were visualized under the microscope using infrared-DIC optics, and recordings were made within the Renshaw cell region (ventral lamina VII) to maximize the possibility of recording from interneurons with both large and small gephyrin-ir clusters. Figure 3A illustrates recordings of mIPSCs in a ventral horn neuron in the presence of TTX, CNQX, and bicuculline to block presynaptic action potentials, AMPA, and GABA-receptor-mediated synaptic currents, respectively. These mIPSCs, recorded at a membrane potential of −60 mV, were confirmed as glycinegic by a complete block with strychnine (1 μM; Fig. 3A). (In 2 of 26 cells, there were infrequent residual synaptic currents, which were blocked by t-tubocurarine and which were most likely nicotinic acetylcholine receptor-mediated synaptic currents. In the remaining 24 cells, there were no detectable residual currents after CNQX, bicuculline, and strychnine).

Examples of recordings of glycine mIPSCs in ventral horn interneurons are illustrated in Fig. 3, B–E, for four different cells. It is evident that there was a large range of mIPSC amplitudes within individual lamina VII interneurons. This variability is demonstrated in the corresponding histograms of mIPSC amplitude and the measures of the CV. A difference in the distribution of mIPSC amplitudes also was observed between cells. While some cells exhibited exclusively small mIPSCs (histograms in Fig. 3, B and D), other cells exhibited an extensive range of amplitudes, with many large mIPSCs (histograms in Fig. 3, C and E).

Figure 4 summarizes the mIPSC results for 26 ventral horn interneurons. The locations of the cells from which recordings were made are illustrated in Fig. 4A with the mean amplitude of the mIPSCs recorded in each cell indicated by the size of the circle. It is evident from this diagram that cells with both small and large mIPSCs were intermingled, although there was a tendency for cells with large mIPSCs to be concentrated toward the gray-white matter border in a region of lamina VII that contains a higher density of Renshaw cells (Fyffe 1990). The mean peak mIPSC amplitude varied between 16.8 and 174.0 pA with a mean mIPSC amplitude of 76.1 ± 42.9 (Fig. 4B). The range of mIPSCs amplitudes that occurs between cells is illustrated in the cumulative probability distributions for mIPSC amplitudes in Fig. 4C. These distributions indicate

**FIG. 2.** Variability in gephyrin-ir cluster size between ventral horn interneurons. A and B: histograms of gephyrin-ir cluster size from neurons showing small clusters (mean cluster size = 0.25 μm², n = 6 cells) and neurons showing large clusters (mean cluster size = 0.59 μm², n = 6 cells). C: summary data showing positive correlation between SD of cluster size and mean cluster size. Note the wide range in mean cluster size among cells. Cells with large cluster sizes and distinctive morphology are putatively labeled as Renshaw cells (A). All data from 13-day-old animals.

**FIG. 3.** Whole cell patch recordings of miniature inhibitory postsynaptic currents (mIPSCs) in ventral horn interneurons. A: characterization of mIPSCs. Left: mIPSCs recorded at −60 mV with a CsCl-based internal solution in the presence of TTX (1 μM), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM), and bicuculline (10 μM). Right: mIPSCs blocked by addition of strychnine (1 μM). B–E: examples of mIPSC recordings in individual neurons. Some neurons showed mostly small mIPSCs (B and D), whereas others showed a predominance of large mIPSCs (C and E). Corresponding histograms show variability of mIPSCs within individual neurons. Scale bar applies to all mIPSCs.
that the major difference between cells was in the range of mIPSC amplitudes. Although some cells exhibited only small mIPSCs, others exhibited a wide range of mIPSCs, from small to very large amplitudes. Figure 4D illustrates a positive linear correlation between mean mIPSC amplitude and the standard deviation, reflecting the fact that the range of mIPSC amplitudes was greater in cells that exhibited a large mean mIPSC amplitude \((r = 0.88; P < 0.0001)\). The linear correlation also indicates that the coefficient of variation was similar over the range of mean amplitudes \((\text{mean CV} = 0.9 \pm 0.3, n = 26)\). In addition, the mIPSC distributions were highly skewed (mean skew = 2.4 ± 1.3, \(n = 26\)), and this skew was not correlated with mean mIPSC amplitude \((P = 0.84)\).

The peak amplitude of mIPSCs may be influenced by many factors, such as electrotonic attenuation in the dendritic tree, the properties of the postsynaptic receptors, and the number and density of receptors at each release site. Electrotonic effects would be expected to produce a correlation between mIPSC time course and peak amplitude for the population of mIPSCs recorded in the same cell. Figure 5, A and B, illustrates the relationship between the rise time and the peak amplitude of all mIPSCs detected in two individual cells. Figure 5A illustrates the plot for a cell with small mIPSC amplitudes, and Fig. 5B shows the plot for a cell with large mIPSC amplitudes. Although no correlation was evident for the cell with small mean mIPSCs, an obvious correlation was seen for the cell with large mean mIPSC amplitudes \((P < 0.0001)\). This correlation was due to the influence of a population of small mIPSCs that exhibited slow rise times. However, the distribution also shows that the relationship between rise time and mIPSC amplitude was flat over a 10-fold range for mIPSCs above ~100 pA (see also exponential fit in Fig. 5B). This suggested that electrotonic influences do not account for most of the large range of mIPSC amplitudes recorded in this cell. (Lowering the detection threshold from 4 to 2.5 SD of the noise for the data in Fig. 5A resulted in the detection of an additional population of small but artifically fast mIPSCs due to the background noise; data not shown.)

To investigate further the correlation between mIPSC time course and peak amplitude, the population of cells was divided into two groups (of approximately equal number). The two groups were made up of all cells with mean mIPSC amplitudes <70 pA \((n = 14\) cells; Fig. 5C) and >70 pA \((n = 12\) cells; Fig. 5D). The individual mIPSC measurements for all cells in each group then were combined, and the rise times were plotted against mIPSC amplitude. If mean mIPSC amplitude is the only difference between cells exhibiting small versus large mIPSCs, then the two distributions should scale linearly and maintain a similar shape if plotted on a different amplitude scaling. As shown in Fig. 5, C and D, the shape of these distributions was different due to the notable absence in Fig. 5C of a population of small, slow rise time mIPSCs evident in Fig. 5D. There are several possible explanations for this difference. One possibility is that the mIPSCs in cells with small mean mIPSC amplitude all were generated over a restricted range of electrotonic locations. This seems unlikely because there is an extensive soma-dendritic synaptic coverage for all ventral horn interneurons, including those with both large and small gephyrin-ir cluster sizes (also see Combined Immunolabeling and Electrophysiology). The most plausible explanation is that there was a population of very small, slow rise time mIPSCs that are below detection threshold for reliable time-course measurements in the cells with small mean mIPSC amplitudes. In this situation, the distribution of small mIPSCs (Fig. 5C) would appear
as a scaled but truncated version of the large mIPSC distribution (Fig. 5D), and this appeared to be the case.

**Combined immunolabeling and electrophysiology**

The gephyrin immunolabeling and electrophysiological results demonstrate that there were large differences within and between cells in both mean gephyrin-ir cluster size and mean glycine mIPSC amplitude. We therefore performed experiments in which immunolabeling and electrophysiological measurements were obtained in the same cells. Recordings of mIPSCs were obtained in visualized ventral horn interneurons using patch pipettes containing Lucifer yellow or Neurobiotin (0.2 mg/ml) for subsequent identification of the cells. After recordings of mIPSCs, slices were fixed and immunolabeled for gephyrin and Lucifer yellow/Neurobiotin (see METHODS). Gephyrin-immunolabeling of recorded cells was always fainter than nonrecorded cells. In only 5 of 26 cells did we obtain gephyrin-immunolabeled clusters that were sufficiently bright and sensitive for accurate morphological analysis (Fig. 6). The reason for unsatisfactory immunolabeling for many identified cells is unknown but was not due to poor tissue penetration because excellent immunolabeling was obtained for the surrounding unidentified cells in the same sections.

Figure 6 illustrates a successfully labeled and identified cell together with the complement of gephyrin-ir clusters and mIPSC recordings. The cell exhibited small gephyrin-ir clusters (Fig. 6, B and C) and contained small mIPSC amplitudes (Fig. 6D). As illustrated in the histograms, the mean cluster area was 0.19 μm² and the mean mIPSC peak amplitude was 44 pA. A profuse coverage of gephyrin-ir clusters was observed over the soma and dendrites of this cell, indicating that glycineric synapses are not preferentially located at distal dendritic locations in cells with small mean mIPSC amplitudes (see **DISCUSSION**).

The results for five identified neurons with successfully combined gephyrin immunolabeling and electrophysiological recording of glycine mIPSCs are summarized in Fig. 7. All five cells exhibited both small gephyrin clusters and small mean mIPSC amplitudes. Figure 7A illustrates, for all five cells, that the variability in mIPSC amplitudes was greater than the variability in gephyrin-ir cluster size, as measured by a comparison of the coefficient of variation in these two parameters. The mean CV for cluster area (0.45) was 73% of the mean CV for

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**FIG. 6.** Combined gephyrin-immunolabeling and electrophysiological recording in an identified ventral horn interneuron. A and B: confocal images showing a reconstruction of the top half of a Neurobiotin-filled interneuron (A) and gephyrin-immunoreactivity for the same cell (B). To increase clarity, the complement of gephyrin-ir clusters on the surface of this cell was isolated from gephyrin clusters in the neighboring neuropil using an image algorithm in Adobe Photoshop 4.0. C and D: corresponding histograms demonstrate that this interneuron exhibited small gephyrin-ir clusters and small mIPSCs. Four example spontaneous currents are shown in D.
mIPSC amplitudes (0.62), measured in the same cells (n = 5, P < 0.05). This result is supported by our measurements in different cells of mean cluster size variability (0.55; n = 17) and mean mIPSC amplitude variability (0.85; n = 26), indicating that the CV for cluster areas was 65% of the CV for mIPSC amplitudes (P < 0.0001).

In Fig. 7B, the mean gephyrin cluster area is compared with the mean mIPSC amplitude for each cell. The shaded area indicates the ranges of values for cluster size and mIPSC amplitude obtained from our separate immunolabeling and electrophysiological experiments in unidentified cells. The shaded area thus represents the theoretically allowable range for mean cluster size and mean mIPSC amplitude, if there were no correlation between these two parameters. The results from our five successfully labeled and identified cells indicate that interneurons with small cluster areas jointly exhibited small mIPSC amplitudes.

**Discussion**

The size and density of receptor clusters at postsynaptic sites has been proposed as an important determinant of the functional properties of a synapse although there is little direct experimental evidence on a potential link between receptor morphology and synaptic efficacy (Nusser et al. 1997; Pierce and Mendell 1993; Schikorski and Stevens 1997; Walmsley et al. 1998). One of the most important parameters determining synaptic efficacy is the postsynaptic current generated in response to the presynaptic release of a quantum of transmitter. In the present experiments, we have obtained experimental evidence on the size and morphology of glycine receptor clusters and on the amplitude and time course of quantal glycineergic postsynaptic currents in rat ventral horn interneurons. Our results demonstrate that there is a considerable variability in the size of gephyrin-ir clusters over the surface of ventral horn interneurons in the rat spinal cord. This supports previous evidence on glycineergic synapses in the spinal cord of the adult cat (Alvarez et al. 1997). Interestingly, the results also indicate that there are considerable differences between interneurons in both mean cluster size and the range of cluster sizes. There is a strong positive correlation between mean cluster size and variability (standard deviation) in cluster size. That is, cells exhibiting a large mean cluster size also exhibit a large range of cluster sizes, whereas cells with a small mean cluster size exhibit only a restricted range of cluster sizes.

These structural observations are paralleled closely by our results on glycineergic mIPSCs recorded in ventral horn interneurons. Within a particular neuron, a large range of mIPSC amplitudes is observed. However, there is also a considerable difference in mean mIPSC amplitude between cells and a strong positive correlation between mean mIPSC amplitude and the range of mIPSCs recorded in the same cell. The range of mean peak currents obtained in our sample of lamina VII interneurons is greater than the range of mean cluster sizes, indicating that other factors such as receptor density may influence the size of the miniature postsynaptic current. In this context, it is interesting that clustering of receptor molecules has been proposed to alter the affinity of the glycine receptor for their ligands (Takagi et al. 1992).

A relationship between postsynaptic receptor cluster area and mIPSC amplitude has been proposed for GABAAergic synapses in cerebellar stellate cells (Nusser et al. 1997). Nusser et al. (1997) found that the distributions of mIPSC amplitudes and postsynaptic cluster sizes were similarly shaped non-Gaussian curves and concluded that the major contribution to GABAAergic mIPSC amplitude variability was site to site variation in the number of postsynaptic receptors. Our results also show highly skewed distributions of receptor cluster areas and mIPSC amplitudes at glycine synapses. Although this evidence does not prove a causal relationship between cluster area and mIPSC amplitude, such a relationship is supported further by our observations on the similarity in cell-to-cell variability in the distributions of glycine receptor cluster area and mIPSC amplitude. Furthermore, our combined immunolabeling and electrophysiological results clearly demonstrate that ventral horn interneurons with small receptor clusters jointly exhibit small mean mIPSC amplitudes.

In addition to site to site differences in receptor number, variability in the amplitude of mIPSCs may be due to a variety of other factors, such as electrotonic attenuation of mIPSCs, differences in receptor subunit composition between different sites, and intrinsic variability at each release site (Clements 1996; Takahashi et al. 1992; Walmsley 1995; Walmsley et al. 1998). Our immunolabeling and electrophysiological results demonstrate that the variability in mIPSC amplitudes is larger than the variability in cluster areas. Thus electrotonic attenuation and intrinsic variability also may contribute significantly to the variability of mIPSC amplitudes recorded in the same cell. Electrotonic influences should be evident in a negative correlation between mIPSC amplitude and rise time, which was observed in a proportion of cells. In all cases, this correlation was due to the influence of a population of small, slow mIPSCs (<50 pA). However, the slope of the relationship between rise time and mIPSC amplitudes above ~100 pA was not significantly different to zero. Thus electrotonic attenuation is unlikely to explain most of the range in mIPSC amplitudes. Kinetic and transmitter binding properties of the postsynaptic receptors may be different between interneurons due to either a different subunit composition or a different phosphorylation state of the receptors. With regard to subunit composition, Takahashi et al. (1992) have demonstrated a developmental correlation between subunit composition and IPSC decay time constant for rat spinal cord neurons. A developmental switch between fetal and adult forms of the glycine receptors has been shown to occur gradually in the spinal cord during the second...
postnatal week (Becker et al. 1988), and a gradual decrease in mean decay time was observed from 0 to 16 days postnatal (Takahashi et al. 1992). In the latter study, it was shown that a reduction in glycine IPSC decay time constant corresponded to a developmental reduction in the proportion of α2 to α1 glycine receptor subunits. Thus some of the cell-to-cell variability in mean mIPSC amplitude may be due to differences in the receptor properties between cells. Although there also may be a correspondence between cluster morphology and receptor subunit composition, the morphological differences observed in cluster size between cells are maintained in the adult spinal cord (Alvarez et al. 1997), well after the developmental switch from immature α2 to mature α1 subunits.

In summary, our results suggest that the large variability in glycine mIPSC amplitude within an individual ventral horn interneuron is due to a combination of site-to-site variability in receptor numbers, variable electrotonic attenuation, and intrinsic fluctuations at each release site. On the other hand, the large difference in mean mIPSC amplitudes among interneurons is likely to be due to a combination of differences in mean receptor cluster size and, to a lesser extent, receptor properties. The results provide additional support for a role of postsynaptic receptor clustering in the regulation of synaptic strength (Calverley and Jones 1990; Collinge and Froehniger 1998; Geinisman et al. 1996; Kirsch and Betz 1998; Nusser et al. 1997; Oda et al. 1995; Pierce and Mendell 1993; Schikorski and Stevens 1997; Siekewitz 1985; Walmsley 1991; Walmsley et al. 1998).

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