Parasagittally Aligned, mGluR$_1$-Dependent Patches are Evoked at Long Latencies by Parallel Fiber Stimulation in the Mouse Cerebellar Cortex \textit{In Vivo}

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Abstract

The parallel fibers (PFs) in the cerebellar cortex extend several millimeters along a folium in the medio-lateral direction. The PFs are orthogonal to and cross several parasagittal zones defined by the olivocerebellar and corticonuclear pathways and the expression of molecular markers on Purkinje cells (PCs). The functions of these two organizations remain unclear, including whether the bands respond similarly or differentially to PF input. Using flavoprotein imaging in the anesthetized mouse in vivo, this study demonstrates that high frequency PF stimulation, which activates a beam-like response at short latency, also evokes patches of activation at long latencies. These patches consist of increased fluorescence along the beam at latencies of 20-25 s with peak activation at 35 s. The long-latency patches are completely blocked by the mGluR$_1$ antagonist, LY367385. Conversely, the AMPA and NMDA glutamate receptor antagonists, DNQX and APV, have little effect. Organized in parasagittal bands the long-latency patches align with zebrin II-positive PC stripes. Additional Ca$^{2+}$ imaging demonstrates that the patches reflect increases in intracellular Ca$^{2+}$. Both the PLC$\beta$ inhibitor, U73122, and ryanodine receptor inhibitor, ryanodine, completely block the long-latency patches, indicating that the patches are due to Ca$^{2+}$ release from intracellular stores. Robust, mGluR$_1$-dependent long-term potentiation (LTP) of the patches is induced using a high frequency PF stimulation conditioning paradigm that generates LTP of PF-PC synapses. Therefore, the parasagittal bands, as defined by the molecular compartmentalization of PCs, respond differentially to PF inputs via mGluR$_1$-mediated release of internal Ca$^{2+}$.

Keywords: Purkinje cell, metabotropic glutamate receptors, intracellular calcium release, flavoprotein imaging, parallel fibers, and parasagittal zones.
Introduction


A parasagittal compartmentalization is present on PCs at the molecular level, exemplified by the parasagittal bands of zebrin II/aldolase C (Brochu et al. 1990; Sillitoe and Hawkes 2002; Ahn et al. 1994). Numerous other molecules are found on PCs in either a zebrin II-positive or the complimentary zebrin II-negative banding pattern (Apps and Hawkes 2009; Hawkes and Herrup 1995). Climbing and mossy fiber afferents show spatial correspondence with these markers (Voogd et al. 2003; Apps and Garwicz 2000; Sugihara and Quy 2007; Voogd and Ruigrok 2004) as do the responses evoked by peripheral inputs (Hallem et al. 1999; Chockkan and Hawkes 1994; Chen et al. 1996). The full functional significance of this parasagittal architecture remains unknown (Apps and Hawkes 2009).

Of interest for this study is the parasagittal organization of type 1 metabotropic glutamate receptors (mGluR$_1$) and downstream signaling cascade. Implicated in regulating PC signaling and cerebellar function (Hartmann and Konnerth 2009), mGluR$_1$ receptors are expressed heavily on PCs (Lein et al. 2007; Grandes et al. 1994). Activation of mGluR$_1$ receptors leads to
activation of phospholipase Cβ (PLCβ) with the production of inositol triphosphate (IP3) and diacylglycerol (DAG). In turn, IP3 binds to IP3 receptors on the endoplasmic reticulum, releasing Ca²⁺ from intracellular stores (Takechi et al. 1998; Finch and Augustine 1998; Linden et al. 1991; Llano et al. 1991a). The PLCβ3 isoform is found in a subset of PCs in a zebrin II-positive banding pattern while the PLCβ4 isoform and the splice variant, mGluR1b, are found in a complementary pattern on zebrin II-negative PCs (Sarna et al. 2006; Mateos et al. 2001). Interestingly, the mGluR1b isoform has less potency in coupling to the PLC downstream signaling pathway than the mGluR1a isoform (Prezeau et al. 1996; Joly et al. 1995). The functional consequences of the parasagittal compartmentalization of the mGluR1 isoforms and intracellular signaling pathway are not known. However, it has been hypothesized that these differences in mGluR1 signaling pathways may result in differential synaptic plasticity among the parasagittal zones (Mateos et al. 2001; Paukert et al. 2010).

The parasagittal bands are crossed by the parallel fibers (PFs) in the molecular layer that extend for 3-5 mm in the medio-lateral direction and make glutamatergic synapses with the dendrites of PCs and cerebellar interneurons. The PFs are hypothesized to play a central role in cerebellar functioning (Braitenberg et al. 1997; Ito 2006; Thach et al. 1992). While generally assumed that PFs provide for relatively uniform, short-latency activation of their postsynaptic targets (Braitenberg 2002; Eccles et al. 1967), the effects of PF input may differ between bands as shown recently by the parasagittal compartmentalization of molecular layer inhibition (Gao et al. 2006). This study demonstrates that high frequency PF stimulation activates patches at long latencies that align with the zebrin II and PLCβ3-positive zones. These patches are due to the release of Ca²⁺ from intracellular stores mediated through the mGluR1 signaling cascade.
Materials and Methods

Animal Preparation

All animal procedures were approved by and conducted in conformity with the Institutional Animal Care and Use Committee of the University of Minnesota and in accordance with the American Physiological Society’s Guide to Principles in the Care and Use of Laboratory Animals. Experimental details on the animal preparation and optical imaging techniques are briefly described as the details have been provided in previous publications (Reinert et al. 2004; Gao et al. 2006; Wang et al. 2009).

Male FVB mice, ages 5-8 months (Charles River Laboratories, Wilmington, MA), were anesthetized by induction with acepromazine (2.0 mg/kg, i.m.), followed by urethane (1.5 mg/kg, i.p.). The electrocardiogram and response to pinch were monitored to assess the depth of anesthesia, supplementing anesthetics as needed. The mice were mechanically ventilated and body temperature feedback-regulated. The animal was placed in a stereotaxic frame, a craniotomy exposed Crus I and II and a watertight chamber of dental acrylic was created around the exposed folia. The chamber was filled and periodically rinsed with a gassed Ringer’s solution (Reinert et al. 2004; Gao et al. 2006). Various drugs in normal Ringer’s solution were applied to the exposed cerebellar surface, including: 1) the AMPA receptor antagonist, DNQX (6,7-dinitroquinoxaline-2,3-dione disodium salt), 2) mGluR1 receptor antagonist, LY367385, and 3) NMDA receptor antagonist, APV (D-(-)-2-amino-5-phosphono pentanoic acid). The glutamate receptor antagonists were purchased from Tocris Bioscience (Ellisville, MO). To block downstream targets of the mGluR1 signaling pathway that release Ca$^{2+}$ from intracellular stores the following drugs were used: 1) PLCβ inhibitor, U73122 (1-(6-(((17b)-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione) and 2) ryanodine (RyR) receptor
antagonist, ryanodine (Tocris Bioscience, Ellisville, MO). These latter two drugs were initially
dissolved in ethanol and then diluted in Ringer’s solution to a final ethanol concentration of 1.0-
1.5%.

To complement the flavoprotein autofluorescence imaging, Ca$^{2+}$ imaging was also used. Using a
glass micropipette, pressure microinjections (2 s with 100 kPa, PLI-100, Medical Systems Corp.)
are made into the molecular layer with a solution consisting of Oregon Green 488 BAPTA-1/AM
dissolved in DMSO plus 20% Pluronic F-127 solution and diluted in normal Ringer’s solution to
a final concentration of 0.5 mM (Sullivan et al. 2005; Gao et al. 2006; Sarkisov and Wang 2008).
The dye injections are visualized in the microscope, allowing for precise placement and control.
Multiple injections at a depth of ~200 µm were made to stain the exposed surface of Crus II with
the Oregon Green solution.

**Optical Imaging**

Images were acquired with a high-speed, cooled CCD camera (Quantix 57 with a 535 X 512
CCD chip at 12 bit digitization or a Quantix 512SC with a 512 X 512 CCD chip at 16 bit
digitization). Binning on the CCD chip is done to achieve a pixel resolution of ~10 X 10 µm.
The camera is focused ~100 µm below the cerebellar surface. The light sources used a stabilized
power supply.

Flavoprotein autofluorescence imaging used a band pass excitation filter (455 ± 35 nm), an
extended reflectance diachronic mirror (500 nm), and a > 515 nm long pass emission filter
(Reinert et al. 2004). Ca$^{2+}$ imaging used an excitation band-pass filter of 490 - 510 nm, a 515 nm
diachronic mirror, and a band-pass filter of 520 - 530 nm (Sullivan et al. 2005; Gao et al. 2006).
Parallel fiber stimulation and LTP protocol

Stimulation of the PFs was delivered by a parylene coated microelectrode (2-5 MΩ) placed just below the surface of the cerebellar cortex (Reinert et al. 2004; Gao et al. 2006). Typical stimulation parameters were a train of 10 pulses (200 µA, 100 µs) at 100 Hz. In one series of experiments, the frequency of stimulation was varied from 10 to 500 Hz. At each frequency, 10 pulses were delivered and all other stimulation parameters kept constant. In another experiment, the amplitude of PF stimulation was varied from 50 to 400 µA, keeping the other stimulation parameters constant (10 pulses at 100 Hz, 100 µs duration). The effectiveness of 3 and 5 pulses at 100 Hz (50 µA and 100 µs) was also tested in three animals.

We also tested whether the long-latency patches undergo long-term potentiation (LTP) using a high frequency induction paradigm that generates LTP of the PF-PC synapses and PC receptive fields in vivo (Wang et al. 2009; Jörntell and Ekerot 2002). The first 30 min was used to establish the baseline response to the PF test stimulation (5 min intervals). The “test” stimulation consisted of a train of 10 pulses at 100 Hz (175 µA, 150 µs duration). Following this baseline period, the PF conditioning stimulation was applied (t = 0 min). The “conditioning” stimulation consisted of 15 pulses (175 µA, 150 µs duration) at 100 Hz every 3 seconds for 5 min (Wang et al. 2009; Jörntell and Ekerot 2002). To evaluate the effect of the conditioning stimulation, the PF test stimulation was applied at 5 min intervals for 120 min.

Analysis of the optical responses

As detailed in previous publications (Chen et al. 2005; Dunbar et al. 2004), an image series consisting of 425 sequential frames was acquired (exposure time of 200 ms for each frame) in
relation to PF stimulation. The first 20 frames collected before PF stimulation (control frames) provide a measure of the background fluorescence. The first step in the analysis is to generate a series of “difference” images by subtracting the average of the 20 control frames from each frame. These difference images are then divided by the average of the control frames on a pixel-by-pixel basis and converted into a percentage (ΔF/F), in which the intensity value of each pixel reflects the change in fluorescence intensity relative to the average of the control frames. Several methods are used to display the responses including showing images of the ΔF/F, either using a grey scale or pseudocoloring. To display the optical responses in relation to the anatomy of the folia, the images were thresholded to highlight pixels above or below the mean ± 1.5 SD of the fluorescence in a region of the image of similar area without a response (i.e., typically Crus I). The thresholded pixels were then displayed on an image of the background fluorescence of the folia (Gao et al. 2003).

To quantify the responses to PF test stimulation, a region of interest (ROI) defined by the evoked beam or the long-latency patches was visually determined. The beam-like response to the PF test stimulation consists of an initial period of increase in fluorescence (light phase) followed by a longer duration decrease (Reinert et al. 2004; Reinert et al. 2007). The former results from the oxidation of mitochondria flavoproteins in the postsynaptic neurons activated by glutamate and is tightly coupled to the strength of the stimulation (Reinert et al. 2004; Reinert et al. 2007; Shibuki et al. 2003; Brennan et al. 2006). For a beam ROI, 5 frames (1 s) centered on the peak amplitude were averaged and the average ΔF/F within the ROI was determined. For a patch ROI, 25 frames (5 s) were averaged around the peak. The same ROI was used throughout an experiment to quantify changes in the fluorescence. An ANOVA was used to statistically assess the effect of a treatment on the response amplitude of the beam or patches (within subject design
To analyze the effects of the LTP conditioning stimulation, the responses in the baseline period
were compared with the responses following the conditioning stimulation (Wang et al. 2009).
The latter was divided into early (0 – 60 min) and late (65 – 120 min) phases. The flavoprotein
responses within the ROI at each 5 min interval were normalized to the average response during
the baseline. Using an ANOVA (within-subject design with repeated measures), we tested for
significant differences between the baseline period and the early and late phases ($\alpha = 0.05$).

**Field potential recordings**

Field potential recordings in the molecular layer of the responses to PF stimuli provided an
electrophysiological assessment of the effect of several pharmacological agents. It is important to
determine if the PF volley and the short-latency post-synaptic response are affected by the
blockers of the release of Ca$^{2+}$ from internal stores (i.e., U73122 and ryanodine). Field potentials
were recorded using glass microelectrodes (2 M NaCl, 2-5 M$\Omega$), digitized at 25 KHz and
averaged (responses to 16 single PF stimuli at 1 Hz). The P$_1$/N$_1$ component was used as a
measure of the presynaptic responses and the N$_2$ component as a measure of the postsynaptic
response (Eccles et al. 1967; Reinert et al. 2004; Gao et al. 2003). The field potentials are
monitored before, during and after washout of the drug and analyzed using an ANOVA (within-subject design with repeated measures).

**Histology and immunohistochemistry**
A series of animals were used to determine the relation between the bands of decreased fluorescence and the parasagittal zonation revealed by anti-zebrin II and PLCβ3 immunostaining.

As outlined above, optical imaging in Crus II was used to determine the locations of beam and long-latency patches evoked by PF stimulation. Next, lesions were generated on the surface of the molecular layer to serve as fiduciary makers by passing a DC current (50 µA for 30 sec) using a PF stimulating electrode. The lesions were targeted to the center of the patches.

Following transcardiac perfusion with PBS containing 4% paraformaldehyde and coronal sectioning (40 µm), immunostaining of zebrin II was carried out (Eisenman and Hawkes 1993; Gao et al. 2006). Cerebellar sections were incubated at room temperature overnight with anti-zebrin II (1:100) and then with peroxidase-conjugated rabbit/anti-mouse IgG for 1 hour (Dako, Denmark). Immunoactivity was revealed by using DAB as the peroxidase substrate. Sections with the lesions were recovered and the zebrin II staining compared to the locations of the long-latency patches evoked by PF stimulation. Also, immunostaining of PLCβ3 (anti-PLCβ3 at 1:200) was performed on alternating sections with the immunostaining for zebrin II to verify that these two molecules are in register as shown previously (Sarna et al. 2006).

Results

High frequency PF stimulation evokes long-latency patches

Using flavoprotein imaging (Gao et al. 2006; Reinert et al. 2004), PF stimulation evokes a beam-like response that consists of an initial increase in fluorescence (on-beam light phase) followed by a long-latency decrease in fluorescence (dark phase). In addition, PF stimulation at 100 Hz evokes “patches” of increased fluorescence (Fig. 1A). The patches are located along the shorter latency, on-beam response region. In the example shown in Figure 1A, 4 patches were evoked (right most image). Typically, 2-5 patches are evoked in Crus II with average amplitude of 0.53
± 0.17% \Delta F/F. The patches develop after the fluorescence returns to the baseline following the dark phase (Fig. 1B). While the number and amplitude of the patches varies in different mice (Fig. 1C), we attribute a large fraction of the variability to the limitations in imaging the entire folium. Crus II varies in orientation, shape and contour among animals. As the focus was optimized to the center of the folium, the more medial and lateral aspects were not always in the plane of focus or in view and therefore, the responses in these regions more difficult to image. The latency of the patches is 20-25 s with peak activation at 35.0 ± 4.9 s (n = 5 mice, Fig. 1B and C). Although there is also variability in the peak time, there is no obvious correlation between the peak time and the patch location on the folium (Fig. 1C). Also, there is no evidence that the location of the patches have any preferred spatial relationship to the surface vasculature (Figs. 1 and 3-7). We will refer to these novel responses as “long-latency patches”.

The long-latency patches are activated by a wide range of PF stimulation intensities. Long-latency patches were evoked at the lowest amplitude tested (50 µA, Fig 2). For the example shown, two patches of increased fluorescence are evident using 10 pulses at 100 Hz (Fig. 2A, top images). At this stimulation amplitude, the beam and patch responses are reduced and the beam is quite narrow. In three additional mice, PF stimulation using 5 and 3 pulses (50 µA) at 100 Hz also evoked long-latency patches (Fig. 2A, middle and bottom images). In 6 mice, the amplitude of the PF stimulation was varied, keeping the other parameters constant. From 50 to 250 µA, the amplitude of the beam and long-latency patches increases in parallel (Fig. 2B). However, the beam and long-latency patches appear to plateau between 250 and 300 µA. The amplitudes of the beam and long-latency patches did not increase further at 350 and 400 µA stimulation and were not significantly different from the responses at 300 µA (beam and patch at 350 vs 300 µA: F(1,3) = 2.0, p = 0.25 and F(1,3) = 1.3, p = 0.33 and beam and patch at 400 vs 300 µA: F(1,3) =
1.4, p = 0.33 and F(1,3) = 4.3, p = 0.13). These results demonstrate that the long-latency patches are evoked at the lowest PF stimulation intensities that evoke the short-latency beam.

The long-latency patches depend on the frequency of PF stimulation. The patches are evoked by 20 Hz but not 10 Hz PF stimulation, increase in amplitude as the frequency of PF stimulation increases (F(7,4) = 3.7, p = 0.0002), and are maximal with 100-200 Hz stimulation (Fig. 3). The amplitude of the patches is significantly different at each frequency (ANOVA followed by post-hoc Bonferroni t-test, p < 0.05, Fig. 3A and D, n = 5 mice). The peak time increases with stimulation frequency (F(6,24) = 10.31, p < 0.0001, Fig. 3B and C). The beam response is also dependent on the frequency of PF stimulation (F(7,4) = 12.8, p < 0.0001), as reported previously (Dunbar et al. 2004; Gao et al. 2006). However, the frequency dependence of the beam is less than that of the long-latency patches (Fig. 3D), as the beam amplitude increased by 35.3% between 20 Hz and 200 Hz and the amplitude of the three patches increased on average 257%.

The long-latency patches were not evident in our initial studies because typically low frequency PF stimulation (10 Hz) and a briefer imaging time were used (Gao et al. 2006; Reinert et al. 2004; Reinert et al. 2007).

**Long-latency patches are mediated by mGluR$_1$ receptors**

The frequency dependence of the long-latency patches prompted the hypothesis that mGluR$_1$ receptors are involved as activation of these receptors requires high frequency PF input (Finch and Augustine 1998; Batchelor et al. 1997; Tempia et al. 1998). The mGluR$_1$ receptor antagonist, LY367385 (200 μM, n = 6 mice) abolishes the long-latency patches (Fig. 4A and D), decreasing the amplitude from 0.46 ± 0.13 to -0.05 ± 0.05 ΔF/F (F(1,5) = 60.93, p = 0.0006). The amplitude of the beam also decreased significantly by 19.5% (F(1,5) = 22.81, p = 0.005),
reflecting that the on-beam response evoked by PF stimulation also has an mGluR₁ component as reported previously (Wang et al. 2009). The percentage decrease for the beam was much less than for the patches, consistent with the larger AMPA and smaller mGluR₁ contributions to the evoked beam (Reinert et al. 2004; Wang et al. 2009). Blocking AMPA receptors with DNQX (50 μM) produced a small (0.42 ± 0.16 to 0.28 ± 0.08, n = 5) but non-significant reduction in the amplitude of the patches (F(1,4) = 5.04, p = 0.09, Fig. 4B and E). In contrast, DNQX results in a 55.6% decrease in the amplitude of the beam (F(1,4) = 108.43, p = 0.0005), similar to previous findings (Wang et al. 2009). Blocking NMDA receptors with APV (250 µM) has no effect on the amplitude of the patches (F(1,4) = 0.11, p = 0.75, n = 5) or the beam (F(1,4) = 0.75, p = 0.44, Fig. 4C and F). Therefore, the long-latency patches are highly mGluR₁-dependent.

**Long-latency patches are evoked in parasagittal zones**

Next we investigated the topography of the long-latency patches. The experiment involved stimulating the PFs at several anterior-posterior positions on Crus II and determining the locations of the long-latency patches. As shown for the example in Figure 5A, four patches were evoked at each PF stimulation position (only 4 of 6 PF stimulation positions are shown). The position of the beam and patches shift with the different stimulation positions. The composite image of the beams (red) and long-latency patches (blue) at the different PF stimulation locations shows that the patches are aligned in parasagittal bands (Fig. 5B). The composite images in Figure 5C – E illustrate the parasagittal zonation of the long-latency patches in three additional mice. Similar parasagittal banding for the long-latency patches was documented in 7 mice.

To assess whether the parasagittal zones reflect the known parasagittal compartmentalization of PCs, the position of the long-latency patches were compared with the banding pattern obtained
with anti-zebrin II staining in Crus II. As described in the Methods this involved: (1) evoking the
long-latency patches and determining their positions, (2) making a small DC lesion in the center
of one or two patches, and (3) staining with anti-zebrin II antibodies after sectioning. An
example showing the correspondence between the patches and zebrin II is shown in Figure 6A
and B. In this example, the two lateral patches were lesioned (labeled A and B in Fig. 6A) and
after immunostaining shown to be aligned with the two lateral zebrin II bands (labeled a and b in
Fig. 6B). These two lateral zebrin II bands correspond to the P6+ and P5+ in the terminology of
Hawkes and colleagues (Sillitoe and Hawkes 2002; Eisenman and Hawkes 1993). Two more
medial and smaller long-latency patches were also evoked by PF stimulation (labeled C and D in
Fig. 6A) and these patches are aligned with two smaller zebrin II bands (c and d, Fig. 6B).

Another example of the correspondence between the location of the long-latency patches and
zebrin II staining is shown in Figure 6C. In this example the lesions were slightly deeper in the
granular layer (arrows) below the P6+ and P5b+ bands. These findings were replicated in 6 mice.
Also, correspondence between the zebrin II and the PLCβ3 zones was found in Crus II in four
mice (two examples are shown in Fig. 6D), as shown previously (Sarna et al. 2006). Therefore,
the long-latency patches are structural and in register with zebrin II and PLCβ3 bands.

We previously demonstrated that low frequency PF stimulation activates molecular layer
inhibition in parasagittal zones that were also aligned with zebrin II-positive bands (Gao et al.
2006; Moseley et al. 2006). Therefore, we examined the spatial relationship between the
inhibitory bands and the long-latency patches. Three examples are shown in Figure 7 in which
the first column in each row shows the beam and bands evoked by 10 Hz PF stimulation. In the
second column the inhibitory bands are outlined in blue. In each animal, PF stimulation at 100
Hz evoked three or more long-latency patches (third column, outlined in red). The outlines of
the inhibitory bands and patches are superimposed onto a background fluorescence image of the folium in the last column. The inhibitory bands and long-latency patches are aligned spatially with the long-latency patches in the center of the inhibitory bands. Similar results were obtained in 6 mice.

**Long-latency patches are due to intracellular Ca\(^{2+}\) release**

Given that the long-latency patches are evoked by high frequency PF stimulation and are mGluR\(_1\)-dependent, we hypothesized that the long-latency patches are caused by mGluR\(_1\)-mediated release of Ca\(^{2+}\) from intracellular stores. Therefore, we examined the long-latency patches using Oregon Green 488 (see Methods). High frequency PF stimulation evoked patches of increased fluorescence that occur at long-latencies (Fig. 8). As shown in the example, 100 Hz PF stimulation evokes both the beam and long-latency patches (Fig. 8A and B). Single pulse or 10 Hz PF stimulation did not evoke the patch response (data not shown). Similar results were obtained in 4 mice using Ca\(^{2+}\) imaging.

Next, the PLC\(\beta\) inhibitor, U73122 (50 \(\mu\)M), was tested and found to completely block the long-latency patches (Fig. 9A and B). As evident in the example and population data, U73122 abolished the long-latency patches (F(1,4) = 33.33, p = 0.005, n = 5). There was also a modest, but significant, 21% reduction in the short-latency, on-beam response (F(1,4) = 33.46, p = 0.004). A reduction in the on-beam component is expected as PF stimulation results in a short-latency release of internal Ca\(^{2+}\) via activation of the mGluR\(_1\) receptors (Finch and Augustine 1998; Takechi et al. 1998). Washout of the U73122 resulted in some recovery of the long-latency patches, although no recovery of the beam (Fig. 9B), likely reflecting that U73122 acts intracellularly. Even in the slice preparation, U73122 is poorly reversible (Bell et al. 1998). The
washed-out period was extensive and for drugs that are reversible and do not act intracellularly this
typically results in substantial recovery of the responses (for example, see Fig. 4). Therefore, we
are confident that the U73122 was removed from the extracellular space.

Field potential recordings were used to test the integrity of PF-PC synaptic transmission after
application of the U73122. Neither the parallel fiber volley (P1/N1, F(1,5) = 5.9, p = 0.06, n = 6)
or the postsynaptic response (N2, F(1,5) = 0.02, p = 0.89) was significantly altered (Fig. 9C and
D). An additional control experiment was undertaken because dissolving the U73122 required
ethanol. In three mice, adding 1.5% ethanol to the Ringer’s resulted in no significant change in
the amplitude of the beam (F(1,2) = 0.50, p = 0.55, n = 3) or the long-latency patches (F(1,2) =
9.5, p = 0.10). Also, 1.5% ethanol did not alter the PF volley (F(1,2) = 0.01, p = 0.92, n = 3) or
post-synaptic response (F(1,2) = 0.02, p = 0.90). Therefore, the block of the long-latency
patches by U73122 is not due to the ethanol depressing the long-latency patches or changes in
the excitability of the PF-PC circuit.

A final test of the intracellular Ca$^{2+}$ release hypothesis used the RyR antagonist, ryanodine.
Elevated intracellular Ca$^{2+}$ triggers additional release via activation of ryanodine receptors
(Llano et al. 1994; Kano et al. 1995). The type 1 RyR is the main ryanodine receptor type in PCs
and RyR1 and IP$_3$ receptors share a common Ca$^{2+}$ pool in PCs (Klein et al. 2007; Waniewski and
Martin 1998; Martin et al. 1998; Khodakhah and Armstrong 1997). Ryanodine (100 μM)
blocked the long-latency patches as shown for the example (Fig. 9E) and the population data
((F(1,4) = 38.1, p = 0.004, n = 5), Fig. 9F). Ryanodine did not produce a significant reduction in
the on-beam response ((F(1,4) = 6.9, p = 0.5). Again, there was some recovery of the long-latency
patches but not the beam with washout (Fig. 9F). As discussed above for U73122, this likely
reflects that ryanodine acts intracellularly and while there is washout from the extracellular space, washout from the intracellular compartment is difficult to achieve in vivo. Field potential recordings (Fig. 9G and H) show that ryanodine did not significantly alter the PF volley (P1/N1, F(1,3) = 0.08, p = 0.79, n = 4) or post-synaptic responses (N2, F(1,3) = 0.13, p = 0.74); therefore, the loss of the long-latency patches is not due to a change in PF-PC synaptic transmission. The results of the U73122 and ryanodine experiments strongly support the hypothesis that the long-latency patches are due to release of Ca^{2+} from intracellular stores.

**Long-latency patches exhibit LTP**

Recently, we demonstrated that high-frequency burst PF stimulation induces a postsynaptic, mGluR1-dependent LTP of the on-beam response (Wang et al. 2009). The long-latency patches exhibit LTP using the same high frequency conditioning stimulation (Fig. 10). The example shows the LTP of the beam and the long-latency patches induced by this high frequency conditioning stimulation (Fig. 10A). The patches are highly potentiated for at least two hours with an increase in amplitude of 227 ± 86% above the baseline, in contrast to the 127 ± 7% increase of the beam (compare long-latency patch data in Fig. 10B and the beam data in C). The amplitude of the patches is potentiated for at least two hours in both the early phase (F(1,4) = 9.8, p = 0.035) and the late phase 60 min following conditioning (F(1,4) = 35.4, p = 0.004). In a control experiment, monitoring of the long-latency patches throughout the same period, but without the high frequency conditioning (gray curve), shows the amplitude of the patches remains constant and is not different from the baseline period (F(1,2) = 0.02, p = 0.91 early phase; F(1,2) = 0.61, p = 0.51 late phase, Fig. 10B).
We tested whether the LTP of the patches is mGluR$_1$-dependent (Wang et al. 2009). Application of LY367385 (200 μM) during the conditioning stimulation (Fig. 10E) completely suppresses the patches (compare Fig. 10E with Fig. 4D). On removal of LY367385 the long-latency patches recovered to baseline amplitude; however, there was no potentiation of the patches either in the early (F(1,4) = 2.6, p = 0.19) or late phase (F(1,4) = 0.28, p = 0.63). The control experiment in which the LY367385 is applied without the conditioning stimulation shows the expected suppression of the long-latency patches followed by return to baseline after washout of the mGluR$_1$ antagonist (gray curve). Application of the AMPA antagonist, DNQX (50 μM), does not block the induction of LTP of the patches (Fig. 10D). During the early phase following induction stimulation, the amplitude of the patches increased 175 ± 50% above baseline (F(1,4) = 38.4, p = 0.004). However, the duration of the LTP of the long-latency patches was shortened and returned to baseline at 80 min. The control experiment applying the DNQX without the conditioning stimulation shows no changes in the long-latency patches during or following washout (gray curve).

The final experiment evaluated whether the maintenance of long-latency patch LTP was dependent on mGluR$_1$ receptors. Following the protocol described for Figure 10, LTP of the beam and long-latency patches was induced by high frequency PF stimulation. The amplitude of the patches increased 191 ± 72% above baseline (F(1,3) = 14.1, p = 0.03) for the 50 min period following LTP induction. The beam amplitude increased by 125 ± 12% (F(1,3) = 20.2, p = 0.02) for the same period. At 50 min following the conditioning stimulation, mGluR$_1$ receptors were blocked with LY367385 (200 μM) for 30 minutes and then washed out (Fig. 11). As the example images (Fig. 11A) and population data (Fig. 11B) show, the LY367385 completely suppressed the long-latency patches (F(1,3) = 46.0, p = 0.007), demonstrating that the
potentiated patches are mGluR$_1$-dependent. During application of the LY367385 the amplitude of the long-latency patches is actually less than the baseline control because the patches are blocked and normally occur in the dark phase (Reinert et al. 2004). The dark phase is evident in the example image at 65 min during the LY367385 application (Fig. 11A). The potentiation of the beam (Fig. 11C) was also mGluR$_1$-dependent as the amplitude was suppressed during the LY367385 ($F(1,3) = 77.3$, $p = 0.003$). The amplitude of the beam was also smaller than the baseline ($F(1,3) = 718.6$, $p < 0.001$), reflecting the mGluR$_1$ component of the baseline response to PF stimulation (Wang et al. 2009). On washout the amplitude of the long-latency patches returned to, and was not significantly different than, the baseline level ($F(1,3) = 1.8$, $p = 0.27$), implying that maintenance of the LTP requires continual mGluR$_1$ activation. The LTP of the beam exhibited the same sensitivity to mGluR$_1$ in that following washout of the LY367385, the amplitude returned to baseline control level and the responses during the baseline and washout periods were not significantly different ($F(1,3) = 0.1$, $p = 0.76$).

**Discussion**

*Properties of the long-latency patches*

This study is the first report that PF stimulation evokes long-latency patches of activity in addition to the well described beam of activity. The long-latency patches are dependent on high frequency PF stimulation, suggesting the involvement of mGluR$_1$ receptors on PCs (Klein et al. 2007; Shigemoto et al. 1992; Finch and Augustine 1998; Batchelor et al. 1994; Tempia et al. 1998). The application of glutamate receptor antagonists confirms that the long-latency patches are dependent on the activation of mGluR$_1$ receptors and not on the activation of AMPA or NMDA receptors. As mGluR$_1$ receptors are not found on granule cell axons or PFs (Kinoshita et
al. 1996; Mateos et al. 1999; Mateos et al. 1998), the evidence demonstrates that the long-latency patches are generated postsynaptically.

Given that the long-latency patches are postsynaptic, we hypothesize that the patches originate in neurons, predominantly in PCs. The patches consist of increased fluorescence, consistent with a neuronal origin (Reinert et al. 2004; Brennan et al. 2006; Reinert et al. 2007). Conversely, activation of glia generates a decrease in fluorescence and is responsible for the dark phase. The dark phase is thought to be the result of increased glycolysis in glia with the production of lactate, a reducing equivalent (Reinert et al. 2007; Reinert et al. 2004; Gao et al. 2009; Kasischke et al. 2004; Pellerin and Magistretti 1994). Approximately 85-90% of PFs synapse on PCs (Harvey and Napper 1988; Palay and Chan-Palay 1974) and mGluR$_1$ receptors are the major mGluR receptor type on these neurons (Shigemoto et al. 1992; Lein et al. 2007). The location of the long-latency patches corresponds with zebrin II and PLC$\beta$3-positive bands on PCs. To our knowledge, glia cells have not been reported to show such a molecular compartmentalization. Furthermore, both U73122 and ryanodine abolish the long-latency patches and both IP$_3$ and RyR receptors are heavily expressed in PCs (Sharp et al. 1993; Lein et al. 2007; Kuwajima et al. 1992).

Molecular layer interneurons are also activated by mGluR$_1$ receptors (Karakossian and Otis 2004; Llano and Marty 1995) that results in intracellular Ca$^{2+}$ release (Collin et al. 2009). However, activation of these increases in intracellular Ca$^{2+}$ requires long duration application of agonists as opposed to the short duration PF stimulation used to evoke the long-latency patches. Furthermore, U73122 and ryanodine have very modest effects on the Ca$^{2+}$ transients evoked in molecular layer interneurons (Collin et al. 2009), in sharp contrast to the complete blockade of
the long-latency patches reported here. Although we do not rule out involvement of cerebellar 
interneurons, the evidence suggests PCs are the major source of the long-latency patches.

We acknowledge that electrical stimulation generates a synchronous activation of PFs that is not 
physiological. However, it should be noted that PF stimulation has provided many insights into 
the organization and function of the cerebellar circuitry and continues to be widely used for both 
in vitro and in vivo studies. The experiments using different amplitudes of stimulation show that 
long-latency patches are evoked by the lowest amplitudes tested. Although an exhaustive search 
for the lowest levels of PF stimulation that can evoke the patches was not conducted, as few as 3 
pulses at 50 µA evoked patches. Also, the amplitudes of the beam and patches plateau between 
250 and 300 µA. These findings suggest that the long-latency patches are activated by 
approximately similar numbers of PFs as those needed to activate and detect a beam using 
flavoprotein imaging.

Long-latency patches are evoked in parasagittal bands

Stimulation of the PFs at different anterior-posterior locations on Crus II showed that the long-
latency patches are evoked in a parasagittal distribution. The location of the patches corresponds 
to the zebrin-II positive staining pattern and that of PLCβ3 (Sarna et al. 2006; Mateos et al. 
2001). The observation that the patches align with zebrin II-positive and not zebrin II-negative 
bands may reflect the structural and physiological differences between mGluR₁a and mGluR₁b 
isoforms (for review see (Conn and Pin 1997)). The mGluR₁a isoform has a long carboxyl-
terminal intracellular domain and mGluR₁b isoform a short one (Tanabe et al. 1992; Pin et al. 
1992). Overall, the mGluR₁b isoform exhibits less potency in coupling to PLC than does the 
mGluR₁a isoform. Compared to mGluR₁a, the mGluR₁b isoform has slower activation of Cl⁻
currents, markedly less constitutive activity, requires a higher concentration of agonists to
activate, and results in lower levels of intracellular Ca$^{2+}$ (Conn and Pin 1997; Prezeau et al. 1996;
Joly et al. 1995; Pin et al. 1992). Therefore, selective expression of mGluR$_{1b}$ in zebrin II-
negative bands is likely to contribute to the lack of long-latency patches in these regions.

Long-latency patches and intracellular Ca$^{2+}$ release

Activation of mGluR$_1$ receptors on PCs activates an inward cation current (slow, excitatory
postsynaptic current) at a much shorter latency (several hundred ms) and faster time course (~
0.5 - 1 s) than that observed from the long-latency patches (Batchelor and Garthwaite 1997;
Takechi et al. 1998; Batchelor et al. 1994). The slow excitatory current is due to a C-type
transient receptor potential (TRPC) cation channel, specifically the TRPC3 cation channel (for
review see (Hartmann and Konnerth 2009)). Furthermore, Ca$^{2+}$ entry through TRPC3 channels
does not account for the Ca$^{2+}$ release from internal stores (Hartmann et al. 2008; Canepari and
Ogden 2006). Therefore, the long-latency patches are likely to involve a mechanism other than
the slow postsynaptic current generated in PCs by TRPC3 channels. Blocking either PLC$\beta$ or
RyR receptors completely suppresses the long-latency patches, demonstrating the patches are
generated by Ca$^{2+}$ release from intracellular stores via the IP$_3$ pathway and Ca$^{2+}$-mediated Ca$^{2+}$
release (Finch and Augustine 1998; Takechi et al. 1998; Linden et al. 1994; Llano et al. 1994;
Kano et al. 1995).

The release of Ca$^{2+}$ from intracellular stores has a host of effects on PC excitability as well as on
short and long-term plasticity. The effects include controlling PC excitability through the gating
of SK channels (Netzeband and Gruol 2008) and BK channels (Canepari and Ogden 2006). The
release of Ca$^{2+}$ from intracellular stores following mGluR$_1$ activation is also involved in the
production of endocannabinoids that transiently decrease transmitter release from PFs (Maejima et al. 2005; Maejima et al. 2001; Duguid et al. 2007). Furthermore, IP$_3$-mediated release is required for long-term depression (LTD) at PF-PC synapses \textit{in vitro} (Miyata et al. 2000). The long latency and protracted time course of the patches provides a mechanism by which a very brief PF input modulates PC intracellular activity and excitability for tens of seconds.

The mechanism for the long delay in activating the patches is not known. One possibility is a release-refill-release type mechanism from intracellular Ca$^{2+}$ stores found in both neuronal and non-neuronal systems (Tsien and Tsien 1990; Berridge 1998). In this scenario, PF stimulation results in an initial release of Ca$^{2+}$ from intracellular stores (Finch and Augustine 1998; Takechi et al. 1998) and could account for the observed reduction in the beam response by U73122 (Fig. 9). This initial short-latency release is followed by refilling of the internal stores and a second release of Ca$^{2+}$. In heterologous expression systems, activation of mGluR receptors can generate repetitive increases in Ca$^{2+}$ from intracellular stores with a periodicity of tens of seconds (Kawabata et al. 1998). Another possible mechanism is that the long-latency patches are caused by oscillations in excitability of PCs driven by molecular layer interneurons. Recently, it was shown in the cerebellar slice that long duration stimulation of molecular layer interneurons with receptor agonists evokes oscillations of internal Ca$^{2+}$ release and corresponding oscillations in the excitability of these interneurons (Collin et al. 2009). The average oscillation period of the Ca$^{2+}$ transients was reported to be 37.9 s, similar to the 35.0 s peak time observed for the patches. If brief PF stimulation results in oscillation in the interneurons \textit{in vivo}, even for only one additional cycle, the molecular layer interneurons would be expected to generate changes in excitability in PCs on these long time scales. However, it needs to be reiterated that the oscillations observed in the molecular layer interneurons are not blocked by U73122 or
ryanodine (Collin et al. 2009), further evidence that the long-latency patches are generated in
PCs and not in molecular layer interneurons.

Plasticity of the long-latency patches

The long-latency patches exhibit marked LTP. The amplitude of the patches increases ~200%
above the baseline response compared to the ~130% increase in the beam amplitude using the
same induction paradigm (Wang et al. 2009). The LTP of the long-latency patches greatly
exceeds the amplitude of the LTP reported in cerebellar slices, either the presynaptic (Linden and
Ahn 1999; Hirano 1991; Salin et al. 1996) or postsynaptic forms (Belmeguenai and Hansel 2005;
Coesmans et al. 2004; Lev-Ram et al. 2002). The induction of the LTP is mGluR₁-dependent,
further implicating mGluR₁ receptors and downstream signaling as the main pathway in the
generation and control of the long-latency patches. The observation that LY367385 application
following LTP induction completely suppressed the long-latency patches shows that the
potentiated patches are mediated by mGluR₁ receptors. The return of the amplitude of the
patches to baseline following washout of the LY367385 suggests that continual mGluR₁ activity
is needed for the maintenance of the LTP. The LTP of the beam exhibits the same increase in
mGluR₁ responsiveness and mGluR₁-dependent maintenance.

The mechanism underlying the marked potentiation of the long-latency patches is unresolved.
The previously described LTP of the beam induced by the high frequency induction paradigm
may contribute (Wang et al. 2009), with the increase in the synaptic response augmenting
internal Ca²⁺ release. Although the degree of the synaptic potentiation is markedly smaller than
that of the long-latency patches, amplification could be achieved through the intracellular
signaling cascade. It is also possible that changes in the mGluR₁ signaling cascade, independent
of the changes in synaptic transmission, leads to potentiation of the patches. Given the important role of protein kinase C (PKC) in PF-PC synaptic plasticity (Linden and Connor 1991; Aiba et al. 1994; Kano et al. 1997; De Zeeuw et al. 1998), we hypothesize that PKC activation is involved in LTP of the long-latency patches. The release of Ca$^{2+}$ from intracellular stores is dependent on the phosphorylation state of mGluR receptors, which is under the control of PKC (Kawabata et al. 1998; Kawabata et al. 1996). Irrespective of the mechanism, the LTP of the long-latency patches provides a novel type of plasticity that can alter excitability and therefore, information processing in PCs in spatially restricted bands. As noted above, many studies have implicated mGluR$_1$ receptors in synaptic plasticity and motor learning in the cerebellum by knocking out or inhibiting mGluR$_1$ receptors (De Zeeuw et al. 1998; Aiba et al. 1994; Kano et al. 1997). This raises the possibility that the effects observed in these previous studies, may have involved interfering with the long-latency patches.

The spatial correspondence between the long-latency patches and the zebrin II-positive bands provides a further demonstration of differential synaptic plasticity among the parasagittal zones (Paukert et al. 2010; Sarna et al. 2006; Wadiche and Jahr 2005). The mGluR$_1$ cascade may provide one possible mechanism for a parasagittally distributed plasticity as some isoforms of mGluR$_1$ and PLC$\beta$ have zebrin II staining patterns as well as different properties (Sarna et al. 2006; Mateos et al. 2001; Conn and Pin 1997). Another possible mechanism underlying the differential plasticity is the sequestration of glutamate by neuronal excitatory amino acid transporters (EAATs). The PC-specific EAAT4 transporter is expressed in a zebrin II band pattern (Nagao et al. 1997; Dehnes et al. 1998; Gincel et al. 2007). PF-PC LTD is dependent on activation of mGluR$_1$ receptors (Aiba et al. 1994; Ichise et al. 2000; Hartell 1994); however, LTD of PF-PC synapses is greatly reduced in folia with high levels of EAAT4 expression.
The loss of LTD in these folia is attributed to the EAAT4 removing the released glutamate, resulting in a limited activation of the mGluR1 receptors. Intriguingly, the long-latency patches and their plasticity described in this report are located within bands with high EAAT4 expression, providing an example in vivo of differential responsiveness and plasticity in relation to neuronal transporters. Whether there is a mechanistic connection between the long-latency patches and EAAT4 remains to be investigated.

The role of AMPA receptors is less clear as DNQX did not block the induction of LTP but the duration of the LTP of the patches was shortened. The mechanism underlying this AMPA-mediated effect is not known, but may involve the large decrease in PF-PC synaptic transmission caused by blocking AMPA receptors (Konnerth et al. 1990; Llano et al. 1991b). The overall reduction in PC excitability may alter the activation of PKC and/or other potential mediators of the LTP. Clearly, this needs further study.

**Coupling between the transverse and parasagittal architectures**

These results provide for coupling between the transverse architecture of the PFs and the parasagittal organization of the cerebellar cortex, specifically to the molecular compartmentalization of PCs. Previously, we demonstrated that PF stimulation activates molecular layer inhibition in parasagittal bands and that the inhibitory bands can control the spatial aspects of the responses in the cerebellar cortex (Gao et al. 2006; Moseley et al. 2006). Climbing fiber inputs to zebrin II-positive bands release more glutamate and generate larger, longer-duration AMPA-mediated excitatory currents in PCs than in zebrin II-negative zones (Paukert et al. 2010). The present study greatly expands these observations on the differential physiological properties of the parasagittal zones, showing that PFs also activate long-latency
patches organized in parasagittal zones. Furthermore, the inhibitory bands and the long-latency patches are aligned and both correspond spatially with the zebrin II and PLCβ3-positive bands on PCs. The inhibitory bands and long-latency patches are optimally activated by different frequencies of PF stimulation, the inhibitory bands by low and the long-latency patches by high frequency stimulation. As discussed above, the LTP of the long-latency patches exemplifies that the parasagittal zones vary in their synaptic plasticity (Paukert et al. 2010; Sarna et al. 2006; Wadiche and Jahr 2005). Therefore, different parasagittal bands have highly differentiated responses and plasticity that is likely to underlie distinct functional roles.

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Harvey RJ and Napper RM. Quantitative study of granule and Purkinje cells in the


Hirano T. Differential pre- and postsynaptic mechanisms for synaptic potentiation and
depression between a granule cell and a Purkinje cell in rat cerebellar culture. *Synapse* 7:

Ichise T, Kano M, Hashimoto K, Yanagihara D, Nakao K, Shigemoto R, Katsuki M and
Aiba A. mGluR1 in cerebellar Purkinje cells essential for long-term depression, synapse


Ji Z and Hawkes R. Topography of Purkinje cell compartments and mossy fiber terminal
fields in lobules II and III of the rat cerebellar cortex: spinocerebellar and cuneocerebellar

Joly C, Gomeza J, Brabet I, Curry K, Bockaert J and Pin JP. Molecular, functional, and
pharmacological characterization of the metabotropic glutamate receptor type 5 splice


Kinoshita A, Ohishi H, Nomura S, Shigemoto R, Nakanishi S and Mizuno N.


Maejima T, Oka S, Hashimoto Y, Ohno-Shosaku T, Aiba A, Wu D, Waku K, Sugiura T and Kano M. Synaptically driven endocannabinoid release requires Ca$^{2+}$-assisted...
metabotropic glutamate receptor subtype 1 to phospholipase Cbeta4 signaling cascade in the cerebellum. *J Neurosci* 25: 6826-6835, 2005.


Voogd J, Pardoe J, Ruigrok TJH and Apps R. The distribution of climbing and mossy fiber collateral branches from the copula pyramidis and the paramedian lobule: Congruence


Figure Legends

Figure 1. PF stimulation evokes long-latency patches. A) Thresholded optical responses in Crus II overlaid on the background fluorescence shown at 3 time frames relative to onset of PF stimulation as noted in upper left corner. The typical biphasic, beam-like response was followed by long-latency patches of fluorescence increase located along the shorter latency beam. PF stimulation parameters consisted of 10 pulses of 200 \( \mu \)A, 100 \( \mu \)s, at 100 Hz and the same parameters were used throughout unless otherwise noted. The pseudocolor scale bar is shown at the bottom. The grey stippled region in the middle reflects that only pixels either greater or less than the mean \( \pm \) SD of the background fluorescence were displayed (i.e., thresholded). B) The time courses of the evoked fluorescence response is from the experiment shown in A, including 4 patches and an “off-patch” region between patches “3” and “4” as noted in the rightmost image in A. Numbering of the patches assigned the lateral-most patch as “1”, the next most lateral patch as “2”, etc. C) Peak time versus the peak amplitude of the evoked patches in 5 animals.

Figure 2. Long-latency patches as a function of the PF stimulation intensity. A) Stimulation at 50 \( \mu \)A using 10 (top), 5 (middle) and 3 (bottom) pulses of 100 \( \mu \)s at 100 Hz evokes both the short-latency beam and the long-latency patches (see arrows in top image). The images of the long-latency patch responses (right) are from 32 s following the onset of PF stimulation. Responses are shown as pseudo-colored images of the \( \Delta F/F \) as denoted by the scale bar. B) Amplitude (mean \( \pm \) SD) of the beam and long-latency patch responses as a function of the amplitude (50 to 400 \( \mu \)A) of PF stimulation keeping other stimulation parameters constant (10 pulses of 100 \( \mu \)s at 100 Hz). The population data in B is based on 6 mice studied at PF stimulations from 50 to 300 \( \mu \)A; 4 of these mice were also tested at 350 and 400 \( \mu \)A PF stimulation.
Figure 3. Frequency-dependence of the long-latency patches. A) Beam (top) and long-latency patches (bottom) evoked by different frequencies of PF stimulation as indicated (only 5 of 8 tested frequencies are shown). All other stimulation parameters were kept constant (10 pulses of 100 μs and 200 μA). The patches were not evoked by 10 Hz stimulation, were present at 30 Hz and peaked at 100 Hz. B) Time courses of the evoked response in patch “3” (labeled in 50 Hz panel in A) at different PF stimulation frequencies. C) The peak time of patches evoked by different PF stimulation frequencies (n = 5 mice). D) Amplitude (mean ± SD) of the beam and patches as a function of stimulation frequency in 5 mice. Both the beam and the long-latency patches exhibit frequency dependence; however, the patches were not evoked at 10 Hz and showed a more pronounced dependence on the frequency of PF stimulation.

Figure 4. Long-latency patches are mGluR₁-dependent. A) mGluR₁ receptor antagonist LY367385 (200 μM) abolished the long-latency patches completely and slightly reduced the beam. B) Non-NMDA receptor antagonist DNQX (50 μM) markedly reduced the beam, but did not affect the long-latency patches. C) NMDA receptor antagonist APV (250 μM) did not affect the amplitude of the patch and beam responses. D-F) Population data for the effects of the different GluR antagonists (LY367385, DNQX and APV) on the beam and long-latency patches. An * denotes a significant change (p < 0.05, see Results for statistical details for each drug). Stimulation parameters were 10 pulses of 200 μA, 100 μs at 100 Hz.

Figure 5. Long-latency patches evoked by PF stimulation are organized in parasagittal zones. A) Experiment showing the position of the evoked beam and long-latency patches in response to PF stimulation at four anterior-posterior locations. B) Composite image of the beams (red) and
patches (blue) evoked by PF stimulation at 6 anterior-posterior positions on Crus II for the experiment in A. The beam and patches shift position with the site of stimulation and show that the long-latency patches are aligned in parasagittal bands. C-E) Composite beam and patches results from similar experiments in three additional mice. Stimulation parameters were 10 pulses of 200 μA, 100 μs at 100 Hz.

**Figure 6.** Long-latency patches are aligned with zebrin II and PLCβ bands. A) Thresholded image showing the beam and long-latency patches evoked by PF stimulation (10 pulses of 200 μA, 100 μs at 100 Hz). The two lateral patches (A and B) were lesioned with DC current. B) Section through the folium in A stained with zebrin II antibodies. Three of the zebrin II bands, P6+, P5b+ and P4a+ ) are labeled (arrowheads). Inset shows that the locations of the lesions (a and b) correspond with the zebrin II-positive bands. C) Another example showing the location of the lesions relative to zebrin II staining. The arrows show the lesions were in the granule cell layer below the P6+ and P5b+ zebrin II bands. D) Two examples of the correspondence between zebrin II and PLCβ3 staining in Crus II. The P6+, P5b+ and P4a+ bands are labeled.

**Figure 7.** Inhibitory bands and long-latency patches are in spatial register. A) Left-most is a pseudocolor image of the response to low frequency PF stimulation (200 μA, 100 μs at 10 Hz for 10 s) taken at the end of stimulation (10 s). In the second image the inhibitory bands are outlined in blue. The third image shows that high frequency PF stimulation (10 pulses of 200 μA, 100 μs at 100 Hz) evokes long-latency patches at 40 s (outlined in red). Right-most image shows superimposition of the outlined patches and bands on a background image. B and C) Two
additional examples of the spatial relation between the inhibitory bands and the long-latency patches using the same conventions and PF stimulation parameters as in A.

**Figure 8.** Ca\(^{2+}\) imaging of the long-latency patches. A) Crus II was loaded with Oregon Green and the responses to PF stimulation imaged. B) Timecourse of a patch (red) and off-patch (blue) response in Ca\(^{2+}\) imaging from the same experiment. A beam-like response was evoked early, followed by long-latency patches. C-F) Two additional examples of the long-latency patches evoking by PF stimulation using Ca\(^{2+}\) imaging. Stimulation parameters for each example were 15 pulses of 200 \(\mu\text{A}\), 100 \(\mu\text{s}\) at 100 Hz.

**Figure 9.** Long-latency patches are due to release of Ca\(^{2+}\) from internal stores. A) Example of the beam and long-latency patches evoked by PF stimulation before (baseline) and after the application of U73122 (50 \(\mu\text{M}\)). Long-latency patches are completely blocked. B) Population data for effects of U73122 on the evoked beam and long-latency patches (n = 5). C) Example field potential recording of the response to PF stimulation before and during the U73122. The PF volley (P\(_1/N_1\)) and the postsynaptic response (N\(_2\)) are not altered by U73122. D) Population data for the effects of U73122 on the evoked field potentials (n = 6). E) Example of the effects of ryanodine (100 \(\mu\text{M}\)) on the beam and long-latency patches. Ryanodine completely suppresses the patches. F) Population data for the effect of ryanodine on the beam and patches (n = 5). G) Example of the effect of ryanodine on the PF volley (P\(_1/N_1\)) and the postsynaptic component (N\(_2\)). H) Population data for the effect of ryanodine on the evoked field potentials (n = 4). Optical responses were evoked by 10 pulses of 200 \(\mu\text{A}\), 100 \(\mu\text{s}\) at 100 Hz. Field potentials were evoked by PF stimulation using 100 \(\mu\text{A}\), 100 \(\mu\text{s}\) at 1 Hz.
Figure 10. Induction of the long-latency patch LTP is mGluR1-dependent. A) Example images of the LTP evoked by the conditioning stimulus for both the evoked beam (top) and long-latency patches (bottom). PF conditioning stimulation is denoted by the grey vertical bar (15 pulses at 175 μA, 150 μs at 100 Hz every 3 s for 5 min). Each image shows the beam or patch response evoked by the “test” PF stimulation (10 pulses at 175 μA, 150 μs at 100 Hz). B) Population data for the PF conditioning stimulation evoked LTP of the patches. The potentiation was more than 200% above the baseline and persisted for 120 minutes (black). Average patch response in animals without conditioning stimulation (gray). C) Population data for LTP of the beam response using the same conditioning paradigm. D) LTP of the long-latency patches was not blocked by AMPA receptor antagonist (50 μM DNQX, black, n = 5); however, the LTP duration was shortened. Control responses with DNQX in gray (n = 4). Error bars are ± 1 SD. E) The mGluR1 receptor antagonist, LY367385 (200 μM) completely suppresses the induction of LTP of the long-latency patches (black, n = 5). Control data shows responses with LY367385 but without the conditioning stimulation (gray, n = 4).

Figure 11. Maintenance of the long-latency patch LTP is mGluR1-dependent. A) Example of beam and patch LTP evoked by conditioning PF stimulation (grey vertical bar, 15 pulses at 175 μA, 150 μs at 100 Hz every 3 s for 5 min). Test PF stimulation as in Fig. 10. At 50 min following the conditioning stimulation, LY367385 (200 μM) was introduced into the chamber for 30 min and then washed out. Test PF stimulation was done during the application of the drug and for 60 min following washout. Example images show the LTP of the long-latency patches and beam and that LY367385 suppressed the LTP of the patches and beam. On washout of LY367385 the long-latency patches and beam only recovered to baseline levels. B and C)
Population data (n = 4) for the effects of LY367385 on the maintenance of the LTP of the long-latency patches and the beam (B and C, respectively).
A  

Beam | Patches

10 pulses | Caudal

5 pulses

3 pulses

1 mm

-1.5 % | 0 % | 1.5 % ΔF/F

B  

ΔF/F (%)  

Stimulation amplitude (μA)

n = 6

Beam | Patch
A

10 Hz 30 Hz 50 Hz 100 Hz 200 Hz

1 mm

-2.0 -1.0 0 1.0 2.0 ΔF/F (%)

B

ΔF/F (%)

0 20 40 60 80

Time (s)

10 Hz 30 Hz 50 Hz 100 Hz 200 Hz

PF stim

C

Peak time of patches (s)

0 25 50 75 100 125 150

Frequency (Hz)

n = 5

10 Hz 20 Hz 100 Hz 200 Hz 500 Hz

D

ΔF/F (%)

0.0 0.3 0.6 1.2

10 20 30 40 50 100 200 500

Frequency (Hz)

n = 5

Beam Patch 1 Patch 2 Patch 3
A. 
Baseline | 20 minutes | 50 minutes | LY367385 65 minutes | 120 minutes
---|---|---|---|---
Beam |  |  |  | 
Caudal |  |  |  | 
Medial |  |  |  | 
Patches |  |  |  | 

Scale: 1 mm

100 Hz, 15 pulses, 3 s for 5 min

B. 

Optical response (%) vs. Time (min)

-40 -20 0 20 40 60 80 100 120

LY367385 (200 µM)

Patches n = 4

C. 

Optical response (%) vs. Time (min)

-40 -20 0 20 40 60 80 100 120

LY367385 (200 µM)

Beam n = 4