IPSC Kinetics at Identified GABAergic and Mixed GABAergic and Glycinergic Synapses onto Cerebellar Golgi Cells

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In the rat cerebellum, Golgi cells receive serotonin-evoked inputs from Lugaro cells (L-IPSCs), in addition to spontaneous inhibitory inputs (S-IPSCs). In the present study, we analyze the pharmacology of these IPSCs and show that S-IPSCs are purely GABAergic events occurring at basket and stellate cell synapses, whereas L-IPSCs are mediated by GABA and glycine. Corelease of the two transmitters at Lugaro cell synapses is suggested by the fact that both GABA_A and glycine receptors open during individual L-IPSCs. Double immunocytochemical stainings demonstrate that GABAergic and glycinergic markers are coexpressed in Lugaro cell axonal varicosities, together with the mixed vesicular inhibitory amino acid transporter. Lugaro cell varicosities are found apposed to glycine receptor (GlyR) clusters that are localized on Golgi cell dendrites and participate in postsynaptic complexes containing GABA_A receptors (GABA_AR) and the anchoring protein gephyrin. GABA_AR and GlyR/gephyrin appear to form segregated clus-

ters within individual postsynaptic loci. Basket and stellate cell varicosities do not face GlyR clusters. For the first time the characteristics of GABA and glycine cotransmission are compared with those of GABAergic transmission at identified inhibitory synapses converging onto the same postsynaptic neuron. The ratio of the decay times of L-IPSCs and of S-IPSCs is a constant value among Golgi cells. This indicates that, despite a high cell-to-cell variability of the overall IPSC decay kinetics, postsynaptic Golgi cells coregulate the kinetics of their two main inhibitory inputs. The glycinergic component of L-IPSCs is responsible for their slower decay, suggesting that glycinergic transmission plays a role in tuning the IPSC kinetics in neuronal networks.

Key words: serotonin; cerebellum; GABA; glycine; VIAAT; GlyT2; inhibitory cotransmission; Golgi cell; Lugaro cell; receptor

GABAergic and glycinergic neurotransmission coexist in many structures of the CNS. Colocalization of the two neurotransmitters is prominent in the spinal cord (Todd and Sullivan, 1990; Ornung et al., 1994; Taal and Holstege, 1994; Yang et al., 1997) and other sensory and motor centers (Wenthold et al., 1987; Ottersen et al., 1988; Wentzel et al., 1993). The observation of GABAergic terminals facing glycine receptor (GlyR) clusters (Triller et al., 1987) and of mixed boutons presynaptic to domains containing GABA_A receptor (GABA_AR) and probably GlyR (Todd et al., 1996) first suggested that the two transmitters might be coreleased at the same synapse. Indeed, coactivation of the GABA_AR and GlyR at a single contact, and by the content of a single vesicle, was demonstrated recently at interneuron–motoneuron connections (Jonas et al., 1998; O'Brien and Berger, 1999). Copackaging of the two neurotransmitters in vesicles is probably achieved by a single vesicular inhibitory amino acid transporter (VIAAT) (McIntire et al., 1997; Sagné et al., 1997).

In the spinal cord and brainstem, the only places where cotransmission of GABA and glycine is formally demonstrated (Jonas et al., 1998; O'Brien and Berger, 1999), pure glycinergic transmission and pure GABAergic transmission also occur (Ornung et al., 1994; Yang et al., 1997). Thus, networks of inhibitory interneurons using GABA and glycine as their transmitters have a complex and versatile functional organization. Analyzing this organization at the cellular and synaptic levels is a prerequisite to understand the specific roles played by GABA and glycine. The cerebellar cortex provides a good model for this study, because it is composed of a small number of well defined cell types (Palay and Chan-Palay, 1974) and contains both neurotransmitters. GABA is the predominant inhibitory neurotransmitter in this structure, but glycine is also found in inhibitory interneurons of the granule cell layer and in fibers of the molecular layer (Wilkin et al., 1981; Ottersen et al., 1987, 1988). The neuronal glycine transporter GlyT2 (Borowsky et al., 1993; Liu et al., 1993), a marker for glycinergic neurons (Poyatos et al., 1997; Spike et al., 1997), is expressed in the granular layer, in Golgi cell axonal varicosities, and in some varicose fibers of the molecular layer (Zafra et al., 1995). Several GlyR subunit mRNAs are detected in the cerebellar cortex (Malosio et al., 1991; Racca et al., 1998), and functional glycine receptors are present in immature granule cells (Kaneda et al., 1995) and in Golgi cells (Dieudonné, 1995).

In a previous study (Dieudonné and Dumoulin, 2000) we demonstrated the existence of a new connection between the cerebellar Lugaro cells and the Golgi cells. In the present work we show that Golgi cells receive both pure GABAergic inhibition

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from basket cells (S-IPSCs) and mixed GABAergic and glycinergic inhibition from Lugaro cells (L-IPSCs). Lugaro cells corelease the two transmitters on apposed postsynaptic arrays of GABA_A and glycine receptors, the kinetics of which is coregulated by the postsynaptic Golgi cell. Therefore, the specific functions of GABA and glycine transmission may result from the different kinetics of their IPSCs.

MATERIALS AND METHODS

Slice preparation. Cerebellar thin slices were prepared from male Wistar rats, aged 11–21 d, following the method originally described by Linás and Sugimori (1980) with slight modifications (Llano et al., 1991). Briefly, the cerebellum was dissected and immediately cooled to 0°C. A parasagittal cut was made in the paravermis, and parasagittal slices (180–300 μ m thick) were cut from the vermis with a microslizer (Dosaka, Kyoto, Japan, or Leika, Nussloch, Germany). The slices were kept at 34°C for 1–9 hr before being transferred to the recording chamber. On some occasions slices were allowed to cool slowly from 34°C to room temperature 1 hr after slicing.

Slices were visualized using a 40 $\times$ water-immersion objective (0.75 numerical aperture; Axioskop; Carl Zeiss) and infrared optics (illumination filter of 750 nm and a Sony CCD camera from which the infrared blocking filter had been removed). Golgi cells were selected for recording both on visual criteria, as explained previously (Dieudonné, 1995), and on the basis of their characteristic passive electrical properties (Dieudonné, 1998). Recordings were restricted to the Golgi cells of lobules I–VIII. The lobules IX and X, which belong functionally to the vestibulocerebellum, were discarded for this study to limit the variability associated with the specialization of the different regions of the cerebellar cortex.

Patch-clamp recording. All experiments were performed at room temperature (20–25°C). The recording chamber was continuously perfused at a rate of 1.5 ml/min with a saline solution, pH 7.4, containing (in mM): 125 NaCl, 2.5 KCl, 2 CaCl₂, 1 MgCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃, and 25 glucose, bubbled with a mix of 95% O₂ and 5% CO₂. The same solution was used during dissection and slicing. Strychnine (Sigma, St. Louis, MO) and gabazine (Research Biochemicals, Natick, MA) were bath applied. Serotonin (Sigma) was also bath applied, usually for 3 min, and washed for at least 15 min between applications to avoid desensitization.

Cells were recorded with an Axopatch 200B (Axon Instruments) in the voltage-clamp mode of the amplifier and held at –70 mV. The access resistance and pipette capacitance were carefully compensated. Pipettes had a resistance of 2–4 M Ω and were filled with the following internal solution (in mM): 142 CsCl, 10 HEPES, 1 EGTA, 5 MgCl₂, 0.1 CaCl₂, 4 Na-ATP, and 0.4 Na-GTP; pH was adjusted to 7.3 with *N*-methyl-D-glucamine. Recordings of spontaneous synaptic activity were stored on a digital audio tape (DTR-1204; BioLogic) and digitized off-line.

Electrical stimulations were performed using a patch pipette (3 M Ω) filled with the following solution (in mM): 140 NaCl, 2.5 KCl, 2 CaCl₂, 1 MgCl₂, and 30 HEPES; pH was adjusted to 7.3 with NaOH. Minimal stimulations of basket cell axons could be obtained in the vicinity of Purkinje cell bodies with voltage pulses of a few volts and 30–50 μ sec duration.

Data analysis. PClamp softwares (Axon Instruments) were used for the acquisition of all recordings. Data were filtered at 2 kHz and digitized at 10 kHz. Spontaneous synaptic currents were detected automatically using the ACS software kindly provided by P. Vincent (Institut des Neurosciences, Paris). All detected events were subjected to visual inspection, and EPSCs were discarded on the basis of their fast decay time course [their time constant is faster than 3 msec (Dieudonné, 1998)]. Selected events could then be transferred in a file with the format of clampex files and further analyzed using the Clampfit program of the PClamp package. To fit the decay of the IPSCs, the offset was forced to 0 pA, and two exponential functions were tried first. The fit was accepted when the ratio of the time constants was >3 or when it was between 2.5 and 3, if the two components were of comparable amplitude (20–80% of the decay). In other cases a single exponential function was fitted to the data. More precise values for the time constants of decay were obtained by fitting the integral of the averaged IPSCs with one or two exponential functions. These values were used for the linear regression plots (see Fig. 8).

Fluorescence immunocytochemistry. Female Sprague Dawley rats (200 gm; Janvier) were deeply anesthetized with pentobarbital (60 mg/kg

body weight, i.p.) and perfused intracardially with 4% paraformaldehyde (PFA) in PBS, pH 7.4. The cerebellum was removed, post-fixed for 12–15 hr in 4% PFA, and cut into 30- μ m-thick sections with a vibratome in sagittal or transverse planes. Sections were immersed for 20 min in 50 mM ammonium chloride in PBS and rinsed. An additional fixation with a mixture of methanol:acetic acid (95:5) for 10 min at –20°C was performed when using the mAb 4a. After a preincubation step of 30 min in 0.1% gelatin and 0.1% Triton X-100 in PBS, overnight incubation was performed at 4°C in the latter solution with a monoclonal anti-GlyR α /b antibody alone (mAb 4a; 1:200) (Pfeiffer et al., 1984) or in combination with a polyclonal antibody against (1) VIAAT (1:500) (Dumoulin et al., 1999), (2) the GABA_AR γ 2 subunit (1:100) (Somogyi et al., 1996), (3) calretinin (1:2000; Swant, Bellinzona, Switzerland) (Schwaller et al., 1993), or (4) GlyT2 (1:2500; Chemicon). The anti-GABA_AR γ 2 antibody was also combined with (5) a monoclonal anti-gephyrin antibody (mAb 7a; 1:200; Boehringer Mannheim, Indianapolis, IN) (Pfeiffer et al., 1984). A monoclonal anti-GAD65 antibody (1:500; Boehringer Mannheim) (Gottlieb et al., 1986) was combined with (6) the anti-calretinin antibody or (7) the anti-GlyT2 antibody. Finally, two of the polyclonal antibodies, raised in different species, were combined: the anti-calretinin antibody (rabbit) and the anti-GlyT2 antibody (goat) (8). After rinses, sections were incubated for 2 hr with the secondary antibodies. The mAb 4a was revealed by an indocarbocyanine (CY3)-coupled goat anti-mouse IgG. For the revelation of different combinations (1–8), the CY3-coupled goat anti-mouse IgG was associated with an FITC-coupled goat anti-rabbit IgG for combination 1 or with a biotin-coupled goat anti-rabbit IgG for combinations 2, 3, and 6. For the latter combinations, an additional incubation of 90 min in FITC-coupled streptavidin was performed after the rinses. For combination 4, we used a CY3-coupled donkey anti-mouse IgG and an FITC-coupled donkey anti-sheep IgG; for combination 5, we used an FITC-coupled goat anti-mouse IgG and a CY3-coupled goat anti-rabbit IgG; and for combination 7, we used an FITC-coupled horse anti-mouse IgG and a CY3-coupled donkey anti-sheep IgG. Finally, combination 8 was revealed using an FITC-coupled donkey anti-rabbit IgG and a CY3-coupled donkey anti-sheep IgG. All secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were used at 1:200. Sections were mounted with Vectashield (Vector Laboratories, Burlingame, CA) before examination with a standard epifluorescent microscope (Leica DMR). No staining was observed when primary antibodies were omitted. In addition, sections from spinal cord were used as a control for the specificity of antibody labeling.

Immunocytochemical data quantification. To investigate the proportion of Lugaro axonal varicosities that were GABAergic, glycinergic, or both, we quantified the results of the calretinin–GAD65, calretinin–GlyT2, and GlyT2–GAD65 double immunostainings. Only Lugaro cell varicosities that could be identified along a fiber were counted on digitalized images. The images corresponding to the second marker were then superimposed, and the colocalization was analyzed. In each case, between 150 and 200 varicosities (*n*) were counted, and the results are expressed as a percentage. We similarly quantified on GlyT2–GlyR α /b double immunostainings the proportion of Lugaro axonal varicosities associated with the GlyR and, conversely, the proportion of GlyR clusters associated with GlyT2-positive terminals.

Double stainings of GlyR α /b and GABA_AR γ 2 subunits revealed an association without colocalization of the two subunits. The absence of colocalization is not caused by an image shift between the two wavelengths. First, strict colocalizations are displayed (see Fig. 4, all panels). Second, as noted in Results, GABA_AR γ 2 clusters were often surrounded by, and not juxtaposed to, GlyR α /b clusters. Extensive quantification of the apposition could not be achieved for two technical reasons. First, because of the uneven detection of GABA_AR γ 2 clusters in the sample, when combined with methanol fixation, areas with strong staining had to be selected for observation. Second, proper quantification should involve a measure of the distance between clusters along Golgi cell dendrites, which is impossible to perform without staining the dendrite itself.

RESULTS

Pharmacological profile of spontaneous and serotonin-evoked IPSCs recorded from Golgi cells

IPSCs were recorded from Golgi cells in the whole-cell configuration of the patch-clamp technique. As described previously (Dieudonné and Dumoulin, 2000), Golgi cells receive a spontaneous inhibitory input composed of small IPSCs (S-IPSCs) oc-

curing at low frequency and a serotonin (5-HT)-dependent input composed of large IPSCs evoked by bath application of serotonin (L-IPSCs). Whereas L-IPSCs can be attributed to the activation by 5-HT of the Lugaro neurons, a neglected interneuron type of the cerebellar cortex (Lugaro, 1894), the synaptic origin of S-IPSCs is not known. A characteristic feature of L-IPSCs is their occurrence at regular intervals, which reflect the rhythmic firing of presynaptic Lugaro cells at several Hertz. The periodicity of L-IPSCs and, in many cells, their large amplitude allow them to be distinguished from S-IPSCs, which are always small and occur at random intervals. In any cell the frequency of L-IPSCs can be calculated simply by subtracting the frequency in the baseline, before serotonin application, from the frequency of IPSCs in the presence of serotonin (Dieudonné and Dumoulin, 2000).

To determine the nature of the receptors involved in S-IPSCs and in L-IPSCs, we performed bath applications of gabazine (20–30 μ M) and strychnine (0.3–1 μ M), which are selective inhibitors of GABA_A and glycine receptors, respectively, in Golgi cells (Dieudonné, 1995). Gabazine blocked entirely the S-IPSCs in 11 of 12 Golgi cells (Fig. 1*A*). In the remaining cell the gabazine-resistant S-IPSCs were small (<30 pA). Their frequency matched the frequency of a distinct population of large IPSCs (recorded in control conditions), which were similar in amplitude to L-IPSCs evoked in this cell by serotonin (data not shown). This suggests that gabazine-resistant S-IPSCs in this cell represent the spontaneous activity of a presynaptic Lugaro cell.

To strengthen the conclusion that the vast majority of S-IPSCs are purely GABAergic, lower concentrations (2 μ M) of gabazine were used in two cells. The frequency of S-IPSCs was decreased from 10 to 0.07 Hz, and the mean amplitude of the remaining IPSCs was 3.4 times smaller than that of control IPSCs. The threshold for the detection of the IPSCs was set at –6 pA, at which the simultaneous opening of two glycine receptors would be detected. It can be concluded that >99% of the S-IPSCs are mediated by GABA_A receptors only.

When serotonin was applied in the continuous presence of 30 μ M gabazine, it evoked IPSCs in 11 of 14 Golgi cells (Fig. 1*A*). These 5-HT-evoked IPSCs were blocked by 300 nM strychnine ($n = 3$), confirming that they are glycinergic IPSCs (Fig. 1*B*). Their mean amplitude was 34 ± 20 pA (mean $\pm$ SD; $n = 10$). Reciprocally, serotonin evoked GABAergic IPSCs in 9 of 13 Golgi cells recorded in the presence of 1 μ M strychnine (Fig. 1*C*). These L-IPSCs had a mean amplitude of 90 ± 80 pA (mean $\pm$ SD; $n = 9$). Therefore Golgi cells receive purely GABAergic spontaneous IPSCs and glycinergic (L-IPSCs_{Gly}) and GABAergic (L-IPSCs_{GABA}) IPSCs evoked by serotonin.

Basket cell→Golgi cell synapses are GABAergic

Synapses between basket cells and Golgi cells have been unequivocally identified at the electron microscopic level (Palay and Chan-Palay, 1974). In a previous paper (Dieudonné and Dumoulin, 2000), we proposed that S-IPSCs recorded in Golgi cells reflect the release of transmitter at these synapses, either miniature or evoked by the spontaneous firing of basket cells in the slice (Vincent et al., 1992; Kondo and Marty, 1998). Extracellular electrical stimulations have been used to confirm this point.

At the age of the animals used in this study, the pinceau-like structures around the cell body and the initial axonal segment of the Purkinje cells are not yet formed, but basket cell axons have already converged onto Purkinje cell bodies (Altman and Bayer, 1997). A relatively selective stimulation of basket cell axons can

Figure 1. Pharmacological characterization of the S-IPSCs and of the L-IPSCs recorded from Golgi cells. *A*, Gabazine blocks spontaneous IPSCs but not serotonin-evoked IPSCs. The *top* trace represents 5 min of recordings during which gabazine (30 μ M) and serotonin (1 μ M) were bath applied at the time indicated by horizontal bars. The asterisk and the filled circle indicate the time at which the *bottom* traces, displayed at a faster time scale, were taken. CNQX (3 μ M) was present during the whole experiment to block all EPSCs. *B*, Summary of a similar experiment in another cell illustrates that strychnine blocks L-IPSCs induced in the presence of gabazine. The cumulative amplitude represents the sum of the peak amplitudes of the IPSCs. *C*, GABAergic L-IPSCs can be evoked in the presence of strychnine. The *top* trace represents 4 min of continuous recording in the presence of strychnine and during application of serotonin (1 μ M). *Bottom* traces display the inhibitory activity, at a faster time scale, at the times indicated by the asterisk and filled circle. In this and the following figures, all of the cells were recorded in the whole-cell configuration of the patch-clamp technique with a CsCl-based internal solution and were held at –70 mV.

thus be obtained by placing the stimulating electrode at the apex of the Purkinje cell bodies. Low-intensity stimulations at this location could in all instances evoke IPSC in Golgi cells (Fig. 2*A*), even when the stimulation electrode was moved to Purkinje cell bodies located 200–300 μ m away from the recorded Golgi cell. Increasing the stimulation intensity induced the recruitment of multiple fibers, indicating the presence of several presynaptic axons in the vicinity of the stimulating electrode (Fig. 2*B*). Attempts to evoke IPSCs from random points of the low molecular layer and in regions of the Purkinje cell layer where Purkinje cell bodies had not been preserved were not always successful, even when using high-intensity stimulations. The electrically evoked IPSCs, as S-IPSCs, were completely blocked by gabazine (Fig. 2*C*). We conclude that basket cells form functional inhibitory contacts with Golgi cells and that the corresponding IPSCs are entirely mediated by GABA_A receptors.

Figure 2. Characterization of the basket cell IPSCs. *A*, Four examples of the IPSCs evoked in a Golgi cell by electrical stimulation of the basket cell axons in the molecular layer just at the top of a Purkinje cell body are shown. *B*, Recruitment curve at the same location shows that the amplitude of the IPSCs continues to increase in discrete steps with the stimulation intensity, even when the probability of failure is null. Multiple fibers were recruited at this location. *C*, Gabazine blocks basket cell IPSCs. Averaged traces of IPSCs before (*top*), during (*middle*), and after (*bottom*) bath application of gabazine (30 μ M) are shown.

Unitary L-IPSCs involve the activation of both GABA_A and glycine receptors

The mixed sensitivity of the L-IPSCs to gabazine and strychnine contrasts with the pure GABAergic nature of the S-IPSCs and suggests that GABA and glycine can both be released by Lugaro cells. It has been shown that a small number of Lugaro cells presynaptic to a given Golgi cell are preserved in slices (Dieudonné and Dumoulin, 2000). In many cases a Golgi cell receives the input from zero or one Lugaro cell (Dieudonné and Dumoulin, 2000). Nevertheless, similar proportions of Golgi cells receive L-IPSCs_{Gly} in the presence of gabazine (79% of 14 cells tested), L-IPSCs_{GABA} in the presence of strychnine (70%; $n = 13$), or L-IPSCs when serotonin is applied in the absence of inhibitors (73%; $n = 26$). This suggests that even when a single presynaptic Lugaro cell is preserved, this cell is able to release both GABA and glycine.

In three cells in which gabazine was added during the application of 5-HT, a glycinergic component of the IPSCs remained. These glycinergic IPSCs disappeared after washout of serotonin from the bath, confirming that they represent L-IPSCs. The converse experiment, that is, applying strychnine during the effect of serotonin, is more difficult to analyze because S-IPSCs are not blocked by strychnine and are mixed with L-IPSCs, the amplitude of which has been decreased by strychnine. In two of these experiments, in which S-IPSCs were sufficiently infrequent to be neglected, strychnine blocked by 28 and 32% the amplitude of the L-IPSCs without any significant effect on their frequency (98 and 101%). These experiments indicate that most L-IPSCs involve the activation of both GABA_A and glycine receptors and that cotransmission is thus the rule at Lugaro cell→Golgi cell synapses.

To confirm this point, we analyzed in detail an experiment (Fig. 3) in which a single presynaptic Lugaro cell was activated by serotonin. In this Golgi cell, the amplitude of L-IPSCs was so much larger than the amplitude of S-IPSCs that they could easily and reliably be discriminated (Fig. 3*A,C*). Whereas >98% of the

S-IPSCs were <60 pA (Fig. 3*B*, *top*, *arrows*), the amplitude of L-IPSCs could be as large as 200 pA. As expected, S-IPSCs during the control period and small (<60 pA) IPSCs during serotonin application occurred both at random intervals and at similar frequencies (Fig. 3*D*). In contrast, the distribution of the intervals between L-IPSCs (IPSCs > 60 pA in the presence of serotonin) was well described by two Gaussian peaks with a preferred frequency of 5.6 Hz (Fig. 3*E*, *left*). This implies that a single presynaptic Lugaro cell is responsible for all of the serotonin-evoked L-IPSCs [see Dieudonné and Dumoulin (2000) for the validity of this criterion]. Although S-IPSCs are not mistaken for L-IPSCs with this threshold method, the contrary is not true because 21% of the large IPSCs were separated by two times the elementary interval (second Gaussian peak), indicating that 17% of the L-IPSCs were <60 pA and have been pooled with S-IPSCs.

In this cell, application of gabazine, after the effect of serotonin had reached its steady state, reduced the frequency of occurrence (Fig. 3*A*, *bottom*) of the IPSCs. As expected, spontaneous and serotonin-evoked activities were differentially affected. Gabazine completely blocked the small and random S-IPSCs, accounting for the reduced frequency of IPSCs, but it decreased the frequency of the rhythmic L-IPSCs only from 5.8 to 5 Hz (Fig. 3*B*). The intervals between the remaining (glycinergic) L-IPSCs showed a multi-Gaussian distribution with equidistant peaks separated by 135 msec, corresponding to a preferred frequency of 7.4 Hz (Fig. 3*E*, *right*). This increase in the rhythmic frequency is caused by a disinhibition of the presynaptic Lugaro cell by gabazine (S. Dieudonné, unpublished results). Inter-IPSC intervals of two and three times the 135 msec period result from the fact that the glycinergic component of some of the IPSCs is so small that it falls under the detection threshold. The number of events in the second and third peaks of the histogram indicates that only 32% of the rhythmic events fell under the detection threshold (–10 pA; which is four glycine channel openings) and that for most of them the sum of two or three glycine channel openings seems to occur (Fig. 3*B*, *asterisks*). These results indicate that for the majority of the L-IPSCs both GABA and glycine are coreleased by the presynaptic Lugaro neuron.

Whether activation of both GABA_AR and GlyR occurs at the same synaptic contacts cannot be inferred from these experiments. Because miniature glycinergic IPSCs are not recorded in this preparation, we could not use them to study whether a single vesicle can release GABA and glycine, as was done in the spinal cord (Jonas et al., 1998) and brainstem (O'Brien and Berger, 1999). We therefore performed immunohistochemical stainings to determine whether markers for GABA and glycine transmission are colocalized at all Lugaro cell→Golgi cell contacts or whether they are segregated at separate subsets of contacts.

Lugaro cell axon terminals express both GABAergic and glycinergic markers

Lugaro cells have typical ovoid somata lying in the upper granular layer, with long dendrites running in the parasagittal plane below and between the Purkinje cell bodies (Lainé and Axelrad, 1996; Dieudonné and Dumoulin, 2000). Two axonal plexi have been described, a parasagittal one that contacts basket cells, stellate cells, and possibly Golgi cells (Lainé and Axelrad, 1996, 1998) and a transverse one, oriented in the same direction as the parallel fibers, that establishes synaptic contacts with Golgi cells and possibly with basket and stellate cells (Dieudonné and Dumoulin, 2000). In a previous study, we demonstrated that this

Figure 3. GABA and glycine are coreleased by single Lugaro cells onto Golgi cells. *A*, The effect of serotonin (1 μ M) on the inhibitory synaptic activity recorded from a Golgi cell is summarized in the *top plot*, where each *dot* represents an IPSC. The mean frequency of IPSCs over bins of 5 sec is plotted at the same time scale in the *bottom graph*. Note the monophasic increase in the amplitude and frequency of the IPSCs after application of serotonin. Gabazine (30 μ M) decreased both the amplitude and the frequency of the IPSCs in the presence of serotonin. *B*, Representative sections (2.5 sec) of recording taken before (*top*; 4 min time point in *A*) and after (*bottom*; 5 min time point in *A*) gabazine application are shown. In the presence of gabazine, IPSCs under the threshold of detection (10 pA) are indicated by an *asterisk*. *C*, Amplitude histograms of the IPSCs during the control period (*left*) and in the presence of 5-HT (*right*) are shown. *D*, Histograms show the distribution of the intervals between two successive events during the control period (*left*) and for the IPSCs <60 pA in the presence of serotonin (*right*). *E*, The same histograms for the IPSCs >60 pA in the presence of serotonin (*left*) and for all the IPSCs recorded after addition of gabazine (*right*) are shown.

transverse plexus of Lugaro axons is composed of thick, calretinin-immunoreactive (-IR) varicose fibers running in the low molecular layer (Dieudonné and Dumoulin, 2000). Calretinin detection was therefore performed in a set of double-immunohistochemistry experiments performed on sections of the vermis cut transversally to the parasagittal plane, parallel to the axis of the folia. The majority of the varicosities (90.4%; $n = 188$) of the calretinin-positive axons were found to be immunoreactive for GAD65 (Fig. 4*A*), as suggested by previous observations indicating a GABAergic phenotype of Lugaro cells (Aoki et al., 1986; Gabbott et al., 1986; Dieudonné and Dumoulin, 2000), and 90.7% ($n = 152$) of them were also positive for the neuronal membrane glycine transporter GlyT2 (Fig. 4*C*). The varicosities identified by their GlyT2-IR were also immunoreactive for GAD65 (90.4%; $n = 168$) (Fig. 4*D*).

The Lugaro cell parasagittal axonal plexus could not be unambiguously identified using anti-calretinin antibodies, because of the small diameter of its fibers and of a background staining of the parallel fibers. This background is very faint in the low molecular layer, where Lugaro transverse axons run, but becomes more important in the rest of the molecular layer, where the parasagittal Lugaro plexus extends (Fig. 4*A*). However, the parasagittal axons could be identified by their GlyT2 immunoreactivity as a sparse plexus of thin parasagittal fibers that can be noticed in both parasagittal and transverse sections (Fig. 4*E*). The varicosities of the latter plexus were also GAD65-IR (87.3%; $n = 182$) (Fig. 4*E*). Moreover, all GAD65-labeled terminals in transverse or sagittal planes were immunoreactive for VIAAT (data not shown), the mixed GABA and glycine vesicular transporter present in GABAergic, glycinergic, and mixed GABA- and glycine-containing terminals (Chaudhry et al., 1998; Dumoulin et al., 1999). Thus, the Lugaro cell is a mixed inhibitory neuron that possesses adequate machinery for GABA and glycine accumulation in, and release from, axonal varicosities.

Cerebellar Golgi cells express glycine receptors

Because Golgi cells receive a glycinergic input, we have investigated the distribution of GlyR in the cerebellar cortex in sections

of the vermis cut in the sagittal plane, which allows the best visualization of the Golgi cell dendritic arborization. Immunoreactivity for GlyR α/β was found in all cerebellar lobules, in both granular and molecular layers (Fig. 5*A*). In the granular layer, GlyR α/β immunoreactivity was detected at some glomerular structures, most presumably corresponding to dendrites of unipolar brush cells (UBCs) (Mugnaini et al., 1994), and on neurons of the granular layer, the neuritic processes of which extended into the molecular layer. These latter neurons had the characteristic morphology and distribution of Golgi cells (Fig. 5*B*). A diffuse cytoplasmic staining was observed within their cell body (Fig. 5*C*), and both basolateral and apical dendrites displayed a "punctated" GlyR α/β labeling (Fig. 5*B,D*). In the molecular layer, Golgi cell dendrites were the only GlyR α/β -IR profiles. They could often be followed from the cell body to their end at the pial surface (Fig. 5*B*). In this layer, simultaneous detection of GlyR α/β and VIAAT, used as a specific marker of inhibitory terminals, showed a close apposition between the two immunoreactivities (Fig. 5*E*). This indicated that GlyR-IR clusters have a postsynaptic location on Golgi cell dendrites; Golgi cells are therefore the only ubiquitous cell type in the cerebellar cortex to receive an inhibitory glycinergic input (UBCs, associated with vestibular afferents, are concentrated in specific lobules).

Simultaneous detection of glycine and GABA_A receptors on Golgi cell dendrites

Because Lugaro cells release both GABA and glycine, we studied whether GABA_ARs colocalized with GlyRs on the dendrites of Golgi cells. An antibody against the GABA_AR γ 2 subunit was chosen for this experiment because this subunit is involved in the postsynaptic localization of major GABA_AR subtypes (Essrich et al., 1998). In agreement with previous reports in the literature (Fritschy et al., 1992; Gutiérrez et al., 1994; Sassoe-Pognetto et al., 2000), GABA_AR γ 2 immunoreactivity was found in the three layers of the cerebellar cortex (data not shown). In parasagittal sections of the vermis, in which Golgi cell dendrites can be followed throughout their course in the molecular layer, simultaneous detection of GlyR α/β and GABA_AR γ 2 subunits showed

Figure 4. Immunocytochemical characterization of Lugaro cell transverse and sagittal axons in the molecular layer. Transverse (*A–D*) or sagittal (*E*) sections were double-stained with anti-calretinin antibody (green in *A1*, *B1*, *C1*) and anti-GAD65 (red in *A2*, *B2*) or anti-GlyT2 (red in *C2*) or with anti-GAD65 antibody (green in *D1*, *E1*) and anti-GlyT2 (red in *D2*, *E2*). *A3*, *B3*, *C3*, *D3*, and *E3* are the superimposed images of *A1* and *A2*, *B1* and *B2*, *C1* and *C2*, *D1* and *D2*, and *E1* and *E2*, respectively. *A*, Low magnification of a transverse section immunoreacted with calretinin (CR) and GAD65 is shown. Lugaro cell axons (arrowheads) run in the low molecular layer (low molec) of the cerebellar cortex, above the Purkinje cell bodies (*P*) that are surrounded by GABAergic terminals (arrows). Note the stronger background for the CR staining (asterisk) in the upper molecular layer. *B*, Varicosities of the calretinin-labeled transverse axons of the Lugaro cell show GAD65 immunoreactivity (arrowheads). Arrows indicate GAD65-positive terminals devoid of calretinin. *C*, Transverse Lugaro cell axon stained for GlyT2 is shown. This is mainly visible in its varicosities (arrowheads). Thin fibers, probably parallel fibers, ascending in the molecular layer show faint calretinin immunoreactivity but are GlyT2 negative (arrows). *D*, GAD65 is also detected in GlyT2-immunoreactive axonal varicosities (arrowheads). In a few GlyT2-positive spots, GAD65 is not colocalized (double arrowheads). Because of their smaller diameter compared with double-stained varicosities, they most likely represent nonvaricose segments of Lugaro cell parasagittal axons cut transversally. *E*, Expression of GAD65 within varicosities stained for GlyT2 in the sagittal plane is shown. Note that in both transverse (*D1*) and sagittal (*E1*) sections, terminals positive for GAD65 only (arrows) outnumber varicosities coexpressing GABA and glycinergic markers (arrowheads), indicating the presence of numerous purely GABAergic boutons. Scale bars: *A*, 10 μ m; *B–E*, 10 μ m.

that the two immunoreactivities were associated on Golgi cell dendrites (Fig. 6*A*). However, the two receptors displayed a variegated pattern of immunoreactivities rather than an exact overlapping (Fig. 6*A*, inset). GlyR-IR clusters without apparent

association with GABA_AR immunoreactivity were exceptionally found on Golgi cell dendrites (data not shown). This may result from reduced GABA_AR γ 2 epitope detection by this antibody when an additional methanol fixation of the sections was used to

Figure 5. Glycine receptor immunoreactivity of the cerebellar Golgi cell. *A*, Low-magnification micrograph of a cerebellar section cut in the sagittal plane and immunoreacted with the mAb 4a. Golgi cell somata (arrows) in the granular layer and dendrites (arrowheads) in both granular and molecular layers appear stained for GlyR α/β , as well as some glomerular structures of the granular layer (crossed arrows). *B*, High magnification of a Golgi cell (reconstruction) located in the upper granule cell layer, showing diffuse staining of the cell body (arrow) and punctate labeling over the dendritic tree in the molecular layer (arrowheads). Note that only Golgi cell dendrites are stained in the molecular layer. The asterisk indicates a Golgi cell body out of focus. *C*, Diffuse intracellular staining of the Golgi cell body and initial dendritic portion, in contrast with the intense, punctiform labeling of dendrites (arrowheads). Note that the nucleus (*n*) is unstained. *D*, Labeled dendrites of Golgi cells in the molecular layer (arrowheads). *E*, Double immunodetection of GlyR α/β (*E1*) and VIAAT (*E2*) in the molecular layer. The slight shift in the immunoreactivity of the two markers indicates their close apposition (arrowheads show the location of VIAAT-positive presynaptic terminals). VIAAT-labeled terminals (arrows) not associated with glycine receptor clusters are thought to be involved in the numerous GABAergic synapses in the molecular layer. *g*, Granular cell layer; *m*, molecular layer; *P*, Purkinje cell layer; *w*m, white matter. Scale bars: *A*, 50 μ m; *B*, 25 μ m; *C*, 10 μ m; *D*, 20 μ m; *E*, 5 μ m.

detect GlyR immunoreactivity, as compared with paraformaldehyde fixation alone.

Gephyrin is a cytoplasmic protein associated with, but not exclusively located at, glycinergic synapses in the CNS (Sassoe-Pognetto et al., 1995, 1999, 2000; Craig et al., 1996; Giustetto et al., 1998; Lévi et al., 1999). In contrast with what is seen for GlyR, immunodetection of gephyrin showed a widespread labeling of the molecular layer that was not selective for Golgi cell dendrites. Dendrites outlined by gephyrin-IR clusters could be followed from the Golgi cell body to the molecular layer but were often lost

there because of the staining of multiple profiles in this layer (data not shown). Double detection showed association, but not colocalization, of GABA $_A$ R γ 2 immunoreactivity with gephyrin-positive clusters in profiles that had the course and location of Golgi cell dendrites between or just above Purkinje cell bodies (Fig. 6*B*).

From these results, we conclude that cotransmission involving GABA $_A$ R and GlyR occurs at single synaptic sites on Golgi cell dendrites. The juxtaposition of the two receptors on Golgi cell dendrites in both the low and the upper molecular layer indicates

Figure 6. *A, B*, Colocalization of GlyR and gephyrin with GABA_AR clusters at Golgi cell dendrites in the low molecular layer is shown. Sagittal sections were double-stained with anti-GABA_ARγ2 antibody (green in *A1*; red in *B1*) and anti-GlyRα/β (red in *A2*) or anti-gephyrin (green in *B2*) antibodies. *A3* and *B3* are the superimposed images of *A1* and *A2* and of *B1* and *B2*, respectively. *A*, Clusters positive for the two receptors are intermingled at single postsynaptic sites (*A3*, arrowheads). *B*, Clusters of GABA_ARγ2 are closely associated to, but do not colocalize with, gephyrin aggregates at putative postsynaptic sites on Golgi cell dendrites (arrowheads). In the insets (solid rectangles) of *A3* and *B3*, a 2.5× magnification of a region where the labeling is representative (dashed rectangle) is shown enlarged. *C–E*, Lugaro to Golgi cell axonal contacts are shown. Transverse (*C, D*) or sagittal (*E*) sections were double-stained with anti-calretinin (green in *C1*) or anti-GlyT2 (green in *D1, E1*) antibodies and with an anti-GlyRα/β antibody (red in *C2, D2, E2*). *C3, D3*, and *E3* are the superimposed images of *C1* and *C2*, *D1* and *D2*, and *E1* and *E2*, respectively. *C, D*, Axonal calretinin-stained (*C*) and GlyT2-stained (*D*) varicosities are found apposed to GlyRα/β-immunoreactive clusters (arrowheads). The insets in *C*, taken from another section, show a contact at a higher magnification (5×). Note that a few terminals are not associated with GlyR (arrows). *E*, A GlyT2-immunoreactive varicose fiber from the Lugaro cell sagittal axonal plexus is shown. Some GlyT2 terminals are apposed to GlyR clusters (arrowheads), whereas others are not (arrows). The latter probably correspond to contacts between the Lugaro cell sagittal axon and molecular layer interneurons. *F*, Double immunostaining of parvalbumin (*F1*) and glycine receptor (*F2*) in the molecular layer is shown. Clusters of glycine receptor (arrows) are not associated with parvalbumin-immunoreactive profiles, belonging either to basket, stellate, or Purkinje cells (double arrowheads). Asterisks indicate the cell bodies of the molecular layer interneurons. *Pva*, Parvalbumin. Scale bars: *A, B, F*, 5 μm; *C–E* (bar in *F*), 10 μm.

that the parasagittal plexus of the Lugaro cell axons contributes to the inhibition of Golgi cells, as shown by Lainé and Axelrad (1998), in addition to the transverse plexus, which is restricted to the lower one-third of the molecular layer (see Dieudonné and Dumoulin, 2000).

Lugaro cell axon varicosities are apposed to glycine receptors on Golgi cell dendrites

To confirm that Lugaro cells are the interneurons presynaptic to the mixed GlyR–GABA_AR clusters present on Golgi cell dendrites, we performed simultaneous immunodetection of calretinin or GlyT2 and GlyR α/β . GlyR α/β clusters were found associated with calretinin-positive (Fig. 6C) and GlyT2-positive terminals (Fig. 6D,E). Quantification of the latter shows that 69.8% ($n = 200$) of the GlyT2-positive terminals in the transverse plexus associate with GlyR α/β clusters, but only 53.4% ($n = 177$) of these terminals do in the parasagittal plexus. These results confirm a major participation of the Lugaro transverse plexus in the connection with Golgi cells and a significant participation of the parasagittal axons to it, although less important. Besides, the number of associations in the transverse plane may have been underestimated because of the difficulty of focusing on the same plane the Lugaro axon and the Golgi dendrites, which have perpendicular orientations. For both plexi, the remaining terminals, which are not apposed to GlyR α/β clusters, are thought to represent contacts with basket and stellate cells, which were demonstrated at the electron microscopic level (Lainé and Axelrad, 1998). Thus, GlyT2 would also be present in axon terminals where transmission is purely GABAergic. Conversely, it was found that most GlyR α/β -immunoreactive clusters present in Golgi cell dendrites in the molecular layer were apposed to GlyT2 immunoreactivities in both transverse (89.5%; $n = 192$) and parasagittal (86.8%; $n = 175$) sections.

Finally, to confirm the absence of a GlyR component at basket cell→Golgi cell synapses, we performed a double immunodetection of parvalbumin [a marker of molecular layer interneurons and Purkinje cells (Kosaka et al., 1993)] and GlyR. In contrast to the results obtained with Lugaro cell markers, parvalbumin-positive profiles were not associated with GlyR clusters on Golgi cell dendrites (Fig. 6F).

Taken together, the morphological and electrophysiological results establish that the mixed serotonin-induced IPSCs recorded in Golgi cells are caused by corelease of GABA and glycine by the Lugaro cell axonal varicosities onto variegated postsynaptic arrays of GlyR and GABA_AR located on Golgi cell dendrites. In contrast, pure GABAergic transmission at basket cell→Golgi cell synapses accounts for the spontaneous IPSCs recorded from Golgi cells.

Kinetic properties of S-IPSCs and L-IPSCs

The convergence of two identified inputs, one purely GABAergic and one mixed GABAergic and glycinergic, on the same postsynaptic cell offers for the first time the possibility to assess the specific function of glycinergic transmission in central structures dominated by GABAergic inhibitory transmission. For this purpose the decay kinetics of S-IPSCs and L-IPSCs was studied in 26 Golgi cells. IPSCs that were well separated from adjacent synaptic events were selected and averaged during the control period (S-IPSCs) and during serotonin application (L-IPSCs). L-IPSCs were distinguished from contaminating S-IPSCs by their larger amplitude (as in Fig. 3), and only L-IPSCs larger than the largest S-IPSC recorded before addition of serotonin were

Figure 7. Variability and coregulation of S-IPSC and L-IPSC decay kinetics. IPSCs were averaged during the control period and during application of serotonin. L-IPSCs were distinguished by their large amplitude. *A*, Example of averaged S-IPSCs (left; dotted lines) and L-IPSCs (right; solid lines) recorded from two Golgi cells is shown. *B*, S-IPSCs, as well as L-IPSCs, of cell 1 and cell 2 were scaled and superimposed. Both were faster in cell 1 than in cell 2. *C*, Plot of the half decay time of the S-IPSCs recorded from 26 Golgi cells against the age of the animals is shown. *D*, S-IPSCs and L-IPSCs were scaled in cell 1 and in cell 2. L-IPSCs are slower than are S-IPSCs in the same proportion in both cells. *E*, The half decay times of L-IPSCs measured in 12 Golgi cells are plotted against the half decay times of S-IPSCs in the same cells. The solid line represents the linear regression through the points. Its slope is 1.28. The dashed line indicates a slope of 1. L, L-IPSC; PN, Postnatal; S, S-IPSC.

selected. The time course of the mean IPSCs was quantified by measuring either their half-width or the ratio of their total charge over their peak amplitude. Both values showed that the decay kinetics of both types of IPSCs was very variable from cell to cell (Fig. 7A,B). The half-width varied from 9.6 to 29.0 msec for S-IPSCs (18 ± 5 msec; mean $\pm$ SD; $n = 26$) and from 14 to 41 msec for the L-IPSCs (25 ± 8 msec; mean $\pm$ SD; $n = 13$). The charge-over-peak ratio varied from 19 to 50 msec for S-IPSCs (36 ± 10 msec) and from 22 to 65 msec for the L-IPSCs (42 ± 14 msec). In granule cells of the cerebellar cortex, maturation of GABAergic synapses during the third postnatal week has been associated with a decrease of the decay time constants of the GABAergic IPSCs (Tia et al., 1996; Carlson et al., 1998). However, maturation is not the cause of the large variability of IPSCs kinetics in Golgi cells. Over all of the experiments, there was no significant correlation between the age of the animal and the

Figure 8. The glycinergic components of the serotonin-evoked IPSCs account for their slower decay kinetics. *A*, S-IPSCs and the GABAergic component of L-IPSCs have the same decay time course. Averaged S-IPSCs and L-IPSCs_{GABA} recorded from the same cell in control conditions and after addition of serotonin and strychnine (1 μ M) are plotted on the left. Biexponential functions fitting the decay of the IPSCs are displayed (solid lines). The time constants are 14 and 52 msec for the S-IPSCs and 20 and 57 msec for the L-IPSCs_{GABA}. The same IPSCs are scaled to their peak on the right. *B*, The time constants of decay of S-IPSCs and L-IPSCs_{GABA} obtained in the same six cells are plotted against each other. The slopes of the linear regressions (solid lines) are 1.16 and 0.86 for the fast (circles) and slow (triangles) components, respectively. *C*, The glycinergic component of L-IPSCs has a slower decay than S-IPSCs have. Averaged S-IPSCs and L-IPSCs_{Gly} recorded from the same cell in control conditions and after addition of serotonin and gabazine (30 μ M) are plotted on the left. The sum of two exponential functions of time constants of 14 and 40 msec is superimposed on the decay of the S-IPSC, whereas the decay of the L-IPSCs_{Gly} is fitted by a monoexponential function with a time constant of 45 msec. The same IPSCs are scaled to their peak on the right. *D*, A plot the same as that in *B* is shown. In all cells the decay of the mean glycinergic IPSCs was appropriately fitted by a single-exponential function, and the single decay time constant of glycinergic IPSCs is compared with both time constants of control IPSCs. The slope of the linear regressions are 2.3 and 0.68.

kinetics of the IPSCs (Fig. 7C). Furthermore, the S-IPSCs recorded from Golgi cells at postnatal day 13 have half decay times ranging from 12 to 27 msec, a dispersion as high as that of the overall population.

The half decay times of S-IPSCs and L-IPSCs, although highly variable from cell to cell, were in fixed quantitative relation to each other when recorded in the same cell (Fig. 7D). In all of the cells, the L-IPSCs decayed more slowly than did the S-IPSCs, and the ratio of their time course was independent of the time constant of the IPSCs. This is illustrated in Figure 7E, where the half decay time of the L-IPSCs is plotted against the half decay time of the S-IPSCs in the same cells. The half decay times are linearly correlated ($p < 0.0001$) (Fig. 7E) with a slope of 1.28. For the charge-over-peak ratios, the regression slope was 1.34 ($p < 0.0001$). Therefore the decay time course of the IPSCs appears to be a highly variable parameter coregulated by the postsynaptic Golgi cells at the various inhibitory synapses converging onto them.

The kinetics of the GABA_A and of the glycinergic components of inhibitory synapses is coregulated

It appeared likely that the consistent kinetic difference between the S-IPSCs and L-IPSCs was caused by the presence of a glycinergic component in the later synaptic events. We hence compared the kinetics of the GABAergic and glycinergic components of the IPSCs in individual Golgi cells. The successive pharmacological isolation of the two components in the same cell is difficult and unreliable because of the slow and partial reversibility of GABA_A and glycine inhibitors in slices. Therefore S-IPSCs (purely GABAergic) were always recorded in control conditions, to serve as a kinetic standard, before recording L-IPSCs (induced by serotonin) in the presence of either gabazine (30 μ M) or strychnine (1 μ M). We found that L-IPSCs_{Gly} had a significantly slower decay than did S-IPSCs (Fig. 8C) with mean ratios of 1.46 ± 0.14 (mean $\pm$ SEM; $n = 7$) and 1.35 ± 0.13 (mean $\pm$ SEM; $n = 7$) for half-width and charge-over-peak measurements, respectively.

A more detailed analysis was performed. The decay of GABAergic S-IPSCs and of the GABAergic component of L-IPSCs (L-IPSCs_{GABA}) was fitted by the sum of two exponential functions (Fig. 8A). The fast time constants were 14 ± 3 msec for the S-IPSCs and 16 ± 5 msec for the L-IPSCs_{GABA}, and the slow time constants were 53 ± 15 and 46 ± 16 msec, respectively ($n = 6$). Fast and slow time constants measured in the same cell for both types of IPSCs were linearly correlated with slopes of 1.16 and 0.86, respectively (Fig. 8B). The ratio of the amplitudes of the fast and slow components were 1.2 ± 0.2 and 1 ± 0.2 for S-IPSCs and L-IPSCs, respectively ($p > 0.3$, t test). In contrast, the decay of the L-IPSCs_{Gly} was appropriately fitted by a single-exponential function in all of the cells studied ($n = 7$). Its time constant was intermediate between the fast and the slow time constants of decay of the S-IPSCs recorded in the same cells, with regression slopes of 2.3 and 0.68, respectively (Fig. 8D). We conclude that the decay of the L-IPSCs_{Gly} differs from the decay of the L-IPSCs_{GABA} and of S-IPSCs, maintaining the L-IPSCs and S-IPSCs decay time course in a fixed ratio. We propose that the slower decay kinetics of L-IPSCs_{Gly} constitutes a significant functional difference between the mixed Lugaro cell→Golgi cell synapses and the pure GABAergic synapses made by basket cells onto the same Golgi cells.

DISCUSSION

The mixed pharmacology of individual L-IPSCs recorded from Golgi cells indicates that Lugaro cells release both GABA and glycine. The experiment described in Figure 3 demonstrates that a single presynaptic Lugaro cell can release both GABA and glycine at each action potential. Corelease is most likely a general rule at Lugaro cell→Golgi cell synapses because the mean amplitude of L-IPSCs [130 pA (Dieudonné and Dumoulin, 2000)] is the sum of the amplitude of L-IPSCs_{GABA} (90 pA) and of L-IPSCs_{Gly} (34 pA). These electrophysiological data are supported by the immunohistochemical demonstration of the pres-

ence of mixed arrays of GABA_AR and GlyR at postsynaptic loci on Golgi cell dendrites, in front of Lugaro cell axonal varicosities. Colocalized immunoreactivities for both GAD65 and GlyT2 in the varicosities of the Lugaro cells suggest that GABA and glycine are indeed coreleased at single synaptic contacts. GlyT2 can accumulate much higher concentrations of glycine in the cytoplasm of neurons than can those achieved by the glial homolog GlyT1 (Roux and Supplisson, 2000). These high glycine concentrations are probably necessary to compete with GABA for transport by the nonselective vesicular transporter VIAAT (Sagné et al., 1997) and to achieve a substantial loading of the synaptic vesicles with glycine. The data presented in this paper offer the first evidence of the occurrence of functional cotransmission by GABA and glycine outside the spinal cord (Jonas et al., 1998) and brainstem motor nuclei (O'Brien and Berger, 1999), suggesting that inhibitory cotransmission may be more widely used in the CNS than suspected previously.

Receptor expression selectivity

Immunocytochemistry experiments have shown that GABA_AR and GlyR subunits can segregate at different synapses on the same postsynaptic cell (Sassoe-Pognetto et al., 1995; Koulen et al., 1996). We show here that GlyR and GABA_AR γ 2 are present at Lugaro cell→Golgi cell synapses but not at basket cell→Golgi cell contacts and therefore that receptors are segregated according to the identity of the presynaptic element. The presynaptic terminal must thus be instructive for the formation of the adequate postsynaptic site. This idea has been illustrated recently in the hippocampal pyramidal cells where α 1 and α 2 GABA_AR subunits segregate to synapses where two different presynaptic interneurons are involved (Nyiri et al., 2001). The cellular processes involved in this mechanism are still under investigation; they probably involve signals mediated by cadherins and/or catenins (Fannon and Colman, 1996; Benson and Tanaka, 1998; Kohmura et al., 1998) or agrin (Böse et al., 2000). In addition, the present results show that >90% of the Lugaro varicosities displayed both GlyT2 and GAD65 immunoreactivities whereas only 73% of them were apposed to GlyR clusters. Thus, segregation of phenotypes does not seem to occur in this example of inhibitory terminals. This also further indicates that the postsynaptic element may select the type of inhibition it receives by avoiding expression of, or being unable to express, the receptors for one of the transmitters coreleased. Indeed, synapses made by the Lugaro cell axons onto basket and stellate cells lacked postsynaptic GlyR, because the latter is only expressed on Golgi cell dendrites in the molecular layer. The nature of both presynaptic and postsynaptic elements is therefore important in determining the molecular composition of a given inhibitory synapse.

Organization of mixed receptor arrays

Gephyrin has been first considered to be a marker of glycinergic synapses. It is now established that gephyrin is also associated with postsynaptic GABA_AR in the absence of GlyR both *in vitro* and *in vivo* (Sassoe-Pognetto et al., 1995, 1999, 2000; Craig et al., 1996; Giustetto et al., 1998). In the majority of synapses, the presence of GABA_AR γ 2 subunits seems to be a critical factor for the association of GABA_AR with gephyrin. Loss of gephyrin prevents synaptic clustering of γ 2-containing GABA_AR (Kneusel et al., 1999), and loss of GABA_AR γ 2 subunits highly decreases the synaptic localization of GABA_AR and gephyrin in several brain regions (Essrich et al., 1998), a perturbation that can be rescued by overexpression of the γ 3 subunit (Baer et al., 1999).

Our data indicate that GABA_AR, gephyrin, and GlyR can accumulate at the same postsynaptic sites. Similar results have been found in spinal cord cultures, in which GABA_AR β 3 or γ 2 subunits, gephyrin, and GlyR colocalize at the same synapses (Lévi et al., 1999; Dumoulin et al., 2000). At Lugaro cell→Golgi cell synapses, gephyrin and GlyR appear to be preferentially located on the edge of GABA_AR γ 2 postsynaptic domains. However, gephyrin and GABA_AR are found to be exactly colocalized at the majority of inhibitory synapses in the molecular layer (Sassoe-Pognetto et al., 2000). This indicates that the organization of the postsynaptic domains of receptors at the Lugaro cell→Golgi cell synapses is different from the organization at the majority of inhibitory synapses in the molecular layer, which are GABAergic synapses made by Purkinje cell recurrent collateral axons and basket and stellate cells.

Segregation of gephyrin or GlyR and GABA_AR immunoreactivities inside the same postsynaptic domain has been described at other synapses. At Golgi cell→granule cell contacts in the granular layer, gephyrin clusters are embedded in GABA_AR domains (Sassoe-Pognetto et al., 2000). Partial or complete segregation of GABA_AR β 3 subunit and gephyrin labelings has also been observed at the ultrastructural level in putative mixed synapses in the spinal cord (Todd et al., 1996), and in the rat retina, gephyrin and GABA_AR α 2 subunit were found in the same synapse but did not colocalize (Sassoe-Pognetto et al., 1995). Segregation of GABA_AR and gephyrin at some synapses suggests that γ -containing GABA_ARs are not recruited by a direct interaction with gephyrin. This is coherent with the lack of evidence of direct protein–protein interaction between gephyrin and GABA_AR (Meyer et al., 1995).

Although our data strongly suggest that GABA_AR and GlyR clusters are segregated at mixed synapses onto Golgi cells, further ultrastructural investigation would be necessary to determine the spatial organization of mixed synapses, in particular to demonstrate formally that the two receptor clusters are facing the same presynaptic varicosity. The detailed organization of mixed synapses may have important functional consequences because the two types of receptor clusters may be located at different distances from the release sites. Consequently, they might be differentially saturated after synaptic release of the two transmitters, as occurs in GABA and glycine cotransmission in the dorsal horn layer I of the spinal cord (Chéry and de Koninck, 1999). In a more extreme model, GABA_AR and GlyR clusters could face different active zones within the same presynaptic bouton, as suggested in the deep cerebellar nuclei (Chen and Hillman, 1993). In both cases the presynaptic neuron could modify rapidly the relative activation of the two types of postsynaptic receptors by changing its release probability. On a slower time scale, the presynaptic neuron could regulate the relative occupancy of both types of postsynaptic receptors by changing the concentration of GABA and glycine inside the synaptic vesicles.

Postsynaptic relevance of inhibitory cotransmission at Lugaro cell→Golgi cell synapses

What is the functional consequence of the variability of the decay time constants of the IPSCs at inhibitory synapses onto Golgi cells? Many data from the literature suggest that there is a correlation between the decay time constant of IPSCs and the period of oscillations in rhythmic neuronal networks (Traub et al., 1998). The difference of IPSC kinetics between Golgi cells may indicate that different parts of the cerebellum use different working frequencies. This would fit with the organization of the

cerebellar vermis into lobules or groups of lobules receiving different afferences (Altman and Bayer, 1997) and with the recording of field oscillations at different frequencies in different cerebellar lobules (Pellerin and Lamarre, 1997; Timofeev and Steriade, 1997; Hartmann and Bower, 1998). Alternatively all parts of the cortex may have a mixed population of Golgi cells, each cell being optimally efficient when the network is oscillating at a frequency adapted to the kinetics of its IPSCs.

In motoneurons, GABAergic IPSCs (IPSCs_{GABA}) decay more slowly than do glycinergic IPSCs (IPSCs_{Gly}) and can be further slowed by acting on allosteric modulatory sites (Jonas et al., 1998). This kinetic difference constitutes the key factor for the demonstration that both receptors are activated during miniature IPSCs. In the cerebellum, IPSCs_{GABA} have a biexponential decay, and IPSCs_{Gly} have a single-exponential decay of time constant intermediate between the two time constants of IPSCs_{GABA}. IPSCs_{Gly} are 1.4 times slower than are IPSCs_{GABA} (half decay time), approximately the inverse of the ratio in the spinal cord (where IPSCs_{Gly} are faster). This number is relatively modest compared with the dispersion of the decay time course between Golgi cells, but it may represent a significant functional difference. The modulation of the decay time course of GABAergic IPSCs by benzodiazepine, although not more profound, is known to have important effects on the function of neuronal networks. In this hypothesis, the main consequence of glycinergic transmission onto Golgi cells would be to give a slower decay time course to Lugaro cell IPSCs, compared with basket cell IPSCs. This would enhance the duration of the inhibition of Golgi cells by Lugaro cells, and therefore the disinhibition of granule cells, and would regulate the number of active granule cells. Because of the presence of putative glycinergic or mixed GABA and glycine neurons in many parts of the CNS, glycinergic transmission may constitute a target for new, more specific, drugs similar in their action to benzodiazepines.

REFERENCES

- Altman J, Bayer SA (1997) Development of the cerebellar system in relation to its evolution, structure, and functions. New York: CRC.
- Aoki E, Semba R, Kashiwamata S (1986) New candidates for GABAergic neurons in the rat cerebellum: an immunocytochemical study with anti-GABA antibody. *Neurosci Lett* 68:267–271.
- Baer K, Essrich C, Benson JA, Benke D, Bluethmann H, Fritschy JM, Luscher B (1999) Postsynaptic clustering of gamma-aminobutyric acid type A receptors by the gamma3 subunit in vivo. *Proc Natl Acad Sci USA* 96:12860–12865.
- Benson DL, Tanaka H (1998) N-cadherin redistribution during synaptogenesis in hippocampal neurons. *J Neurosci* 18:6892–6904.
- Borowsky B, Mezey E, Hoffman BJ (1993) Two glycine transporter variants with distinct localization in the CNS and peripheral tissues are encoded by a common gene. *Neuron* 10:851–863.
- Böse CM, Qiu D, Bergamaschi A, Gravante B, Bossi M, Villa A, Rupp F, Malgaroli A (2000) Agrin controls synaptic differentiation in hippocampal neurons. *J Neurosci* 20:9086–9095.
- Carlson BX, Elster L, Schousboe A (1998) Pharmacological and functional implications of developmentally-regulated changes in GABA(A) receptor subunit expression in the cerebellum. *Eur J Pharmacol* 352:1–14.
- Chaudhry FA, Reimer RJ, Bellocchio EE, Danbolt NC, Osen KK, Edwards RH, Storm-Mathisen J (1998) The vesicular GABA transporter VGAT localizes to synaptic vesicles in sets of glycinergic as well as GABAergic neurons. *J Neurosci* 18:9733–9750.
- Chen S, Hillman DE (1993) Colocalization of neurotransmitters in the deep cerebellar nuclei. *J Neurocytol* 22:81–91.
- Chéry N, de Koninck Y (1999) Junctional versus extrajunctional glycine and GABA(A) receptor-mediated IPSCs in identified lamina I neurons of the adult rat spinal cord. *J Neurosci* 19:7342–7355.
- Craig AM, Banker G, Chang W, McGrath ME, Serpinskaya AS (1996) Clustering of gephyrin at GABAergic but not glutamatergic synapses in cultured rat hippocampal neurons. *J Neurosci* 16:3166–3177.
- Dieudonné S (1995) Glycinergic synaptic currents in Golgi cells of the rat cerebellum. *Proc Natl Acad Sci USA* 92:1441–1445.
- Dieudonné S (1998) Submillisecond kinetics and low efficacy of parallel fibre-Golgi cell synaptic currents in the rat cerebellum. *J Physiol (Lond)* 510:845–866.
- Dieudonné S, Dumoulin A (2000) Serotonin-driven long-range inhibitory connections in the cerebellar cortex. *J Neurosci* 20:1837–1848.
- Dumoulin A, Rostaing P, Bedet C, Levi S, Isambert MF, Henry JP, Triller A, Gasnier B (1999) Presence of the vesicular inhibitory amino acid transporter in GABAergic and glycinergic synaptic terminal boutons. *J Cell Sci* 112:811–823.
- Dumoulin A, Levi S, Riveau B, Gasnier B, Triller A (2000) Formation of mixed glycine and GABAergic synapses in cultured spinal cord neurons. *Eur J Neurosci* 12:1–11.
- Essrich C, Lorez M, Benson JA, Fritschy JM, Luscher B (1998) Postsynaptic clustering of major GABAA receptor subtypes requires the gamma 2 subunit and gephyrin [see comments]. *Nat Neurosci* 1:563–571.
- Fannon AM, Colman DR (1996) A model for central synaptic junctional complex formation based on the differential adhesive specificities of the cadherins. *Neuron* 17:423–434.
- Fritschy JM, Benke D, Mertens S, Oertel WH, Bachi T, Mohler H (1992) Five subtypes of type A gamma-aminobutyric acid receptors identified in neurons by double and triple immunofluorescence staining with subunit-specific antibodies. *Proc Natl Acad Sci USA* 89:6726–6730.
- Gabbott PL, Somogyi J, Stewart MG, Hamori J (1986) GABA-immunoreactive neurons in the rat cerebellum: a light and electron microscope study. *J Comp Neurol* 251:474–490.
- Giustetto M, Kirsch J, Fritschy JM, Cantino D, Sassoe-Pognetto M (1998) Localization of the clustering protein gephyrin at GABAergic synapses in the main olfactory bulb of the rat. *J Comp Neurol* 395:231–244.
- Gottlieb DI, Chang Y-C, Schwob JE (1986) Monoclonal antibodies to glutamic acid decarboxylase. *Proc Natl Acad Sci USA* 83:8808–8812.
- Gutiérrez A, Khan ZU, De Blas AL (1994) Immunocytochemical localization of gamma 2 short and gamma 2 long subunits of the GABAA receptor in the rat brain. *J Neurosci* 14:7168–7179.
- Hartmann MJ, Bower JM (1998) Oscillatory activity in the cerebellar hemispheres of unrestrained rats. *J Neurophysiol* 80:1598–1604.
- Jonas P, Bischofberger J, Sandkühler J (1998) Corelease of two fast neurotransmitters at a central synapse. *Science* 281:419–424.
- Kaneda M, Farrant M, Cull-Candy SG (1995) Whole-cell and single-channel currents activated by GABA and glycine in granule cells of the rat cerebellum. *J Physiol (Lond)* 485:419–435.
- Kneussel M, Brandstätter JH, Laube B, Stahl S, Müller U, Betz H (1999) Loss of postsynaptic GABA(A) receptor clustering in gephyrin-deficient mice. *J Neurosci* 19:9289–9297.
- Kohmura N, Senzaki K, Hamada S, Kai N, Yasuda R, Watanabe M, Ishii H, Yasuda M, Mishina M, Yagi T (1998) Diversity revealed by a novel family of cadherins expressed in neurons at a synaptic complex. *Neuron* 20:1137–1151.
- Kondo S, Marty A (1998) Differential effects of noradrenaline on evoked, spontaneous and miniature IPSCs in rat cerebellar stellate cells. *J Physiol (Lond)* 509:233–243.
- Kosaka T, Kosaka K, Nakayama T, Hunziker W, Heizmann CW (1993) Axons and axon terminals of cerebellar Purkinje cells and basket cells have higher levels of parvalbumin immunoreactivity than somata and dendrites: quantitative analysis by immunogold labeling. *Exp Brain Res* 93:483–491.
- Koulen P, Sassoe-Pognetto M, Grunert U, Wässle H (1996) Selective clustering of GABA(A) and glycine receptors in the mammalian retina. *J Neurosci* 16:2127–2140.
- Lainé J, Axelrad H (1996) Morphology of the Golgi-impregnated Lugaro cell in the rat cerebellar cortex: a reappraisal with a description of its axon. *J Comp Neurol* 375:618–640.
- Lainé J, Axelrad H (1998) Lugaro cells target basket and stellate cells in the cerebellar cortex. *NeuroReport* 9:2399–2403.
- Lévi S, Chesnoy-Marchais D, Sieghart W, Triller A (1999) Synaptic control of glycine and GABA(A) receptors and gephyrin expression in cultured motoneurons. *J Neurosci* 19:7434–7449.
- Liu QR, Lopez-Corcuera B, Mandiyan S, Nelson H, Nelson N (1993) Cloning and expression of a spinal cord- and brain-specific glycine transporter with novel structural features. *J Biol Chem* 268:22802–22808.
- Llano I, Marty A, Armstrong CM, Konnerth AM (1991) Synaptic- and agonist-induced excitatory currents of Purkinje cells in rat cerebellar slices. *J Physiol (Lond)* 434:183–213.
- Llinás R, Sugimori M (1980) Electrophysiological properties of *in vitro* Purkinje cell somata in mammalian cerebellar slices. *J Physiol (Lond)* 305:171–195.
- Lugaro E (1894) Sulle connessioni tra gli elementi nervosi della corteccia cerebellare con considerazioni generali sul significato fisiologico dei rapporti tra gli elementi nervosi. *Rivista Sperimentale di Freniatria* 20:297–331.
- Malosio ML, Marqueze-Pouey B, Kuhse J, Betz H (1991) Widespread expression of glycine receptor subunit mRNAs in the adult and developing rat brain. *EMBO J* 10:2401–2409.

- McIntire SL, Reimer RJ, Schuske K, Edwards RH, Jorgensen EM (1997) Identification and characterization of the vesicular GABA transporter. *Nature* 389:870–876.
- Meyer G, Kirsch J, Betz H, Langosch D (1995) Identification of a gephyrin binding motif on the glycine receptor beta subunit. *Neuron* 15:563–572.
- Mugnaini E, Floris A, Wright-Goss M (1994) Extraordinary synapses of the unipolar brush cell: an electron microscopic study in the rat cerebellum. *Synapse* 16:284–311.
- Nyiri G, Freund TF, Somogyi P (2001) Input-dependent synaptic targeting of $\alpha 2$ -subunit-containing GABA_A receptors in synapses of hippocampal pyramidal cells of the rat. *Eur J Neurosci* 13:428–442.
- O'Brien JA, Berger AJ (1999) Cotransmission of GABA and glycine to brain stem motoneurons. *J Neurophysiol* 82:1638–1641.
- Ornung G, Shupliakov O, Ottersen OP, Storm-Mathisen J, Cullheim S (1994) Immunohistochemical evidence for coexistence of glycine and GABA in nerve terminals on cat spinal motoneurons: an ultrastructural study. *NeuroReport* 5:889–892.
- Ottersen OP, Davanger S, Storm-Mathisen J (1987) Glycine-like immunoreactivity in the cerebellum of rat and Senegalese baboon, *Papio papio*: a comparison with the distribution of GABA-like immunoreactivity and with [³H]glycine and [³H]GABA uptake. *Exp Brain Res* 66:211–221.
- Ottersen OP, Storm-Mathisen J, Somogyi P (1988) Colocalization of glycine-like and GABA-like immunoreactivities in Golgi cell terminals in the rat cerebellum: a postembedding light and electron microscopic study. *Brain Res* 450:342–353.
- Palay SL, Chan-Palay V (1974) Cerebellar cortex: cytology and organization. Berlin: Springer.
- Pellerin JP, Lamarre Y (1997) Local field potential oscillations in primate cerebellar cortex during voluntary movement. *J Neurophysiol* 78:3502–3507.
- Pfeiffer F, Simler R, Grenningloh G, Betz H (1984) Monoclonal antibodies and peptide mapping reveal structure similarities between the subunits of the glycine receptor of rat spinal cord. *Proc Natl Acad Sci USA* 81:7224–7227.
- Poyatos I, Ponce J, Aragon C, Gimenez C, Zafra F (1997) The glycine transporter GLYT2 is a reliable marker for glycine-immunoreactive neurons. *Brain Res Mol Brain Res* 49:63–70.
- Racca C, Gardiol A, Triller A (1998) Cell-specific dendritic localization of glycine receptor α subunit messenger RNAs. *Neuroscience* 84:997–1012.
- Roux MJ, Supplisson S (2000) Neuronal and glial glycine transporters have different stoichiometries. *Neuron* 25:373–383.
- Sagné C, El Mestikawy S, Isambert MF, Hamon M, Henry JP, Giros B, Gasnier B (1997) Cloning of a functional vesicular GABA and glycine transporter by screening of genome databases. *FEBS Lett* 417:177–183.
- Sassoe-Pognetto M, Kirsch J, Grunert U, Greferath U, Fritschy JM, Mohler H, Betz H, Wassle H (1995) Colocalization of gephyrin and GABA_A-receptor subunits in the rat retina. *J Comp Neurol* 357:1–14.
- Sassoe-Pognetto M, Giustetto M, Panzanelli P, Cantino D, Kirsch J, Fritschy JM (1999) Postsynaptic colocalization of gephyrin and GABA_A receptors. *Ann NY Acad Sci* 868:693–696.
- Sassoe-Pognetto M, Panzanelli P, Sieghart W, Fritschy JM (2000) Colocalization of multiple GABA(A) receptor subtypes with gephyrin at postsynaptic sites. *J Comp Neurol* 420:481–498.
- Schwaller B, Buchwald P, Blumcke I, Celio MR, Hunziker W (1993) Characterization of a polyclonal antiserum against the purified human recombinant calcium binding protein calretinin. *Cell Calcium* 14:639–648.
- Somogyi P, Fritschy JM, Benke D, Roberts JD, Sieghart W (1996) The gamma 2 subunit of the GABA_A receptor is concentrated in synaptic junctions containing the alpha 1 and beta 2/3 subunits in hippocampus, cerebellum and globus pallidus. *Neuropharmacology* 35:1425–1444.
- Spike RC, Watt C, Zafra F, Todd AJ (1997) An ultrastructural study of the glycine transporter GLYT2 and its association with glycine in the superficial laminae of the rat spinal dorsal horn. *Neuroscience* 77:543–551.
- Taal W, Holstege JC (1994) GABA and glycine frequently colocalize in terminals on cat spinal motoneurons. *NeuroReport* 5:2225–2228.
- Tia S, Wang JF, Kotchabhakdi N, Vicini S (1996) Developmental changes of inhibitory synaptic currents in cerebellar granule neurons: role of GABA(A) receptor alpha 6 subunit. *J Neurosci* 16:3630–3640.
- Timofeev I, Steriade M (1997) Fast (mainly 30–100 Hz) oscillations in the cat cerebellothalamic pathway and their synchronization with cortical potentials. *J Physiol (Lond)* 504:153–168.
- Todd AJ, Sullivan AC (1990) Light microscope study of the coexistence of GABA-like and glycine-like immunoreactivities in the spinal cord of the rat. *J Comp Neurol* 296:496–505.
- Todd AJ, Watt C, Spike RC, Sieghart W (1996) Colocalization of GABA, glycine, and their receptors at synapses of the rat spinal cord. *J Neurosci* 16:974–982.
- Traub RD, Spruston N, Soltesz I, Konnerth A, Whittington MA, Jefferys GR (1998) Gamma-frequency oscillations: a neuronal population phenomenon, regulated by synaptic and intrinsic cellular processes, and inducing synaptic plasticity. *Prog Neurobiol* 55:563–575.
- Triller A, Cluzeaud F, Korn H (1987) Gamma-aminobutyric acid-containing terminals can be apposed to glycine receptors at central synapses. *J Cell Biol* 104:947–956.
- Vincent P, Armstrong CM, Marty A (1992) Inhibitory synaptic currents in rat cerebellar Purkinje cells: modulation by postsynaptic depolarization. *J Physiol (Lond)* 456:453–471.
- Wentholt RJ, Huie D, Altschuler RA, Reeks KA (1987) Glycine immunoreactivity localized in the cochlear nucleus and superior olivary complex. *Neuroscience* 22:897–912.
- Wentzel PR, De Zeeuw CI, Holstege JC, Gerrits NM (1993) Colocalization of GABA and glycine in the rabbit oculomotor nucleus. *Neurosci Lett* 164:25–29.
- Wilkin GP, Csillag A, Balázs R, Kingsbury AE, Wilson JE, Johnson AL (1981) Localization of high affinity [³H]glycine transport sites in the cerebellar cortex. *Brain Res* 216:11–33.
- Yang HW, Min MY, Appenteng K, Batten TF (1997) Glycine-immunoreactive terminals in the rat trigeminal motor nucleus: light- and electron-microscopic analysis of their relationships with motoneurons and with GABA-immunoreactive terminals. *Brain Res* 749:301–319.
- Zafra F, Aragon C, Olivares L, Danbolt NC, Gimenez C, Storm-Mathisen J (1995) Glycine transporters are differentially expressed among CNS cells. *J Neurosci* 15:3952–3969.