Only a minority of the inhibitory inputs to cerebellar Golgi cells originates from local GABAergic cells

GABAergic inhibition of cerebellar Golgi cells

Mark D. Eyre1 and Zoltan Nusser1

Laboratory of Cellular Neurophysiology, Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest 1083, HUNGARY

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Correspondence should be addressed to Zoltan Nusser or Mark D. Eyre, Laboratory of Cellular Neurophysiology, Institute of Experimental Medicine, Szigony str. 43, 1083 Budapest, Hungary. e-mail: nusser@koki.hu or eyre@koki.hu

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Mark D. Eyre and Zoltan Nusser

1 Laboratory of Cellular Neurophysiology, Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest 1083, HUNGARY

MDE and ZN resigned the experiments; MDE and ZN performed research; MDE and ZN wrote the manuscript

Correspondence should be addressed to Zoltan Nusser or Mark D. Eyre, Laboratory of Cellular Neurophysiology, Institute of Experimental Medicine, Szigony str. 43, 1083 Budapest, Hungary e-mail: nusser@koki.hu or eyre@koki.hu

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Abstract
Cerebellar Golgi cells (GoCs) efficiently control the spiking activity of granule cells through GABA_A receptor-mediated tonic and phasic inhibition. Recent experiments provided compelling evidence for the extensive interconnection of GoCs through electrical synapses, but their chemical inhibitory synaptic inputs are debated. Here, we investigated the GABAergic synaptic inputs of GoCs using in vitro electrophysiology and quantitative light- (LM) and electron microscopy (EM). We characterized GABA_A receptor-mediated IPSCs in GoCs and Lugaro cells (LuCs) and found that IPSCs in GoCs have lower frequencies, smaller amplitudes and much slower decay kinetics. Pharmacological and LM immunolocalization experiments revealed that GoCs express α3, whereas LuCs express α1 subunit-containing GABA_A receptors. The selective expression and clustered distribution of the α3 subunit in GoCs allowed the quantitative analysis of GABAergic synapses on their dendrites in the molecular layer (ML). EM and LM experiments in rats, wild type and GlyT2-GFP transgenic mice revealed that only one third of axon terminals establishing GABAergic synapses on GoC dendrites contain GlyT2, ruling out LuCs, globular cells and any non-cortical glycinergic inputs as major inhibitory sources. We also show that axon terminals of stellate/basket cells very rarely innervate GlyT2-GFP-expressing GoCs, indicating that only a minority of the inhibitory inputs to GoCs in the ML originates from local interneurons, and the majority of their inhibitory inputs exclusively releases GABA.
Significance Statement

Golgi cells are essential for controlling the activity of granule cells in the cerebellum by releasing the inhibitory neurotransmitter GABA, but very little is known about the sources of their own inhibition. We used functional and morphological techniques to demonstrate that the inhibitory postsynaptic receptors on Golgi cells are unique among the cell types in the cerebellar cortex, and used these unique GABA<sub>A</sub> receptors to visualize GoC inhibitory synapses and their presynaptic inputs. This study extends our understanding of cerebellar microcircuits by demonstrating that only a minority of the inhibitory inputs to Golgi cells originate from GABAergic cells of the cerebellar cortex.
The essential role of GoCs in higher cerebellar functions was elegantly demonstrated by Watanabe et al. (1998) by showing severe deficits in motor coordination following their selective pharmacological ablation. To understand the way these GABAergic interneurons (INs) fulfill their roles in circuit dynamics, their intrinsic properties and synaptic inputs and outputs need to be quantitatively determined. GoCs are located in the granule cell layer (GCL) of the cerebellar cortex and comprise a molecularly heterogeneous population of cells (Vincent et al., 1985; Ohishi et al., 1994; Neki et al., 1996; Singec et al., 2003; Simat et al., 2007). Despite this heterogeneity, their classifying morphological features are an axonal arborization restricted to the GCL and a characteristic dendritic arbor that extends into the molecular layer (ML; Eccles et al., 1967; Palay and Chan-Palay, 1974). Although there are additional GABAergic INs in the GCL (LuCs and globular cells), GoCs provide the sole source of GABAergic inhibition to granule cells (GrCs) and efficiently control the spiking activity of GrCs through GABA\textsubscript{A} receptor-mediated tonic and phasic inhibition (Brickley et al., 1996; Farrant and Nusser, 2005; Silver, 2010). Golgi cells receive feed-forward and feed-back excitatory inputs from mossy fibers and parallel fibers in the GCL and ML, respectively (Eccles et al., 1967; Dieudonne, 1998; Vos et al., 1999; Kanichay and Silver, 2008). It is also widely accepted that they are richly interconnected through electrical synapses (Dugue et al., 2009; Vervaeke et al., 2010), underlying the synchronization or desynchronization of spontaneously active GoC networks.

Based on the location of GoC dendrites in all layers of the cerebellar cortex, all GABAergic cells that have axonal arbors in the cortex could, in principle, provide GABAergic synaptic inputs to GoCs. Synaptic GABAergic inhibition onto GoCs has historically been assumed to arise from ML INs (MLIs; i.e. stellate and basket cells), based on the EM observation of symmetric synapses onto GoC dendrites (Palay and Chan-Palay, 1974) and the presence of IPSCs following electrical stimulation in the ML (Dumoulin et al., 2001). However, a recent study using selective light-mediated activation of MLIs expressing channelrhodopsin2 challenged this view by demonstrating...
a lack of functional connectivity between MLIs and GoCs (Hull and Regehr, 2012). Several studies 
have reported the lack of chemical inhibitory connectivity between GoCs, ruling this out as the 
major source of their inhibitory inputs (Dieudonne, 1998; Dugue et al., 2009; Vervaeke et al., 
2010), although some connectivity among GoCs has been reported (Hull and Regehr, 2012). 

Dieudonne and Dumoulin (2000) demonstrated that the application of serotonin (5-HT) evokes 
action potential-dependent mixed GABAergic and glycinergetic IPSCs in GoCs, which are likely to 
arise from LuCs. Lugaro cells are GCL INs with horizontal dendritic arbors running parallel with 
the Purkinje cell layer (PCL) and axons arborizing mainly in the ML and to a lesser extent in the 
GCL. LuCs have been shown to form synapses onto IN dendrites in the ML (Laine and Axelrad, 
1998), but little is known about their exact postsynaptic target cells or their presynaptic inputs, apart 
from the presence of calbindin-immunoreactive boutons surrounding their somata that were 
assumed to be Purkinje cell local axon collaterals (Laine and Axelrad, 2002; Simat et al., 2007). 

Considerable variability has been observed in the morphological features of GCL INs. In addition 
to GoCs and LuCs, globular and candelabrum cells have also been discerned (Laine and Axelrad, 
1994; Hirono et al., 2012). There is also heterogeneity among GoCs; five subtypes have been 
identified based on soma size and molecular content (e.g. glycine transporter type 2 (GlyT2), 
GAD67, mGluR2, neurogranin; Simat et al., 2007), but the functional consequences of this 
heterogeneity are unknown. Because LuCs, globular and most GoCs express GlyT2, we have used 
mice in which eGFP is expressed by bacterial artificial chromosome insertion under the control of 
the GlyT2 promoter (Zeilhofer et al., 2005) as a marker to investigate the inhibitory inputs and 
outputs of GCL INs.
**Materials and Methods**

*Animals.* Male and female mice heterozygous for the bacterial artificial chromosome insertion of enhanced green fluorescent protein (eGFP) under the control of the glycine transporter type 2 gene (Zeilhofer et al., 2005; henceforth GlyT2-GFP mice), wild-type C57Black6J mice (henceforth WT mice) and Wistar rats were sacrificed by decapitation, in accordance with local laws and with the ethical guidelines of the Authors’ University.

*Acute slice preparation.* GlyT2-GFP mice (n = 30, mean age 26.7 ± 5.2 days) were deeply anesthetized with isoflurane (Abbott Laboratories). After decapitation, the brain was removed and placed into a sucrose-based ice-cold artificial cerebrospinal fluid (ACSF) containing the following (in mM): 230 sucrose, 2.5 KCl, 25 glucose, 1.25 NaH2PO4, 24 NaHCO3, 4 MgCl2, and 0.5 CaCl2, bubbled continuously with 95% O2 and 5% CO2, resulting in a pH of 7.4. In order to investigate the factors contributing to the spontaneous activity of GoCs, we conducted experiments from two male Wistar rats (age 20 and 21 days) using the above solution, and also two GlyT2-GFP mice (age 25 days - male, and age 26 days - female), two male WT mice (age 21 and 24 days) and three male Wistar rats (age 18, 19 and 24 days) using a different, K-gluconate-based ice-cold cutting solution. This contained the following (in mM): 130 K-gluconate, 15 KCl, 0.05 EGTA, 20 HEPES, 25 glucose, 3 kynurenic acid, pH adjusted to 7.4 with NaOH. In all cases, parasagittal slices from the cerebellar vermis were cut at 250 μm thickness with a Vibratome (VT1000S; Leica) and were stored in ACSF containing the following (in mM): 126 NaCl, 2.5 KCl, 25 glucose, 1.25 NaH2PO4, 24 NaHCO3, 2 MgCl2, and 2 CaCl2, bubbled continuously with 95% O2 and 5% CO2, resulting in a pH of 7.4. After a 30 min recovery period at 33°C, slices were further incubated at room temperature until they were transferred to the recording chamber.

*Electrophysiological recordings.* Somatic whole-cell recordings were performed at 26.7 ± 0.9°C using IR-DIC on an Olympus BX51WI microscope with a 40x water-immersion objective. All voltage- and current-clamp recordings were performed using a mixed K-gluconate- and KCl-based intracellular solution containing the following (in mM): 65 K-gluconate, 70 KCl, 2.5 NaCl, 1.5
MgCl₂, 0.025 EGTA, 10 HEPES, 2 Mg-ATP, 0.4 Mg-GTP, 10 creatinine phosphate, and 8 biocytin, pH = 7.33, 270–290 mOsm. The reversal potential for chloride ions was calculated as -15.3 mV (http://www.physiologyweb.com/calculators/nerst_potential_calculator.html). All recordings were performed in ACSF in the presence of 3 mM kynurenic acid to inhibit ionotropic glutamate receptors. We investigated the spontaneous neuronal activity of GoCs recorded from Wistar rats and WT mice prepared using both cutting solutions, and in GlyT2-GFP mouse slices cut in the K-gluconate-based solution, for 3 minutes in cell-attached mode prior to attaining the whole-cell configuration. A series of constant hyper- and depolarizing current pulses with incremental amplitudes was applied to each cell in order to elicit voltage responses and supra-threshold action potential firing patterns. Continuous DC currents were not applied to cells to maintain them at a specified membrane potential. For voltage-clamp recordings of miniature IPSCs (mIPSCs) at a holding potential of -70 mV, 1 μM tetrodotoxin (TTX; Alomone Laboratories) was either included in the ACSF or washed into the bath. After establishing the whole-cell configuration and allowing for a 2 min stabilization period, a period of 4 min was recorded for each cell (‘baseline’). For pharmacological experiments, the perfusion solution was changed to one containing ACSF, 100 nM 2',4-Difluoro-5'-[8-fluoro-7-(1-hydroxy-1-methylethyl)imidazo[1,2-a]-pyridin-3-yl]-[1,1'-biphenyl]-2-carbonitrile (TP003; Tocris) or 100 nM zolpidem (Sigma). After a 12 min period of drug equilibration, a second 4 min period (‘steady-state’) was recorded. In a subset of recordings, the drug solution was then changed to one containing the drug plus SR95531 (20 μM; Sigma). In some experiments, spontaneous IPSCs (sIPSCs) were recorded for 1 minute after establishing the whole-cell configuration, and then TTX was washed into the bath. All recordings were performed with MultiClamp 700A and 700B amplifiers (Molecular Devices). Patch pipettes were pulled (Universal Puller; Zeitz-Instrumente Vertriebs) from thick-walled borosilicate glass capillaries with an inner filament (1.5 mm outer diameter, 0.86 mm inner diameter; Sutter Instruments). Data were digitized online at 20 kHz and filtered at 3 kHz with a low-pass Bessel filter. For voltage recordings, membrane potential values (RMP, steady-state responses used to calculate the input...
resistance and the Sag ratio i.e. ratio of peak versus steady state of the hyperpolarizing voltage response) uncorrected for liquid junction potential were measured manually; action potential properties (threshold, amplitude, etc.) were detected using a custom-made software written in Python (AP threshold was defined as the voltage at which the first derivative of the voltage trace reaches 10% of its peak; AP amplitude was defined as the difference between the AP threshold voltage and the most depolarized voltage of the AP; AHP amplitude was defined as the difference between the AP threshold voltage and the most hyperpolarized voltage of the AP). For current recordings, individual mIPSCs were detected as inward current changes above a variable threshold for 1.2 ms, referenced to a 2.5 ms baseline period, and analyzed offline using EVAN 1.5 (Nusser et al., 2001). The detection thresholds were similar between cell types (Golgi: 1.78 ± 0.23 pA, range 0.5 - 3.0 pA; Lugaro: 2.14 ± 0.24 pA, range 1.5 - 3.0 pA). Traces containing overlapping synaptic currents in their decaying phase were discarded from the analysis of decay times. Access resistance (Ra) was subject to 70% compensation and was continuously monitored. If Ra changed >20% during the recording, the cell was discarded from the analysis. All recordings were rejected if the uncompensated Ra became > 20 MΩ. After recordings, slices were fixed in 0.1 M phosphate buffer (PB) containing 2% paraformaldehyde (PFA; Molar Chemicals) and 15 v/v% picric acid (PA) for 24 h before post hoc visualization of the biocytin-filled cells.

Post hoc visualization of biocytin-filled cells. Slices were washed several times in 0.1 M PB, embedded in agar, and re-sectioned at 60 μm thickness with a Vibratome. Sections were then washed in Tris-buffered saline (TBS), blocked in TBS containing 10% normal goat serum (NGS) for 1 h, and then incubated in TBS containing rabbit anti-GFP (1:1000; Millipore), 2% NGS and 0.1% Triton X-100 overnight at 24°C. Sections were then washed three times in TBS, incubated in TBS containing Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:500; Life Technologies), Cy3-conjugated streptavidin (1:500; Jackson ImmunoResearch), 2% NGS and 0.1% Triton X-100 for 2 h, followed by washing and mounting on glass slides in Vectashield (Vector Laboratories). In some cases, other primary antisera were used: mouse anti-GFP (1:1000; NeuroMAb) and rabbit anti-
calbindin IgGs (1:1000, Swant), and the secondary antisera used were Alexa Fluor 488-conjugated goat anti-mouse (1:500; Life Technologies) and Cy5-conjugated goat anti-rabbit (1:500; Jackson ImmunoResearch) IgGs. Images were acquired using a confocal laser scanning microscope (FV1000; Olympus) with a 20x (numerical aperture (NA) = 0.7) or a 60x (NA = 1.35) objective. Z-stack images were acquired for cell identification, morphological reconstruction and quantification with the Neurolucida system (Micro-BrightField). The axons of MLIs were fully reconstructed from the point where they emerged from the soma, and markers were placed at axonal varicosities.

**Immunofluorescent reactions and quantification.** Male adult (n = 4, age 70 days, for neurogranin immunolabeling) or juvenile (n = 2, age 21 and 28 days, for GlyT2 immunolabeling) GlyT2-GFP mice, three male adult WT mice (age 35, 35 and 68 days) or two male adult Wistar rats (both age 42 days, for GlyT2 immunolabeling) were anesthetized initially with isoflurane, followed by Ketamine (www.vetcentre.com; 0.5ml/100g body weight; intraperitoneal injection), and were then transcardially perfused with 0.9% saline for 2 min followed by 2% PFA in 0.1 M sodium acetate buffer (pH = 6.0) for 15 minutes. The cerebella were dissected and then washed three times in PB. Vibratome sections were cut at 60 μm. All sections were then washed in TBS, followed by blocking in TBS containing 10% NGS for 1 h. The sections were then incubated in a solution containing single or mixtures of primary antisera made up in TBS containing 0.1% Triton X-100 and 2% NGS overnight at 24°C. Next, sections were incubated in appropriate secondary antisera made up in TBS containing 2% NGS for 2 h, then washed and mounted in Vectashield (Vector Laboratories). The following primary and secondary antisera were used for experiments illustrated in Fig. 3A-B: rabbit anti-GABA₆R α3 (1:1000; Synaptic Systems) and Alexa488-conjugated goat anti-rabbit (1:500; Invitrogen/Molecular Probes); guinea-pig anti-neuroligin-2 (1:500; Frontier Institute) and Cy3-conjugated donkey anti-guinea pig (1:500; Jackson ImmunoResearch); mouse anti-GABA₆R β3 (1:1000; Neuromab) and Cy5-conjugated goat anti-mouse (1:1000; Jackson ImmunoResearch). The following primary and secondary antisera were used for experiments illustrated in Fig. 3C-E: mouse anti-GFP (1:1000; NeuroMAb) and Alexa488-conjugated goat anti-mouse (1:500;
Invitrogen/Molecular Probes); guinea pig anti-GABA\(_A\)\(\alpha\)3 subunit (1:500; Synaptic Systems) and
Cy3-conjugated donkey anti-guinea pig (1:500; Jackson ImmunoResearch); rabbit anti-neurogranin
(1:1000; Millipore) and Cy5-conjugated goat anti-rabbit (1:1000; Jackson ImmunoResearch). The
following primary and secondary antisera were used for experiments illustrated in Fig. 5: guinea pig
anti-GlyT2 (1:5000; Millipore) and Cy3-conjugated donkey anti-guinea pig (1:500; Jackson
ImmunoResearch); rabbit anti-GABA\(_A\)\(\alpha\)3 subunit (1:1000; from Prof. W. Sieghart, Vienna,
Austria) and Cy5-conjugated goat anti-rabbit (1:500; Jackson ImmunoResearch). Z-stack images
were acquired in a random manner using a confocal laser scanning microscope (FV1000) with a
20x or a 60x objective. Golgi cell dendrites were reconstructed and GABA\(_A\)\(\alpha\)3 immunoreactive
puncta were indicated by markers using the Neurolucida software. Two adjacent edges and the
upper focal plane were used as exclusion boundaries for stereological optical disector counting of
markers. The number of markers divided by the volume of the confocal stack was used to calculate
synapse density estimates.

**Electron microscopy.** Female adult GlyT2-GFP mice (n = 5, age 38.4 ± 10.5 days) were
anesthetized initially with isoflurane inhalation, followed by Ketamine, and then transcardially
perfused with 0.9% saline for 2 min followed by a fixative containing 4% PFA and 15 v/v% PA in
0.1 M PB with either 0%, 0.01% or 0.1% glutaraldehyde for 15-30 min. The cerebella were then
washed three times in PB and Vibratome sections were cut at 70 μm. Sections were cryoprotected
in 10% sucrose in PB for 1 hour and then 30% sucrose overnight at 4°C followed by freezing in
liquid nitrogen and thawing in PB. Sections were then treated with 1% H\(_2\)O\(_2\) in PB for 10 minutes,
incubated with mouse anti-GFP IgG (1:500, NeuroMAb) diluted in TBS with 2% NGS overnight at
24°C, followed by biotinylated goat anti-mouse (1:50, Vector Laboratories) antibody diluted 1:50 in
TBS with 2% NGS for 2 h, and then an avidin-biotin complex (ABC; Vector Laboratories) diluted
in TBS overnight at 24°C, and then with 3_3-diaminobenzidine tetrahydrochloride (DAB; 0.05%
solution in Tris buffer, pH 7.4) as chromogen and 0.01% H\(_2\)O\(_2\) as oxidant for 2 min. Sections were
then postfixed in 1% OsO\(_4\) for 20 min, stained with 1% uranyl acetate for 25 min, dehydrated in a
graded series of ethanol and embedded in epoxy resin (Durcupan). Sections were re-embedded, and serial sections were cut at 70 nm thickness using an ultramicrotome (Ultracut; Leica Microsystems) and collected onto copper pioloform-coated slot grids. Sections were viewed using a JEOL1011 microscope, and digital images of all labeled profiles present in a section were captured with a cooled CCD camera (Cantega G2 camera, Olympus Soft Imaging Solutions GmbH).

Statistical procedures. All data are expressed as mean ± standard deviation throughout this manuscript. All statistical comparisons were made with Statistica 11 software (Scientific Computing). Intrinsic electrical properties datasets failing the Shapiro–Wilk normality test were compared using the non-parametric Mann-Whitney U test; data passing this test were compared by one-way parametric ANOVA. Comparisons of data pertaining to the spontaneous firing of GoCs in the three different genotypes were made using either a non-parametric Kruskal-Wallis test or a one-way parametric ANOVA, according to the results of the Shapiro-Wilk normality tests. sIPSC and mIPSC properties and GABAAR pharmacology data passed the Shapiro–Wilk normality test and were compared by one-way parametric ANOVA, with ACSF ‘baseline’ and drug ‘steady state’ conditions in the same cells treated as repeated measures (rm-ANOVA). Where appropriate, data were further assessed by conducting a post hoc test (Tukey’s unequal n honestly significant difference post hoc test: Tukey’s unequal n; or the Kruskal-Wallis multiple comparisons test). Differences were considered significant if p < 0.05.
Results

Because GFP is expressed in distinct types of GABAergic INs of the GCL in GlyT2-GFP mice (GoCs, LuCs and globular cells; Zeilhofer et al., 2005; Simat et al., 2007) and their cell type-specific features cannot be easily identified in acute slices due to the high density and extensive overlap among the numerous GFP-containing axonal and dendritic processes, we post hoc identified all of our recorded and biocytin-filled INs. We successfully recovered 39 neurons with sufficient amounts of axons and dendrites for unequivocal identification as either GoCs (n = 30; Fig. 1A) or LuCs (n = 9; Fig. 1B). GoCs had somata of variable sizes, several basal dendrites and apical dendrites crossing the PCL and ramifying to different extents in the ML. Their axons extensively arborized in the GCL. LuCs had horizontally elongated somata and dendrites running parallel with and under the PCL in the GCL, and a sparsely ramifying axon in the ML. The axons of LuCs were frequently truncated. Neither globular cells nor LuCs with somatic locations deep in the GCL were present in our sample.

Differences in GoCs (n = 30) and LuCs (n = 9) responses to hyper- and depolarizing current injections were readily observed (Fig. 1A,B). Quantitative comparisons indicated that current rheobase was significantly larger (98.0 ± 58.0 pA vs. 30.0 ± 21.2 pA; p = 0.0002, Mann-Whitney U test) and input resistance was significantly lower (207.0 ± 89.8 MΩ vs. 556.3 ± 239.5 MΩ; p = 0.0001, Mann-Whitney U test; Fig. 1C) in GoCs versus LuCs. Action potential (AP) parameters also varied between cell types: GoCs had a significantly more negative AP threshold (-35.8 ± 4.1 mV vs. -29.7 ± 5.9 mV; p = 0.0041, Mann-Whitney U test), larger AP amplitude (56.6 ± 8.0 mV vs. 42.6 ± 6.6 mV; p = 0.00003, One-way ANOVA) and larger afterhyperpolarization amplitude (25.3 ± 4.8 mV vs. 20.3 ± 2.7 mV; p = 0.0059, One-way ANOVA; Fig. 1C) than LuCs. In contrast, the resting membrane potential (-69.0 ± 7.7 mV vs. -72.4 ± 9.1 mV; p = 0.34, One-way ANOVA) and sag ratio (ratio of peak versus steady state of the hyperpolarizing voltage response; 1.08 ± 0.09 vs. 1.03 ± 0.03; p = 0.46, Mann-Whitney U test; Fig. 1C) were not significantly different between GoCs and LuCs. However, by far the most reliable difference between the two cell types was the
much higher frequency of spontaneous synaptic potentials in LuCs (Fig. 1A,B; see also Hirono et al., 2012).

We therefore recorded sIPSCs and mIPSCs from a subset of these GoCs (n = 12) and LuCs (n = 7) in the whole-cell voltage-clamp mode. All data passed the Shapiro-Wilk test and was compared with a rm-ANOVA followed by Tukey’s unequal n when appropriate. Spontaneous IPSCs were significantly less frequent (0.4 ± 0.2 Hz vs. 10.9 ± 5.0 Hz; p = 0.0002), significantly smaller in amplitude (10.5 ± 3.1 pA vs. 21.1 ± 7.2 pA; p = 0.0033) and had a significantly larger mean weighted decay time constant (τw; 20.3 ± 10.1 ms vs. 5.5 ± 0.6 ms; p = 0.0042) in GoCs compared to LuCs, consistent with our qualitative assessment based on current-clamp recordings. Application of TTX had a minimal effect on the frequency of IPSCs in GoCs (reduced to 0.3 ± 0.2 Hz; n = 12, p = 1.00), but significantly reduced the frequency in LuCs (reduced to 6.6 ± 3.1 Hz; n = 7, p = 0.0026). In contrast, neither IPSC peak amplitude (10.3 ± 2.7 pA for GoCs; 21.7 ± 7.2 pA for LuCs; p = 0.52, rm-ANOVA) nor τw (21.6 ± 7.7 ms for GoCs; 5.5 ± 0.6 ms for LuCs; p = 0.69, rm-ANOVA) were significantly affected for either cell type. The lack of TTX effect on the frequency of IPSCs in GoCs suggests that a large fraction of cell bodies providing the inhibitory inputs to GoCs was either spontaneously silent or was present outside of our in vitro slice. In contrast, LuCs receive inhibitory inputs from at least one spontaneously active local source, consistent with the results of Hirono et al. (2012).

Several studies have reported the presence or absence of spontaneous spiking activity of GoCs in acute slices (Dieudonne, 1998; Forti et al., 2006; Dugue et al., 2005, 2009; Hirono et al., 2012; Vervaeke et al 2010; Rudolph et al., 2015) and suggested that the lack of spontaneous activity might reflect deficiencies in the quality of the slices (Rudolph et al., 2015). In our initial dataset in GlyT2-GFP mice, we observed that only 7% of GoCs (2/30) displayed spontaneous activity, whereas spontaneous firing was not observed in LuCs. We investigated GoC spontaneous spiking activity in more detail by using two different cutting solutions (sucrose- or K-gluconate-based) and compared GlyT2-GFP mouse, WT mouse (C57Black6J) and Wistar rat GoCs, measuring the spontaneous
firing frequencies in the cell-attached configuration for 3 minutes prior to attaining the whole-cell configuration. We found that the occurrence of spontaneous activity was low in GoCs from GlyT2-GFP (21%; 6/28 cells) and WT (13%; 1/8 cells) mouse slices cut in K-gluconate (median and maximum frequencies in GlyT2-GFP: 0 Hz and 0.58 Hz; in WT: 0 Hz and 2.16 Hz). In contrast, Wistar rat GoCs frequently exhibited spontaneous activity, either using sucrose- (69%; 9/13 cells) or K-gluconate-based (79%; 11/14 cells) cutting solutions (median and maximum frequencies: sucrose: 2.9 Hz and 9.9 Hz; gluconate: 3.2 Hz and 11.3 Hz). When we quantitatively analysed the data from slices prepared with the K-gluconate solution across genotypes, the mean spontaneous frequency of rat GoCs (3.4 ± 3.1 Hz, n = 14) was significantly higher than that found in both GlyT2-GFP and WT mouse GoCs (0.1 ± 0.2 Hz, n = 15; p = 0.0048 and 0.3 ± 0.8 Hz, n = 8; p = 0.0023, respectively, Kruskal-Wallis multiple comparisons post-hoc test).

We also measured the frequency, peak amplitude and kinetic parameters of sIPSCs and found no significant differences between genotypes for any of these measured parameters (e.g. sIPSC $\tau_w$; rat, 21.0 ± 11.1 ms; GlyT2-GFP mouse, 24.4 ± 10.0 ms; WT mouse, 27.6 ± 9.3 ms; p = 0.15, one-way ANOVA), which were very similar to those found in GlyT2-GFP mouse GoCs prepared using the sucrose-based cutting solution (see above). All of these data taken together indicate species-specific variability in the spontaneous activity of GoCs in acute slices, but our data does not rule out any additional mechanism. Because the $\tau_w$ of sIPSCs in GoCs recorded from GlyT2-GFP mice, WT mice and rats were very similar, we performed most of the following experiments in GlyT2-GFP mice.

The large difference in the decay time constant of mIPSCs between GoCs and LuCs indicates either a different contribution of GABAergic and glycinergic components to the IPSCs, a different subunit composition of the postsynaptic receptors or differences in dendritic filtering of the synaptic currents. To test the first possibility, we applied the selective GABA$_A$R antagonist SR95531 (20 $\mu$M) and observed a complete block of mIPSCs in both GoCs and LuCs (Fig. 2A), indicating that the presynaptic inhibitory cells either release only GABA that activates postsynaptic GABA$_A$Rs, or
if they co-release GABA and glycine, then glycine does not activate postsynaptic glycine receptors in these cells at this time point of development. To investigate potential differences in the subunit composition of postsynaptic GABA<sub>A</sub>Rs in the two cell types, we analyzed the pharmacological properties of mIPSCs in GoCs and LuCs (Fig. 2B,C).

We found that 100 nM zolpidem, a selective positive allosteric modulator for α<sub>1</sub> subunit-containing GABA<sub>A</sub>R at this concentration, enhanced the mIPSC τ<sub>w</sub> selectively in LuCs (from 6.0 ± 0.7 ms to 8.5 ± 1.4 ms, n = 4; p = 0.0039; rm-ANOVA then Tukey’s unequal n), whereas 100 nM TP003, a positive allosteric modulator specific for α<sub>3</sub> subunit-containing GABA<sub>A</sub>R, prolonged the mIPSC τ<sub>w</sub> selectively in GoCs (from 18.1 ± 6.1 ms to 21.4 ± 8.5 ms, n = 8; p = 0.0091; rm-ANOVA then Tukey’s unequal n). There was no significant effect of zolpidem or TP003 on the frequency or amplitude of mIPSCs in either cell type (Fig. 2C). The fast decay times of mIPSCs in LuCs is also consistent with postsynaptic α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub> subunit-containing GABA<sub>A</sub>Rs, whereas the four-fold slower decay time of mIPSCs in GoCs is consistent with α<sub>3</sub> subunit-containing receptors (Eyre et al., 2012). We also fitted exponentials to the decay of individual mIPSCs recorded from GoCs and LuCs and found that the distributions differed significantly (p < 0.0001; Mann-Whitney U test) between the two cell types (Fig. 2B). In addition, the mean 10-90% rise times of mIPSCs in LuCs (0.6 ± 0.1 ms) and GoCs (1.2 ± 0.6 ms) were comparable to those recorded from neuronal populations expressing either only α<sub>1</sub> or only α<sub>3</sub> as α subunits, respectively (Eyre et al., 2012), suggesting the lack of a major effect of dendritic filtering on the differences in τ<sub>w</sub> between these two cell types.

Given the apparently selective expression of the GABA<sub>A</sub>R α<sub>3</sub> subunit by GoCs, we performed immunofluorescent labeling to investigate GABAergic synapses on GoCs. Immunofluorescent experiments following low pH-mediated antigen retrieval indicated that the α<sub>3</sub> subunit was present in the cerebellar cortex as intensely fluorescent clusters. These clusters were much less frequent than those labeled for neuroligin-2 or the GABA<sub>A</sub>R α<sub>1</sub>, β<sub>2</sub>, β<sub>3</sub> or γ<sub>2</sub> subunits. Triple labeling experiments revealed that the α<sub>3</sub> subunit immunopositive clusters were also immunoreactive for the
GABA<sub>A</sub>R β3 subunit, the usual β subunit partner of the α3 subunit, and for neuroligin-2, indicating that these clusters correspond to GABAergic synapses in both the ML (Fig. 3A) and the GCL (Fig. 3B). Many of the neuroligin-2 puncta were not labeled for the α3 or β3 subunits, representing the much more abundant inhibitory synapses on Purkinje cell dendrites and on MLIs that express mainly the α1 and β2 subunits. Next, we performed immunofluorescent labelling for the α3 subunit in GlyT2-GFP mice, and colocalized this with neurogranin, a marker selective for a subpopulation of GoCs. Immunofluorescent reactions for the α3 subunit revealed punctate labelling of the GCL and ML in GlyT2-GFP mice, similar to WT mice and rats, and demonstrated that these clusters were associated with both GlyT2-GFP-expressing and neurogranin immunopositive dendrites (Fig. 3C-F). Quantification of the α3 subunit immunopositive puncta in the ML of lobule 8 in GlyT2-GFP mice revealed that 36.0% of all α3 positive puncta were associated with dendrites expressing GlyT2-GFP (0.48 x 10<sup>6</sup> puncta per mm<sup>3</sup>), but only 15.8% of these puncta had an adjacent, presynaptic GlyT2-GFP-expressing axon-like structure (Fig. 3C-F). In contrast, quantification indicated that 11.0% of all α3 positive puncta were associated with dendrites immunoreactive for neurogranin. In these dendrites, 41.2% of the α3 positive puncta were associated with presynaptic GlyT2-GFP-expressing axonal structures (Fig. 3C-F), demonstrating that axons synapsing onto neurogranin-labeled GoCs are more likely to express GlyT2-GFP. This difference was also evident when we quantified the labelling in lobule 5: 19.0% of all observed α3 puncta were associated with dendrites expressing GlyT2-GFP, of which 32.5% had an adjacent, presynaptic GlyT2-GFP-expressing axon-like structure, whereas 11.2% of all α3 puncta were associated with dendrites immunoreactive for just neurogranin, of which 71.1% had an adjacent, presynaptic GlyT2-GFP-expressing axon-like structure. In addition, 10.6% of all observed α3 puncta were associated with dendrites expressing both GlyT2-GFP and neurogranin, of which 72.3% had an adjacent, presynaptic GlyT2-GFP-expressing axon-like structure.

We observed that, in lobule 8, 32.3% of all α3 puncta were associated with GlyT2-GFP-expressing structures that could not be unambiguously categorized as dendrites, and the remaining 20.7% of
puncta were not associated with either GlyT2-GFP-expressing or neurogranin immunoreactive structures. These values were similar in lobule 5; 49.3 % of all observed α3 positive puncta were associated with ambiguous GlyT2-GFP-expressing profiles, and 9.9% were not associated with either GoC marker. This is likely to be the consequence of incomplete labelling of GoC dendrites with neurogranin or GFP due to the extremely mild chemical fixation required for the selective, synaptic labelling of the α3 subunit.

We also reconstructed the dendrites of the labelled GoCs and calculated the density of α3 positive puncta associated with a GlyT2-GFP-expressing axon-like structure or not. In lobule 8, for GlyT2-GFP-expressing dendrites, there were 19 and 102 α3 positive puncta per mm of GoC dendrite with and without GlyT2-GFP-expressing inputs, respectively. For neurogranin immunoreactive dendrites, there were 35 and 50 α3 positive puncta per mm dendrite with and without GlyT2-GFP-expressing inputs, respectively. In lobule 5, for GlyT2-GFP-expressing dendrites, there were 26 and 53 α3 positive puncta per mm dendrite with or without GlyT2-GFP-expressing inputs, respectively. Finally, for neurogranin immunoreactive dendrites in lobule 5, there were 70 and 26 α3 positive puncta per mm dendrite with and without GlyT2-GFP inputs, respectively (Fig. 3F). These data indicate that although there may be subtle regional differences within the cerebellar cortex, the majority of GABAAR α3 subunit-containing synapses on GlyT2-GFP-expressing dendrites do not have a GlyT2-GFP-expressing presynaptic partner. In contrast, the proportion of GlyT2-GFP-expressing inputs onto neurogranin immunoreactive GoCs is substantially higher, consistent with the finding of a recent report demonstrating that these cells receive a selective GlyT2-expressing input from deep cerebellar nuclei (DCN) neurons (Ankri et al., 2015).

Next, we performed another set of experiments for estimating the proportion of GlyT2-GFP-expressing inputs onto GlyT2-GFP-expressing GoC dendrites. We used an immunoperoxidase reaction for GFP and processed the sections for EM (GlyT2-GFP-DAB). We systematically sampled the ML in lobule 8 of GlyT2-GFP-DAB reactions and analyzed 117 dendritic profiles at the ultrastructural level. The vast majority of synaptic contacts onto GlyT2-GFP-DAB dendrites
were asymmetric and were characteristic of glutamatergic parallel fiber synapses (546 out of 570 boutons, 95.8%; 553 out of 578 synapses, 95.7%; Fig. 3G). Of the 24 boutons forming symmetrical synapses onto GlyT2-GFP-DAB dendrites, 20 boutons were not labelled for GlyT2-GFP-DAB (Fig. 3H,I). Only four appositions (16.6%) between GFP-expressing axons and GFP-expressing dendrites were observed; three formed synapses with one active zone (AZ) each and one formed a synaptic contact with two AZs onto the target dendrite (Fig. 3J).

We also observed and examined a total of 54 GlyT2-GFP-DAB boutons, out of which 39 (72.2%) formed 44 AZs onto MLI dendrites and 11 (20.4%) established 18 AZs onto MLI somata (Fig. 4A-C); the remaining 4 boutons contacted GlyT2-GFP-DAB dendrites, as detailed in the previous paragraph. We never observed synapses onto Purkinje cell dendrites or spines, even though the GlyT2-GFP-DAB axons were sometimes observed to come into direct contact with these structures (Fig. 4D). We also observed six instances of contacts between GlyT2-GFP-DAB labelled dendrites, three of which had puncta adherentia-like membrane specializations and potential electrical synapses, although these were difficult to unequivocally identify in our material. In summary, these data corroborate our LM observations, indicating that the majority of inhibitory inputs onto GlyT2-GFP-expressing GoCs in the ML do not originate from GlyT2-GFP-expressing axons.

The GlyT2-GFP mouse line is subject to mosaic expression of the transgene, resulting in a variable degree of GFP expression in glycinergic cells (Husson et al., 2014). In order to test whether the low proportion of α3 positive puncta facing GlyT2-GFP-expressing terminals is the consequence of an incomplete expression of the GFP in glycinergic cells, we used an immunofluorescent reaction for GlyT2 to label glycinergic terminals irrespective of their GFP content in the cerebellar cortex of rats and mice, and combined this with immunofluorescent labeling for the α3 subunit (Fig. 5).

Quantification of the immunoreaction indicated that only a minority of α3 subunit immunoreactive puncta was apposed by GlyT2 immunoreactive varicosities (28.5% in Wistar rat, 31.3% in WT mouse, 33.8% in GlyT2-GFP mouse; Fig. 5B). Conversely, approximately half or less of all GlyT2 immunoreactive terminals were facing α3 subunit immunoreactive puncta (27.0% in Wistar rat,
40.7% in WT mouse, 55.4% in GlyT2-GFP mouse). These results add further evidence for a lack of glycine/GlyT2 in the majority of inhibitory inputs to GoC dendrites located in the ML.

As neither Purkinje nor GoC axons arborize in the ML, the most obvious cells giving rise to GlyT2-GFP negative GABAergic axons that could, in principle, innervate GoC dendrites in the ML are MLIs. Because the existence of MLI to GoC connections is debated, we investigated if this connectivity could explain the high occurrence of GlyT2-GFP unlabeled synapses onto GoC dendrites by recording and intracellularly filling MLIs in acute slices (Fig. 6). From the 8 cells that were recovered with sufficiently intact axonal arbors, we reconstructed a total of 7909 μm of axon with 1870 boutons, but observed that only 19 boutons (1.02%) were closely opposed to GlyT2-GFP-expressing dendrites (Fig. 6C). In contrast, appositions were frequently observed with Purkinje cell dendrites (labeled for calbindin; Fig. 6E) and MLI somata (which transiently express the GlyT2-GFP transgene during their development and are thus weakly labelled in our sample; Fig. 6F). Our data argue against the presence of profound MLI to GoC chemical synaptic connectivity and are consistent with either extremely weak or a lack of innervation (Hull and Regehr, 2012).

However, one percent of an abundant bouton population could still be a significant source of input to an infrequent cell population. From our reconstructions we quantified that, on average, MLIs have 234 ± 37 boutons per cell, which is similar to previous estimates by Sultan & Bower (1998) using Golgi impregnation. To estimate the proportion of GABAergic inputs on GoCs that might originate from these MLIs, we compared the density of MLIs (48 000 / mm³, measured in the present study) and GoCs (~4700 / mm³ GlyT2 positive GoC, as reported in Simat et al., 2007; Billings et al., 2014), giving a ratio of 10:1 (see also Korbo et al., 1993). This implies that a maximum of 23 GABAergic synapses (234 boutons per cell × 1.02 % contacting GoCs × 10:1 cell ratio) could originate from MLIs onto each GlyT2-expressing GoC, which would constitute a maximum of 14% of all GABAergic synapses (163) onto GoC dendrites in the ML (1350 ± 545 μm of dendrite per GoC × 0.121 GABAAR α3 puncta per μm of GoC dendrite).
DISCUSSION

With the aid of GlyT2-GFP animals, patch-clamp recordings and post hoc morphological analysis, we were able to identify GoCs and LuCs, and to investigate their place in the GABAergic circuit of the cerebellar cortex. By identifying the GABA$_3$R $\alpha_3$ subunit as a selective marker for inhibitory synapses on GoC dendrites, we provided LM evidence that GABAergic innervation of GoCs by local inhibitory axon populations is sparse. GlyT2-GFP-expressing axons provide a maximum of 16% of all GABAergic synapses onto GlyT2-GFP-expressing GoC dendrites in the ML, with potential origin from LuCs, globular or DCN cells. Glycine immunoreactive axons not expressing GlyT2-GFP could account for a further 18% of these inputs. Our data argue against the presence of MLI to GoC connections, but we cannot exclude the possibility of a sparse innervation (a maximum of 14%). However, even if this connection exists, more than half of the inhibitory inputs onto GoCs must arise from purely GABAergic sources that are not located within the cerebellar cortex.

Local inhibitory inputs to GoCs

Golgi cell dendrites occupy all layers of the cerebellar cortex, and both basal and apical dendrites contain GABA$_3$R $\alpha_3$ subunit positive puncta, demonstrating that these cells receive inhibitory inputs in all layers. Thus all inhibitory neurons of the cerebellar cortex, as well as any extra-cortical inputs, could provide GABAergic/glycinergic innervation of GoCs. Molecular layer IN axons are confined to the ML, where they innervate each other and provide a powerful inhibition to Purkinje cells (Kondo and Marty, 1998; Vincent and Marty, 1996). It has been assumed that these axon terminals are also responsible for the inhibitory inputs onto GoC dendrites in the ML (Palay and Chan-Palay, 1974). Here we investigated the potential inhibitory connections between MLIs and GoCs using intracellularly filled MLIs and post hoc LM analysis of the spatial relationships between their axon terminals and GlyT2-GFP-expressing GoC dendrites. Our results demonstrate that a maximum of one percent of the MLI boutons are in close apposition with GlyT2-GFP-
expressing GoC dendrites, which could in principle provide direct synaptic inputs. However, due to
the insufficient ultrastructural preservation in our mildly fixed tissue, we could not verify these
appositions as synaptic connections using EM. Even if they were all synapses, and even though
MLIs outnumber GoCs by 10:1, we calculate that a maximum of 14% of all GABAergic inputs onto
GoCs could arise from MLIs in the ML. These results are in agreement with those of Hull and
Regehr (2012) who used optogenetic stimulation techniques and paired recordings to demonstrate
the lack of functional connectivity between MLIs and GoCs.

Lugaro and globular cells are the least studied inhibitory INs of the GCL. Their classifying feature
is that they have a minimal axonal arbor in the GCL, but instead provide a long-range innervation in
the ML. As demonstrated here and in previous studies (Zeilhofer et al., 2005; Simat et al., 2007),
LuCs and globular cells express GFP in these GlyT2-GFP transgenic animals and are likely to be
responsible for at least some fraction of the ~16% GABA/glycinergic inputs of GlyT2-GFP-
expressing GoCs in the ML. Although LuC/globular cell input to GoCs is sparse, they seem to be
sufficient to generate large amplitude rhythmic IPSCs upon 5-HT application (Dieudonne and
Dumoulin, 2000; Hirono et al., 2012). Our EM analysis demonstrated that the GlyT2-GFP-
expressing axon terminals exclusively innervate INs (MLIs and GoCs), but avoid the innervation of
the principal (Purkinje) cells of the cerebellar cortex. GoCs innervate glutamatergic GrCs, and
MLIs clearly innervate Purkinje cells; therefore globular and LuCs are the sole IN-selective INs of
the cerebellar cortex. Such IN-selective INs have been described in many brain regions, including
the hippocampus (Acsady et al., 1996; Gulyas et al., 1996), neocortex (Meskenaite, 1997; Melzer et
al., 2012) and the main olfactory bulb (Eyre et al., 2008), and our data indicate that LuC/globular
cells are also included in this category.

Inhibitory inputs onto GoCs could also arise from Purkinje cell local collaterals arborizing in the
superficial GCL and PCL. Calbindin immunoreactive Purkinje cell local axon collateral boutons
have been shown in close apposition to LuCs and globular cells adjacent to the PCL (Laine and
Axelrad, 2002; Simat et al., 2007). Purkinje cells are spontaneously active in in vitro slices (Hausser
and Clark, 1997), strongly suggesting causality between their cessation of activity and the significant reduction in LuC IPSC frequency we observed after the application of TTX. Consistent with this, globular cells have been shown to receive monosynaptic inhibitory inputs from Purkinje cells (Hirono et al., 2012). In contrast, we observed a very low frequency of sIPSCs in GoCs and no significant change in the frequency following TTX application, indicating that a significant proportion of the GABAergic inputs of GoCs are unlikely to be provided by Purkinje cells, but must instead arise from sources that are either spontaneously inactive or are not present in our acute slices. As stellate and basket cell networks are also spontaneously active in slices (Hausser and Clark, 1997), this gives further support to the idea that MLIs are also unlikely to provide major inhibitory inputs to GoCs.

Factors governing the occurrence of spontaneous activity in GoCs are also not well defined, although the age of the animals and the recording temperature are likely to be critical (Dieudonne, 1998; Forti et al., 2006). We found that spontaneously active GoCs are much more frequently encountered in rat versus mouse slices (Rudolph et al., 2015). Although we cannot entirely rule out the possibility that mouse GoCs are more vulnerable to the slicing procedure, our GoCs in mouse slices appeared healthy; success rates of patching, access resistances and firing patterns were similar to those obtained from rats. The facts that the rheobase current is smaller and the RMP is more depolarized in rat GoCs suggests that they are more excitable than mouse GoCs, although maximum firing rates upon current injection were similar. We suggest that the difference might lie in distinct ion channel expression patterns between rat and mouse GoCs.

Golgi cells have extensive axonal arbors in the GCL and provide phasic and tonic inhibition to GrCs. The issue of whether GoCs provide chemical GABAergic innervation to each other is debated; two recent studies using paired recordings found no evidence for this connection (Dugue et al., 2009; Vervaeke et al., 2010), but Hull and Regehr (2012) demonstrated a GoC to GoC connection probability of about 20%. A potential reason for this discrepancy might be the consequence of tonic presynaptic GABA<sub>B</sub> receptor-mediated inhibition of GABA release from GoC
axons. Dugue et al. (2009) and Vervaeke et al. (2010) did not eliminate GABA_B receptor-mediated presynaptic inhibition, whereas Hull and Regehr (2012) performed their experiments in the presence of a GABA_B receptor antagonist. We did not inhibit GABA_B receptors in our recordings, and thus GoC to GoC synaptic inhibition is unlikely to have contributed to our functional measurements. Anatomically, even if GoCs provide GABAergic innervation to each other, these inputs are confined to the GCL and cannot be responsible for the >50% ‘missing’ inputs on GoC dendrites in the ML. Due to the high density of GlyT2-GFP-expressing axonal and dendritic processes in the GCL, we were unable to discern the extent to which GlyT2-GFP-expressing presynaptic axons are in direct apposition with GlyT2-GFP-expressing GoC basal dendrites at α3 positive puncta. However, we consider it possible that this coverage is similar to that of the apical dendrites, as is suggested by the similar density of GABA_AR α3 puncta in these two regions (rat ML: 0.002 ± 0.001 puncta / µm³; GCL: 0.002 ± 0.0003 puncta / µm³).

**Extra-cortical inhibitory inputs to GoCs**

Recent work by Ankri et al. (2015) elegantly demonstrated that a population of GlyT2- and GAD65-expressing cells in the DCN projects to the cerebellar cortex and targets a subgroup of GoCs that are spontaneously active and express neurogranin, but not GlyT2. In agreement with this report, here we show that 40-70% of α3 immunoreactive synapses on neurogranin-expressing, GlyT2-GFP negative dendrites were contacted by GlyT2-GFP-expressing axons. This proportion is higher than that found on GlyT2-GFP-expressing GoCs (16-34%). A plausible explanation of these data is that GlyT2-GFP-expressing GoCs receive most, if not all of their GlyT2-GFP-expressing inputs from local LuCs/globular cells, whereas the neurogranin positive, GlyT2-GFP negative GoCs receive some of their inputs from LuCs/globular cells and an additional major input from GlyT2-GFP-expressing DCN cells. Because only 40% of DCN glycinergic cells express GFP in GlyT2-GFP mice (Husson et al., 2014), it is possible that the lack of GFP in the majority of the boutons...
contacting α3 positive puncta in GoC dendrites (both GlyT2-GFP-expressing and neurogranin immunopositive populations) could originate from these glycinergic, but GFP negative, DCN neurons. Our results using immunolocalization with an anti-GlyT2 antibody in WT mice and Wistar rats, however, argues against this possibility.

Ankri et al. (2015) demonstrated that the majority of GAD65-expressing DCN cell axons in the cerebellar cortex also express GlyT2, but did not test the expression of GAD67, which is also known to label DCN neurons (Uusisaari et al., 2007). Although the DCN have not been extensively characterized (Uusisaari and De Schutter, 2011), a report (Baurle and Grussner-Cornehls, 1997) indicated that only 23.7% of all DCN cells immunoreactive for GABA also expressed GlyT2. Many small GABAergic cells in the DCN project to the inferior olive (Batini et al., 1989), but this does not rule out the possibility that they also project to the cerebellar cortex, or even that all purely GABAergic DCN neurons are of this type. We therefore predict that a proportion of DCN GABAergic INs directly influences the cerebellar cortex inhibitory networks (Uusisaari and De Schutter, 2011). In summary, our results indicate that the majority of the inhibitory inputs onto GlyT2-GFP-expressing GoCs in the ML must arise from neurons outside the cerebellar cortex that contain GABA, but not glycine.
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FIGURE LEGENDS

**Figure 1.** Morphological and electrophysiological characterization of granule cell layer interneurons in the GlyT2-GFP mouse. **A**, Maximum intensity projection image of a confocal image Z-stack of an *in vitro* recorded and biocytin (red) labelled GoC showing GlyT2-GFP immunoreactivity (green). Scale bar: 20 μm. The neurolucida reconstruction of this cell is shown on the right (dendrites in blue, axon in black). ML – molecular layer; PCL – Purkinje cell layer; GCL – granule cell layer, Scale bar: 20 μm. Voltage responses of this cell to DC current injections are shown on the far right. **B**, As in **A**, but showing a Lugaro cell. Although the GFP signal was not detectable in this cell after the recording, prior to patching this cell was clearly GFP positive, as shown in the epifluorescent greyscale image in the inset (arrow; arrowheads indicate adjacent GlyT2-GFP expressing neurons). The neurolucida reconstruction of this cell is shown on the right (dendrites in red, axon in black). Scale bar: 20 μm. Voltage responses of this cell to DC current injections are shown on the far right. Note the frequent occurrence of spontaneous IPSPs in the traces. The IPSPs are depolarizing due to the elevated Cl⁻ concentration of the intracellular solution.

**C**, Summary of some electrophysiological properties of morphologically identified Golgi (blue) and Lugaro (red) cells. Rin - input resistance; RMP - resting membrane potential; AP - action potential; AHP - afterhyperpolarization; Sag ratio - ratio of peak versus steady state of the hyperpolarizing voltage response; Rheobase - minimum current required to generate at least one action potential. **:** p < 0.01, ***: p < 0.001. RMP and sag ratio were not different between cell types (p = 0.34 and p = 0.46, respectively). Average rheobase current was significantly different between cell types (p = 0.0002). Comparisons were made by Mann-Whitney U test or one-way ANOVA.

**Figure 2.** Golgi (GoC) and Lugaro cells (LuC) have distinct mIPSC properties and different GABAₐR α subunit content. **A**, Continuous current recordings from a GoC (blue) and a LuC (red) in the presence of 1 μM tetrodotoxin before and following the application of 20 μM SR95531 (traces below). Note the greater frequency and larger amplitude of mIPSCs in the LuC, and that all
currents were eliminated by the GABA_A_R antagonist. **B**, Average, peak-scaled traces from example cells before (GoC; blue, LuC; red) and after bath application of 100 nM TP003 (left; GoC - teal, LuC - orange) or 100 nM zolpidem (centre; GoC - grey, LuC - dark red). Note the much faster mIPSC decay and selective prolongation by 100 nM zolpidem in the LuC, compared to the slower decay and selective prolongation by 100 nM TP003 in the GoC. The right panel shows the cumulative probability distributions of τ_w of all individually fitted mIPSCs in GoCs (blue, n = 408 mIPSCs) and LuCs (red, n = 2300 mIPSCs). **C**, Summary of mIPSC mean frequency, peak amplitude and weighted decay time (τ_w) before (GoCs - blue, LuCs - red) and after bath application of 100 nM TP003 or 100 nM zolpidem. Note the large variance in τ_w in the population of GoCs, and that GoCs with a slower decay show a greater prolongation upon TP003 application. ****: p < 0.01 (Repeated measures parametric ANOVA, Tukey’s unequal n HSD post hoc test).

**Figure 3.** GlyT2-GFP-expressing Golgi cell (GoC) dendrites in the molecular layer (ML) are sparsely innervated by GlyT2-GFP-expressing axons. **A, B,** Immunofluorescent labelling for the GABA_A_R α3 subunit (α3, green) in the ML (**A**) and GCL (**B**) of WT mice is sparse, but overlaps with neuroligin-2 (NL2, red) and β3 subunit (β3, cyan) immunopositive puncta, indicating their synaptic enrichment. Maximum intensity projections of two (**A**) or four (**B**) confocal images at 1 μm separation. Scale bar: 2 μm, applies to **A** and **B**. **C,** Immunofluorescent labelling for the GABA_A_R α3 subunit (red) in the ML of GlyT2-GFP mice is evident as multiple puncta, many of which are associated with GlyT2-GFP-expressing (green) or neurogranin-immunoreactive (blue) dendrites. Scale bar: 10 μm. **D,** Maximum intensity projection (five confocal sections at 1 μm separation) of the left-hand boxed region in **C** at a higher magnification showing α3 subunit immunoreactive puncta (arrows) associated with a GlyT2-GFP-expressing dendrite, but lacking a presynaptic GlyT2-GFP-expressing bouton. Scale bar: 1 μm. **E,** Maximum intensity projection (five confocal sections at 1 μm separation) of the right-hand boxed region in **C** at a higher magnification showing intersections between GlyT2-GFP-expressing axons (arrowheads) and a neurogranin immunoreactive dendrite. Note the presence of additional GABA_A_R α3 subunit-immunoreactive
puncta not associated with a presynaptic GlyT2-GFP-expressing bouton (arrow). Scale bar: 1 μm.

**F**, Schematic proportional representation of α3 subunit immunoreactive puncta present on GoCs in lobule 5 (left) and lobule 8 (center), categorized as expressing neurogranin (Ng+, blue cells) or only GlyT2-GFP (GlyT2+, green cells). Each puncta was categorized as either facing a presynaptic, GlyT2-GFP-expressing (green) or GlyT2-GFP negative (hollow) axonal bouton, and total puncta density per mm of GoC dendrite for each GoC type is indicated below each cell. The graph (right) shows the percentage of GlyT2-GFP positive (green) and negative (hollow) boutons contacting α3 positive clusters for each type of GoC in the two lobules. **G**, Electron micrograph showing an asymmetric synapse made by a parallel fiber bouton (PFb) onto a GlyT2-GFP-DAB labelled dendrite (GFP+d). Scale bar: 500 nm, also applies to panels **H-J.** **H, I**, Electron micrographs showing symmetrical synapses formed by unlabeled boutons (b) onto GlyT2-GFP-DAB-labelled dendrites. **J**, Electron micrograph showing a symmetrical synapse formed by a GlyT2-GFP-DAB-labelled bouton (GFP+b) onto a GFP+d. Two PFbs forming asymmetric synapses onto the same dendrite are also visible. Bar graph shows the percentage of symmetrical synapses formed onto GFP+d by GFP positive (GFP+b) and negative (b) boutons.

**Figure 4.** GlyT2-GFP-expressing axons target molecular layer interneurons (MLI). **A**, Electron micrograph showing a GlyT2-GFP-DAB positive axon varicosity (GFP+b) making a symmetrical synapse (arrowhead) with a smooth, thin, non-spiny MLI dendrite (MLId). Scale bar: 500 nm. **B**, Electron micrograph showing a GFP+b establishing a symmetrical synapse with a MLI soma (MLIs). Scale bar: 2 μm. **C**, Electron micrograph of the boxed region in **B** showing the synaptic junctions (arrowheads) at a higher magnification. Scale bar: 500 nm. **D**, Electron micrograph showing a thin GlyT2-GFP-DAB immunoreactive inter-bouton axon segment (black precipitate) adjacent to, but lacking a synapse with, a Purkinje cell dendrite (PCd). Scale bar: 500 nm. Bar graph shows the percentage of synapses formed by GlyT2-GFP-DAB immunoreactive axons onto MLId, MLIs, GlyT2-GFP-DAB immunoreactive dendrites (GFP+d) and PCd.
Figure 5. Only one third of GABA$_A$R $\alpha$3 subunit immunoreactive puncta face varicosities immunolabeled for GlyT2. A, Maximum intensity projection (six confocal sections at 1 µm separation) from a Wistar rat section immunolabeled for GABA$_A$R $\alpha$3 (red) and GlyT2 (cyan). Scale bar: 20 µm. ML – Molecular Layer; PCL Purkinje Cell Layer; GCL – Granule Cell Layer. B, Enlarged view of the boxed region in panel A showing GABA$_A$R $\alpha$3 immunoreactive puncta (left, red) either not associated with (arrows) or closely associated with (double-headed arrow) GlyT2 immunoreactive terminals (middle, cyan), as can be seen in the overlay (right). Quantification of GABA$_A$R $\alpha$3 immunoreactive puncta is shown for three genotypes in the lower part of the overlay panel. All images are maximum intensity projections of four confocal sections at 1 µm separation. Scale bar: 2 µm.

Figure 6. GlyT2-GFP-expressing dendrites receive negligible inputs from molecular layer interneuron (MLI) axons. A, Maximum intensity projection image of a confocal image Z-stack of an in vitro recorded and biocytin (red) labelled MLI with axons spreading amongst GlyT2-GFP-expressing processes (green). Scale bar: 10 µm, also applies to B-C. B, Neurolucida reconstruction of the soma and axon of the recorded cell (red; black circles indicate varicosities; green circles indicate varicosities next to GlyT2-GFP-expressing dendrites). The number of reconstructed cells and the percentage occurrence of each varicosity type are indicated. C, Boxed area in A shown as a series of confocal image planes highlighting a single putative contact (green arrow). Note that most varicosities of the biocytin-filled axon have unlabeled targets, despite numerous GlyT2-GFP-expressing structures in the vicinity. D, Neurolucida reconstruction of a different MLI (soma and dendrites in blue, axon in red). Scale bar: 50 µm. Boxed regions correspond to the fluorescent images shown in E (left) and F (right). E, The axon of the MLI in D makes a bouton in apposition (red) to a calbindin immunoreactive (CB; cyan) Purkinje cell dendrite. F, The same axon also has several boutons in close apposition with a weakly GlyT2-GFP labelled MLI soma (GFP; green). Scale bar: 5 µm, also applies to E.