Tonic activation of GABA-B receptors reduces release probability at inhibitory connections in the cerebellar glomerulus

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ABSTRACT

In the cerebellum, granule cells are inhibited by Golgi cells through GABAergic synapses generating complex responses involving both phasic neurotransmitter release and the establishment of ambient GABA levels. Whereas at this synapse the mechanisms of postsynaptic integration have been clarified to a considerable extent, the mechanisms of neurotransmitter release remained largely unknown. Here we have investigated the quantal properties of release during repetitive neurotransmission revealing that tonic GABA-B receptor activation by ambient GABA regulates release probability. Blocking GABA-B receptors with CGP55845 enhanced the first IPSC and short-term depression in a train while reducing trial-to-trial variability and failures. The changes caused by CGP55845 were similar to those caused by increasing extracellular Ca2+ concentration in agreement with a presynaptic GABA-B receptor modulation of release probability. However, the slow tail following IPSC peak demonstrated a remarkable temporal summation and was not modified by CGP55845 or extracellular Ca2+ increase. This result shows that tonic activation of presynaptic GABA-B receptors by ambient GABA selectively regulates the onset of inhibition bearing potential consequences for the dynamic regulation of signal transmission through the mossy fiber – granule cell pathway of the cerebellum.
**INTRODUCTION**

GABAergic synapses are endowed with a variety of GABA-A receptor subtypes determining, in association with spillover and tonic GABA levels, the intensity and kinetics of inhibitory neurotransmission (Cherubini and Conti, 2001; Farrant and Nusser, 2005; Glykys and Mody, 2007). GABA-B receptors, in turn, can limit GABA release (Morishita and Sastry, 1995; Deisz et al., 1997; Mougnot et al., 1998; Jensen et al., 1999; Fearon et al., 2003; Than and Szabo, 2002; Chen and Yung, 2005; Kaneda and Kita, 2005) through a presynaptic mechanism. It has been proposed that, at central synapses, this mechanism could operate by reducing presynaptic calcium influx and synaptic vesicle recovery (Dittman and Regehr, 1996; Takahashi et al., 1998; Sakaba and Neher, 2003), thereby regulating the dynamics of repetitive neurotransmission (Hefft et al., 2002). However, the impact of this change is likely to depend on the specific property of synapses, including their presynaptic dynamics, the GABA-A receptor subtypes, the amount of neurotransmitter spillover and the tonic GABA level.

Along the mossy fiber pathway of cerebellum, GABAergic synapses are formed between Golgi cells and granule cells inside the glomeruli. In response to stimulation, inhibition arises rapidly with millisecond precision before slowly degrading over about 100 ms (Eccles et al., 1967, in vivo; Maffei et al., 2002, in vitro). The Golgi cells are spontaneously active at low frequency (Forti et al., 2006; Solinas et al., 2008a,b) and respond to punctuate stimulation with brief spike bursts (Vos et al., 1999). The granule cells are activated by the mossy fibers (D’Angelo et al., 1993, 1995, 1999, 2001; Sola et al., 2004; Nieus et al., 2006; Rossi et al., 2006) and their activity is limited by the feed-forward and feed-back inhibitory loops formed by Golgi cells. In granule cells, α1 and α6 subunit-containing GABA-A receptors (Brickley et al., 1999; Farrant and Nusser, 2005) generate fast and slow IPSC components in response to direct release onto postsynaptic densities and spillover from neighboring contacts (Rossi and Hamann, 1998; Hamann et al., 2002; Rossi et al., 2003). In addition, ambient GABA at sub-micromolar concentration activates high affinity α6 GABA-A receptors generating tonic inhibition. This latter has been shown to regulate the input-output relationship of the granule cell (Brickley et al., 1996) and has been proposed to control the mossy fiber – granule cell gain (Mitchell and Silver, 2003). However, it is unknown whether GABA-B receptors, which are present in Golgi cells (Kulik et al., 2002) and have affinities in the same GABA concentration range (Galvez et al., 2000), could also be activated presynaptically and regulate neurotransmitter release. This potential regulation, given the multiple types and different activation modes of postsynaptic receptors, is expected to exert complex effects on repetitive Golgi cell – granule cell neurotransmission.

In this paper we report a novel mechanism for tonic GABA levels in the cerebellar glomerulus. In juvenile rats, GABA-B receptors exerted a tonic depression on release probability at the Golgi cell – granule cell synapse. The main consequence was that of reducing the first response without substantially altering the total inhibitory charge in a train. It is therefore proposed that ambient GABA, by acting through presynaptic GABA-B receptors, selectively regulates the sharpness of the onset of inhibition in granule cells. The potential consequences for signal transmission along the mossy fiber pathway are discussed.

**METHODS**

*Slice preparation and solutions*

Patch-clamp recordings in acute cerebellar slices were performed as previously reported (D’Angelo et al., 1993, 1995, 1999; Armano et al., 2000). Briefly, 17-to-23 day-old Wistar rats were anaesthetised with halothane (Aldrich, Milwaukee, WI) and killed by decapitation. Acute 220-μm-thick slices were cut in the sagittal plane from the cerebellar vermis in cold Krebs’ solution and maintained at 30°C before being transferred to a 1.5 ml recording chamber mounted on the stage of an upright microscope (OLYMPUS BX51WI, Japan). The preparations were perfused with Krebs’
solution (2 ml/min) and maintained at 30°C with a Peltier feedback device (TC-324B, Warner Instr. Corp., Hamden, CT, USA).

Krebs solution for slice cutting and recovery contained (mM): NaCl 120, KCl 2, MgSO4 1.2, NaHCO3 26, KH2PO4 1.2, CaCl2 2, glucose 11, and was equilibrated with 95% O2 and 5% CO2 (pH 7.4). During recordings, the glutamate receptors blockers CNQX, APV, 7Cl-Kyn and AIDA were applied through a local perfusion pipette. In some experiments, slices were pre-incubated with a low-Ca2+ solution and Ca2+ concentration was changed by both local and bulk bath perfusion. Krebs solutions with different Ca2+ concentrations (from 0.5 mM to 4 mM) were prepared maintaining the total concentration of divalent cations by corresponding changes in Mg2+ (with 6 mM Ca2+ no Mg2+ was added). The patch-clamp pipette solution contained (mM): Cs2SO4 81, NaCl 4, MgSO4 2, CaCl2 0.02, BAPTA 0.1, glucose 15, ATP-Mg 3, GTP 0.1, HEPES 15. This solution maintained resting free [Ca2+] at 100 nM and pH was adjusted to 7.2 with CsOH. Patch-clamp pipettes filled with this solution had a resistance of 5-8 MΩ before seal formation. All drugs were obtained from Sigma, except BAPTA tetrapotassium salt (Molecular Probes, Eugene, OR, USA); CNQX, APV, 7Cl-Kyn, AIDA and GABA-A receptor blocker bicuculline (Tocris-Cookson, Avonmouth, UK), and TTX (LATOXAN, Valence, France).

Data recording and analysis

In this paper we have recorded miniature synaptic currents (mIPSCs), spontaneous synaptic currents (sIPSCs) and evoked synaptic currents (eIPSCs) from granule cells. Granule cells were voltage-clamped at -10 mV with an Axopatch 200-B amplifier and currents sampled with a Digitida 1440-A interface (low-pass filter = 10 kHz, sampling rate = 100 kHz). Golgi cell axon bundles were stimulated with a patch-pipette via an isolation unit at a frequency of 0.1 Hz - 0.33 Hz. The acquisition program automatically switched between eIPSC and background activity recordings (for 3 to 10 sec), from which sIPSCs and mIPSCs were detected. IPSCs were digitally filtered at 1.5 kHz and analysed off-line with P-Clamp (Axon Instruments software). Peak amplitude, time to peak (ttp) and rise-time from 10% to 90% of peak amplitude (RT 10-90) and duration at half-width (HW) were computed. The paired-pulse ratio (PPR) between the first and second IPSC in a sequence was PPR=IPSC2/IPSC1. To minimize the impact of stimulus artefact in eIPSC trains, exponential fitting to response decay was extrapolated to find the baseline from which amplitude in the subsequent response was measured.

sIPSC and mIPSC analysis was performed automatically with Clampfit software, setting a proper threshold for event detection; a further visual inspection of detected signals allowed to reject noisy artefacts. A 10 min period was adopted to evaluate mean eIPSC amplitude and coefficient of variation (CV); longer periods did not usually improve the estimate. The error introduced in eIPSC CV by the indirect response, which causes non-zero failures (see Fig. 1D), was eliminated by setting all indirect responses to zero (these were identified because the lack of the transient component caused a distinct population of small and slow IPSCs in amplitude/RT 10-90 plots, data not shown).

Since the stability of synaptic transmission can be influenced by slow modifications of neurotransmitter release and series resistance, response stability was assessed from average eIPSC amplitudes over 3 min periods (Larkman et al., 1992). The average eIPSC amplitude changed by less than 10% over the time-windows used for statistical analysis. The series resistance was monitored by measuring passive current transients induced by 10-mV hyperpolarising voltage steps from a holding potential of -60 mV. The cerebellar granule cell has a compact structure and behaves like a single electrotonic compartment (Silver et al., 1996; D’Angelo et al., 1993, 1995; Cathala et al., 2003). Accordingly, the transients were reliably fitted with a mono-exponential function yielding membrane capacitance Cm= 4.3±0.1 pF (n=29), membrane resistance Rm=2.7±0.3 GΩ (n=29), and series resistance Rs=17.3±1.1 MΩ (n=29). The –3dB cell + electrode cut-off frequency was fVC = (2πRsCm)-1 = 2.4±0.1 kHz (n=29). Accepted deviations of these parameters in current transients recorded over the time-windows used for statistical analysis were less than 10%.
Data are reported as mean ± MSE and, unless otherwise indicated, statistical comparisons are done using Student’s t-test.

**Identification of minimal IPSCs**

At several central synapses, identification of minimal stimulation requires to adopt indirect statistical methods like establishing a certain percentage of successful responses to stimulation, often preventing from an accurate estimate of release failure rate and release probability. The glomerular synapses in the cerebellum, by being composed of a very few (just 2-4; Hamori and Somogyi, 1983; Harvey and Napper, 1991) contacts per granule cell, proves convenient to overcome these difficulties. At the excitatory connection between mossy fibers and granule cells, the intensity of stimulation can be finely tuned by “counting” the discrete levels of the response amplitude and selecting the minimal one (Sola et al., 2004; Saviane and Silver, 2006a, 2006b). At the inhibitory connections investigated here, minimal eIPSCs were similarly obtained by finely tuning the stimulation intensity. In addition, minimal eIPSC amplitude was systematically compared to that of sIPSCs, which are due to the activation of single Golgi cell contacts. Therefore, a post-hoc criterion could be applied in that minimal eIPSCs had to be statistically indistinguishable from sIPSCs, otherwise the recording was not further considered for quantal analysis. An additional advantage was that of identifying stimulation failures from the absence of the slow spillover-dependent “indirect” component”, therefore improving the confidence in release probability estimates and allowing the application of the “failure method” (see eq.3 below). Finally, the limited number of synaptic contacts and the favorable electrotonic properties of recordings allow a reliable estimate of quantum properties from mEPSCs.

**Binomial release statistics**

The quantal parameters of release were obtained by applying to minimal eIPSCs three statistical methods corresponding to eq.1, eq.2 and eq.3 in order to improve the reliability of parameter estimation. These methods derive from binomial statistics and are only schematically described here, since they do not significantly differ from previous applications (for a comprehensive treatment see McLachlan 1978; Clements and Silver, 2000; Clements, 2003; Sola et al., 2004).

The quantal theory states that the mean number of quanta released at each impulse (m, mean quantum content) depends on the number of releasing sites (n) and on the probability (p) that each quantum (q, quantum size) is released, so that eIPSC variance (S²=SD²) and mean amplitude (M=mq) are related through a parabolic function and eIPSC variability depends on the number of released quanta. The contribution of intrinsic quantum variability can be accounted for by identifying intra-site (type-I) and inter-site (type-II) sources. Intra-site variability (cvI) reflects fluctuation in the number of open channels at single sites (cvI-ss) and asynchrony in quantal delay at the eIPSC peak (cvI-qd). Inter-site variability (cvII) reflects differences among postsynaptic densities. Thus, the total quantal variance at the eIPSC peak can be expressed as cv²tot = cv²I + cv²II = (cv²I-ss + cv²I-qd) + cv²II. The variability of mIPSCs (cvq) includes intra-site and inter-site quantal variability, cv²q = cv²I-ss + cv²II. Since cv²I-ss can be assumed to be similar to cv²II (see Clements, 2003, for details), we equally divided the two terms as cv²I-ss ≃ cv²II = ½ cv²q. The limits of this assumption were assessed by calculating the error introduced by setting cv²I-ss at its extreme values (either cv²I-ss = 0 or cv²I-ss = cv²q). The term cvI-qd was obtained by measuring the difference in variance associated with stimulus-aligned eIPSCs compared to onset-aligned quantal eIPSCs in low Ca²⁺ solutions (as proposed by Clements and Silver, 2000). Thus, cv²I ≃ ½ cv²q + cv²I-qd (parameter value estimates are reported under Fig. 1-3).

The relationship between eIPSC S² and M constructed by using different Ca²⁺ concentrations in the extracellular solution was analyzed using a binomial model under the assumption of homogeneous release probability (this was supported by the substantial symmetry of the data distribution, see Clements and Silver, 2000 and Fig. 4A):
The model can also be applied without a knowledge of the whole variance/mean distribution (McLachlan, 1978), since the parameters \( p \) and \( n \) can be calculated from the mean amplitude and coefficient of variation of eIPSCs \( (M=mvq, \text{ and } CV=S/M, \text{ where } S \text{ is eIPSC SD}) \). In this model \( m=np, SD^2=np(1-p) \), and the probability \( p \) is:

\[
S^2 = q_p M(1 + cv^2) - \frac{M^2}{n}
\]

An estimate of \( p \) could also be obtained from the failure rate \( (N_0/N; N_0 \text{ are the failures out of } N \text{ responses}) \), which does not explicitly depend on previous determinations of quantum properties except that the number of releasing sites needs to be calculated beforehand with eq.1 or eq. 2:

\[
p = 1 - \frac{M \cdot CV^2}{q_p (1 + cv^2)}
\]

Although the three methods have different dependencies on experimental measurements and different intrinsic estimation errors (McLachian, 1978), they yielded very similar parameter values (see Fig. 4B) supporting the reliability of \( p \) estimates.

**RESULTS**

In this paper we have investigated Golgi cell – granule cell neurotransmission and its modulation by GABA-B receptors in acute cerebellar slices (the synaptic organization of inhibition in the cerebellar glomerulus is summarized in Fig. 1A). Whole-cell recordings were performed in the presence of glutamate receptor antagonists to block excitatory synaptic transmission.

The granule cells usually showed spontaneous activity (Fig. 1B) characterized by inhibitory synaptic currents occurring at an average frequency of \( 4.22 \pm 0.81 \text{ Hz} \) (\( n=25 \)). Most (>98%) of these events disappeared during application of 1 \( \mu M \) TTX, which suppresses spontaneous Golgi cell activity (Forti et al., 2006). Thus, these events were most likely to represent unitary spontaneous current (sIPSC) generated at individual Golgi cell – granule cell connections. The rare TTX-insensitive spontaneous events \( (0.066 \pm 0.008 \text{ Hz}, n=9, 1.6\% \text{ of all the spontaneous events}) \) were identified as miniature synaptic currents (mIPSCs). Electrical stimulation of the neuropile (Fig. 1C) evoked inhibitory synaptic currents (eIPSC). Any synaptically-related activities were abolished by 10 \( \mu M \) bicuculline and were therefore fully mediated by GABA-A receptors (\( n=5, \text{ data not shown} \)). These results confirm the absence of slow GABA-B receptor-mediated responses in granule cell inhibitory currents (Rossi et al., 2006; for different neurons cf. Misgeld et al., 1995; Dutar and Nicoll, 1998; Nicoll, 2004).

In the glomerulus, numerous synaptic terminals and granule cell dendrites are enwrapped into a glial sheet limiting neurotransmitter diffusion (Fig. 1A). This arrangement was previously shown to cause evident effects on GABA-A receptor mediated neurotransmission: while sIPSCs and a fast transient component of eIPSCs proved dependent on direct release onto the postsynaptic site, the slow eIPSC tail turned out to be generated by indirect activation through spillover (Rossi and Hamann, 1998; Brickley et al., 1996). Consistently, in our recordings (Fig. 1D) sIPSCs lacked the slow sustained tail characterizing eIPSCs \( (HW = 4.44 \pm 0.52 \text{ ms vs. } 13.61 \pm 2.43 \text{ ms, } n=6) \) and the indirect component emerged in isolation when the direct component failed. The transient component arose rapidly, with a time-to peak varying between 0.5 ms and 2 ms (Tia et al., 1996). The duration of the raising phase was not limited by the recording system (which ensured 100 \( \mu s \) resolution, see Methods and Sola et al., 2004), and probably reflected the time needed for receptor opening and asynchronies due to multi-quantal release (see below and Fig.2).
Evidence for multi-quantal evoked release

The glomerular synapse offers a convenient model for assessing quantal properties of neurotransmission, since it allows to easily identify the eIPSCs generated at unitary synaptic connections, to distinguish transmission from stimulation failures, and to directly estimate single quantum properties (see Methods). First, the amplitude of eIPSCs elicited by minimal stimulation was statistically indistinguishable from that of sIPSCs (in the same cells, 13.83 ± 0.95 pA vs. 15.94 ± 1.45 pA; ratio = 1.12 ± 0.07; n=16; p=0.2 paired t-test), consistent with an origin of both sIPSCs and minimal eIPSCs from unitary synaptic connections (Fig. 1C). This condition was assessed systematically: by increasing stimulus intensity, eIPSCs outranged sIPSCs (40.3 ± 4.9 pA, n=13) indicating recruitment of additional connections (Hamori and Somogyi, 1983; Harvey and Napper, 1991). In the continuation of this work, we will exclusively consider eIPSCs elicited by minimal stimulation and therefore presumably arising from single connections. Secondly, the occurrence of the indirect component in isolation allowed to identify release failures at the site under investigation and to exclude potential stimulation failures (Fig. 1D; cf. DiGregorio et al., 2002; Sola et al., 2004). Thirdly, the mIPSCs did not show indents in their raising phase (Fig. 2A), suggesting that they originated from release of single neurotransmitter quanta (e.g. see Cathala et al., 2003), whereas indents could often be observed in the sIPSCs and eIPSCs. The mIPSCs were therefore used to estimate the quantum size \( (q = 8.47±0.94 \text{ pA}, n=9) \) and coefficient of variation \( (cv_q = 0.29±0.12, n=9) \).

The quantal nature of GABAergic neurotransmission at the glomerular synapse was further investigated by perfusing solutions containing variable Ca\(^{2+}/\text{Mg}^{2+}\) proportions (Katz and Miledi, 1967; Dodge and Rahamimoff, 1967; Dittman and Regehr, 1996). In normal extracellular calcium (2 mM Ca\(^{2+}\)), the eIPSCs showed marked trial-to-trial amplitude fluctuations with CV (0.61±0.09, n=6) which was significantly larger than \( cv_q \) (p<0.01, unpaired t-test, n=6 and 9), supporting that eIPSCs were generated by release of multiple quanta (Fig. 2B). In low extracellular Ca\(^{2+}\) (0.5 mM Ca\(^{2+}\)), when release tends to occur at single releasing sites or to fail, eIPSC amplitude (10.58 ± 1.26, n=6, failures excluded) was statistically indistinguishable from that of mIPSC (p=0.19, unpaired t-test, n=6 and 9)(Fig. 2C). The raise of extracellular Ca\(^{2+}\) from 0.5 mM to 6 mM progressively increased the eIPSC mean amplitude (Fig. 3A). Moreover, the eIPSC amplitude distribution became broader and its mode moved to the right. Finally, the failures and coefficient of variation (CV) were reduced (Fig. 3B). These observations indicate a mechanism in which GABA is released in multiple quanta during evoked neurotransmission at unitary synaptic connections and release probability is controlled by calcium (Edwards et al., 1990; Takahashi et al., 1998).
to stimulus-aligned eIPSCs (see Methods). This scatter causes a reduction of the eIPSC peak by 17.5 ± 2.8% (n=6) and allows to estimate of the variability of quantum delay, $c_{\text{vd}}^2 = 0.03 \pm 0.04$ (n=6). This term adds to the intra-site variance, which then becomes $c_{\text{v}}^2 = c_{\text{v,ss}}^2 + c_{\text{v,qd}}^2 \approx \frac{1}{2} c_{\text{q}}^2 + c_{\text{v,qd}}^2 = 0.073$.

The eIPSC variability was investigated by constructing a variance/mean plot, in which eIPSC mean amplitude was changed by varying extracellular Ca$^{2+}$ concentration (multiple-probability fluctuation analysis, MPFA: Silver, 2003; Silver et al., 1996; Clements and Silver, 2000; Sola et al., 2004). The data were pooled from 20 recordings (Fig. 4A) belonging to experiments, in which 2 to 3 different Ca$^{2+}$ concentrations were tested (1 mM, 2 mM, 4 mM, and 6 mM). The plot showed a parabolic shape with downward concavity and was fitted with eq.1 yielding values for the quantum size at eIPSC peak, $q_p = 10.71 \pm 1.29$ pA and the number of releasing sites, $n = 4.66 \pm 0.76$. The calculated average $p$ value in 2 mM Ca$^{2+}$ was 0.31±0.12. The result of MPFA (eq.1) were compared to those obtained using the CV method (eq.2) and the failure method (eq.3). At 2 mM Ca$^{2+}$ (n=5), eq.2 yielded $n=5.76\pm0.53$ and $p=0.33\pm0.03$ ($q$ was taken from minis, see Fig. 1-2). With the $n$ value calculated from eq.2 and the failure rate, eq. 3 yielded $p=0.31\pm0.14$. These calculations were extended over the whole Ca$^{2+}$ concentration range that was investigated and, consistently, all the methods yielded similar $p$ values supporting the reliability of estimates (Fig. 4B).

The impact of the assumption that $c_{\text{v,ss}}^2 \approx c_{\text{v,qd}}^2 = \frac{1}{2} c_{\text{q}}^2$ (Clements, 2003) was evaluated by setting either $c_{\text{v,ss}}=0$ or $c_{\text{v,ss}}=c_{\text{q}}$. With eq.1, the error introduced by setting $c_{\text{v,ss}}=0$ was +1% for $q$ and <1% for both $p$ and $n$; the error introduced by setting $c_{\text{v,ss}}=c_{\text{q}}$ was -7% for $q$ and <1% for both $p$ and $n$. With eq.2, the error introduced by setting $c_{\text{v,ss}}=0$ was +15% for $p$ and -18% for $n$; the error introduced by setting $c_{\text{v,ss}}=c_{\text{q}}$ was -2% for $p$ and 2% for $n$. With eq.3, the dependence on $c_{\text{v}}$ is indirect and passes through the $n$ value estimated with eq.2. The error propagated from eq 2 to eq.3 was 17% by setting $c_{\text{v,ss}}=0$ and -1.6% by setting $c_{\text{v,ss}}=c_{\text{q}}$. Therefore, the assumption about single quantum variability introduced relatively little error on $p$, $n$ and $q$ estimates.

***** Fig. 4 ****

Short-term plasticity during repetitive stimulation

Golgi cells usually respond to punctuate stimulation with short high-frequency spike bursts (Vos e al., 1999; Solinas et al., 2007a, b; Rancz et al., 2007). To imitate native Golgi cell patterns, we tested the effect of 5-impulse 100 Hz stimulus trains (Fig. 5).

In normal calcium (2 mM), the transient eIPSC component showed a clear depression, while the persistent component showed a marked build-up. In the transient component, PPR = 0.50 ± 0.09 (n=8) was measured on the first two eIPSCs. In low calcium (0.5 mM), the initial depression turned into facilitation, yielding a PPR = 1.50 ± 0.35 (n = 5) on the first two eIPSCs. At both calcium concentrations, the eIPSC tended to similar and constant amplitude in the 3rd and 4th response. Thus, as at other synapses (e.g. the neighboring mossy fiber – granule cell synapse, see Sola et al., 2004), facilitation and depression coexisted but, at normal calcium, depression prevailed.

The 5 neurons covered in this section are a sub-population of the larger sample reported in Fig. 4. Consistent with the behaviour reported in Fig. 4, release probability computed using eq. 2 on the first eIPSC in the trains changed from $p = 0.42 \pm 0.06$ in 2 mM calcium to $p = 0.23 \pm 0.05$ in 0.5 mM calcium (n=5). Moreover, the variance/mean points obtained for subsequent eIPSCs during the trains also fell along the parabolic trajectory obtained by changing Ca$^{2+}$ concentration (Fig. 4A). This indicated that a progressive reduction in the number of released quanta was the main determinant of eIPSC short-term depression.

***** Fig. 5 ****

Modulation of inhibitory neurotransmission by GABA-B receptors
At various GABAergic synapses, neurotransmitter release has been reported to undergo negative modulation through GABA-B auto-receptors (Dittman and Regehr, 1996; Takahashi et al., 1998; Sakaba and Neher, 2003). It is therefore possible that GABA-B receptors, which are expressed in Golgi cells (Kulik et al., 2002), also control GABA release from Golgi cell terminals. To investigate this hypothesis, we perfused the high-affinity GABA-B receptor blocker CGP55845, which is reported to act preferentially on presynaptic GABA-B receptors (Yamada et al., 1999; Jensen et al., 1999; Than and Szabo, 2002; Fearon et al., 2003; Chen and Yung, 2005; Kaneda and Kita, 2005). Another GABA-B receptor antagonist, CGP35348 (Morishita and Sastry, 1995; Desiz et al., 1997; Mougnot et al., 1998), was not used since it is known to block postsynaptic GABA-B receptors at the Golgi cell – granule cell synapses and could generate spurious effects on eIPSCs (Rossi et al., 2006). In 5 experiments, low-concentration (1 µM) CGP55845 modified the eIPSC trains in a characteristic and significant manner (Fig. 6A). While the first eIPSC increased by 29.6±9.0% (n=5, p<0.03 paired t-test), the second eIPSC in the train did not and PPR decreased by 55.7±12% (n=5, p<0.05 paired t-test). Then, nearly the same steady-state amplitude as in control trains was attained within 3-4 impulses (Fig. 6B).

***** Fig. 6 *****

GABA-B receptor blockage increases release probability

The fact that GABA-B receptor blockage increased the first eIPSC and decreased PPR suggested that neurotransmitter release was enhanced. In order to investigate the mechanism of CGP55845 action, the PPR and CV changes were plotted vs. the amplitude change (Fig. 6C). In both cases the data showed a significant negative correlation (for PPR, R²=0.91, p<0.01 paired t-test; for CV, R²=0.89 p<0.01 paired t-test), consistent with an increase in release probability as the major factor responsible for the CGP55845 effect. Consistently, in the [M₂/M₁ vs. (CV₁/CV₂)²] plot, the experimental points fell above the diagonal as expected from a raise in quantal release (Fig. 7A). The calculation of p using eq. 2 yielded a raise from p=0.42±0.08 to p=0.67±0.09 (n=5, p<0.05 paired t-test) (Fig. 7B).

The mechanistic relationship between CGP55845 action and a change in release probability was further supported by the similar location, in all these graphs (Fig. 6C and Fig. 7A-B), of points obtained by increasing release probability with a raise in extracellular calcium. To this aim, a comparison is explicitly shown for transition from 0.5 mM to 2 mM calcium, since this matches the eIPSC amplitude variation observed after CGP perfusion, but qualitatively similar results were also obtained by using a change from 2 mM to 4 mM calcium (data not shown). The relationship between CGP55845 and calcium changes was further assessed by using variance-mean analysis. When the variance-mean points for subsequent eIPSC in the trains were reported in Fig. 4A, which was constructed at different calcium concentrations, the points obtained both before and after CGP55845 perfusion fell along the same parabolic trajectory (eq.1). This observation further supported the conclusion that the changes occurring with CGP55845 were caused by a raise in release probability (Fig. 7C; cf. e.g. Sola et al., 2004).

Consistent with an increased release probability, mIPSCs recorded in 3 µM TTX (Fig. 7D) significantly increased their frequency (39.3±7.9%, n=6; unpaired t-test, p<0.008) but their amplitude remained unchanged (0.6±2.8%, n=6; unpaired t-test, p=0.37).

***** Fig. 7 *****

Frequency-dependence of GABA-B receptor blockage and response build-up during repetitive stimulation

Since the trains used in these experiments were delivered every 10 sec, the observed CGP55845 modulation on the first IPSC in the trains occurred at low frequency. However, when IPSC changes were measured in the last IPSC in the trains, almost no modulation was observed.
This effect was reminiscent of that reported at the parent synapse between mossy fibers and granule cells, which is regulated by GABA spillover from the neighboring Golgi cell terminals (Mitchell and Silver, 2000b). We therefore reconstructed the frequency-dependence of CGP55845 effects by reporting the percentage changes at frequencies comprised between 0.1 Hz and 100 Hz (Fig. 8). It turned out that CGP55845 had a much stronger effect at low than at high frequency with a sharp roll-off around 10 Hz. Therefore, these results indicate that GABA-B receptor modulation is prevalent during low frequency transmission.

As noted in Fig. 5A and Fig. 6A, high frequency 100-Hz IPSC trains showed a marked build-up of the response (cf. Rossi et al., 2003). The effectiveness of this temporal summation was monitored through the charge transferred along the trains. No relevant effects of CGP55845 were observed on the total charge transfer (n=5, p=0.66 paired t-test; Fig.6B). Likewise, the charge transfer was similar at both 0.5 mM and 2 mM calcium (n=5, p=0.58 paired t-test; Fig. 5B). Thus, different from IPSC peak amplitude, the transferred charge was almost insensitive to GABA-B receptor blockage and calcium changes. This result indicates that GABA-B receptor regulation of release probability has the initial transient component of the response to a stimulus train as its specific target.

***** Fig. 8 *****

DISCUSSION

In this paper we show that, in the juvenile rat cerebellum, GABAergic transmission at the Golgi cell – granule cell synapse is multiquantal. During repetitive stimulation, GABAergic transmission undergoes short-term depression of a fast transient component, while a slow protracted component shows a remarkable build-up due to temporal summation of (Rossi and Haman, 1998; Rossi et al., 2002). These observations conform to a mechanism of synaptic integration, in which α1 receptors determine a transient (direct) response to released GABA, while α6 receptors determine slow (indirect) spillover-mediated currents (Cherubini and Conti, 2001; Farrant and Nusser, 2005; Glykys and Mody, 2007). The main finding is that release probability was tonically reduced by GABA-B receptor activation. This regulation was typically expressed at low-frequency (0.1-10 Hz) but became undetectable at high frequency (100 Hz). During high-frequency trains, GABA-B receptor-mediated regulation was evident in the first IPSC but no longer so after a few impulses either in the transient or slow component. Thus, ambient GABA in the glomerulus could act presynaptically on Golgi cell GABA-B auto-receptors selectively regulating the onset of inhibition.

Quantal properties of neurotransmission at the Golgi cell – granule cell synapse

The Golgi cell – granule cell synapse conformed to a general model of multi-quantal neurotransmission, in which IPSC amplitude fluctuations are mostly generated by the variable number of quantal events occurring at multiple releasing sites (Edwards et al., 1990; Cherubini and Conti, 2001). Although at other inhibitory synapses minis could be multiquantal (Auger et al., 1998; Auger and Marty, 2000; Glykys and Mody, 2007), this is probably not the case at the Golgi cell – granule cell synapse, since minis have fast non-indented rising phases and coincide with the unicellular eIPSCs measured in low-calcium solutions. Moreover, with a quantum conductance of 214 pS, a single channel conductance of 30 pS and an open probability of 0.6 (for critical review on these parameters see Farrant and Nusser, 2005), it can be estimated that around 10 GABA-A α1 subunit-containing channels are present in the postsynaptic density and about 80% of them are open at peak. With these parameters, a quantum is associated with an intrasite variance (cv1-ss) of 0.16, in line with the expectation that cv1-ss is about half of the quantum variance, cvq (Clements, 2003). Thus, statistical fluctuations of minis amplitude are consistent with the hypothesis that, at the Golgi cell – granule cell synapse, release occurs in single rather than aggregated quanta and suggest a nearly-saturated model of postsynaptic receptor activation.
Assuming independent release of multiple quanta, MPFA yielded an average release probability $p=0.32$ and an average number of releasing sites $n=4.7$ (standard extracellular calcium concentration, 2 mM). The quantum size obtained with MPFA was in fair agreement with the average minis size and $p$ almost coincided with the values yielded by failure rate and CV calculations (see Fig. 4B), providing a cross validation of the different methods and supporting the conformity of the system to simple binomial statistics (e.g. see MacLachian, 1978; Saviane et al., 2006). The consistency of quantal parameter estimates was presumably enhanced by the availability of single quantum properties extracted from independent minis measurements, by the secure identification of minimal eIPSCs through a comparison with sIPSCs and by the selection of transmission failures through the indirect eIPSC component. The multiquantal nature of neurotransmission is supported by the EM observation that Golgi cell axon terminals make multiple contacts containing several synaptic vesicles on different dendritic digits on the same granule cell dendrite (Hámori and Szentagothai, 1966; Hámori and Somogy, 1983; Jakab and Hámori, 1988). Additional EM analysis may be useful to estimate the number of morphologically-identified active zones and postsynaptic receptor density. Moreover, the contribution of GABA-A receptor desensitization to the IPSC trains may be estimated using computational modeling of neurotransmission (T. Nieu and E. D’Angelo, preliminary observations).

The small size of the inhibitory synaptic currents should not be surprising. The single quantum conductance (214 pS) is relatively large compared to the resting whole-cell conductance (around 1000 pS; D’Angelo et al., 1995; Armano et al., 2000; Rancz et al., 2007; Arenz et al., 2008), so that even a single quantum can determine a remarkable inhibitory effect contrasting the depolarization caused by excitatory synapses. The proposed synaptic arrangement and release statistics are similar to those of the neighboring mossy fiber - granule cell synapse (Sola et al., 2004; Saviane and Silver, 2006), indicating that the glomerular system can provide an appropriate excitatory/inhibitory balance to the granule cells.

### Regulation of release probability by tonic GABA-B receptor activation

GABA-B receptors blockage increased release probability and, accordingly, increased the first eIPSC and accentuated short-term depression in eIPSC trains. Moreover, minis frequency (but not minis amplitude) also increased. A prediction from such presynaptic mechanism is that $p$ variations and associated IPSC amplitude changes should be larger with low initial release probability. Another prediction is that PPR and CV changes should be negatively correlated with IPSC amplitude variation. Actually, all these correlations proved statistically significant and points lie down the $m_2/m_1$ vs. $(CV_1/CV_2)^2$ plot fell above the unitary diagonal, as expected from a raise in quantal release. The range of responses to GABA-B receptor blockage may reflect regulation of ambient GABA-levels (Hamann et al., 2002; Farrant and Nusser, 2005) or variable efficiency of the transduction cascades involving GABA-B receptors.

The effects of the GABA-B receptor antagonist CGP55845 were similar to those caused by increasing extracellular Ca$^{2+}$, consistent with the observation that CGP55845 preferentially inhibits presynaptic GABA-B receptors (Yamada et al., 1999; see also Jensen et al., 1999; Than and Szabo, 2002; Fearon et al., 2003; Chen and Yung, 2005; Kaneda and Kita, 2005). Moreover, the action of CGP55845, by occurring in the first response in a train, reveals the blockage of GABA-B receptors that were tonically activated. The estimated ambient GABA level in the cerebellar glomerulus is $10^{-8}-10^{-6}$ M (Farrant and Nusser, 2005) and can therefore activate presynaptic GABA-B receptors, which have EC$_{50}$ in the same concentration range (Galvez et al., 2000). Given the strong analogy with the presynaptic effects of Ca$^{2+}$, it is possible that the $p$ reduction caused by GABA-B receptors involves G-protein-dependent inhibition of voltage-dependent calcium currents, as reported at other central synapses (Dittman and Regehr, 1996; Takahashi et al., 1998). A slowing down of vesicle recycling (Sakaba and Neher, 2003) might occur along the short trains used for synaptic stimulation in our recordings explaining the absence of CGP modulation at high-frequency. Finally, it should be noted that the mechanism of presynaptic GABAergic regulation may not be the same at other brain...
synapses. For instance, at inhibitory synapses of the hippocampus, GABA receptors have been reported to exert their presynaptic effect independent from a change in release probability (Hefft et al., 2002).

In aggregate, these results indicate that GABA-B receptors in Golgi cells (Kulik et al., 2002) are expressed in presynaptic terminals, where they are tonically activated by ambient GABA and negatively regulate neurotransmitter release. Although a tonic presynaptic inhibition of neurotransmitter release by GABA-B receptor was reported also at other central synapses (Morishita and Sastry, 1995; Mougnot et al., 1998; Jensen et al., 1999; Fearon, 2003; Chen and Yung, 2005; Porter and Nieves, 2004), in the cerebellar glomerulus this mechanism would benefit of restricted neurotransmitter diffusion, which can also favor cross-talk between glutamatergic and GABAergic terminals. In particular, mGlu receptors can inhibit GABA release from Golgi cell terminals and GABA-B receptors can inhibit glutamate release from mossy fiber (Mitchell and Silver, 2000a, b). Moreover, GABA-B receptors are also expressed postsynaptically, where they can regulate granule cell input resistance through a modulation of inward rectifier channels (Rossi et al., 2006). The frequency dependence of these processes is also peculiar, in that presynaptic GABA-B receptor-mediated regulation at both excitatory and inhibitory terminals occurs at low-frequency (when only tonic GABA levels are relevant), while that of granule cell conductance occurs at high frequency (therefore requiring GABA build-up during repetitive neurotransmission). Finally, it should be noted that ambient GABA levels have been reported to regulate granule cell input resistance (Brickley et al., 1996) and mossy fiber – granule cell gain (Mitchell and Silver, 2003) through tonic activation of GABA-A receptors. Thus, presynaptic control by GABA-B autoreceptors on Golgi cell terminals appears as a part of a more complex system based on ambient GABA levels and spillover suited to control multiple aspects of glomerular signal processing.

**Functional implications and potential impact on granular layer functions**

The rapid activation of IPSC is well suited to explain Golgi cell – granule cell inhibition reported *in vivo* in the adult cat (see Eccles et al., 1967). Presynaptic GABA-B receptor-mediated control of release probability can be thought as a mechanism to regulate the sharpness of the onset of inhibition. This effect is better conceived within the time-window hypothesis (Kistler and DeZeeuw, 2003; Mapelli and D’Angelo, 2007; D’Angelo and DeZeeuw, 2008), which assumes that Golgi cell feed-forward inhibition can limit the discharge of connected granule cells within about 5 ms. Low ambient GABA and GABA-B receptor activation would allow to sharply delimit the time-window, during which granule cell spikes have the highest probability of being generated. And since the ambient GABA level is proportional to Golgi cells background activity (Rossi et al., 2003), these same granule cells will also have lower GABA-A receptor-mediated noise and leakage (Brickley et al., 1996) resulting in higher precision and intensity of their response to mossy fiber activation. This temporally limited and precise discharge would then improve the pattern recognition process that is thought to take place in Purkinje cells (Brunel et al., 2004; Steuber et al., 2007). The impact of these time-filtering mechanisms needs to be further investigated using large-scale detailed network models and multisite and imaging recordings of network activity.
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Fig. 1. Golgi cell-granule cell inhibitory currents
(A) Schematic drawing of glomerular synapses. In the glomerulus (dashed circle), granule cell (GrC) dendrites make synaptic contact with a mossy fiber terminal (mf) as well as with Golgi cells (GoC) axon fibers (gray). A granule cell dendrite can receive GABAergic inhibition through direct release of GABA from the corresponding GoC synaptic contact or through diffusion of GABA from synapse formed by axonal branches on neighboring dendrites in the glomerulus. Electrical stimuli were applied in the surrounding neuropile activating the Golgi cells axons, while recording from a granule cell. Excitatory transmission was blocked by perfusing glutamate receptors antagonists. (B) Spontaneous activity recorded from a granule cell at -10 mV. The sIPSCs disappeared during 1µM TTX perfusion leaving rare miniature IPSCs (mIPSC, magnified in the inset). Note also that background noise decreased during TTX perfusion, presumably reflecting a reduction of tonic GABA receptor activation by ambient GABA. (C) Evoked IPSCs (eIPSCs) following electrical stimulation (arrows). The eIPSCs were elicited first by using minimal stimulation (set as explained in the Methods) and then after raising the stimulation intensity (from 8V to 10V) in the same cell. The scatter plot shows the amplitude of sIPSCs and eIPSCs for the same tracings shown at left. (D) Minimal eIPSC (black trace) and sIPSC (gray trace) are shown superimposed. Note that sIPSC has faster decay kinetics than minimal eIPSC. The slow eIPSCs is shown at the bottom. These traces are averages of 6 recordings from the same cell.

Fig. 2. Quantal components of the inhibitory response
(A) The mIPSCs (10 traces) show amplitude fluctuations. The average mIPSC is shown in gray and replotted in the next two panels. (B) Two single minimal eIPSCs show a marked indent in their rising phase. The indent overlaps with the average mIPSC. (C) The amplitude of most eIPSCs recorded in 0.5 mM [Ca²⁺]o does not differ significantly from that of mIPSCs, while some of them (dashed traces with arrowheads) could be biquantal. The failure rate in this cell was 50%. The mIPSCs in A have been obtained from a cell different from that in B and C. The arrows indicate the time of synaptic stimulation.

Fig. 3. Dependence of eIPSCs on [Ca²⁺]o
(A) Average minimal eIPSCs (averages of 10 traces) are increased when passing from 1 mM to 4 mM [Ca²⁺]o (the arrow indicates synaptic stimulation). The histograms show the amplitude distributions for the same recordings. Note the decrease in failures and the shift of the distribution to the right when [Ca²⁺]o is increased. Each histogram is made of 125 responses (3 pA bin-size) and no clear quantal peaks can be observed. (B) Histograms of minimal eIPSC peak amplitude (left), coefficient of variation (CV, failures included, middle) and failure rate (right), at the different [Ca²⁺]o tested. At 0.5 mM Ca²⁺ eIPSCs failures were so numerous to preclude CV calculation. Values are reported as mean±MSE.

Fig. 4. Variance-mean plot and p estimates
(A) A variance-mean plot was constructed using minimal eIPSCs taken from 20 granule cells recorded with 1, 2, 4, 6 mM [Ca²⁺]o (open circles are single cell values, filled circles are average values ± MSE, n=5, corresponding to different [Ca²⁺]o). The data were fitted with a parabolic function (eq. 1, gray line; \( q_p = 10.71 \pm 1.29 \text{ pA}, n = 4.66 \pm 0.76, \text{ cv}^2_I = 0.073; R^2=0.87, \ p<0.05 \text{ paired t-test} \)). The points corresponding to 4 subsequent responses in a 100 Hz train (control, filled triangles; CGP55845, open triangles; mean ± MSE, n=5, same cells as in Fig. 6 and 7) fall on the same parabolic trajectory. (B) Release probability estimated with three different methods (eq. 1, open circles; eq.2, filled circles; eq.3, open triangles) at different [Ca²⁺]o. The three estimates were similar for all [Ca²⁺]o values.
Fig. 5. Short-term plasticity during repetitive synaptic stimulation
(A) The traces show the eIPSCs elicited by short 100-Hz trains (averaging of 20 responses) taken at 0.5 mM [Ca$^{2+}$]_o and 2 mM [Ca$^{2+}$]_o from the same cell. While short-term facilitation appears at 0.5 mM [Ca$^{2+}$]_o, the response shows short-term depression of peak amplitude at 2 mM [Ca$^{2+}$]_o. The inset shows exponential fitting to the decay of an IPSC in the train, which was needed to determine the baseline from which the amplitude of the subsequent IPSC could be measured. (B) Plot of normalized eIPSC peak amplitude (mean ±MSE) in the train for 8 experiments like those shown in A (0.5 mM [Ca$^{2+}$]_o n=5, 2 mM [Ca$^{2+}$]_o n=8). The histogram represents the charge transfer during the trains. Despite different short-term dynamics, no relevant difference in charge transfer was observed between 0.5 mM [Ca$^{2+}$]_o (white bar) and 2 mM [Ca$^{2+}$]_o (black bar) (p=0.58 unpaired t-test).

Fig. 6. Modulation of inhibitory transmission by GABA_B receptors
(A) The traces show the minimal eIPSCs elicited by short 100-Hz trains (averaging of 30 responses) taken in control and after perfusion of CGP55845 (1 µM). The control (black) and CGP55845 (gray) trace are superimposed to show the relative changes in the eIPSC amplitude. Note that the 1st eIPSC increased but then the response tended to a common steady state. The inset shows exponential fitting to the decay of an IPSC in the train, which was needed to determine the baseline from which the amplitude of the subsequent IPSC could be measured. (B) Plot of normalized eIPSC peak amplitude (mean ±MSE) in the train for 5 experiments similar to those shown in A. Note again that the first eIPSC increased after CGP55845 but then the response tended to stabilize around the control level (mean ± MSE). The histogram represents charge transfer during the trains, showing no relevant difference between control (white bar) and CGP55845 (black bar) (p=0.88 paired t-test). (C) The PPR and CV changes after CGP55845 perfusion are plotted vs. peak amplitude changes (filled circles). In both plots, the data show a linear negative correlation (gray lines; R$^2$=0.91, p<0.01 paired t-test; R$^2$=0.89, p<0.01 paired t-test). The points corresponding to the average change in PPR and CV obtained by raising [Ca$^{2+}$]_o from 0.5mM to 2mM lay in proximity of the CGP data (open circles, mean ± MSE, n=5).

Fig. 7. The effect of GABA_B receptor modulation on release probability.
(A) The plot shows the relationship between (CV$^2$/CV$^1$)-2 and M2/M1 for eIPSCs recorded in control and during CGP55845 perfusion (filled circles indicate single cells, the filled triangle indicates mean ± MSE, n=5). All points fall above the unitary diagonal, i.e. in the sector compatible with a change in release probability. It should be noted that the point corresponding to recordings obtained by raising [Ca$^{2+}$]_o from 0.5 mM to 2 mM (open circle, mean ± MSE, n=5) lays also in the same sector. (B) The release probability increase during CGP55845 perfusion is plotted for single cells (filled circles) as well as for their average (filled triangle, mean ± MSE, n=5). The open circles show the average release probability estimated by increasing [Ca$^{2+}$]_o from 0.5 mM to 2 mM (mean ± MSE, n=5). (C) The peak amplitude variation during CGP55845 perfusion is plotted vs. the p change. The data show a linear positive correlation (gray line, R$^2$=0.95, p<0.01 paired t-test). The point obtained by raising [Ca$^{2+}$]_o from 0.5mM to 2mM (open circle, mean ± MSE, n=5) lays in proximity of the CGP55845 data. The data used for CGP55845 perfusion and calcium concentration changes are the same in all these panels and Fig. 6. (D) mIPSCs were recorded in the presence of 1 µM TTX either in control or in the presence of CGP55845. mIPSCs did not change their amplitude but increased their frequency (n=15 in control and n = 22 in CGP during 7 min recording). The histograms show average changes in mIPSC amplitude and frequency.

Fig. 8. Frequency-dependence of GABA-B receptor blockage.
The traces on top show eIPSCs elicited at different frequencies in control and during CGP perfusion. Note the eIPAC increase at 0.1 Hz, 1 Hz, and 10 Hz but not at 100 Hz. The plot shows average changes in eIPSC amplitude at different stimulation frequencies.
**A**

Graph showing the action potentials with labels for 1 mM and 4 mM.

**B**

Bar graphs showing peak amplitude (pA), CV, and % failures for different [Ca\(^{2+}\)](mM) concentrations with n=6 for each bar.
A

![Graph A]

**Axes:**
- **Y-axis:** mean eIPSCs variance (pA²)
- **X-axis:** mean eIPSCs amplitude (pA)

B

![Graph B]

**Axes:**
- **Y-axis:** release probability
- **X-axis:** [Ca²⁺], (mM)

**Legend:**
- **CV method** (filled circles)
- **Failure method** (open triangles)
- **V/M plot method** (open circles)

**Graph B:**
- **Curves:**
  - **Solid line:** CV method
  - **Dashed line:** Failure method
  - **Dotted line:** V/M plot method

**Data Points:**
- **Error Bars:** Represent standard error of the mean.
A

0.5 mM $[\text{Ca}^{2+}]_o$

2 mM $[\text{Ca}^{2+}]_o$

10 pA

20 ms

B

normalized ePSC peak amplitude

- $[\text{Ca}^{2+}]_o = 0.5\text{mM}$
- $[\text{Ca}^{2+}]_o = 2\text{mM}$

Charge (pC)

0 1

0 0.4 0.8 1.2 1.6

0 10 20 30 40

time (msec)