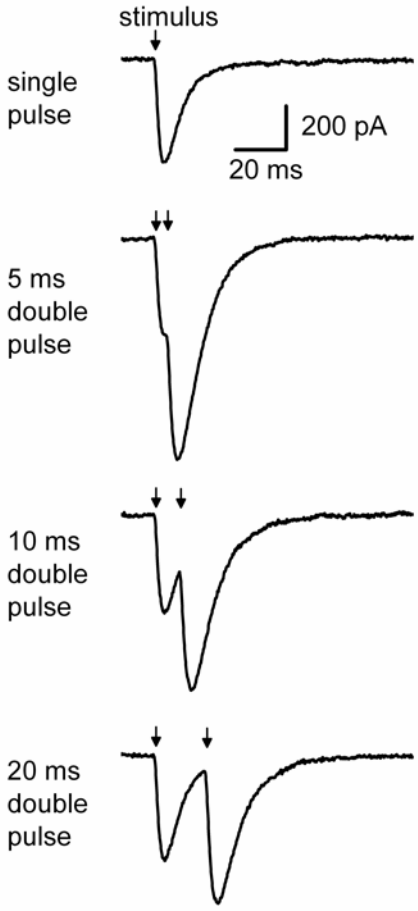
Supplemental Material 1

Glutamate receptor desensitization does not affect the spatiotemporal estimates of glutamate photolysis

The mapping experiments reported require discrete activation of different patches of granule cells. We directly determined the spatial and temporal profile of photoreleased glutamate by using the dendrites of Purkinje cells as sensitive glutamate detectors. EPSCs were recorded in voltage-clamped Purkinje cells in response to glutamate photolysis at various lateral distances from their dendrites and the peak amplitude and kinetics examined (Figure 1B main text). Whereas all EPSCs had comparable kinetics, their amplitudes declined to half maximal with a lateral distance of ≈20 μm in the XY plane. These experiments not only provide an empirical measurement of the XY photolysis point spread function, but also suggest that the diffusion of glutamate from the photolysis spot in the XY plane is negligible. However, interpretation of these data in the context of glutamate diffusion requires that under our experimental conditions the kinetics of the photolysis-evoked responses is not dominated by receptor desensitization.

We thus examined whether under our conditions glutamate receptor desensitization prevented detection of glutamate diffusion by photo-releasing glutamate in rapid succession at the same spot. If desensitization dominated the amplitude and kinetics of the first response, then the amplitude and kinetics of the second pulse should have been markedly different. Currents were recorded in whole-cell voltage-clamped Purkinje cells in response to two,1 ms pulses of UV light (40 μm spot as in main text). The photolysis spot was centered on the

dendritic tree. As can be noted below in sample traces shown, the kinetics and amplitudes of EPSCs resulting from the first and second pulses of glutamate were comparable even with delays as brief as 5 ms, demonstrating that the desensitization of dendritic glutamate receptors is minimal under our experimental conditions. Thus, glutamate receptor desensitization does not affect our estimates and the photolysis XY point spread function empirically determined using the dendrites of Purkinje cells as glutamate detectors provides an accurate description of the spatiotemporal profile of glutamate in the granule cell layer during the mapping experiments.



Supplemental Material 2

The strength of powerful ascending inputs is not underestimated under our experimental conditions because of deviations in the angle of the dendrites of Purkinje cells

If the dendrites of Purkinje cells were significantly angled in the mapping experiments, granule cells making ascending inputs would be located off-center and, as a consequence of being averaged with parallel fibers, the strength of the powerful ascending inputs may have been underestimated. Two potential scenarios with different implications are plausible:

1) The plane of Purkinje cell dendrites is angled and not perfectly perpendicular to the granule cell layer (as depicted in Figs 2 and 3 of the main text), or

2) The plane of the dendrites is not perfectly perpendicular with respect to the surface of the slice.

We considered both possibilities and as detailed below find that under our experimental conditions neither affects the interpretation of the data or the conclusions made.

1) The plane of Purkinje cell dendrites may be angled and not perfectly perpendicular to the granule cell layer

If the dendrites of Purkinje cells are off center then patches of granule cells making ascending inputs may not be arranged in vertical columns as assumed in our photolysis maps (vertical columns were averaged to obtain patch

efficacy). If this were the case, and ascending inputs are much more powerful as suggested, within each row there should be a large difference in the efficacy of the patch containing ascending inputs and its adjoining parallel fiber patch. Moreover, the maximum peak efficacy would systematically shift laterally to the soma of the target Purkinje cell. This shift would be proportional to the extent of deviation of the Purkinje cell dendrite from 90 degrees with respect to the Purkinje cell layer and would be larger for rows located further distances from the Purkinje cell layer. Figure 3B of the main text shows a row by row analysis of the strength of patches of granule cells. As can be seen, in each row the most powerful (potential ascending) patch is no more than 1.4-fold as effective as its adjoining patches (as compared to an expected 3.2-8.5-fold discussed in the main text). Therefore, the collapsed columns are not averaging out or hiding any large differences in the strength of ascending and their adjoining patches and are thus a reasonable representation of the data.

As an additional analysis of this concern, taking into account all of the information related to orientation of the folium and/or filled dendrites for each individual experiment, two investigators independently determined the location of the “ascending” patch located in the row closest to the Purkinje cell layer. This row is least affected by any deviations in the angle of the Purkinje cell dendrite and thus is the most sensitive indicator of any difference in the strength of ascending and parallel fiber patches. As was the case for the previous analysis, the ascending patch was found to be no more than 1.2-fold stronger than its

adjoining patches. This analysis was done for all experimental conditions and is included in Figures 1, 3, and 4 of the main text.

It should be noted that photoreleasing glutamate in a vertical column (z-axis) and thus activating a cylinder of granule cells cannot in any way alter the estimates reported above. This is because if one considers the geometry of the cerebellar cortex, the column of photolysis would activate granule cells of comparable nature (ascending vs. PF).

2) The plane of the dendrites may not be perfectly perpendicular with respect to the surface of the slice.

In principle a complication can arise if the plane of the dendrites is far from perpendicular with respect to the surface of the slice. This was not the case because whole-cell voltage-clamped Purkinje cells were filled with a fluorescent dye and the plane of their dendrites visually ascertained by focusing up and down. Using this approach, the collapsed dendritic width was never more than about 20 µm. Even if one assumes that the dendrites are two-dimensional an angle of 10 degrees will make the collapsed width appear to be 30 µm wide. If one further considers that the dendrites themselves have a finite width (even only assuming 5 µm), this collapsed width would be even larger than 30 µm. Because we never observed collapsed widths of more than 20 µm, we are confident that there was not a major deviation. On the basis of our observations we estimate the maximum deviation to have been less than 8 degrees. Considering such maximal angular deviation and the 100 µm photolysis half width in the z-axis

(Figure 1C main text), the impact on the efficacies of ascending and parallel fiber patches would be insignificant.

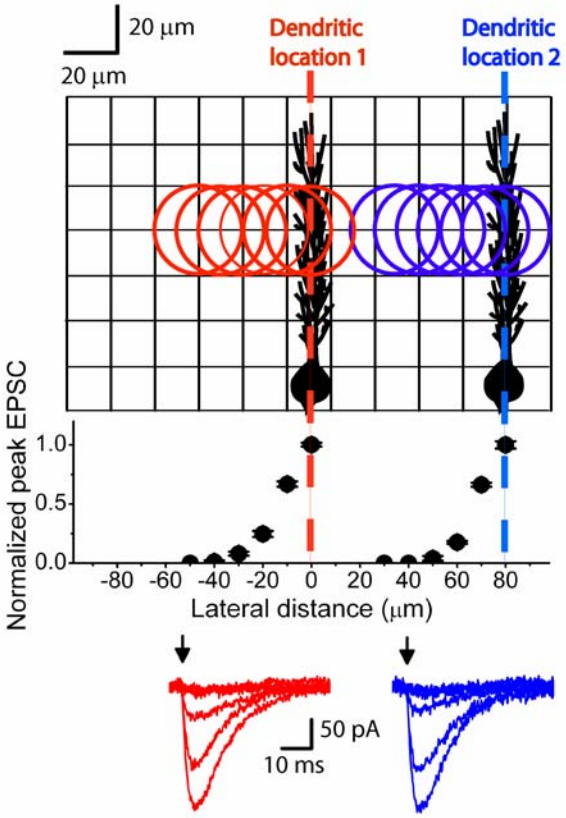
Collectively, therefore, it is extremely unlikely that the similarity in the strength of ascending and their adjoining parallel fiber patches reported here can be an artifact of our experimental conditions.

Supplemental Material 3

Equal volume of excitation at all photolysis locations

During the photolysis mapping experiments glutamate was photoreleased throughout the field of view. An assumption made was that at all locations within the mapped field of view on average the same number of granule cells were activated. However, lenses are not perfect and particularly at the edges could cause significant aberrations resulting in potential photolysis inhomogeneities. We designed our photolysis optical path to minimize such potential aberrations and empirically tested the system to ensure that the same amount of glutamate was photoreleased at all locations, especially those located peripherally.

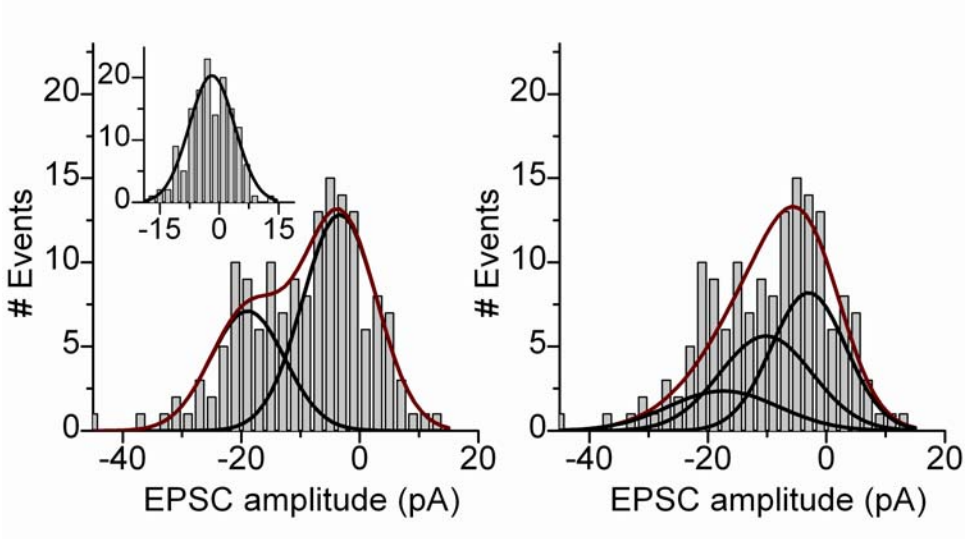
A whole-cell voltage-clamped Purkinje cell in a coronal cerebellar slice was initially positioned in the center of the field of view and currents were recorded in response to glutamate photolysis at various locations with respect to its dendritic tree (in the figure shown photolysis locations are indicated by red circles and currents traces are shown in red at the bottom). The cell was then re-positioned to a peripheral location within the field of view and currents were recorded in response to glutamate photolysis with the UV pulses of identical intensity and duration at the same relative locations with respect to its dendritic tree (blue circles and current traces). As typified by the samples shown, the amplitude and kinetics of the EPSCs recorded from the Purkinje cell were comparable at both the center and peripheral locations. These results confirm that with our photolysis optical design it was possible to photorelease the same amount of glutamate throughout the entire mapped region.


20 μm
20 μm
Dendritic location 1
Dendritic location 2
Normalized peak EPSC
1.0
0.5
0.0
-80 -60 -40 -20 0 20 40 60 80
Lateral distance (μm)
50 pA
10 ms

Supplemental Material 4

Fitting Gaussians to EPSC amplitude histograms

To determine the average amplitude of apparent unitary EPSCs mediated by ascending and parallel fiber inputs, Gaussian distributions were fit to peak EPSC amplitude histograms obtained from the repeated activation of granule cells (Walter and Khodakhah, 2006). The details of the fitting procedure and the constraints used are delineated in full in Methods section of the main text.



The histogram in the left panel shows data from one such experiment together with the fits obtained using the outlined constraints. It is noteworthy that mainly because of the inherent noise of the baseline (shown as an inset histogram) and the small size of the responses there was considerable overlap between the response and failure populations. We explored the possibility that the responses obtained could have been made of smaller events which were undetected because of the overlap of the populations. As an initial quantitative test of this possibility, for each experiment we used the same constraints outlined and increased the number of Gaussians by force-fitting the histogram such that the apparent unitary amplitude was halved. The R^2,

X^2/DoF, and the P value obtained from the X^2 test evaluating the goodness of the fit to the experimental data for both the original and halved apparent unitary amplitude fits were then compared. Within the context of this test, the relative standard commonly used in biological research for accepting a fit is a P value greater than 0.05.

For the experiment shown above, the amplitude histogram was originally fit with a sum of two Gaussians (one for failures and one for responses, left panel), resulting in an absolute peak to peak distance of 15.5 pA. The fit had an R^2 value of 0.91, X^2/DoF of 2.0, and a P value of 0.9 suggesting an excellent fit. When this distribution was force fit using the sum of three Gaussians (one for failures and 2 for responses, right panel), R^2 decreased to 0.86, X^2/DoF increased to 3.2, and the P value reduced to 0.2. The decrease in R^2, the corresponding increase in X^2/DoF, and the decrease in the P value when the histogram was force fit suggests that force fitting the histogram yielded poor fits, and that 15.5 pA, and not 7.7 pA, more accurately represents amplitude of the apparent unitary response.

For all 6 experiments in which granule cells making only parallel fiber inputs were activated, increasing the number of Gaussians fit to the amplitude histograms such that the apparent unitary amplitude was halved yielded poorer fits. On average, R^2 was reduced to 0.85±0.04 from 0.87±0.04, X^2/DoF was increased to 3.0±0.6 from 2.5±0.6, and the P values decreased to 0.13±0.11 from 0.31±0.1. As a separate test we increased the number of Gaussians to force the apparent unitary amplitudes to be on the order of 2-3 pA. This force fitting also decreased the goodness of fit; R^2 decreased to 0.81±0.06, X^2/DoF increased to 3.5±0.6, and the P value decreased to 0.01±0.02. On the basis of the analysis presented, the average amplitude of 12.3±2.3 pA obtained

from the original fits of the histograms (reported in the main text) represents the best estimate of the average absolute apparent unitary parallel fiber EPSC amplitude.

This same procedure also resulted in poorer fits for all 13 histograms obtained from experiments for ascending inputs. Increasing the number of Gaussians fit to the amplitude histograms such that the apparent unitary amplitude was halved decreased R^2 to 0.89±0.03 from 0.93±0.02, increased X^2/DoF to 4.4±1.0 from 3.9±0.9 and decreased the P value to 0.14±0.16 from 0.42±0.33. Again increasing the number of Gaussians used to fit the histograms by forcing apparent unitary amplitudes of 2-3 pA further decreased the goodness of fit; R^2 decreased to 0.85±0.05, X^2/DoF increased to 4.9±1.0, and the P value decreased to 0.03±0.07. Collectively, this analysis suggests that the average absolute amplitude of 9.6±1.2 pA obtained in the main text indeed represents the best estimate of the average absolute apparent unitary ascending input EPSC amplitude.

References

Walter,J.T. and Khodakhah,K. (2006). The linear computational algorithm of cerebellar Purkinje cells. J.Neurosci. *26*, 12861-12872.

Supplemental Material 5

Estimating the strength of granule cells inputs

The experimental data presented in the main text show that ascending and parallel fiber inputs have comparable apparent unitary EPSC amplitudes. However, whether these inputs have similar strengths depends on a number of additional factors: 1) the number of synapses that each input type makes with a single Purkinje cell; 2) the number of release sites per synapse; and 3) whether multivesicular release occurs, and if so, the extent to which it increases the EPSC amplitude. These factors are taken into consideration below.

Parallel fiber inputs

1) Number of synapses. Estimates of the number of synapses made with an individual Purkinje cell were obtained from evaluating the number of Gaussians fit to experimentally determined EPSC amplitude histograms. Given the release probability reported per release site at these synapses (Foster et al., 2005), if a granule cell makes more than one synaptic contact with a target Purkinje cell the non-failure population must be fit with more than one Gaussian. For parallel fibers, the majority of experimentally obtained amplitude histograms (4 of 6) were fit with a sum of two Gaussians function (one for responses and one for failures). As described in detail in Supplemental Material 4, force-fitting all amplitude histograms with increased numbers of Gaussians significantly reduced the goodness of fit in each case. Thus, based on the number of Gaussians used to fit the amplitude histograms, 4 of the 6 parallel fiber inputs examined contained a single synapse and 2 contained two synapses. Thus, on

average, the parallel fiber input makes ≈1.3 synapses, $\frac{(4\times1)+(2\times2)}{6}$. In principle, these are overestimates because a second population of responses could have been the result of the activation and release from synapses originating from multiple cells rather than a single cell.

2) Number of release sites per synapse. As stated above, input strength is also dependent on the number of release sites per synapse. The average number of release sites will be higher than the average number of parallel fiber synapses to a Purkinje cell if each individual synapse contains more than one release site. However, the majority of parallel fiber synapses onto Purkinje cells (90%) have only a single release site (Xu-Friedman et al., 2001).

3) Multivesicular release at each synapse. The input strength of a parallel fiber will also be affected by multivesicular release if it is a common phenomenon and if increasing the number of vesicles released significantly increases the ESPC amplitude. As discussed multivesicular release is not a major contributing factor. This is because even though multivesicular release occurs, the probability that two vesicles are released simultaneously from the same release site is quite low (≈0.1) under physiological calcium concentration and temperature (Foster et al., 2005). Moreover, because of receptor saturation, release of two vesicles instead of one does not double the EPSC amplitude, but only marginally (≈1.4 x) increases it (Foster et al., 2005).

Collectively, the above considerations suggest that the average parallel fiber input strength is simply obtained by multiplying the parallel fiber apparent unitary EPSC amplitude by the average number of synapses made by each parallel fiber with an individual Purkinje cell. Using the upper estimate of 1.3 synapses obtained from our

electrophysiological data, the average parallel fiber EPSC input strength is 16.0 pA (12.3 pA x 1.3 synapses).

Ascending inputs

1) Number of synapses. Similar to that considered for parallel fibers, for ascending inputs the majority of experimentally obtained amplitude histograms (10 of 13) were fit with a sum of two Gaussians function (one for responses and one for failures) and thus the average number of synapses made was ≈1.2, $\frac{(10\times1)+(3\times2)}{13}$.

2,3) Number of release sites per synapse and multivesicular release. As for parallel fiber inputs, to calculate the average ascending input EPSC strength, the number of release sites per ascending synapse and the extent of multivesicular release must be considered. As opposed to a parallel fiber synapse, which as discussed above contains a single release site, there are no estimates of the number of release sites at an ascending synapse. However, the area of the post-synaptic density of a synapse formed by an ascending or parallel fiber input is indistinguishable (Gundappa-Sulur et al., 1999) and therefore it is likely that ascending synapses also contain a single release site. Given that the areas of the post-synaptic densities are comparable, it also follows that the extent of receptor saturation is likely to be similar at the two synapses. Therefore, as observed at the parallel fiber synapse, the impact of multivesicular release under physiological conditions should be minimal. Taken together, the average ascending EPSC input strength is 11.5 pA (9.6 pA x 1.2 synapses), a value comparable to that obtained for the parallel fiber input.

References

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Gundappa-Sulur,G., De Schutter,E., and Bower,J.M. (1999). Ascending granule cell axon: an important component of cerebellar cortical circuitry. J.Comp Neurol. *408*, 580-596.

Xu-Friedman,M.A., Harris,K.M., and Regehr,W.G. (2001). Three-dimensional comparison of ultrastructural characteristics at depressing and facilitating synapses onto cerebellar Purkinje cells. J.Neurosci. *21*, 6666-6672.

Supplemental Material 6

Greater than 10 release sites per granule cell input results in a failure rate of less than 0.003

The histograms below are simulations using a binomial distribution representing the probability of releasing 0, 1, … vesicles from a granule cell input containing either 4 (top row) or 11 (bottom row) release sites. The graphs on the left show discrete probabilities, whereas those on the right show amplitude histograms with noise incorporated. The specific parameters used are delineated in the figure legend below and were based on the data reported by Sims and Hartell (Sims and Hartell, 2005). As can be noted, while a failure rate (0.1) comparable to that reported by Isope and Barbour is obtained when 4 release sites are simulated (Isope and Barbour, 2002), increasing the number of release sites to 11 to achieve a larger average EPSC amplitude (as suggested by Sims and Hartell, 2005) results in a failure rate less than 0.003. This failure rate is incompatible with the minimum of 0.1 reported for this input (Foster et al., 2005; Isope and Barbour, 2002).

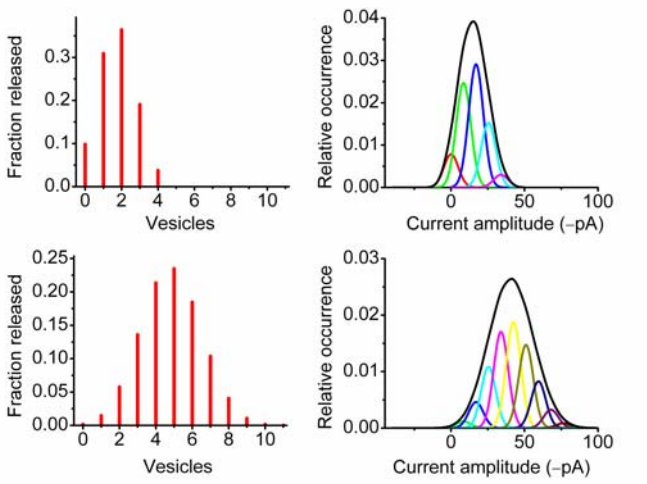

Figure legend

Simulations assumed a binomial distribution with a release probability per site of 0.44, a quantal amplitude of 8.5 pA, and 4 (top) or 11(bottom) release sites. Noise is incorporated in the histograms on the right (5 pA).

References

Foster,K.A., Crowley,J.J., and Regehr,W.G. (2005). The influence of multivesicular release and postsynaptic receptor saturation on transmission at granule cell to Purkinje cell synapses. J.Neurosci. *25*, 11655-11665.

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