

GABA Spillover from Single Inhibitory Axons Suppresses Low-Frequency Excitatory Transmission at the Cerebellar Glomerulus

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GABA type B receptors (GABA_B-Rs) are present on excitatory terminals throughout the CNS, but surprisingly little is known about their role in modulating neurotransmission under physiological conditions. We have investigated activation of GABA_B-Rs on excitatory terminals within the cerebellar glomerulus, a structure where glutamatergic excitatory and GABAergic inhibitory terminals are in close apposition and make axodendritic synapses onto granule cells. Application of the GABA_B-R agonist baclofen depressed evoked mossy fiber EPSCs by 54% at 1 Hz. The amplitude of miniature EPSCs recorded in tetrodotoxin was unchanged in the presence of baclofen, but the frequency was significantly reduced, indicating a purely presynaptic action of baclofen under our recording conditions. At physiological temperature (37°C) presynaptic GABA_B-Rs were not tonically acti-

vated by spontaneous GABA release from Golgi cells, which fire at ~8 Hz in slices at this temperature. However, tonic activation could be induced by blocking GABA uptake or by lowering temperature. GABA_B-Rs were activated at physiological temperature when Golgi cell firing was increased above the basal level by stimulating a single inhibitory Golgi cell input at 50 Hz, suppressing the mossy fiber-evoked EPSC by 24% at 1 Hz. Furthermore, glutamate release was selectively inhibited at low-frequency mossy fiber inputs (<10 Hz) during Golgi cell stimulation. Our findings suggest that GABA spillover in the glomerulus modulates sensory input to the cerebellar cortex.

Key words: transmitter spillover; GABA_B; heteroreceptor; presynaptic; glutamate; synaptic transmission

Presynaptic GABA type B receptors (GABA_B-Rs) modulate synaptic transmission throughout the nervous system by altering the probability of transmitter release. GABA_B-Rs present on inhibitory terminals (autoreceptors) can be activated homosynaptically by GABA released from the same synapse (Deisz and Prince, 1989; Davies et al., 1991). GABA_B-Rs are also found on excitatory terminals in brain regions where direct presynaptic and reciprocal inhibitory synapses are absent (Shepherd, 1998). Several studies have established that GABA_B-Rs on excitatory terminals in hippocampus and cerebellar cortex can be activated by spillover of GABA from interneurons (Isaacson et al., 1993; Dittman and Regehr, 1997; Vogt and Nicoll, 1999). Synchronous activation of many interneurons is likely during extracellular stimulation in these preparations so it is unclear whether GABA release from a single input or synchronous release from many inputs is necessary for heterosynaptic activation. Studying single inhibitory inputs is therefore important for assessing the extent to which presynaptic GABA_B heteroreceptors are activated *in vivo*.

Heterosynaptic activation of presynaptic receptors requires spillover of transmitter. The distance over which transmitter can travel from the release site and activate receptors at other synapses is dependent on diffusion and the efficacy of transmitter uptake in the locality (Isaacson et al., 1993; Barbour and Häusser, 1997; Rusakov et al., 1999). Heteroreceptor activation is therefore likely to be strongly dependent on temperature, because uptake mechanisms have a large Q_{10} (~3; Wadiche and Kavanaugh, 1998). However, most of the experiments examining GABA spillover onto presynaptic heteroreceptors have been performed below body temperature (26–33°C; Isaacson et al., 1993; Dittman and Regehr, 1997; Yamada et al., 1999; Aroniadou-Anderjaska et al., 2000). This

raises the question of whether the extrasynaptic concentration of GABA is sufficient to produce significant tonic and/or phasic activation of GABA_B-Rs on excitatory inputs at body temperature (Isaacson et al., 1993). The fraction of GABA_B-Rs occupied at physiological temperature is a key parameter because it influences glutamate release probability and thus determines the frequency-dependent behavior of the synapse (Brenowitz et al., 1998; Brody and Yue, 2000; Kreitzer and Regehr, 2000). Quantifying the inhibition of EPSCs by synaptically released GABA is therefore an important prerequisite for inferring how presynaptic GABA_B-Rs modulate synaptic efficacy under physiological conditions.

We have examined activation of presynaptic GABA_B-Rs at excitatory mossy fiber–granule cell synapses in the cerebellum. This preparation has several advantages for investigating presynaptic modulation (Mitchell and Silver, 2000). The presynaptic elements that innervate granule cells form a glomerular structure that consists of a single large excitatory mossy fiber terminal and an inhibitory Golgi cell axon (Eccles et al., 1967; Jakab and Hámori, 1988). The whole structure, including the ends of the granule cell dendrites, are ensheathed in a glial coat (Jakab and Hámori, 1988) which may localize spillover of transmitter (Brickley et al., 1996; Wall and Usowicz, 1997; Rossi and Hamann, 1998; Mitchell and Silver, 2000). Each of the three to five granule cell dendrites is innervated by a different mossy fiber, and up to 50 different granule cells make synapses within each glomerulus (Eccles et al., 1967; Jakab and Hámori, 1988). The low density of mossy fibers and Golgi cells in the granule cell layer allow the activation of single inputs using local extracellular stimulation (Silver et al., 1996). Here we demonstrate that GABA_B-Rs on mossy fiber terminals are activated when single Golgi cell inputs are stimulated at physiological temperature (~37°C). Furthermore, we find that GABA_B-Rs depress EPSCs only when mossy fiber firing rate is low.

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MATERIALS AND METHODS

Sagittal slices of cerebellum (250 μm) were prepared from 12- to 13-d-old Sprague Dawley rats as previously described (Silver et al., 1998). The external recording solution contained (in mM): 125 NaCl, 2.5 KCl, 2 CaCl₂, 1 MgCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃, and 25 glucose, pH 7.3 when bubbled with 95% O₂ and 5% CO₂; 310 mOsm. All recordings were made at 36.5 ± 0.1°C (range,

35–38°C; $n = 41$) except where stated otherwise. We added 15 μM bicuculline methobromide, 10 μM D-2-amino-5-phosphonopentanoic acid (D-AP-5), 20 μM 7-chlorokynurenic acid (Tocris Cookson, Bristol, UK), and 0.5 μM strychnine to the perfusate to block GABA_A, NMDA, and glycine receptors. Baclofen (50–100 μM) and CGP35348 (500 μM) were added to activate and antagonize GABA_B-Rs, respectively, and NO711 (50 μM) was added to block GABA uptake. Recordings were made using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA), and 7–10 M Ω micropipettes made from standard wall borosilicate glass. Some of the experiments in which GABA_B-Rs were activated pharmacologically were recorded with an internal solution containing (in mM): 110 CsF, 30 CsCl, 10 CsHEPES, 10 CsEGTA, 2 NaCl, and 2 MgATP, adjusted to pH 7.3 with CsOH; 290 mOsm. However, the majority were recorded with 140 CsCl, 10 CsHEPES, 10 CsEGTA, 2 NaCl, and 2 MgATP, adjusted to pH 7.3 with CsOH; 290 mOsm. This gave a reversal potential of ~ 0 mV for EPSCs and IPSCs. In experiments in which excitatory and inhibitory inputs were activated onto the same granule cell, a pipette solution with a chloride reversal potential of -24 mV was used (75 CsMethylsulphonate, 50 CsCl, 10 CsHEPES, 10 CsEGTA, 2 NaCl, and 2 MgATP) allowing EPSCs and IPSCs to be viewed separately. Golgi cell and mossy fiber axons were stimulated using patch pipettes filled with recording solution and driven by isolated constant voltage devices (Digitimer, Welwyn Garden City, UK). A single fiber was identified with minimal stimulation on the basis of an all-or-none postsynaptic current elicited by a stimulus of graded intensity and the presence of a single peak in the average current. The stimulation voltage was set 5–10 V above threshold to ensure reliable fiber stimulation (Silver et al., 1996). Data were acquired at 10–25 kHz after low-pass filtering at 2–5 kHz (4 pole Bessel filter) using Axograph 4 software (generous gift of John Clements, Australian National University, Canberra, Australia) and an Instrutech ITC-18 interface (Instrutech, Port Washington, NY). Miniature EPSCs were detected with a template detection method implemented in Axograph (Clements and Bekkers, 1997). The time stability of synaptic currents was assessed using Spearman rank order correlation analysis. Means are expressed \pm SEs. Groups were compared using a paired two-tailed Student's t test except where stated. Results were considered significant at $p < 0.05$.

RESULTS

Pharmacological activation of GABA_B-Rs on mossy fiber terminals

We first established whether presynaptic GABA_B-Rs were present on mossy fiber terminals by using a conventional pharmacological approach. Application of the GABA_B-R agonist baclofen (100 μM) reduced the mean amplitude of the evoked non-NMDA receptor-mediated EPSC by $54 \pm 5\%$, in a reversible manner at 37°C (1 Hz, $p < 0.001$, $n = 12$; Fig. 1*A,B*). The coefficient of variation (CV; SD/mean) of the EPSC increased in the presence of baclofen ($200 \pm 20\%$ of control, $p = 0.002$, $n = 12$; Fig. 1*B*), consistent with the EPSC depression being mediated, at least in part, by a reduction in glutamate release onto the granule cell. We investigated the locus of the GABA_B-R action further by examining the effect of baclofen on miniature currents recorded in the presence of tetrodotoxin (0.5 μM). Under our experimental conditions, using a cesium-based internal solution to block postsynaptic potassium currents, the mean amplitude of miniature EPSCs in the presence of baclofen was similar to control ($p > 0.05$, $n = 6$; Fig. 1*C*), but the miniature frequency was significantly reduced ($50 \pm 12\%$, $p = 0.01$, $n = 6$; Fig. 1*D*), as expected for a purely presynaptic mechanism.

We examined the effect of presynaptic GABA_B-R activation on the EPSC over a range of mossy fiber stimulation frequencies that were similar to the firing rates observed *in vivo* (van Kan et al., 1993). Under control conditions the steady-state amplitude of the evoked mossy fiber EPSC declined as the stimulation frequency increased (Fig. 2*A,B*). In the presence of a saturating concentration of baclofen (100 μM ; Takahashi et al., 1998) the frequency dependence of the EPSC was flattened because of selective depression at

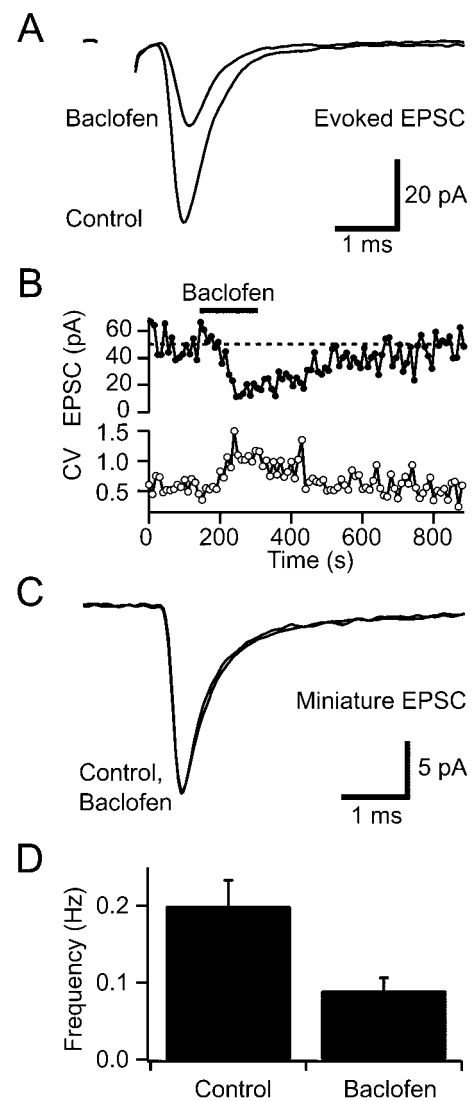


Figure 1. Pharmacological activation of presynaptic GABA_B-Rs depresses glutamate release from mossy fibers. *A*, Average non-NMDA receptor-mediated EPSCs evoked by mossy fiber stimulation at 1 Hz in control solution and in 100 μM baclofen. *B*, Bath application of 100 μM baclofen reduced the EPSC amplitude in a reversible manner (each point is the average of 10 EPSCs). The coefficient of variation (CV) of the EPSC amplitude (bottom panel) increased during depression, indicating reduced glutamate release. *C*, Superimposed average spontaneous miniature EPSCs recorded in tetrodotoxin (0.5 μM) in control solution and in the presence of 100 μM baclofen. *D*, Histogram showing mean frequency of miniature EPSCs in control and in 100 μM baclofen ($n = 6$).

frequencies < 10 Hz ($p < 0.001$, $n = 8$; Fig. 2*B*). However, at stimulation frequencies of ≥ 10 Hz GABA_B-R activation had no significant effect on the amplitude of steady-state EPSC ($p \geq 0.28$, $n = 8$; Fig. 2*B*).

Tonic GABA release, presynaptic GABA_B-R activation, and GABA uptake

In the absence of stimulation, Golgi cells in slices fire spontaneously at 3–6 Hz at room temperature (Dieudonne, 1998; Misra et al., 2000) and at 8 Hz at 37°C (Mitchell and Silver, 2000), which is comparable to the range of basal rates observed *in vivo* (8–15 Hz; Edgley and Lidieth, 1987; van Kan et al., 1993; Vos et al., 1999). The resulting GABA release contributes to a GABA_A receptor-mediated tonic leak current in granule cells (Brickley et al., 1996; Tia et al., 1996; Wall and Usowicz, 1997) and can be detected as spontaneous IPSCs that are blocked by the GABA_A antagonist bicuculline (data not shown). We sought to determine whether this spontaneous release of GABA from Golgi cell terminals could also

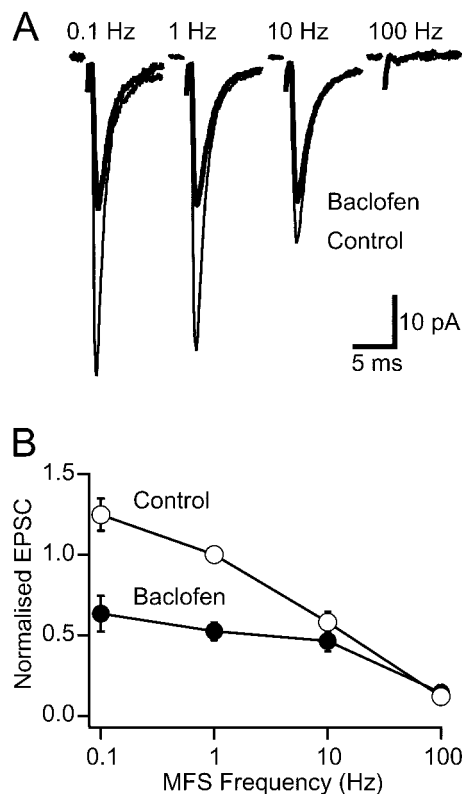


Figure 2. Frequency dependence of GABA_B-R-mediated EPSC depression. *A*, Averaged steady-state EPSCs from the same cell recorded at different stimulation frequencies in control solution (thin trace) and in the presence of 100 μ M baclofen (thick trace). *B*, Steady-state frequency dependence of EPSC amplitude in control (open symbols) and in the presence of 100 μ M baclofen (filled symbols). The relationship shows the average of eight cells normalized to the EPSC amplitude at 1 Hz mossy fiber stimulation (MFS) in control solution.

tonically activate GABA_B-Rs on mossy fibers by examining the effect of a GABA_B antagonist on the EPSC amplitude. No change in the EPSC amplitude was observed during application of 500 μ M CGP35348 at mossy fiber stimulation frequencies between 1 Hz ($97 \pm 7\%$ of control, $p = 0.7$, $n = 5$; Fig. 3*A*) and 100 Hz ($p \geq 0.3$, $n = 3$; Fig. 3*B*) at 37°C. These results indicate that GABA_B-Rs are not tonically activated at physiological temperatures.

Because tonic activation of GABA_B-Rs has been reported in other preparations at lower temperature (Kombian et al., 1996; Aroniadou-Anderjaska et al., 2000) (see also Parnas et al., 1999), we decided to investigate whether the lack of tonic activation at the mossy fiber could simply reflect our recording temperature. When we performed similar experiments at room temperature, the GABA_B antagonist CGP35348 (500 μ M) potentiated EPSCs by $15 \pm 3\%$ (1 Hz, $p = 0.01$, $n = 4$; Fig. 3*C,D*), suggesting that GABA_B-Rs tonically depress glutamate release at this temperature. What mechanism might underlie this difference in GABA_B-R activation at different temperatures? The most likely candidate is transmitter uptake because transporters have a high Q_{10} (see also Terrian et al., 1987; Wadiche and Kavanaugh, 1998). We tested this possibility by examining whether tonic GABA_B-R activation could be induced at physiological temperatures when GABA uptake was blocked. We used NO711 to block GABA uptake because it is not a substrate for uptake (Zeevalk and Nicklas, 1997) and thus does not induce GABA release by heteroexchange. Furthermore, NO711 specifically blocks the GAT-1 transporter, which is known to be present in cerebellar glomeruli (Itouji et al., 1996). It may also inhibit GAT-3, which is present in the glial sheath that partially surrounds glomeruli at this age (Itouji et al., 1996). Application of 50 μ M NO711 produced a tonic depression of the EPSC at physiological temperature ($24 \pm 5\%$, $p = 0.01$, $n = 5$) that was blocked

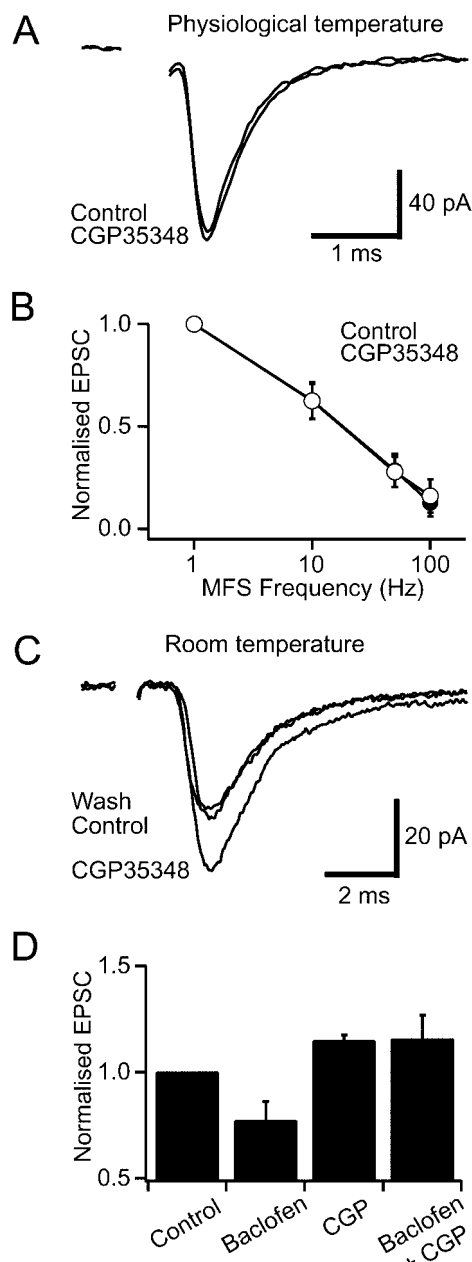


Figure 3. Tonic activation of GABA_B-Rs is temperature-dependent. *A*, Averaged EPSCs evoked at 1 Hz in control and in 500 μ M CGP35348 at 37°C. *B*, Steady-state frequency dependence of EPSCs for control solution (open symbols) and in the presence of 500 μ M CGP35348 (filled symbol) at 37°C. The relationship shows the average of three cells normalized to the 1 Hz EPSC amplitude in control solution. *C*, Averaged EPSCs evoked at 1 Hz mossy fiber stimulation (MFS) in control solution and in the presence of the GABA_B antagonist CGP35348 (500 μ M) at room temperature. *D*, Histogram of normalized EPSC amplitudes during control, 50 μ M baclofen, 500 μ M CGP35348, and 500 μ M CGP35348 plus 50 μ M baclofen at room temperature ($n = 4$).

by the presence of 500 μ M CGP35348 ($6 \pm 5\%$ reduction, $p = 0.22$, $n = 5$; Fig. 4). These results suggest that at physiological temperature uptake maintains the basal GABA concentration below that necessary to activate presynaptic GABA_B-Rs.

Activation of GABA_B-Rs on mossy fibers by stimulating single Golgi cell inputs

We examined whether GABA_B-Rs on mossy fiber terminals are activated at physiological temperatures when GABA release is increased by stimulating single Golgi axons. We stimulated independent mossy fiber and Golgi cell inputs onto a granule cell

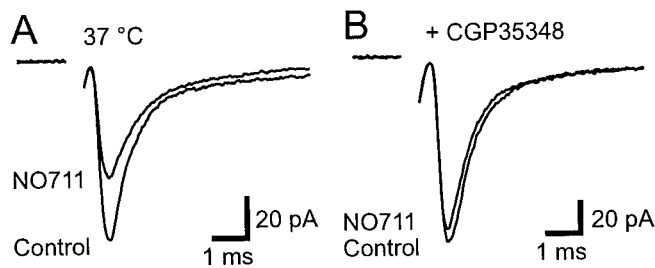


Figure 4. Inhibition of GABA uptake induces tonic activation of GABA_B-Rs at physiological temperature. *A*, Averaged EPSCs evoked at 1 Hz in control solution and in the presence of the GABA uptake blocker NO711 (50 μ M) at 37°C. *B*, Averaged EPSCs evoked at 1 Hz in the GABA_B antagonist CGP35348 (500 μ M) and in the presence of both 500 μ M CGP35348 and 50 μ M NO711 at 37°C.

(illustrated in Fig. 5*A*) with minimal stimulation. Care was taken to ensure that stimulation of EPSCs and IPSCs was independent and that they originated from single fibers by checking that PSC amplitude was independent of stimulation voltage (Silver et al., 1996) and there were no temporally distinct components. Evoked EPSCs and monosynaptic IPSCs were distinguished by their kinetics and reversal potential (Mitchell and Silver, 2000).

The efficacy of the excitatory mossy fiber input was monitored from the amplitude of evoked non-NMDA receptor-mediated EPSCs during a stimulation train delivered at 1 Hz after block of ionotropic GABA_A receptors with bicuculline. At the start of the train, the EPSC amplitude declined exponentially ($\tau = 1.2$ sec; Fig. 5*C*, *dashed line*) because of frequency-dependent depression. Once the EPSC amplitude had stabilized, the inhibitory Golgi cell input was stimulated for a sustained period at a frequency within the range observed *in vivo* during limb movement (van Kan et al., 1993), locomotion (Edgley and Lidieth, 1987), and facial stimulation (Vos et al., 1999). Stimulation of the Golgi cell input for 5 sec

at 50 Hz caused a marked depression in the EPSC amplitude (Fig. 5*B*). Although this depression was present when all experiments were averaged (Fig. 5*C*, *top trace*), it is clear from the bimodal distribution of responses (Fig. 5*D*) that no EPSC depression occurred in some experiments. Individual cells were therefore classified into responsive or nonresponsive groups by testing whether the EPSC amplitude was significantly different during Golgi cell stimulation (GoS), from the prestimulation amplitude (Fig. 5*C*; single tailed *t* test). This approach indicated that 61% (19 of 31 cells) of granule cell recordings exhibited EPSC depression during GoS (Fig. 5*D*, *solid line*). Averaging the nonresponsive cells gave no residual response, confirming our separation procedure was effective (Fig. 5*C*, *bottom trace*).

The average time course of EPSC depression during GoS was relatively slow with the rising phase detectable after 1 sec of Golgi cell stimulation ($p < 0.001$; $n = 19$; Fig. 6*A*). The decay of the EPSC depression could be approximated with a single exponential with a time constant of 3 sec, which is comparable to GABA_B-R-mediated depression of EPSCs and presynaptic calcium currents at other central excitatory synapses (Isaacson et al., 1993; Pfrieger et al., 1994; Dittman and Regehr, 1997). At the peak of the response, the EPSC amplitude was depressed by $24 \pm 3\%$ during 5 sec GoS at 50 Hz ($p < 0.001$, $n = 19$, responsive cells; Fig. 6*A*). During depression the coefficient of variation of the EPSC increased (0.43 ± 0.05 to 0.58 ± 0.07 , $n = 19$, $p = 0.001$; Fig. 6*A,B*), indicating that it was caused by a reduction in probability of glutamate release. We also tested a brief stimulation protocol (80 Hz for 250 msec) that mimicked the duration of Golgi cell activity during joint flexion (van Kan et al., 1993). However, this protocol produced no change in the EPSC amplitude when mossy fibers were evoked at 1 Hz ($101 \pm 1\%$ of control; $n = 5$; $p = 0.44$).

If the mossy fiber axon was directly activated by the Golgi cell stimulation electrode, it is possible that the EPSC depression could be caused by "depletion" of the terminal rather than via activation of presynaptic receptors. However, examination of the traces dur-

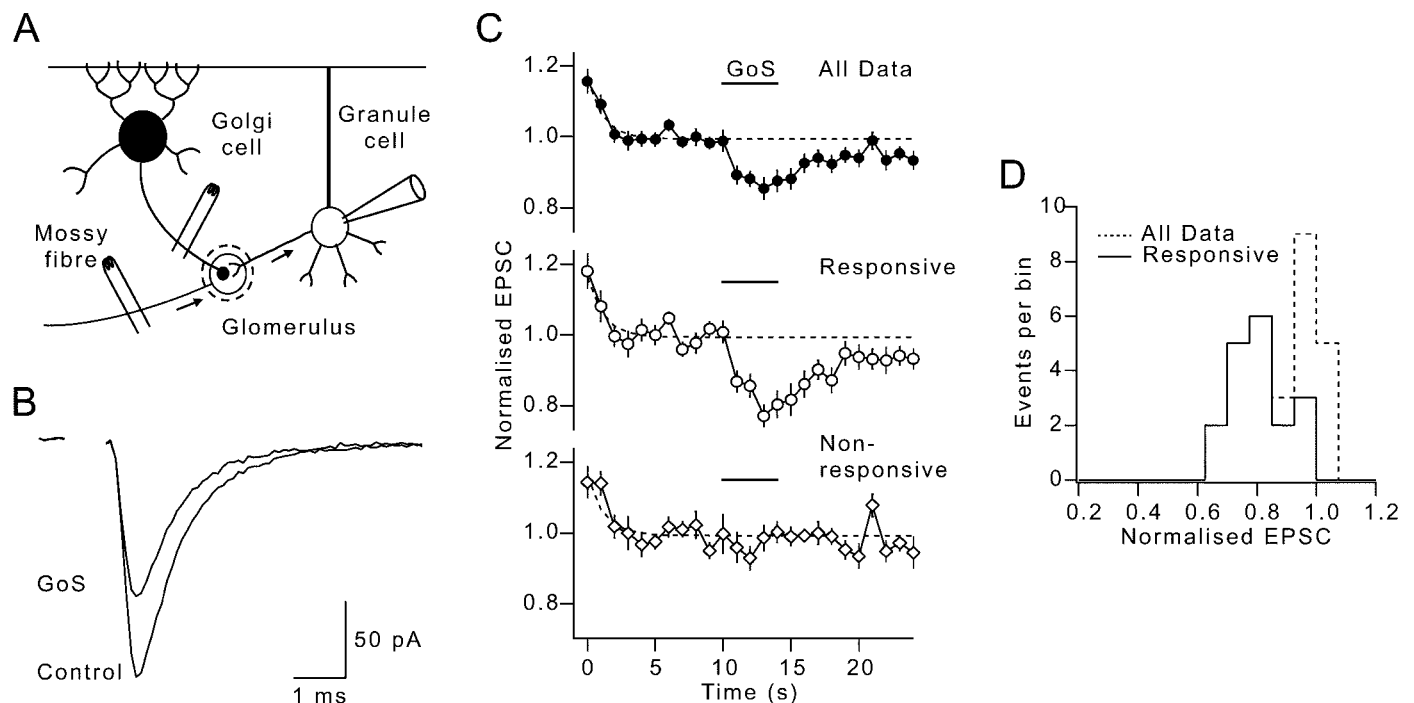


Figure 5. Stimulation of single Golgi cell input suppresses mossy fiber EPSCs. *A*, Recording configuration: whole-cell patch-clamped granule cell and stimulation of independent inhibitory and excitatory inputs. *B*, Average non-NMDA EPSC waveforms from control period and during Golgi cell stimulation (GoS). *C*, Traces show average non-NMDA EPSCs amplitudes (normalized to pre-GoS baseline) evoked by 1 Hz mossy fiber stimulation in the presence of ionotropic GABA_A receptor antagonist. *Bar* shows timing of 5 sec burst of 50 Hz GoS. *Dashed lines* show extrapolated single exponential fit to pre-GoS frequency-dependent depression for all data. *Top trace* shows average of all 31 cells. *Middle trace* shows average of 19 cells that responded to GoS (*Responsive*). *Bottom trace* shows average of 12 cells that did not respond to GoS (*Non-responsive*). *D*, *Dashed line* shows distribution of mean EPSC amplitudes during GoS for all cells. *Solid line* shows distribution for responsive cells.

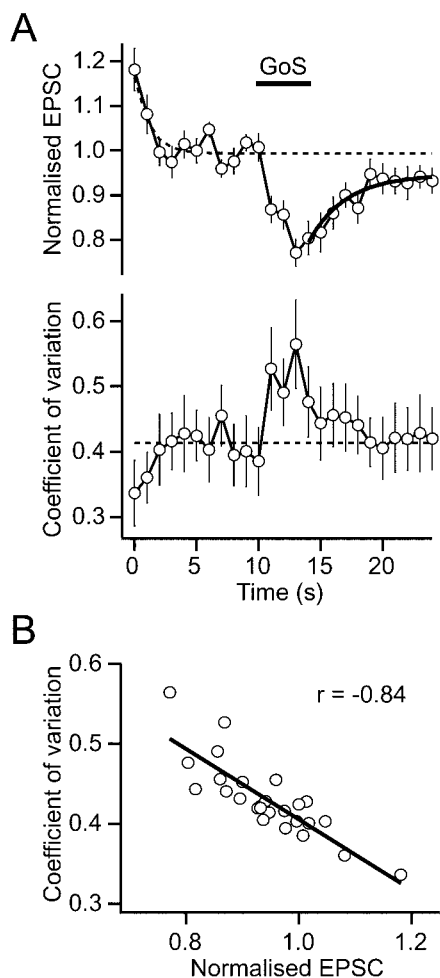


Figure 6. Golgi cell-induced suppression of mossy fiber EPSCs is presynaptic. *A*, Top panel, Normalized EPSC amplitudes for responsive cells during 1 Hz Mossy fiber stimulation in presence of ionotropic GABA_A receptor antagonist (same data as Fig. 5C, middle trace). Bar shows timing of 5 sec burst of 50 Hz GoS. Dashed lines show time course of frequency-dependent depression from all data (Fig. 5C, top trace). Bold line shows fit of single exponential function to EPSC recovery ($\tau = 3$ sec). Bottom panel, CV during EPSC depression ($n = 19$). Dashed line shows pre-GoS baseline. *B*, Relationship between CV and normalized peak EPSC. Negative correlation (Spearman correlation coefficient; $r = -0.84$) indicates a reduction in release probability during EPSC depression.

ing Golgi cell stimulation showed that no synaptic currents were evoked in the presence of GABA_A blockers (Fig. 7A,B), ruling out this possibility. Furthermore, we tested whether GABA_B-Rs were involved in EPSC depression by examining the effect of sustained 50 Hz GoS in the presence of a GABA_B-R antagonist. In 500 μ M CGP35348 the Golgi cell stimulation-induced depression of the EPSC was completely blocked ($98 \pm 6\%$ of control, $p = 0.74$, $n = 5$; Fig. 7C), providing direct evidence that depression of glutamate release from the mossy fiber was caused by GABA_B-R activation.

To better understand the physiological role of presynaptic GABA_B-Rs and to test whether the frequency dependence of GABA_B-R-mediated depression occurs during endogenous activation, we examined the effects of Golgi cell stimulation on EPSCs evoked at two different frequencies. Consistent with the frequency dependence of pharmacologically activated GABA_B-Rs (Fig. 2), inhibition of EPSCs during Golgi cell stimulation was significantly greater during 1 Hz mossy fiber stimulation ($22 \pm 6\%$) than during 10 Hz mossy fiber stimulation ($10 \pm 17\%$, $p = 0.02$, $n = 4$; Fig. 8A,B). Indeed, EPSCs evoked at 10 Hz showed no significant depression during sustained Golgi cell stimulation at 50 Hz ($p = 0.30$). These results demonstrate that presynaptic GABA_B-Rs selectively depress low-frequency mossy fiber transmission during

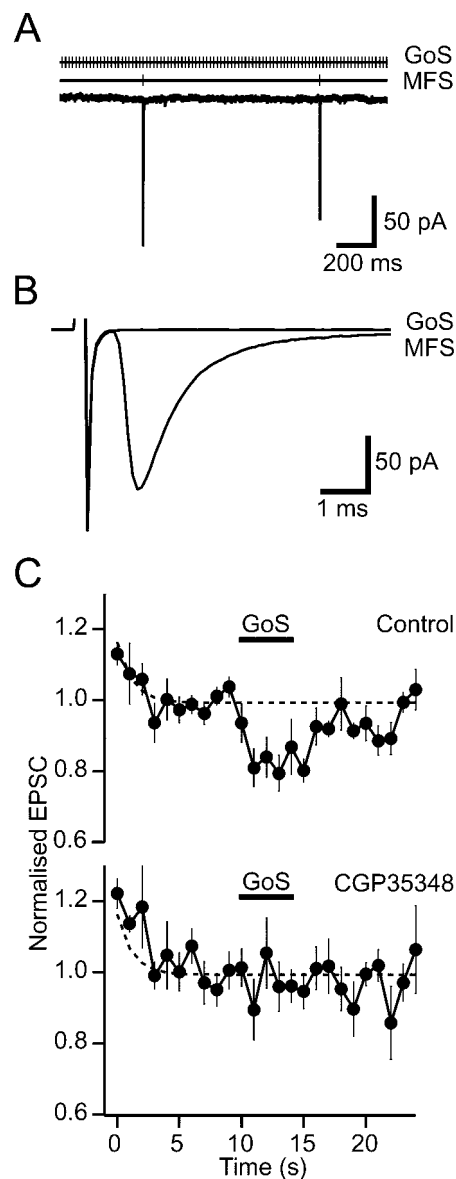


Figure 7. GABA_B-Rs mediate Golgi cell stimulation-induced EPSC depression. *A*, Simultaneous 50 Hz Golgi cell stimulation (GoS) and 1 Hz mossy fiber stimulation (MFS) in the presence of GABA_A receptor blockers. EPSCs were evoked by MFS, whereas no currents were associated with the GoS. *B*, Stimulus averaged currents: MFS indicates EPSC evoked at 1 Hz, and GoS shows IPSCs were not evoked during 50 Hz GoS. *C*, Normalized evoked EPSC amplitudes from the same cells before, during, and after 50 Hz GoS for control and in the presence of the GABA_B-R antagonist CGP35348 (500 μ M; $n = 5$). Dashed lines show time course of frequency-dependent depression from all data (Fig. 5C, top trace).

activation by synaptically released GABA at physiological temperature.

DISCUSSION

Our results show that when individual Golgi cells fire at sustained rates above their basal level, GABA spills over onto excitatory mossy fiber terminals. GABA spillover activates presynaptic GABA_B heteroreceptors at physiological temperature and depresses glutamate release in a frequency-dependent manner. This suggests that mossy fibers firing at low frequency are selectively inhibited, whereas high-frequency inputs remain unaffected.

GABA spillover at physiological temperature

Our results demonstrate that functional GABA_B-Rs are present on the mossy fiber terminal, consistent with immunohistochemical

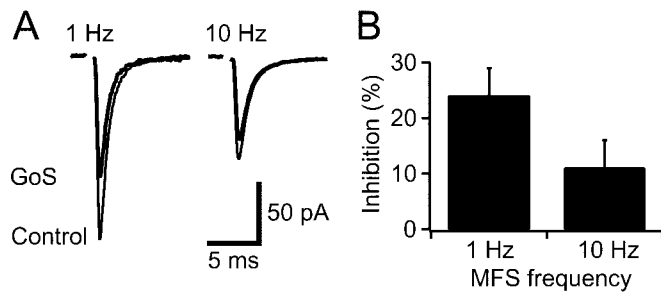


Figure 8. Physiologically activated GABA_B-Rs suppress low-frequency excitatory transmission. *A*, Averaged EPSCs from the same cell evoked at 1 and 10 Hz mossy fiber stimulation (MFS) during 50 Hz Golgi cell stimulation (GoS). GABA_B-R-mediated EPSC depression was more pronounced at 1 Hz than at 10 Hz. *B*, Histogram of mean EPSC inhibition during GoS for four cells.

evidence that shows staining for the R1b splice variant of the GABA_B-R in the cerebellar glomerulus (Poorkhalkali et al., 2000). Activation of GABA_B-Rs on mossy fiber terminals by synaptically released GABA is likely to be locally restricted within glomeruli because the concentration of GABA falls rapidly as it diffuses from the release site (for estimates for glutamate diffusion, see Barbour and Häusser, 1997; Rusakov et al., 1999), GABA transporters are present in the glomerulus (Itouji et al., 1996), and glomerular synapses are partially encapsulated by glia at this age (Hámori and Somogyi, 1983). Previous studies indicate that GABA spillover occurs in the cerebellar glomerulus. The basal level of GABA produces a postsynaptic leak conductance by tonically activating $\alpha 6$ -containing GABA_A receptors (Brickley et al., 1996; Tia et al., 1996; Wall and Usowicz, 1997). These high-affinity receptors, which are located both synaptically and extrasynaptically (Nusser et al., 1998), also detect evoked GABA released from neighboring synapses, generating slow IPSCs (Rossi and Hamann, 1998). Our results showing that presynaptic GABA_B-Rs are activated tonically at room temperature are consistent with these previous studies and the observation that GABA_B-Rs are tonically active at excitatory synapses in rat supraoptic nucleus and olfactory bulb (Kombian et al., 1996; Aroniadou-Anderjaska et al., 2000). However, we find that presynaptic GABA_B-Rs are not tonically activated at physiological temperature ($\sim 37^{\circ}\text{C}$), suggesting GABA spillover is dependent on temperature. Several processes could underlie this temperature dependence, including changes in GABA release, modulation, or uptake. Reduced GABA release seems unlikely because Golgi cell firing rate increases with temperature (Dieudonne, 1998; Misra et al., 2000; Mitchell and Silver, 2000), and most spontaneous IPSCs in granule cells are sensitive to tetrodotoxin (Brickley et al., 1996; Wall and Usowicz 1997). A decrease in the efficacy of the modulatory mechanism is also unlikely because the degree of inhibition of the EPSC by 100 μM baclofen did not change with temperature ($45 \pm 1\%$, $n = 3$ at room temperature; $54 \pm 5\%$, $n = 12$ at 37°C ; $p = 0.41$, unpaired t test). In contrast, our findings that tonic activation of presynaptic GABA_B-Rs can be revealed at 37°C when uptake is inhibited, suggest that GABA uptake is likely to underlie this temperature sensitivity. Furthermore, GABA_B-Rs can be activated at physiological temperature when Golgi cell stimulation is sustained for ≥ 1 sec, suggesting that uptake can be overcome when the amount of GABA release is increased. These results suggest that GABA spillover is more restricted at physiological temperatures than at lower temperatures because uptake is more effective at removing extracellular GABA. Temperature dependence of heterosynaptic GABA_B-R activation has also been observed in hippocampus (Isaacson et al., 1993), highlighting the importance of uptake when investigating the physiological role of transmitter spillover.

GABA release from a single Golgi cell input activates heterosynaptic GABA_B-Rs

Several studies have established that presynaptic GABA_B-Rs on excitatory terminals can be activated by spillover of GABA re-

leased from interneurons (Isaacson et al., 1993; Dittman and Regehr, 1997; Vogt and Nicoll, 1999; Yamada et al., 1999; Aroniadou-Anderjaska et al., 2000). However, it is unclear whether GABA release from a single input can activate presynaptic heteroreceptors or whether synchronous release from many axons is necessary. We have tested this by examining whether GABA release during stimulation of single inhibitory inputs onto cerebellar granule cells can activate heteroreceptors. Activation of an individual synaptic input is feasible with local extracellular stimulation in this preparation (Silver et al., 1996) because Golgi cells and mossy fibers are present at a low density in the granule cell layer (Palay and Chan-Palay, 1974), and one Golgi cell axon is thought to innervate an individual glomerulus (Eccles et al., 1967; Ito, 1984; Jakob and Hámori, 1988). Even if more than one Golgi cell axon occasionally innervates glomeruli, the minimal stimulation method will ensure only one input is activated. Furthermore, if one of the axons is not synaptically connected to the granule cell from which we recorded, slow spillover-mediated IPSCs (Rossi and Hamann, 1998) should be present when the stimulation voltage is lowered to just below the threshold of the synaptically connected axon. This was not observed. Our results therefore show that GABA_B-Rs on excitatory mossy fiber terminals can be activated by stimulating single inhibitory inputs at physiological temperature and frequencies observed *in vivo* (Edgley and Lidieth, 1987; van Kan et al., 1993; Vos et al., 1999). The EPSC depression obtained during physiological activation of GABA_B-Rs was significantly smaller than the depression observed with a high concentration of baclofen. Because GABA and baclofen are both full agonists (Kaupmann et al., 1997), this result suggests that GABA_B-Rs are only partially occupied by GABA during sustained Golgi cell firing at 50 Hz. It is possible that the strength of GABA_B-R modulation changes with development because there are considerable changes in the morphology of the glomerulus between the juvenile animals studied here (P13) and mature rats (P45) (Hámori and Somogyi, 1983).

Frequency dependence of GABA_B-R modulation of the EPSC

Physiological activation of presynaptic GABA_B-Rs reduced the EPSC by 24% (at 1 Hz mossy fiber stimulation) during sustained Golgi cell stimulation. At a higher mossy fiber stimulation frequency of 10 Hz, GABA spillover did not induce significant EPSC depression, consistent with the frequency dependence observed with baclofen. Modulation of tonically firing mossy fibers by presynaptic GABA_B-Rs will therefore be restricted to low-frequency inputs. This contrasts with the behavior of avian auditory synapses where pharmacological activation of presynaptic GABA_B-Rs causes EPSC depression up to 100 Hz and potentiation at even high frequencies (Brenowitz et al., 1998). These differences may reflect functional heterogeneity in GABA_B modulation at different central synapses.

GABA_B-Rs are thought to inhibit glutamate release via G-protein-mediated downregulation of presynaptic calcium currents (Dittman and Regehr, 1996; Isaacson, 1998; Takahashi et al., 1998) and of the release process (Capogna et al., 1996; Dittman and Regehr, 1996). If GABA_B-Rs simply modulate the initial release probability (P_{r1}), then the frequency dependence may arise from the release process in a manner analogous to that observed after cortical long-term plasticity (Markram and Tsodyks, 1996). Simple vesicular depletion models predict that as stimulation frequency is increased, the release probability becomes dependent on the rate of recovery of the readily releasable pool and independent of the vesicle fusion rate and thus P_{r1} (Abbott et al., 1997; Tsodyks and Markram 1997; Matveev and Wang 2000). If this mechanism contributes to other forms of presynaptic modulation, differences in the frequency dependence of GABA_B-R modulation of excitatory mossy fibers and mGluR modulation of inhibitory Golgi cells (Mitchell and Silver, 2000) could simply reflect the values of P_{r1} and the rate of recovery of the release sites at these two synapses. Another mechanism that might underlie the frequency dependence include voltage-dependent relief of G-protein inhibition (Bean,

1989; Brody et al., 1997; Dolphin, 1998) at higher mossy fiber firing frequencies because bursts of action potential waveforms relieve inhibition of calcium channels in human embryonic kidney 293 cells (Brody et al., 1997). Activation of mGluRs on the Golgi cell terminals during mossy fiber stimulation (Mitchell and Silver, 2000) might also influence the frequency dependence of physiologically activated GABA_B-R-mediated EPSC depression by reducing GABA release at higher mossy fiber stimulation frequencies. However, this mechanism is not likely to be limiting, because the frequency dependence of EPSC depression observed with GABA spillover and baclofen was similar. Last, GABA_B-R-mediated depression could be occluded by autoreceptor-mediated EPSC depression, which is also likely to be frequency-dependent. Although investigation of the precise mechanisms underlying the frequency dependence GABA_B-Rs is beyond the scope of this study, characterization of the steady-state frequency dependence of EPSCs during GABA_B-R activation is relevant for understanding transmission at mossy fiber inputs that fire relatively regularly, as observed for “tonic” inputs encoding joint angle (van Kan et al., 1993). Furthermore, our steady-state data provide the basis for a detailed investigation of the effects of GABA_B-R modulation on short-term plasticity by determining the level of GABA_B-R activation under physiological conditions.

Bidirectional spillover of transmitter in the glomerulus: implications for information processing in the cerebellar cortex

Spillover of GABA onto GABA_B-Rs on mossy fiber terminals shown here mirrors glutamate spillover onto mGluRs on Golgi cell axon terminals (Mitchell and Silver, 2000). The two components of these bidirectional spillover-mediated processes have opposite yet complementary effects in terms of information processing. Activation of mGluRs on Golgi cell terminals by glutamate has an excitatory action by suppressing GABA release onto granule cells. Because the size of this effect depends on the firing frequency of the excitatory input, it will tend to boost the postsynaptic effects of mossy fibers firing at higher rates and may compensate for frequency-dependent depression of the EPSC. In contrast, activation of GABA_B-Rs on mossy fibers by GABA spillover depresses low-frequency excitatory inputs, making these inputs less effective. These two spillover-mediated presynaptic mechanisms are likely to be effective under different network conditions: the mGluR system will be most effective when the Golgi cell firing rate is low, whereas the GABA_B system will be most effective when it is high. The spatial characteristics of the mGluR and GABA_B-R-mediated effects are also different. GABA_B-R-mediated inhibition of the EPSC is set by the Golgi cell axonal arbor, which contacts ~5000 granule cells in the region (Ito, 1984), whereas mGluR-mediated Golgi cell disinhibition is set by the distribution of the ~20 glomeruli (per folium) associated with an individual mossy fiber (Eccles et al., 1967; Ito, 1984). Our results suggest that presynaptic heteroreceptors in the glomerulus modulate the efficacy of synaptic input onto granule cells over a range of network activity and play a role in processing sensory information as it enters the cerebellar cortex.

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