SUPPLEMENTAL MATERIAL

to the paper

The spatial organization of long-term synaptic plasticity at the input stage of cerebellum

by

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Spatial correlation between electrode responses in MEA recordings

MEA recordings allow to correlate the amplitude and response times in different slice locations in the granular layer following mossy fiber activity.

Measuring N_1 delay between two different points yielded a mossy fiber conduction velocity of 2.05±0.43 m/s (12 slices and 236 electrodes), so that, even for the furthest electrodes, conduction times are negligible. In the same slices, N_{2a} amplitude was 265.6±17.6 μV at <700 μm and 245.5±18.3 μV at >700 μm (p=0.43, paired *t*-test). Thus, there is no loss of activity due to distance, suggesting the integrity of functional connections.

A detailed analysis is reported for a single slice in **Fig. SM1**. Neither N_{2a} amplitude nor delay were well correlated with distance from the stimulating electrode. There was instead a significant negative correlation between N_{2a} amplitude and delay. This reflects the fact that those granule cells, which, by chance, receive a stronger synaptic drive, anticipate spike generation (cf. Fig. 3 and 4 in main text and see D'Angelo et al., 1995; Nieus et al., 2006).

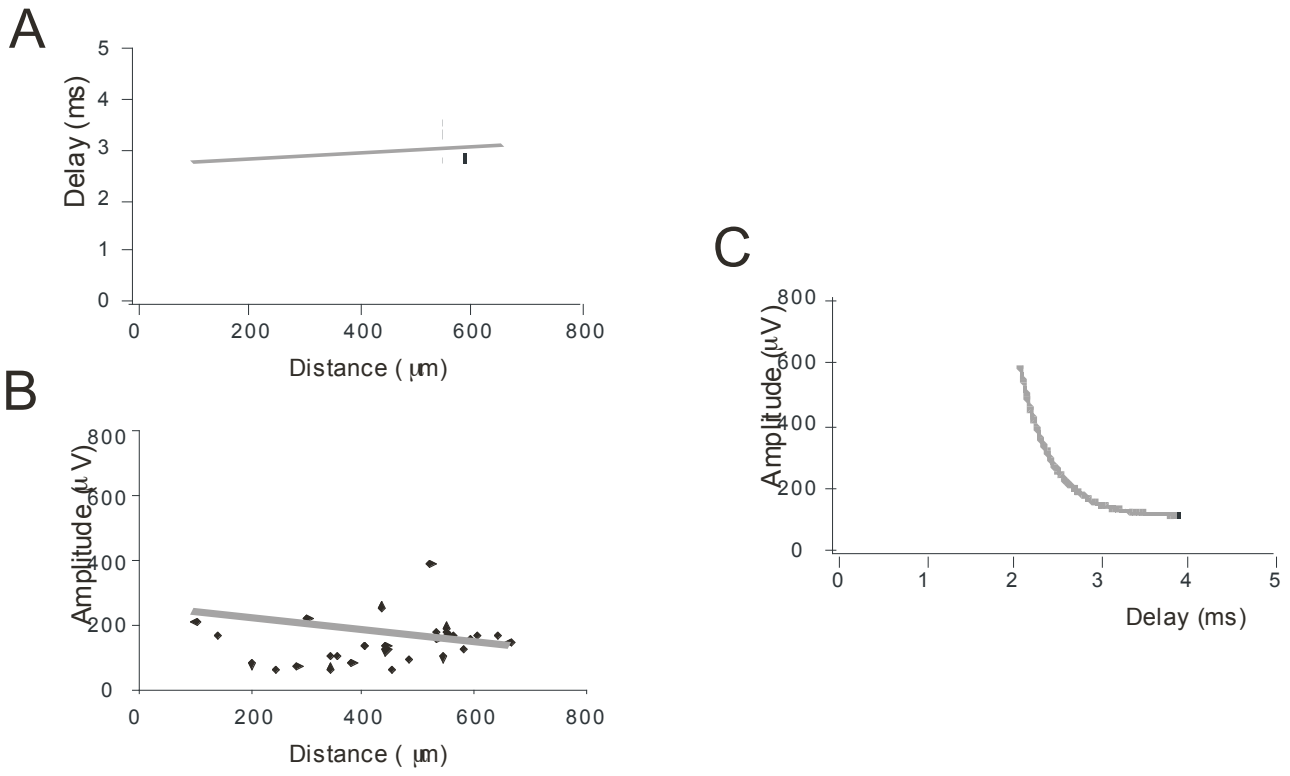

Fig. SM1. Correlation between amplitude, delay, and distance from the stimulating electrode. The example shows data taken from a single slice showing 30 active electrode in the granular layer. **(A)** The plot shows that N_{2a} peak delay is poorly correlated with distance from the stimulation site (linear fit, R^2=0.07). **(B)** The plot shows that N_{2a} peak amplitude is poorly correlated with distance from the stimulation site (linear fit, R^2=0.05). **(C)** The plot shows that N_{2a} peak amplitude is correlated with N_{2a} peak delay (exponential fit, R^2=0.62).

On the cellular mechanisms involved in field potential generation: synaptic inhibition and its control of granule cell discharge

We have shown in the main paper (Figs 1-4) that field potentials generated in the granular layer by mossy fiber stimulation consist of two postsynaptic waves, N_{2a} and N_{2b}, which are differentially affected by GABA and NMDA receptor activation. The nature of these waves, which was inferred from physiological and pharmacological experiments, suggests complex interactions between excitatory and inhibitory mechanism. A first question arises since there are two types of GABAergic inhibition, tonic and phasic (Brickley et al., 1996; Hamann et al., 2002). The fact that only N_{2b} was affected by GABA-A receptor blockage may be explained through a delayed circuit-driven Golgi cell activation corresponding to the phasic process early reported by Eccles et al. (1967). However, it is less intuitive to understand why tonic inhibition could affect N_{2b} but not N_{2a}. A second issue is raised by NMDA receptor activation, which is voltage-dependent and occurs with delay due to slow kinetics of the NMDA synaptic response (D'Angelo et al., 1995). So one may hypothesize that the NMDA current is controlled by inhibition occurring through the Golgi cell circuit.

To the aim of illustrating the principles governing the interaction of synaptic excitation and inhibition in granule cells, we have used detailed simulations of granule cell membrane potential dynamics. With the model it is possible to isolate the action of phasic and tonic inhibition, both of which use a common furosemide-sensitive mechanism based on α6 GABA-A receptor subunits precluding their pharmacological dissection in physiological experiments. Moreover, the model allows to evidence the dependence of NMDA receptor activation from the inhibitory mechanisms. Finally, since granule cell depolarization depends both on the number of mossy fibers and on resting membrane potential, a precise interpretation of the events requires a combination of techniques. With LCA we preserve the resting membrane potential, similar as in MEA recordings, but cannot assess the number of active mossy fibers. The opposite occurs in WCR. The model allows to combine this information and to predict how the system reacts for certain combinations of resting membrane potential and mossy fiber activity.

Methods

For the simulations we have implemented the model developed by D'Angelo et al. (2001) and extended by Nieus et al. (2006) with the addition of inhibitory synapses. Inhibition was simulated as the sum of a tonic and a phasic Cl^--mediated conductances with reversal potential at –65 mV (-62.9 mV estimated with the gramicidin perforated patch by Brickley et al., 1996). *Tonic inhibition* was reproduced as a voltage-independent leakage accounting for 34% of the basal granule cell input resistance (Armano et al., 2000; this value may be even larger if older animals are considered, e.g. Brickley et al., 1996; Rossi et al., 2003). In order to simulate *phasic inhibition* we adopted a simplified C=O=D scheme activated with a GABA pulse (1 mM-1 ms). The maximum conductance [G_{max}=1250 pS] and kinetic constants [$k_{(C=O)on}$=4.2 $ms^{-1}mM^{-1}$, $k_{(C=O)off}$=0.033 ms^{-1}, $k_{(O=D)on}$=0.032 ms^{-1}, $k_{(O=D)off}$=0.07 ms^{-1}] were determined by fitting the model to IPSCs obtained using minimal-stimulation in whole-cell recordings (Mapelli L., Rossi P. and D'Angelo E., unpublished results). It should be noted that, although biophysically accurate schemes (see for example the 8-state kinetic scheme used to simulate quantal release in granule cells in culture; Barberis et al., 2005) and the contribution of spillover (Hamann et al., 2002) could also be modeled, the present 3-state scheme coupled to a GABA pulse was sufficient to generate the synaptic conductance change occurring in response to single mossy fiber impulses.

Summary of the mechanisms of granule cell synaptic activation

The granule cell relay of cerebellum is among the simplest and most investigated in the whole brain. There are 4 mossy fibers on average per granule cell (Jakab and Hamory, 1988; Harvey and Napper, 1991), and more than 1 is needed to activate an action potential from rest

(D'Angelo et al., 1995; Chadderton et al., 2004). Moreover, there are 3-4 inhibitory synapse per granule cell (Jakab and Hamory, 1988; Harvey and Napper, 1991).

The AMPA and NMDA receptor-mediated currents have complex effect on the time course of synaptic responses. The AMPA current generates the fast depolarization determining most of the EPSP peak (e.g. Chatala et al., 2003). This, however, also receives a non-negligible contribution from the NMDA current (D'Angelo et al., 1995). The AMPA current, in addition to the fast component, also has a slow component determined by glutamate spillover in the glomerulus (Mitchell and Silver, 2003). This knowledge is essential to interpret the pharmacology of N_{2a} and N_{2b} in Fig.4. On the one hand we observe that the effect of the NMDA component is much stronger on N_{2b} that N_{2a}. The fact that NMDA receptors marginally contribute to the first component simply reflects the marginal (but not negligible) activation of NMDA receptors at this time (e.g. see also Fig.2 in Maffei et al. 2002). On the other hand, the AMPA current can extend its action therefore contributing to doublet generation.

The model used here was previously shown to account for these multiple mechanisms (Nieus et al., 2006). In particular, the model is able to generate EPSPs, single spikes or spike doublets using the appropriate number of mossy fibers starting from a resting membrane potential of -70 mV (**Fig. SM2**). Differences of ±5 mV around this value (e.g. D'Angelo et al., 1995; Brickley et al., 1996; Chadderton et al., 2004) simply changed the ability of the model to generate spikes and doublets with a given number of active mossy fibers (data not shown). This simulation is therefore consistent with the observations obtained in loose cell-attached and field recordings reported in the main text (cf. Fig. 3).

Both tonic and phasic inhibition affect generation of the second but not first spike

The model can be used to illustrate how tonic and phasic inhibition contribute to regulate granule cell excitation and doublet generation.

We will first consider the effect of *tonic inhibition* on the response to a mossy fiber impulse (**Fig. SM2A**). Sub-threshold EPSPs were just slightly (<5%) increased by blocking tonic inhibition. In supra-threshold responses, generation of the first spike was scantly affected, whereas doublet generation was markedly enhanced.

Phasic inhibition was elicited by activating Golgi cell – granule cell synapses with a delay of 4 ms (**Fig. SM2B**), accounting for the time needed for Golgi cell activation and subsequent Golgi – granule cell neurotransmission (Armano et al., 2000). Due to this delay, phasic inhibition did not affect the first spike, but remarkably depressed generation of the second spike. Increasing the number of active inhibitory synapses strengthened the effect.

Given this picture, and also assuming varying initial membrane potential in the physiological range (data not shown), blocking tonic and phasic inhibition does not substantially affect generation of the first spike but increases the percentage of secondary spikes. This simulation is therefore consistent with the observations obtained in loose cell-attached and field recordings reported in the main text (cf. Fig. 4).

Tonic inhibition acts by controlling NMDA channel unblock and the late phase of EPSPs

Tonic inhibition, with a reversal close to the resting potential, acts as a shunt increasing membrane conductance and reducing membrane time constant (Brickley et al., 1996; Hamann et al., 2002; Cavelier et al., 2004; Farrant and Nusser, 2006). Interestingly, as predicted by this simulation, furosemide application markedly enhances the generation of doublets sin slice in slice experiments (Hamann et al., 2002). The mechanism of this action is examined here in some detail.

In the granule cell model, injection of a brief current pulse (2 ms, 25 pA) causes a steep subthreshold membrane charging followed by a nearly-exponential relaxation. In the presence of tonic inhibition, membrane charging is unchanged, while decay accelerates. Similar to a current pulse, the AMPA current (Cathala et al., 2003) rapidly charges the membrane determining the EPSP rising phase (**Fig. SM3A**) followed by a nearly-exponential relaxation. However, the EPSPs peak

region and decay phase are extended by protracted inward currents generated by AMPA and NMDA receptors (Nieus et al., 2006). When tonic inhibition is activated, the EPSP rising phase is unchanged, while the decay phase accelerates and the peak region narrows. Thus, tonic inhibition acts by controlling the late phase of EPSP generation.

The intervention of the NMDA current in controlling EPSP time course is shown in **Fig. SM3B**. Blocking the NMDA current slightly reduces EPSP peak and strongly depresses the late EPSP phase (Fig. SM2B), which conforms to the time course imposed by the membrane time constant (D'Angelo et al., 1995). Tonic inhibition, by accelerating EPSP decay, interrupts NMDA channel unblock. Thus, the action of tonic inhibition on the EPSP is amplified by regulation of the NMDA current.

These simulations support the notion that, during activation of a single EPSP, the effect of tonic inhibition mainly depends on shortening of membrane time constant and block of the NMDA current. It should be noted that tonic inhibition could also affect steady-state firing and synaptic gain in response to a long series of stimuli (Brickley et al., 1996; Hamann et al., 2002; Mitchell and Silver, 2003).

Conclusions

The present simulations show that the interpretation given to granular layer field responses conforms to current knowledge on synaptic and cellular physiology. To summarize:

1) Generation of the second spike in a doublet occurs within a physiological range of parameters and can be selectively controlled by GABAergic inhibition. Conversely, inhibition does not affect the 1st spike remarkably. Tonic and phasic inhibition have a synergistic effect.

2) Generation of the 2nd spike exploits voltage-dependent NMDA channel unblock, which is depressed by inhibition. NMDA receptors also marginally contribute to EPSP peak amplitude and therefore to generation of the 1st spike.

Beside the mechanistic implications for the interpretation of MEA recordings, these simulations support the view that the the inhibitory circuit can regulate NMDA channel unblock and doublet generation at the mossy fiber - granule cell relay. The importance of this mechanism for generation of plasticity at the granular layer input has been highlighted in the main text. Doublets may also be important at the granular layer output, determining the subsequent induction of parallel fiber – Purkinje cell LTD Casado et al., 2003).

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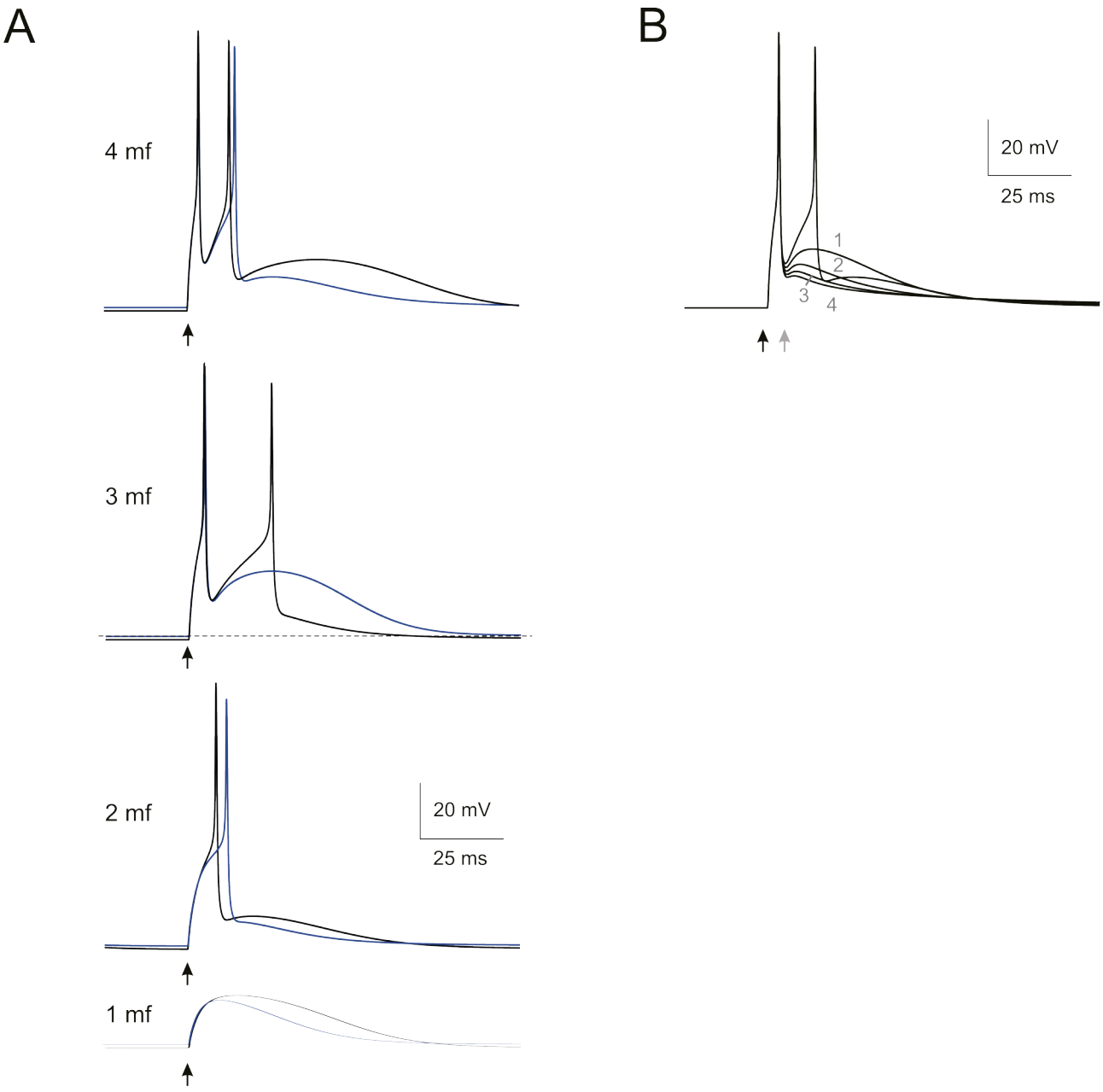

Fig. SM2. ***The effect of inhibition on EPSP and spike generation.*** (A) *Tonic inhibition*. In this simulation, 1-to-4 mossy fibers activate the granule cell model either with tonic inhibition off (black trace) or on (blue traces) from the membrane potential of –70 mV. With 4 active mossy fibers, the EPSP generates a doublet whatever tonic inhibition is on or off. With 3 active mossy fibers, the EPSP generates a doublet but, when tonic inhibition is on, the second spike disappears. With 2 active mossy fibers, there is just one spike in control, whose delay is increased by tonic inhibition. With 1 active mossy fiber, the control EPSP shows an extended growth and protracted decay phase: when tonic inhibition is on, the EPSP terminates earlier and its decay is faster. The black arrows indicate mossy fiber stimulation. (B) *Phasic inhibition*. In this simulation, 4 mossy fibers are activated in the presence of tonic inhibition. Subsequent activation of 1-to-4 Golgi cell – granule cell synapses determines a marked inhibition of the second spike. The Golgi cell – granule cell synapses (gray arrow) are activated 4 ms after the mossy fiber – granule cell synapses (black arrow).

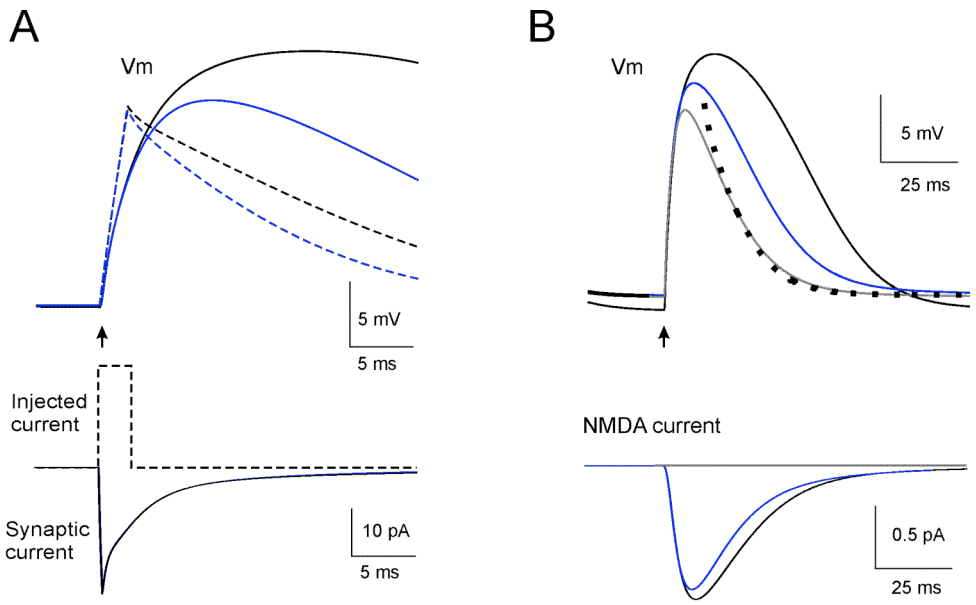

Fig. SM3. ***The action mechanisms of tonic inhibition on EPSP generation***. (A) The response to passive current injection (dotted traces) or synaptic conductance changes (continuous traces) is shown with tonic inhibition off (black traces) or on (blue traces). The corresponding currents are shown at the bottom. When tonic inhibition is on, the response to current injection shows similar amplitude and raising phase but faster decay. (B) The EPSP is shown in control (black trace), after activating tonic inhibition (blue trace), and after blocking the NMDA current (gray trace). The corresponding NMDA current is shown at the bottom (same color notation). Tonic inhibition accelerates EPSP decay, and the effect is accentuated by NMDA current blockage. When both tonic inhibition and NMDA currents are blocked, the EPSP decays conforms to passive membrane decay (dots, replotted from A).