

# Activation of Presynaptic GABA<sub>A</sub> Receptors Induces Glutamate Release from Parallel Fiber Synapses

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The parallel fibers relay information coming into the cerebellar cortex from the mossy fibers, and they form synapses with molecular layer interneurons (MLIs) and Purkinje cells. Here we show that activation of ionotropic GABA receptors (GABA<sub>A</sub>Rs) induces glutamate release from parallel fibers onto both MLIs and Purkinje cells. These GABA-induced EPSCs have kinetics and amplitudes identical to random spontaneous currents (sEPSCs), but, unlike sEPSCs, they occur in bursts of between one and five successive events. The variation in amplitude of events within bursts is significantly less than the variation of all sEPSC amplitudes, suggesting that the bursts result from repetitive activation of single presynaptic fibers. Electron microscopy of immunogold-labeled  $\alpha$ -1 subunits revealed GABA<sub>A</sub>Rs on parallel fiber terminals. We suggest that the activation of these receptors underlies the increased amplitude of parallel fiber-evoked Purkinje cell EPSCs seen with application of exogenous GABA or after the release of GABA from local interneurons. These results occur only when molecular layer GABA<sub>A</sub>Rs are activated, and the effects are abolished when the receptors are blocked by the GABA<sub>A</sub>R antagonist gabazine (5  $\mu$ M). From these data, we conclude that GABA<sub>A</sub>Rs located on parallel fibers depolarize parallel fiber terminals beyond the threshold for Na<sup>+</sup> channel activation and thereby induce glutamate release onto MLIs and Purkinje cells.

**Key words:** cerebellum; GABA<sub>A</sub> receptors; AMPA receptors; synaptic terminal; parallel fiber; Purkinje cell

## Introduction

Recent data demonstrating the influence of subthreshold activity at synaptic terminals (Awatramani et al., 2005; Alle and Geiger, 2006; Shu et al., 2006) has made the subject of presynaptic ionotropic receptors a topic of much interest. This work has shown that small voltage changes at the terminal have substantial effects on transmission at the synapse and therefore indicate that similar changes in voltage induced by presynaptic receptors could have major influences on synaptic signaling and neuronal processing. Many types of receptors have been found on presynaptic membranes of several different cell types and in several different brain regions (MacDermott et al., 1999; Kullmann et al., 2005). Of these receptors, GABA<sub>A</sub> receptors (GABA<sub>A</sub>Rs) are of particular interest because they can be either excitatory or inhibitory depending on several different factors (Alger and Nicoll, 1982; Staley et al., 1995; Chavas and Marty, 2003; Gullledge and Stuart, 2003; Szabadics et al., 2006). GABA<sub>A</sub>Rs have been found previously in the brain on axonal membranes of hippocampal granule cells (Ruiz et al., 2003; Jang et al., 2006; Alle and Geiger, 2007), the

molecular layer interneurons (MLIs) of the cerebellum (Pouzat and Marty, 1999), cortical pyramidal cells (Szabadics et al., 2006), as well as on nerve terminals of the posterior pituitary (Zhang and Jackson, 1993) and calyx of Held (Turecek and Trussell, 2002). Outside of the brain, GABA<sub>A</sub>Rs have also been found on the axonal membranes of neurons in the peripheral nervous system (MacDermott et al., 1999). The reports on the influence of these receptors on excitability are different in these regions and even vary from inhibitory (Ruiz et al., 2003) to excitatory (Nakamura et al., 2007) within the same preparation.

In the granule cells of the cerebellum, in which extrasynaptic receptors have been well documented (Farrant and Nusser, 2005; Kullmann et al., 2005), GABA<sub>A</sub>Rs have not yet been found outside of the somatodendritic compartment. We report here the presence of GABA<sub>A</sub>Rs in the axonal compartment of granule cells, and we suggest that the response of the receptor to exogenous or endogenous GABA induces depolarization of the synaptic terminals. The depolarization is enough to cross the threshold for Na<sup>+</sup> channel activation and thereby induce release of glutamate from the synaptic terminals onto postsynaptic targets. Thus, these presynaptic receptors are excitatory and likely regulate transmission through parallel fiber synapses.

## Materials and Methods

**Slice preparation.** Wistar rats between postnatal day 13 (P13) and P29 were anesthetized with halothane and decapitated before removal of the cerebellum. Two-hundred-micrometer-thick sagittal or 400- $\mu$ m-thick coronal slices were made using a Leica (Nussloch, Germany) VT 1000S vibratome while the cerebellum was bathed in an ice-cold artificial CSF (ACSF) that was bubbled with 95% oxygen and 5% carbon dioxide. The ACSF was composed

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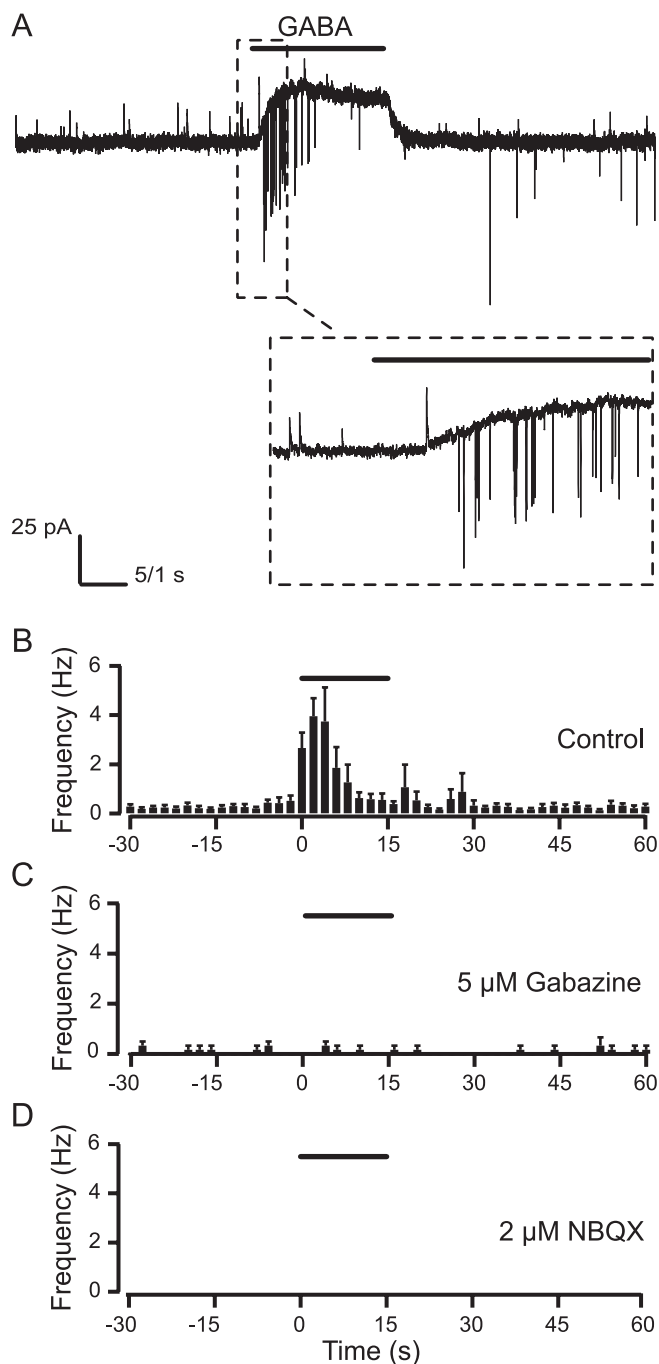
of the following (in mM): 130 NaCl, 2.4 KCl, 1.3 NaH<sub>2</sub>PO<sub>4</sub>, 1 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub>, 10 glucose, 26 NaHCO<sub>3</sub>, and 1–3 kynurenic acid.

**Whole-cell recordings.** The molecular layer was visualized using an Axioskop microscope (Zeiss, Oberkochen, Germany) equipped with a 63× water immersion objective. Recordings were made at room temperature using an EPC-10 amplifier (HEKA Elektronik, Lambrecht, Germany). During recordings, the slices were perfused with the same ACSF as they were cut in, without kynurenic acid. Unfilamented borosilicate glass pipettes were pulled using a HEKA Elektronik pipette puller and had an open tip resistance of ~7 MΩ for interneurons and ~3 MΩ for Purkinje cells when filled with an internal solution containing the following (in mM): 150 K-gluconate, 4.6 MgCl<sub>2</sub>, 10 K-HEPES, 1 K-EGTA, 0.1 CaCl<sub>2</sub>, 4 Na-ATP, and 0.4 Na-GTP, pH 7.3. A second slightly larger “puff” pipette was filled with ACSF and supplemented with either 50 μM GABA or 10 μM muscimol. The puff was generated using a pump to regulate the amount of pressure (~50 millibars) applied to the back of the pipette and was triggered using a voltage pulse.

**Extracellular stimulation.** A platinum wire was inserted into a patch pipette that was filled with ACSF and was connected to an Isolated Pulse Stimulator (model 2100; A-M Systems, Carlsborg, WA) that was set to produce constant voltage for 20 μs after each trigger. The pipette was placed in the parallel fibers of the 400-μm-thick coronal slices, and voltage was then varied (~5–20 V) to produce a single EPSC of ~300 pA. The voltage was then left unchanged for the duration of the experiment while the stimulator was triggered by a computer. For experiments shown in Figure 8, two stimulation electrodes were positioned on opposite sides of the recorded Purkinje cell. To localize the stimulus, these electrodes were constructed from theta class with opposite poles of the stimulator in each barrel of the pipette. One electrode (electrode A) was used to deliver test pulses before and after the train (stim i and stim ii), whereas the other (electrode B) was used to deliver the train itself (Dittman and Regehr, 1997). A stimulation protocol was performed at the beginning and end of each experiment to verify that fibers stimulated with electrode A were distinct from the fibers stimulated with electrode B. Because parallel fiber synapses show paired-pulse facilitation, we could easily determine whether stimuli evoked from electrode B induced any facilitation in the responses recorded from stimuli evoked from electrode A. The positions of the electrodes were adjusted until no facilitation was produced and therefore indicated that the fibers stimulated by the two different electrodes were distinct populations.

**Analysis.** Spontaneous EPSCs (sEPSCs) were detected using EVAN (version 1.2; courtesy of Dr. Istvan Mody, University of California, Los Angeles, Los Angeles, CA) or Neuromatic (version 1.91; courtesy of Dr. Jason Rothman, thinkrandom.com), and all analyses were performed in IGOR Pro (version 5.0.3; WaveMetrics, Lake Oswego, OR) except for the fitting of individual EPSC decay times with exponential equations, which was done in EVAN.

**Immunocytochemistry.** P18 Sprague Dawley rats were anesthetized with pentobarbital (60 mg/kg body weight) and intracardially perfused with 4% (w/v) paraformaldehyde and 0.1% glutaraldehyde (Polysciences, Warrington, PA) in PBS. After dissection, the cerebellum was kept overnight in 4% paraformaldehyde at 4°C. Transverse vibratome sections (200 μm) of the cerebellum were treated with ammonium chloride (0.13 g/50 ml of PBS) for 30 min at 4°C. After washing in PBS, the samples were gradually dehydrated in methanol and embedded in low-cryl Hm20 (Polysciences) in an AFS Reichert apparatus (Leica, Wien, Austria) after a progressive lowering of temperature. After polymerization under UV light at -45°C during 48 h, pale yellow sections were incubated for 30 min in goat gold conjugates blocking solution (Aurion, Wageningen, The Netherlands). The sections were washed (three times for 5 min each) in an incubation buffer made of PBS with 0.2% bovine serum albumin-c (Aurion). Sections were subsequently incubated overnight at 4°C in the same buffer with an antibody directed against the α<sub>1</sub> GABA subunit (Zezula et al., 1991; Nusser et al., 1995). After extensive washes (six times for 5 min each), the sections were incubated 1 h at room temperature in gold-conjugated secondary antibodies against rabbit IgG (1:50; British Biocell International, Cardiff, UK) and then washed (six times for 5 min each) in the incubation buffer and then in PBS (two times), followed by a 5 min fixation in 2% glutaraldehyde in PBS. After a



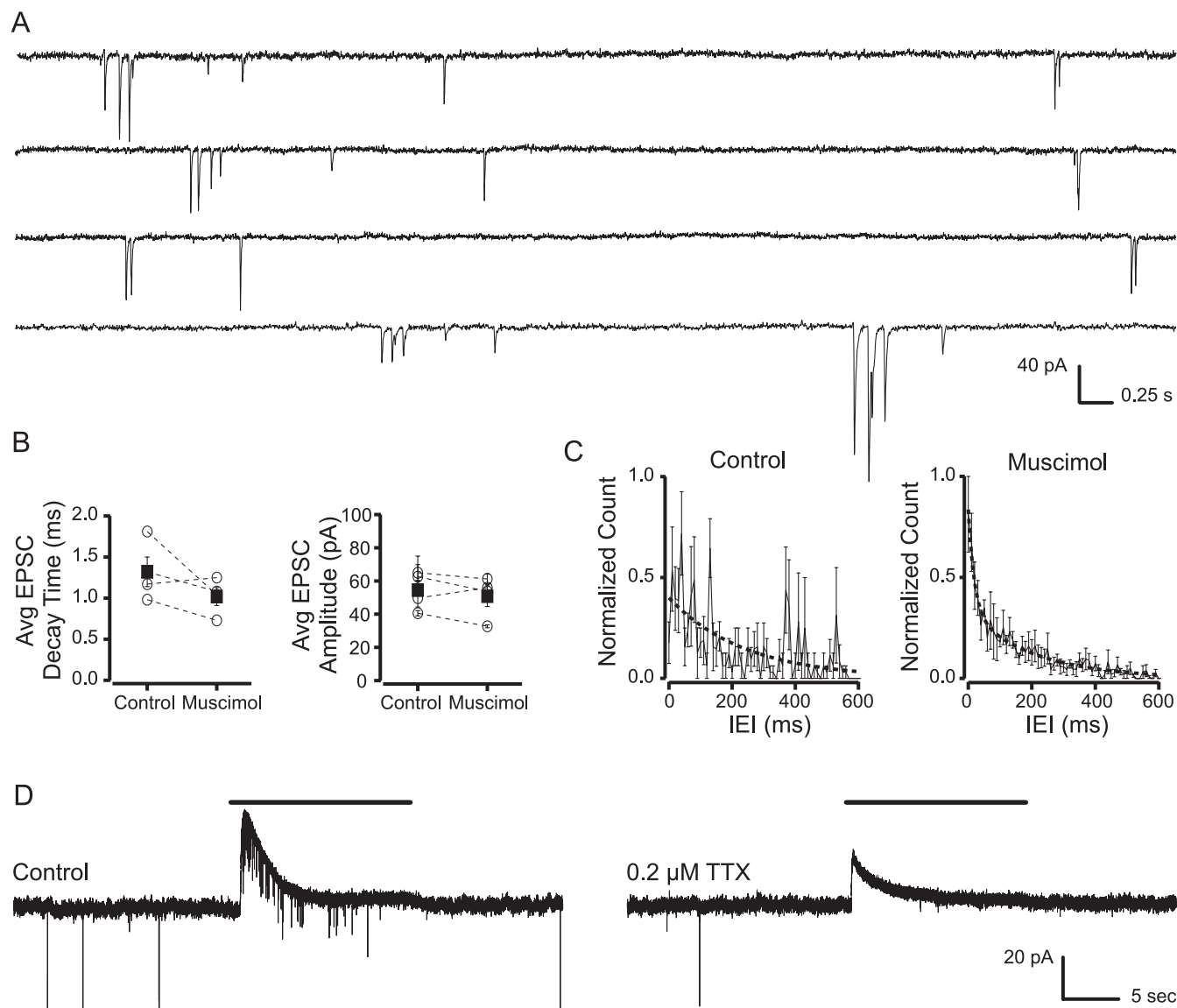
**Figure 1.** GABA<sub>A</sub>R activation induces EPSCs in MLIs. **A**, Spontaneous activity recorded in a molecular layer interneuron during the application of 50 μM GABA (black line). The expansion shows the presence of a flurry of fast inward currents recorded during the first seconds of GABA application. **B–D**, Histograms of the average frequency of inward events recorded during the same time period shown in **A**, with the black bar signifying the application of either 10 μM muscimol or 50 μM GABA. **B**, Under control conditions ( $n = 19$  cells). **C**, In the presence of 5 μM gabazine ( $n = 3$  cells). **D**, In the presence of 2 μM NBQX ( $n = 4$  cells).

wash of 5 min in PBS and six washes of 2 min in distilled water, sections were counterstained with uranyl acetate and lead citrate for inspection with a Philips Tecnai 12 electron microscope (FEI, Eindhoven, The Netherlands).

## Results

### GABA<sub>A</sub>R activation induces EPSCs in interneurons

Using a pressure pipette, we applied 50 μM GABA to parallel fibers in cerebellar slices while monitoring spontaneous activity



**Figure 2.** GABA<sub>A</sub>R activation produces bursts of EPSCs. *A*, Individual 8 s samples of synaptic activity recorded during four separate applications of muscimol. Note that EPSCs occur in bursts of between one and four events. *B*, Average decay times and amplitudes of sEPSCs and EPSCs evoked by muscimol. Open circles are individual cells, and black squares are the averages of all the cells. Dashed lines connect data from the same cell. *C*, Histograms of the average interevent intervals for sEPSCs and EPSCs evoked by muscimol application ( $n = 4$  cells). The histogram of the control events is fit by a monoexponential equation with  $\tau$  of 241 ms, and the histogram of the events induced by muscimol is fit by a biexponential equation with  $\tau_1$  of 207 ms (39%) and  $\tau_2$  of 19 ms (61%). *D*, Synaptic activity recorded in one cell before (left) and after (right) the bath application of 0.2  $\mu$ M TTX. The downward deflections are EPSCs. The bars indicate the application of 10  $\mu$ M muscimol. Spontaneous EPSCs happened to be on average larger than muscimol-evoked EPSCs in these traces, but this was not a general rule.

in MLIs under whole-cell voltage-clamp conditions. The application of GABA induced a slow outward current in the interneurons as predicted from the holding potential ( $-60$  mV) and the  $[Cl^-]_i$  (10 mM) (Fig. 1*A*). However, GABA also induced a flurry of fast inward currents that coincided with the onset of the application of the agonist (Fig. 1*A*; average time course in *B*). This phenomenon is not an artifact caused by the pressure pipette because it is blocked by the specific GABA<sub>A</sub>R antagonist gabazine (5  $\mu$ M). In three of three cells, the antagonist completely blocked both the slow outward current as well as the fast inward currents (Fig. 1*C*). In two of these three cells, gabazine was washed from the bath and both currents returned (data not shown).

To determine whether the effect also requires the activation of GABA<sub>B</sub> in addition to GABA<sub>A</sub> receptors, we made recordings in the presence of 1  $\mu$ M CGP 55845 [2*S*]-3-[(1*S*)-1-(3,4-dichlorophenyl)ethyl]amino-2-hydroxypropyl(phenylmethyl)phosphinic acid]

and found that the fast inward currents remained (data not shown). The fast inward currents were also induced when GABA was replaced by the highly specific GABA<sub>A</sub>R agonist muscimol (10  $\mu$ M) (see Figs. 2, 4, 5). Therefore, the fast inward currents result from the specific activation of GABA<sub>A</sub>Rs.

In MLIs, IPSCs and EPSCs can be easily distinguished based on the decay kinetics of the two currents. The waveform of the IPSC decay is best fit by a biexponential equation whose fastest time constant is  $\sim 8.7$  ms, whereas the decay of EPSCs is best fit by a monoexponential equation with a time constant of  $\sim 1.3$  ms (Llano and Gerschenfeld, 1993). The fast inward currents that we elicited when puffing GABA were distinguishable from IPSCs because they were fit by monoexponential equations with time constants  $< 2$  ms and were absent when the AMPA receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[*f*]quinoxaline (NBQX) (2  $\mu$ M) was washed onto the slice. This concentration of

NBQX completely blocked the fast inward currents and had no effect on the slow outward current, indicating that the fast currents were EPSCs originating from brief activations of AMPA receptors (Fig. 1D).

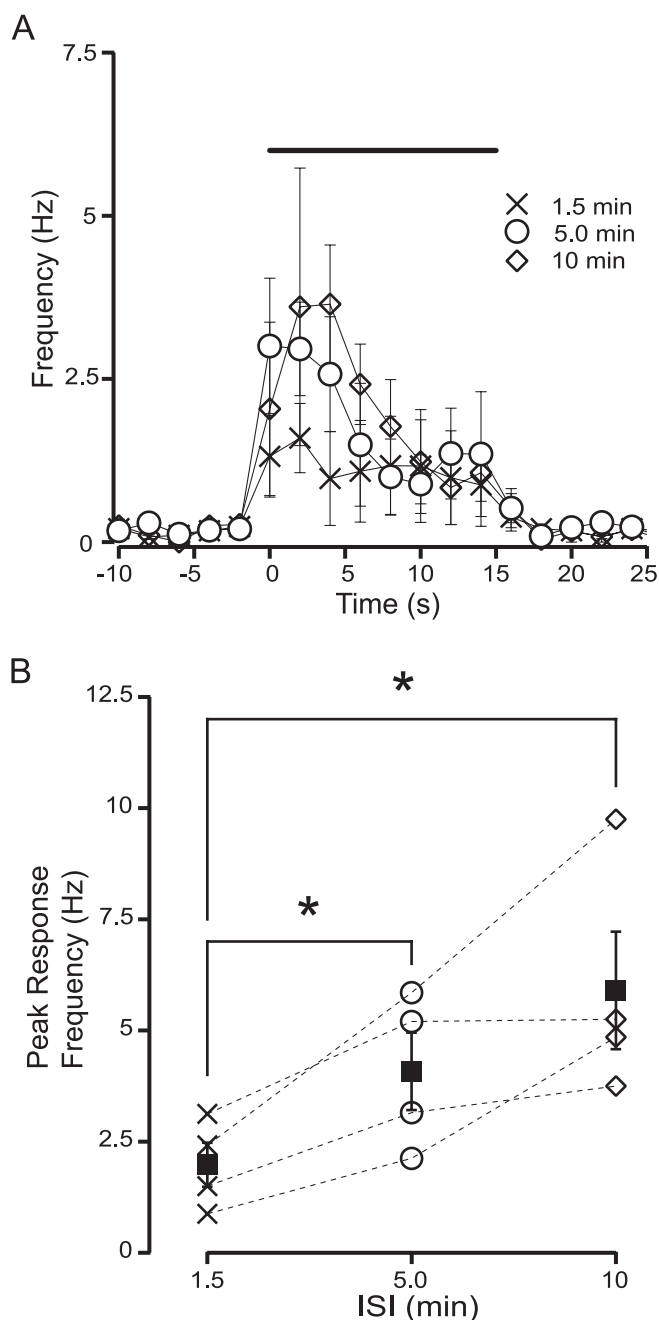
#### GABA<sub>A</sub>R-induced EPSCs are similar to sEPSCs

GABA<sub>A</sub>R-induced EPSCs (Fig. 2A) have properties that are nearly identical to spontaneous EPSCs recorded in the same cells under control conditions. The decays were fit by single exponentials with  $\tau$  values averaging  $1.0 \pm 0.1$  ms compared with  $1.3 \pm 0.2$  ms in control conditions ( $p > 0.05$ , paired Student's  $t$  test;  $n = 4$ ) (Fig. 2B) and amplitudes averaging  $50.9 \pm 6.3$  pA compared with  $54.5 \pm 5.7$  pA in control conditions ( $p > 0.05$ ;  $n = 4$ ) (Fig. 2B). However, unlike in control conditions, the GABA<sub>A</sub>R-induced EPSCs appeared in short bursts (Fig. 2A). When the inter-EPSC intervals from data recorded under control conditions are plotted in a histogram, they are fit by a single-exponential equation ( $\tau$  of 241 ms), whereas two exponentials were required to fit the data collected when muscimol was present ( $\tau_1$  of 207 ms,  $\tau_2$  of 19 ms,  $A_1$  of 39%, and  $A_2$  of 61%) (Fig. 2C). The need for a second exponential indicates that the EPSCs occurred in bursts, and the parameters of the fast component indicate that the average time in between events within the bursts is 19 ms. The burst pattern of Figure 2A, in which the response is clearly composed of several distinct bursts, is incompatible with schemes in which release would occur randomly in a large number of presynaptic fibers. It suggests instead that no more than one fiber was active at any given time and that each active fiber gave rise to a series of EPSCs, either because it fired repetitively or because it remained depolarized for some time ( $\sim 200$  ms, the duration of a burst). To verify whether the individual bursts which compose the response originated from repetitive activation of the same synapse, we looked at the variability of the amplitudes within bursts and compared them with the variability of all sEPSCs combined, because EPSCs originating from the same synapse are predicted to have similar properties whereas EPSCs from different synapses should show greater variability (Forti et al., 1997; Auger and Marty, 2000). We found that the coefficient of variation of amplitudes recorded during bursts was significantly smaller than the total coefficient of variation from all EPSCs ( $0.36 \pm 0.02$  vs  $0.61 \pm 0.04$ ;  $p < 0.05$ , paired Student's  $t$  test), consistent with bursts having originated from single synapses.

To determine whether repetitive presynaptic firing is part of the mechanism responsible for generating the bursts, we tested for the involvement of Na<sup>+</sup> channels. To do this, we repeated the above experiments and washed 0.2  $\mu$ M TTX onto the slice after inducing the effect under control conditions. In four of four cells, TTX completely abolished the GABA<sub>A</sub>R-induced EPSCs (Fig. 2D), indicating that the effect requires Na<sup>+</sup> channel activation and subsequent firing of presynaptic action potentials.

#### GABA<sub>A</sub>R-induced EPSCs show long-lasting fatigue

Despite the prolonged presence of the GABA<sub>A</sub>R agonist (15 s), the overall period of bursting was variable, lasting from  $\sim 0.5$  to 5 s (Figs. 1A, 2D), with each individual burst lasting only for a few hundred milliseconds (Fig. 2A). This indicates that GABA<sub>A</sub>R activation is sufficient to induce the bursting but is not sufficient to maintain it. During activation, GABA<sub>A</sub> receptors enter desensitized states (Jones and Westbrook, 1995), and this could explain why the increased EPSC frequency is not maintained despite prolonged presence of muscimol. To gain insight into the mechanism responsible for stopping the bursting and perhaps the mechanism responsible for producing it, we determined the



**Figure 3.** Induction of GABA<sub>A</sub>R-activated EPSCs shows long-lasting fatigue. **A**, Histogram of the frequency of EPSCs recorded after waiting 1.5 min (crosses), 5 min (circles), or 10 min (diamonds) between muscimol applications. The black line represents the application of 10  $\mu$ M muscimol. **B**, Peak response recorded in each cell as a function of the three different time intervals. Symbols are the same as in **A**, and black boxes correspond to the averages. Lines connect data from individual cells. \* $p < 0.05$ , paired Student's  $t$  test.

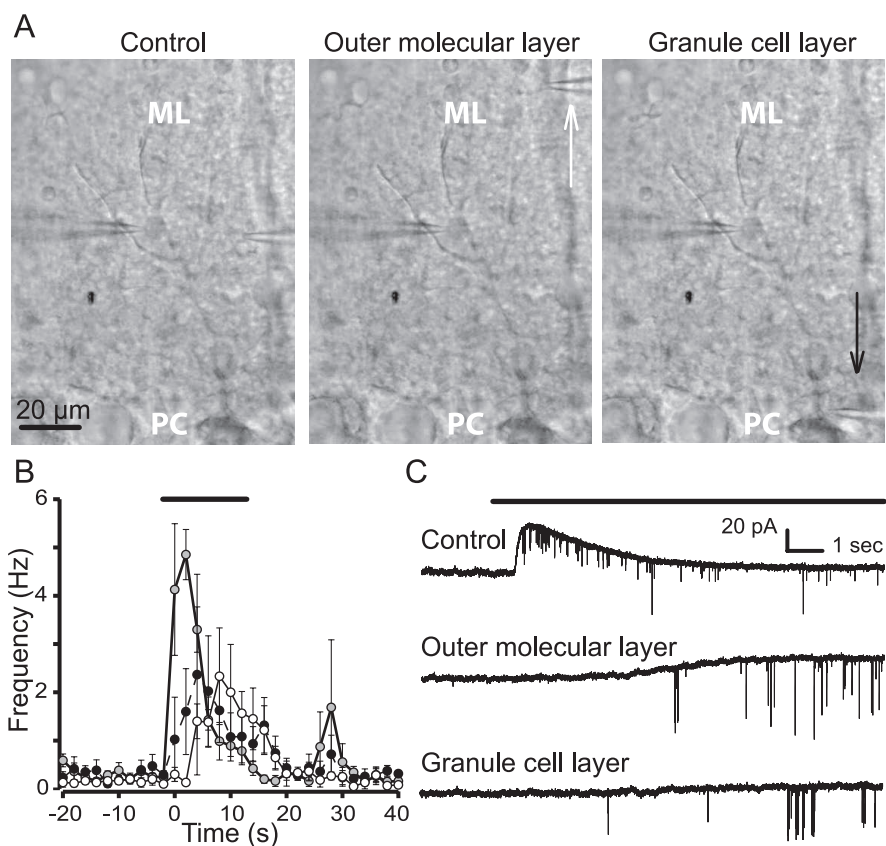
amount of time that is required to fully recover the effect between agonist applications. We found that, when compared with using 5 min intervals between applications, repeating the agonist application every 1.5 min either abolished the response altogether or severely attenuated its magnitude ( $p < 0.05$ , Student's  $t$  test;  $n = 4$ ) (Fig. 3A,B). However, waiting 10 min between agonist applications showed a trend toward increasing the response without producing a significantly different response than when 5 min intervals were used ( $p > 0.05$ , Student's  $t$  test;  $n = 4$ ) (Fig. 3A,B). After long-lasting GABA pulses, GABA<sub>A</sub>R have been shown to

recover from desensitization with time constants on the order of at most  $\sim 13$  s (Jones and Westbrook, 1995). Therefore, because the increase in EPSC frequency was not fully recovered after 90 s, GABA<sub>A</sub>R desensitization is unlikely to provide the entire explanation for its transience. It has been noted in another context that responses to long GABA applications recover more slowly than expected from GABA<sub>A</sub>R desensitization properties (Segal, 1993). An attractive explanation is that the responses involve changes in  $[Cl^-]_i$  and take time to recover. Changes in  $[Cl^-]_i$  are particularly likely because of the surface to volume ratio of the axon domain, but testing for  $[Cl^-]_i$  redistribution with direct measurements from the axons will await additional study.

#### GABA<sub>A</sub>R activation occurs in the molecular layer

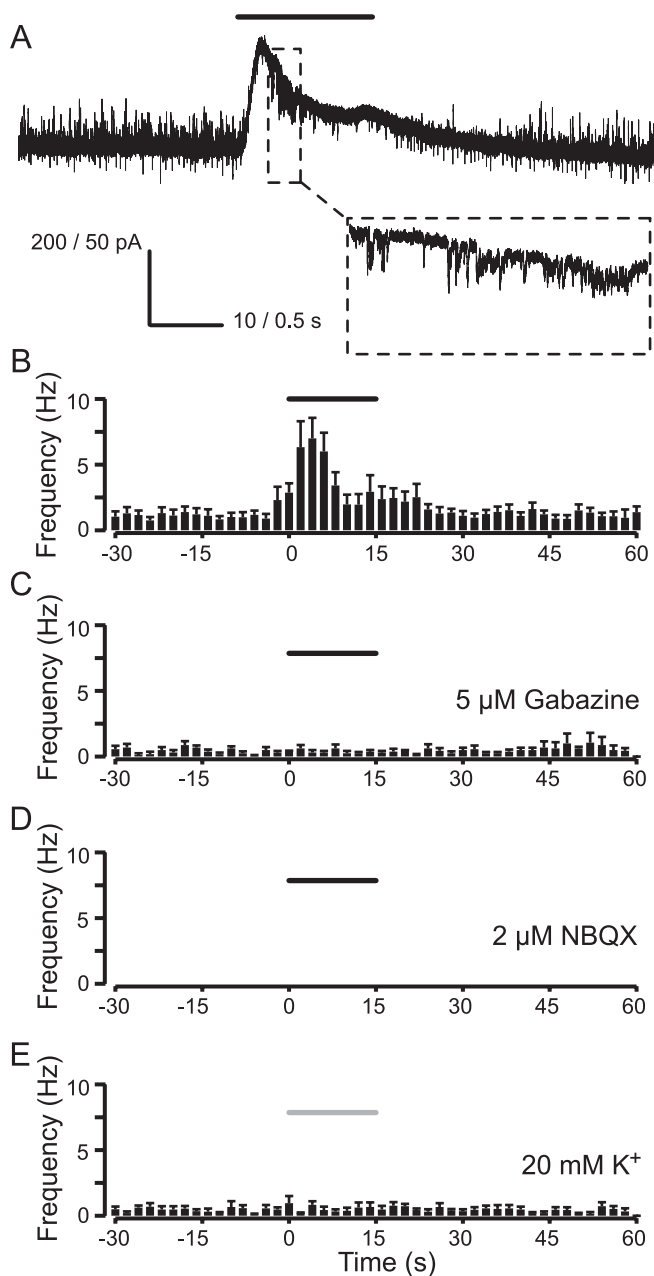
It is not obvious how GABA<sub>A</sub>R activation induces activity in parallel fibers, but one possibility is that the agonist that was released from our puffer pipette was not localized to the molecular layer and instead diffused into the granule cell layer. If this was the case, then several scenarios could explain why EPSCs are induced by the GABA<sub>A</sub>R agonists. The cell bodies of granule cells contain GABA<sub>A</sub>Rs (Pirker et al., 2000), and, despite hyperpolarized  $E_{GABA}$  with respect to the threshold for voltage-gated  $Na^+$  channels (Brickley et al., 1996), it is feasible that prolonged activation of the GABA<sub>A</sub>Rs on the cell bodies could induce spiking in granule cells via a biphasic response to GABA<sub>A</sub>R activation similar to that seen in other cell types (Alger and Nicoll, 1982; Staley et al., 1995; Kaila et al., 1997; Perkins and Wong, 1997; Price and Trussell, 2006). If diffusion of our GABA<sub>A</sub>R agonists into the granule cell layer induced such a depolarization in the cell bodies of the granule cells, then it could have possibly generated a train of action potentials in granule cell somata and led to the burst of postsynaptic EPSCs we recorded in postsynaptic targets. In fact, Golgi cells, whose cell bodies are also in the granule cell layer, have been shown to increase their synaptic output in response to application of GABA (Kaneda et al., 1995), perhaps attributable to a similar mechanism.

To determine whether this or any other scenarios involving the diffusion of the agonist away from the tip of the pipette to the granule cell layer was causing the induction of EPSCs, we designed an experiment in which different locations of the puffer pipette were used to release muscimol. We reasoned that, if we moved the pipette closer to the site of induction of the EPSCs, then the muscimol would have a shorter distance to diffuse and the effect would be stronger and recorded after a shorter delay, whereas moving the pipette away from the site of induction would increase the delay and attenuate the response. Therefore, after inducing the effect in the control position with the puffer pipette next to the patch-clamped interneuron (Fig. 4, left), we used the laminar organization of the cerebellar cortex as a guide



**Figure 4.** GABA<sub>A</sub>R activation occurs in the molecular layer. **A**, Photographs of the placement of the puffer pipette relative to the interneuron that was recorded. In all three photos, the recording pipette is on the left and the puffer pipette is on the right. The left photo shows the puffer pipette in the control position. In the center photo, the puffer pipette (indicated by the white arrow) is moved away from the Purkinje cells (PC) to the outer molecular layer (ML), and, in the right photo, the puffer pipette (black arrow) is moved toward the Purkinje cells. **B**, Histograms of the average EPSC frequency recorded from the three positions of the puffer pipette. The biggest response is recorded when the pipette is in the control position (gray circles), and a diminished and delayed response is recorded when the puffer pipette is in the outer molecular layer (white circles) or the Purkinje cell layer (black circles). **C**, Representative traces of responses recorded when the puffer pipette is in the three different positions. Note that an outward current appears in all three traces but is much reduced and delayed when the puffer pipette is in the outer molecular layer or granule cell layer. This indicates that the agonist is eventually diffusing back to the interneuron from which we are recording, and it is not until this occurs (and the outward current appears) that the EPSC frequency begins to increase.

to move the pipette to two additional positions. We placed the puffer pipette in a position closer to the granule cells in the Purkinje cell layer (Fig. 4A, middle) as well as in a position the same distance away from the control position on the other side of the patch-clamped interneuron (Fig. 4A, right). A significantly larger response was recorded when the puffer pipette was placed at the control position in the center of the molecular layer ( $p < 0.05$ , Student's *t* test;  $n = 5$ ) (Fig. 4B, C). The weak response recorded when the pipette was moved away from the control position coincided with a small, slow, outward current in the interneuron. This current occurred after a much longer delay from the beginning of the puffer application than the outward current that was recorded when the puffer pipette was in the control position (Fig. 4C). This delayed outward current is indicative of muscimol diffusion back to the interneuron in the molecular layer and was recorded when the pipette was either moved closer to or farther from the granule cell layer. Therefore, the small delayed EPSC response recorded when the pipette is moved away from the control position probably results from muscimol diffusion away from the tip of the puffer pipette back to the parallel fibers. Together, these data indicate that the site of induction is in the molecular layer and not on the granule cell somata.



**Figure 5.** GABA<sub>A</sub>R activation induces EPSCs in Purkinje cells. **A**, Spontaneous activity recorded in a Purkinje cell held at  $-60$  mV during the application of  $10 \mu\text{M}$  muscimol (black line). The expected reversal potentials for IPSCs and EPSCs are  $-70$  and  $0$  mV, respectively. The expansion shows the presence of bursts of fast inward currents as recorded in the MLs. Note that, whereas the frequency of (inward) EPSCs is greatly increased, the (outward) IPSCs are abolished after several seconds of muscimol application, probably attributable to desensitization of GABA<sub>A</sub>Rs. **B–E**, Histograms of the average EPSC frequency recorded during the time period shown in **A**. **B–D**, Black bar again signifying the application of muscimol. **B**, Under control conditions ( $n = 13$  cells). **C**, In the presence of  $5 \mu\text{M}$  gabazine ( $n = 4$  cells). **D**, In the presence of  $2 \mu\text{M}$  NBQX ( $n = 4$  cells). **E**, In control conditions, with the gray bar representing the application of  $20 \text{ mM}$   $\text{K}^+$  instead of muscimol.

#### GABA<sub>A</sub>R activation induces EPSCs in Purkinje cells

Having determined that activation of GABA<sub>A</sub>Rs in the molecular layer induces release of glutamate from parallel fibers, we next performed whole-cell recordings from Purkinje cells to test whether the effect could also be recorded in other cell types targeted by parallel fibers. We found that the fast inward currents also occurred in Purkinje cells (Fig. 5*A,B*), and, as in the inter-

neurons, the response was blocked with  $5 \mu\text{M}$  gabazine ( $n = 4$ ) (Fig. 5*C*) or  $2 \mu\text{M}$  NBQX ( $n = 3$ ) (Fig. 5*D*). Therefore, the response recorded in Purkinje cells can also be attributed to GABA<sub>A</sub>R-induced activation of AMPA-mediated currents. Along with the results of the experiments in which the position of the puffer pipette was varied, these data indicate that GABA<sub>A</sub>R activation in the molecular layer leads to release of glutamate from the parallel fibers.

#### EPSC induction does not result from $\text{K}^+$ -mediated volume transmission

The possibility remains that the GABA<sub>A</sub>Rs are not directly on parallel fibers but instead are on another class of cells in the molecular layer that cause indirect depolarization of parallel fibers. It has been demonstrated that GABA<sub>A</sub>R stimulation can lead to increased  $[\text{K}^+]_o$  of up to  $\sim 8 \text{ mM}$  (Barolet and Morris, 1991; Avoli et al., 1996; Kaila et al., 1997). Another explanation for the ability of GABA<sub>A</sub>R agonists to activate parallel fibers in our study could be that, by applying GABA to the molecular layer, we activated GABA<sub>A</sub>Rs on other cells in the vicinity of the parallel fibers (e.g., interneurons, Purkinje cells, or glia) and caused the subsequent release of  $\text{K}^+$  from these cells, which then depolarized parallel fibers beyond the voltage-gated  $\text{Na}^+$  channel threshold. To test this hypothesis, we performed experiments in which we recorded from Purkinje cells and induced EPSCs using  $10 \mu\text{M}$  muscimol and then attempted to reproduce the EPSC induction by exchanging the puffer pipette with one containing ACSF supplemented with  $20 \text{ mM}$   $\text{K}^+$ . We found that exchanging muscimol for  $20 \text{ mM}$   $\text{K}^+$  produced a small inward current in the Purkinje cell but did not increase the frequency of sEPSCs ( $n = 4$ ) (Fig. 5*E*). Therefore, a secondary pathway involving the release of  $\text{K}^+$  from neighboring cells does not appear to be responsible for the GABA<sub>A</sub>R-induced EPSCs.

#### GABA<sub>A</sub>R $\alpha_1$ subunits are located on parallel fiber terminals

Having determined that GABA<sub>A</sub>Rs located on parallel fibers are responsible for the muscimol-induced activation of their synapses, we next investigated the localization of GABA<sub>A</sub>Rs on these fibers. Using electron microscopy of immunolabeled  $\alpha_1$  subunits, we imaged the molecular layer and found that 27% of all asymmetrical synapses had  $\alpha_1$  subunits associated with the plasma membrane at either or both the presynaptic (Fig. 6*A1–A4*) and postsynaptic (Fig. 6*B1,B2*) sides of the synapse. These asymmetric synapses were considered to be originating from parallel fibers because parallel fibers form  $>99\%$  of the excitatory, asymmetric synapses in the molecular layer of the cerebellum (Palay and Chan-Palay, 1974; Silver et al., 1998; Foster et al., 2002). The immunogold particles were primarily associated with the active zone, although occasional membrane labeling occurred away from the synapse (Fig. 6*B1,B2*, crossed arrows). A particle density profile performed orthogonally with respect to the active zone indicated that most labeling occurred within  $25 \text{ nm}$  of the center of the synaptic cleft (Fig. 6*C*). This distribution is consistent with a direct association of the antibody to the presynaptic membrane in 74% of the cases and to the postsynaptic membrane in 26% of the cases. Although the presence of postsynaptic GABA<sub>A</sub>Rs at glutamatergic synapses in the cerebellum is consistent with a previous study (Nusser et al., 1998), their presence on the presynaptic membrane is a novel finding. A lateral distribution analysis further determined that gold particles were found more often within the synapse than in the extrasynaptic membrane (59 vs 33 particles, respectively, in the presynaptic membrane and 26 vs 17 particles, respectively, in the postsynaptic membrane) (Fig.

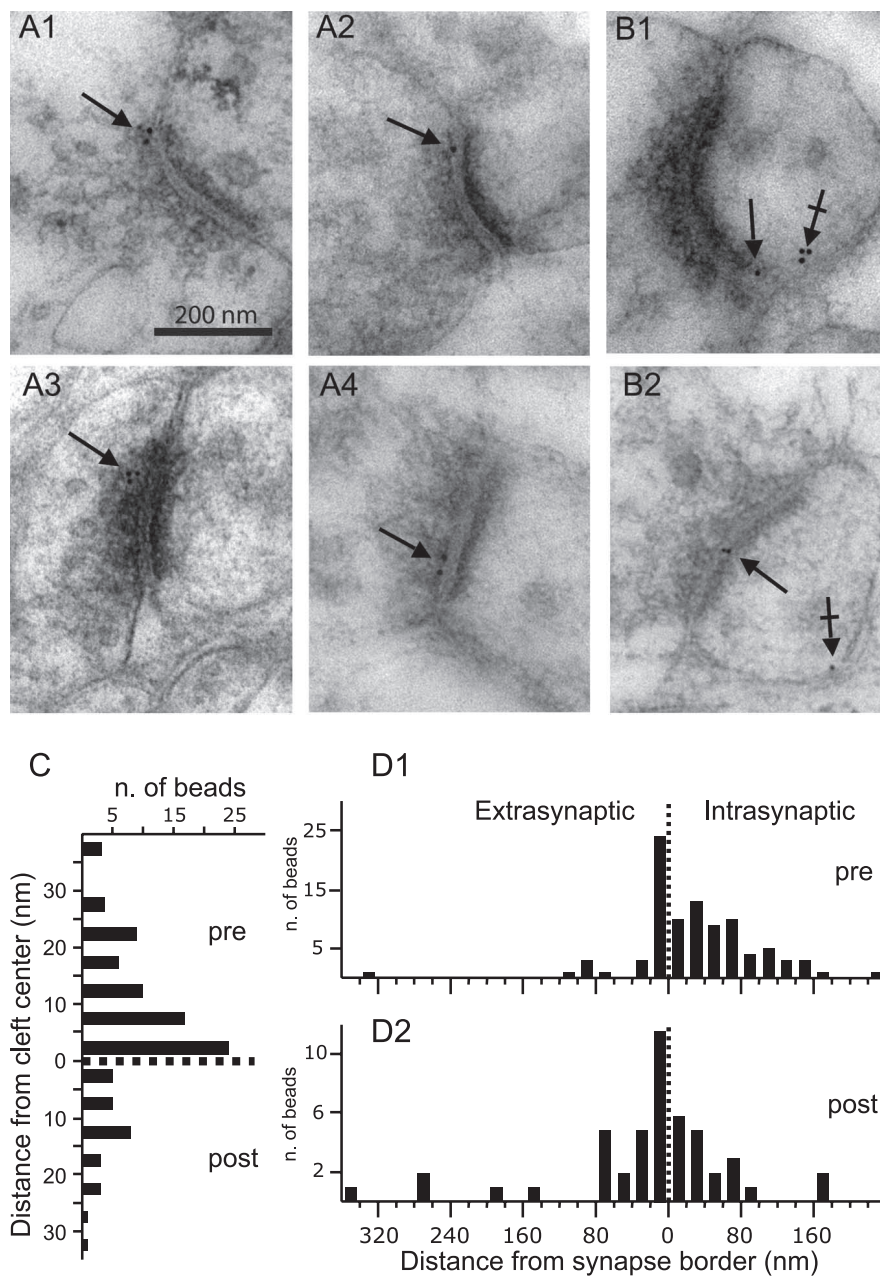
6D1,D2). Both for presynaptic and postsynaptic labeling, it is striking that the majority of particles were close to the edge of the synaptic densities. One possibility for this finding is that lateral diffusion of receptors on the cell membrane (Triller and Choquet, 2005) may be slower at the presynaptic terminals and at the postsynaptic density than in the extrasynaptic membrane, and this could underlie the enrichment of  $\alpha 1$  subunits in these areas. Incomplete preservation of morphology after preparation of the tissue for EM precluded reliable identification of parallel fiber axons, but it is certainly possible that receptors exist on the axon as well. Altogether, these results indicate that  $\alpha 1$  subunits are targeted to the presynaptic side of parallel fiber synapses, and they support the hypothesis that functional GABA<sub>A</sub>Rs underlie the muscimol-induced depolarization of these synapses.

#### GABA<sub>A</sub>R activation increases parallel fiber transmission

If GABA<sub>A</sub>Rs cause depolarization of the presynaptic terminal, then it would be predicted that transmission at parallel fiber synapses should be increased when the receptors are activated (Awatramani et al., 2005). To test whether transmission is affected, we evoked EPSCs in Purkinje cells by stimulating parallel fibers and compared EPSCs evoked in control conditions with EPSCs evoked in the presence of muscimol. To achieve this, EPSCs were evoked in coronal slices under control conditions by placing an extracellular stimulating electrode in the molecular layer  $\sim 300 \mu\text{m}$  from the patch-clamped Purkinje cell. A puffer pipette was placed in the parallel fibers approximately half way between the stimulation electrode and the patch-clamped Purkinje cell, and  $10 \mu\text{M}$  muscimol was puffed after baseline EPSCs were recorded for 30 s. Figure 7A shows that the amplitudes of the resulting EPSCs recorded in Purkinje cells were increased during application of muscimol and returned to control amplitudes after muscimol was washed away. Muscimol increased evoked EPSC amplitudes by  $42.1 \pm 6.6\%$  (Fig. 7A,B), and the effect was completely blocked by  $5 \mu\text{M}$  gabazine (data not shown). These results indicate that muscimol application increased the amplitude of parallel fiber EPSCs by activating presynaptic GABA<sub>A</sub>Rs.

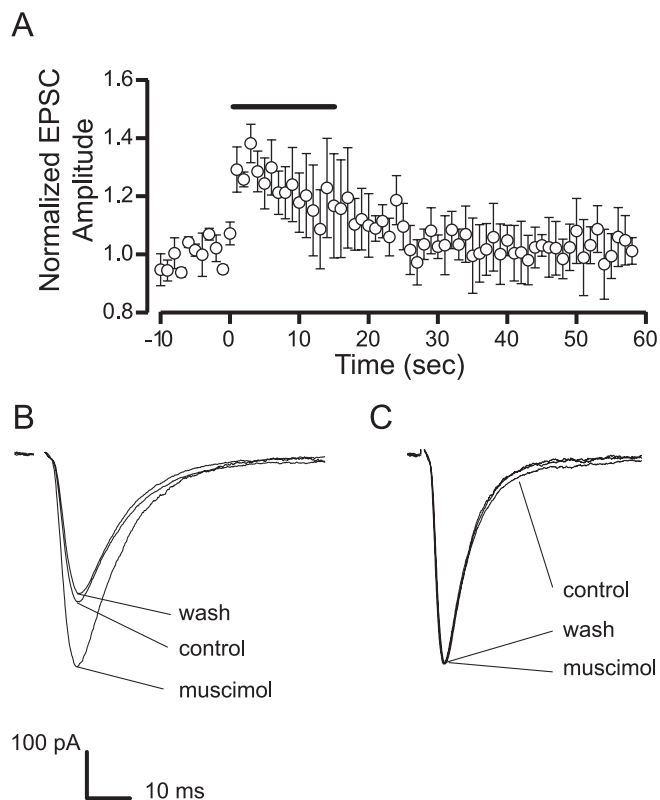
#### Presynaptic receptors activated by synaptically released GABA potentiate parallel fiber transmission

The experiments above demonstrate that activation of the presynaptic receptors with exogenously applied GABA agonists potentiate parallel fiber transmission, but the concentrations and



**Figure 6.** Localization of  $\alpha 1$  GABA<sub>A</sub>R subunit. **A1–A4**, Examples of gold particles (arrows) associated with the presynaptic element at the border of the presynaptic active zone (**A1, A2**) or within the presynaptic active zone (**A3, A4**). **B1, B2**, Examples of gold particles associated with the postsynaptic differentiation (arrows) or with the extrasynaptic membrane (crossed arrows). **C**, Histogram of the distances of particles (100 particles from 82 boutons) from the cleft center (0, dotted line). Note that immunolabeling predominates on the presynaptic side. **D1, D2**, Histograms of the distances from the synaptic border of the gold particles observed in the presynaptic (**D1**) and the postsynaptic element (**D2**). Distances were measured from the nearest border of the synapse (0, dotted line). The left portion of the graph indicates the distance outside of the synapse (extrasynaptic), and the portion on the right indicates distance within the synapse (intrasynaptic).

time course we used for the application of the agonist in those experiments are obviously different from those that the receptors would encounter in normal cerebellar function. We therefore wanted to determine whether activation of the receptors by GABA released from neurons has the same effect on parallel fiber transmission. Previously, Dittman and Regehr (1997) demonstrated that GABA<sub>B</sub>Rs on parallel fibers can be activated by GABA released from nearby interneurons. By recording evoked EPSCs in Purkinje cells using a stimulation paradigm identical to the one used in their study but using a GABA<sub>B</sub>R antagonist (see Materials

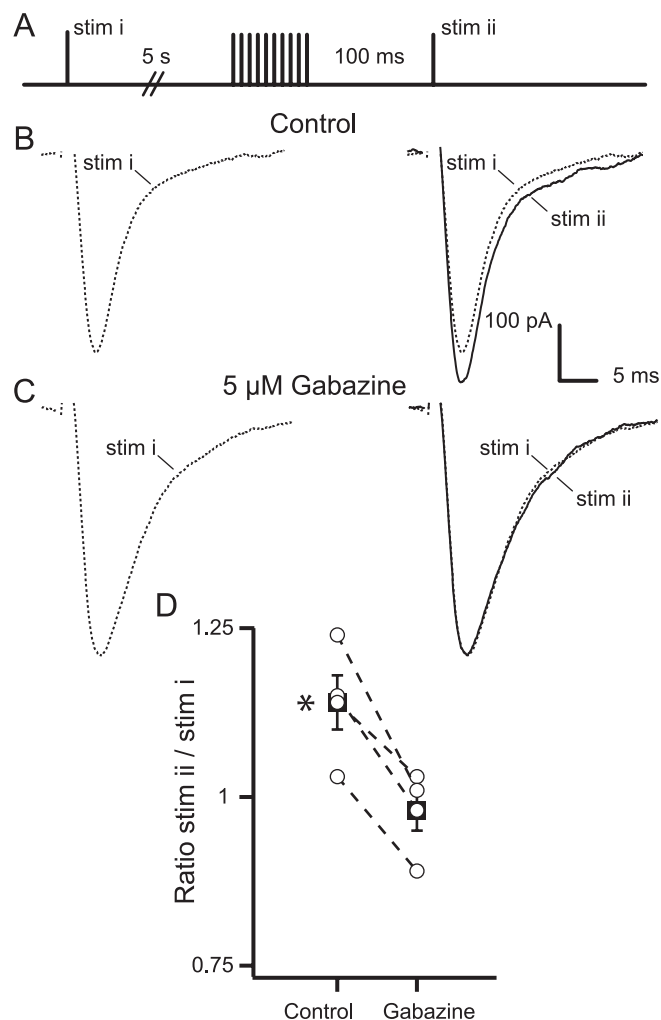


**Figure 7.** GABA<sub>A</sub>R activation increases the amplitude of EPSCs evoked in Purkinje cells. **A**, Time course of evoked EPSC amplitudes normalized to the average amplitude recorded in each cell during the 30 s before the application of 10  $\mu$ M muscimol (black bar). EPSCs were recorded in Purkinje cells and were evoked by giving an extracellular stimulus to the parallel fibers every second. **B**, Representative traces of average EPSCs evoked in control conditions, in the presence of muscimol, and after muscimol was washed from the bath. **C**, Traces from **B** normalized to show no change in the voltage clamp during or after muscimol application.

and Methods) (Fig. 8A), we show that the GABA released from interneuron activity also activates GABA<sub>A</sub>Rs. In Figure 8B, we show that EPSCs evoked 100 ms after a train of 10 stimuli at 100 Hz is given to the parallel fibers have amplitudes that are  $14 \pm 4\%$  greater than EPSCs that are evoked before the train ( $n = 4$ ;  $p < 0.05$ , paired Student's *t* test) (Fig. 8D). However, washing 5  $\mu$ M gabazine onto the slice abolished the difference in amplitudes of the events evoked before and after the train (Fig. 8C,D). This effect is attributable to the sequential activation by the stimulus train of parallel fibers and their postsynaptic MLIs. The GABA released by the MLIs activates the GABA<sub>A</sub>Rs on the presynaptic parallel fibers and facilitates the EPSC recorded postsynaptically. However, when the presynaptic receptors are blocked by 5  $\mu$ M gabazine, the GABA released by the interneurons has no effect and the postsynaptically recorded EPSC is unchanged by the stimulus train. These experiments demonstrate that activation of presynaptic GABA<sub>A</sub>Rs by GABA released from nearby MLIs induces a facilitation of parallel fiber transmission similar to that recorded when receptors are activated with exogenously applied agonists.

## Discussion

Our results show that activation of GABA<sub>A</sub>Rs in the cerebellar molecular layer causes transmitter to be released from glutamatergic synapses onto Purkinje cells and MLIs. Several lines of evidence indicate that the GABA<sub>A</sub>Rs responsible for activating these synapses are located on parallel fibers. (1) Activation of



**Figure 8.** Interneuron activity increases the amplitude of EPSCs evoked in Purkinje cells. **A**, Illustration of a stimulus paradigm in which two individual sets of parallel fibers were stimulated using two separate stimulation electrodes to separate the fibers that were activated by a train of 10 stimuli at 100 Hz (see Materials and Methods). stim i was timed to produce an EPSC in the postsynaptic Purkinje cell 5 s before the train of stimuli given to nearby parallel fibers, and then stim ii was delivered 100 ms after the termination of the train. The entire sequence was repeated every 30 s for 10 min before 5  $\mu$ M gabazine was washed onto the slice. **B**, Sample traces of averaged EPSCs from the control period before gabazine was perfused. The average EPSC produced from stim ii (right, solid line) has an increased amplitude compared with the average EPSC produced by stim i (left and right, dotted line). **C**, Sample traces of the average EPSCs recorded after gabazine had been perfused for 5 min onto the same cell as in **B**. The average EPSC produced from stim ii (right, solid line) is now identical to the average EPSC produced by stim i (dotted line, left and right). **D**, Group data from four cells showing that perfusion of gabazine onto the slice systematically abolishes the increase in EPSC amplitude caused by the train of stimuli given to nearby parallel fibers. Note the absence of any facilitation in gabazine indicating that the parallel fibers stimulated with the train are indeed distinct from the parallel fibers stimulated by the two test stimuli. All recordings were made in the presence of a blocker of GABA<sub>B</sub>Rs (2  $\mu$ M CGP 55845).

GABA<sub>A</sub>Rs induces EPSCs in both Purkinje cells and MLIs, which are both innervated by parallel fibers. (2) In MLIs, the size and time course of the EPSCs that are induced by GABA<sub>A</sub>R activation are the same as those occurring at rest [these events are classically considered to reflect random quantal release from parallel fibers (Carter and Regehr, 2002)]. (3) That the EPSCs arise from climbing fiber terminals seems highly unlikely because of the rapid kinetics of the GABA-evoked EPSCs we recorded in MLIs (Jornell and Ekerot, 2003; Szapiro and Barbour, 2007), and the features of the response in Purkinje cells (a flurry of fast, quantal



events) is incompatible with previous reports of EPSCs originating from climbing fibers. (4) Our EM micrographs show  $\alpha_1$ -containing GABA<sub>A</sub>Rs on parallel fiber terminals. (5) We demonstrate that the GABA<sub>A</sub>Rs responsible for the effect are only located in the molecular layer (activation of GABA<sub>A</sub>Rs outside of the molecular layer does not produce the response) (Fig. 4). (6) A secondary mechanism involving K<sup>+</sup> released from other cells in the molecular layer (via a GABA-mediated depolarizing nonsynaptic potential) (Voipio and Kaila, 2000) can be ruled out because circumventing activation of GABA<sub>A</sub>Rs and directly applying K<sup>+</sup> to the parallel fibers does not reproduce the response. Together, these points indicate that GABA<sub>A</sub>Rs are present on the terminals of parallel fibers and that opening the receptors activates parallel fiber synapses.

Our results also give several important clues about the presynaptic mechanism by which agonist application to the receptors activates parallel fiber synapses. One step in the process involves voltage-gated Na<sup>+</sup> channels, as is evidenced by the inability to record GABA-induced EPSCs in the presence of TTX. This indicates that Na<sup>+</sup> channel activation is a necessary component of the mechanism, but, when we made cell-attached recordings from granule cell bodies, we did not record action potentials resulting from application of GABA<sub>A</sub>R agonists to the molecular layer (data not shown). One possible explanation for this result is that GABA<sub>A</sub>Rs are only expressed on a subset of parallel fibers and that we were unable to locate the particular subset of cell bodies that correspond to those fibers. This is, however, unlikely because many such trials have been made in both coronal and sagittal slice preparations. Another more likely explanation is that, if action potentials are generated in the parallel fiber axons or terminals, they do not propagate back to the cell body. The lack of action potential propagation in the presence of GABA<sub>A</sub>R agonists could be attributable to a shunting effect of GABA<sub>A</sub>R-induced currents that prevents the current carried by a local action potential to passively depolarize the axonal membrane at the next potential site of propagation (Segev, 1990).

More insight into the mechanism comes from the observation that EPSCs recorded postsynaptically usually occur in short bursts lasting several hundreds of milliseconds, with between two to five EPSCs in each burst. These bursts can be explained by either coordinated release from several presynaptic parallel fibers or rapid repetitive firing of a single fiber. It is unlikely that the precision of EPSC coordination within bursts results from several fibers firing at almost the same time because the bursts occur randomly during the first few seconds of agonist application.

The mechanism of GABA-induced activation of parallel fibers that begins to emerge from the results is the following: activation of GABA<sub>A</sub>Rs on parallel fibers depolarizes the membrane, either because  $E_{\text{GABA}}$  is more depolarized than the membrane potential or because  $E_{\text{GABA}}$  is hyperpolarized, but continued Cl<sup>-</sup> accumulation collapses the Cl<sup>-</sup> gradient and induces a biphasic GABA response (Staley et al., 1995). We favor the explanation of depolarized  $E_{\text{GABA}}$  because the depolarization from exogenously applied agonists occurs almost instantaneously, because synaptically released GABA also causes the depolarization (Fig. 8B), and because recent studies have shown higher Cl<sup>-</sup> concentrations in axons than in somata (Price and Trussell, 2006; Szabadics et al., 2006). Our results recorded in the absence of TTX indicate that the depolarization is sufficient to cross the threshold for voltage-dependent Na<sup>+</sup> channels, but our experiments in the presence of TTX indicate that they do not depolarize beyond the threshold of synaptic Ca<sup>2+</sup> channels. This is not unexpected because Ca<sup>2+</sup> channels at parallel fiber to Purkinje cell synapses are composed

of various high-voltage-activated Ca<sup>2+</sup> channels (Mintz et al., 1995) and open at more depolarized values than Na<sup>+</sup> channels (Nowycky et al., 1985; Fox et al., 1987; Usowicz et al., 1992; Hille, 2001). Therefore, Na<sup>+</sup> channel activation is necessary for the activation of these Ca<sup>2+</sup> channels and is the likely explanation for why the GABA<sub>A</sub>-induced EPSCs are TTX sensitive.

A positive feedback system in which parallel fiber activation of interneurons results in increased excitability of parallel fibers could be a mechanism to ensure that information coming into the granule cells from mossy fibers is reliably relayed and processed in Purkinje cells. The mechanism described here predicts that bursts of activity in granule cells, as is recorded in response to sensory stimuli *in vivo* (Chadderton et al., 2004; Jorntell and Ekerot, 2006), will activate postsynaptic MLIs, which will increase the activation of presynaptic GABA<sub>A</sub>Rs and thereby the efficacy of the later portion of the burst. Therefore, the positive feedback is likely to be a mechanism to ensure that bursts of activity in granule cells is highly distinguishable from random spiking, and modulation of these presynaptic receptors could play a crucial role in affecting the salience of the bursts and cerebellar physiology.

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