Title: Developmental Changes in Agonist-induced Retrograde Signaling at Parallel Fiber-Purkinje Cell Synapses: Role of Calcium Induced Calcium Release.

Francis CREPEL and Hervé DANIEL

Pharmacologie de la Synapse, I.B.B.M.C, Bât. 430, Université Paris-Sud, 91405 Orsay Cedex, France

Abbreviated title: Glutamate and endocannabinoids as retrograde messengers.

Corresponding author: Pr. François Crépel; Pharmacologie de la Synapse, I.B.B.M.C, Bât. 430, Université Paris-Sud, 91405 Orsay Cedex. France. Phone: (33) 1 69 15 63 27. E-mail: francis.crepel@ibbm.u-psud.fr

Abstract: 250 words
Text pages: 52
Figures: 10
Tables: 0
Supplementary results: 1
Supplementary Figures: 2
ABSTRACT

In cerebellar Purkinje cells (PCs), activation of post-synaptic mGluR1 receptors inhibits parallel fiber (PF) to PC synaptic transmission by retrograde signaling. However, results were conflicting with respect to whether endocannabinoids (Maejima et al., 2001) or glutamate (Glu) (Levenes et al., 2001) is the retrograde messenger involved. Experiments in cerebellar slices from 10 to 12 day-old rats and mice confirmed that suppression of PF-EPSCs by mGluR1 agonists was entirely blocked by cannabinoid receptor antagonists at this early developmental stage. In contrast, suppression of PF-EPSCs by mGluR1 agonists was only partly blocked by cannabinoid receptor antagonists in 18 to 22 day-old rats, and the remaining suppression was accompanied by an increase in paired-pulse facilitation. This endocannabinoid-independent suppression of PF-EPSCs was potentiated by the Glu uptake inhibitor DL-TBOA and blocked by the desensitizing kainate (KA) receptors agonist SYM 2081, by non saturating concentrations of CNQX (but not by GYKI), and by dialyzing PCs with GDP-βS. An endocannabinoid-independent suppression of PF-EPSCs was also present in nearly mature wild type mice but was absent in GluR6 -/- mice. The endocannabinoid-independent suppression of PF-EPSCs induced by mGluR1 agonists as well as the KA-dependent component of depolarization-induced suppression of excitation (DSE) (Crepel, 2007) were blocked by ryanodine acting at a pre-synaptic level. We conclude that retrograde release of Glu by PCs participates in mGluR1 agonist-induced suppression of PF-EPSCs at nearly mature PF-PC synapses, and that Glu operates through activation of pre-synaptic KA receptors located on PFs and prolonged release of calcium from pre-synaptic internal calcium stores.
INTRODUCTION

In cerebellar Purkinje cells (PCs), activation of mGluR1 post-synaptic metabotropic glutamate receptors by selective agonists (Galante and Diana, 2004; Levenes et al., 2001; Maejima et al., 2001) or by sustained parallel fiber (PF) stimulation (Brown et al., 2003; Maejima et al., 2001; Neale et al., 2001) inhibits both excitatory and inhibitory inputs to these neurons by retrograde signaling (Brown et al., 2003; Galante and Diana, 2004; Levenes et al., 2001; Maejima et al., 2001; Marcaggi and Attwell, 2005). Endocannabinoids have been favored as the retrograde messenger involved in this signaling (Galante and Diana, 2004; Maejima et al., 2001), as was previously shown for depolarization-induced suppression of inhibition (DSI) (Diana et al., 2002; Glitsch et al., 1996; Glitsch et al., 2000; Kreitzer and Regehr, 2001a; Llano et al., 1991a; Ohno-Shosaku et al., 2001; Pitler and Alger, 1992; Pitler and Alger, 1994; Vincent et al., 1992; Wang and Zucker, 2001; Wilson and Nicoll, 2001; Wilson et al., 2001) and for depolarization-induced suppression of excitation (DSE) (Brenowitz and Regehr, 2003; Kreitzer and Regehr, 2001b; Safo and Regehr, 2005). However, the study by Levenes et al. (2001) contrasts with these results, suggesting instead that retrograde release of glutamate (Glu) by PCs was responsible for the observed agonist-dependent suppression of PF-EPSCs, and this, through activation of pre-synaptic ionotropic Glu receptors borne by PFs. Since most studies on the role of endocannabinoids in retrograde signaling at PF-PC synapses have been performed in juvenile rats and mice (but see Safo and Regehr, 2005) whereas the study by Levenes et al. (2001) was performed in nearly mature rats, the apparent discrepancy on the nature of retrograde messengers involved in agonist-induced suppression of excitation at PF-PC synapses might be related to developmental differences. Indeed, we now know that DSE at PF-PC synapses is entirely mediated through
retrograde release of endocannabinoids in juvenile rodents whereas it also involves retrograde
release of Glu in nearly mature animals (Crepel, 2007).

Therefore, the present experiments were designed to determine whether such distinct
mechanisms are also involved in suppression of PF-EPSCs by activation of post-synaptic
mGluR1 in juvenile and nearly mature rats and mice. Since pre-synaptic kainate (KA)
receptors are involved in DSE in nearly mature PF-PC synapses (Crepel, 2007), emphasis was
made on a possible role of these receptors in agonist-induced suppression of PF-EPSCs in
nearly mature PF-PC synapses, as well as on a possible participation of pre-synaptic calcium-
induced calcium release in this process.
METHODS

Experimental procedures complied with guidelines of the French Animal Care Committee. They were performed on juvenile (10 to 12 day-old) and on nearly mature (18 to 22) day-old male rats (Sprague-Dawley). Additional experiments were also performed on 22 to 24 day-old C56BL/6 and GluR6−/− (on a hybrid 129Sv x C57BL/6 background) mice as well as on juvenile (10 to 12 day-old) C56BL/6 mice. In all cases, animals were stunned before decapitation and parasagittal slices, 250 μm thick, were cut in ice-cold saline solution (see below) from the cerebellar vermis with a vibroslicer. Slices were incubated at room temperature in saline solution equilibrated with 95% O₂ / 5% CO₂ for at least 1 hour. The recording chamber was perfused at a rate of 2 ml/minute with oxygenated saline solution containing in mM: NaCl, 124; KCl, 3; NaHCO₃, 24; KH₂PO₄, 1.15; MgSO₄, 1.15; CaCl₂, 2; glucose, 10, and the GABAₐ antagonist bicuculline methochloride (10 μM, Sigma Aldrich, St. Quentin Fallavier, France), osmolarity 320 mOsm, final pH 7.35 at 27-28°C except when otherwise specified. PCs were directly visualized with Nomarski optics through the X 40 water-immersion objective of an upright microscope (Zeiss).

Drugs were added to the superfusate. (S)-3,5-dihydroxyphenylglycine (DHPG), domoate, DL-threo-beta -Benzyloxyaspartate (DL-TBOA), 6-cyano-7-nitroquinoxaline-2-3-dione (CNQX), GYKI 52466 hydrochloride (GYKI), D-2-amino-5-phosphopentanoic acid (D-APV), (2S,4R)-4-Methylglutamic Acid (SYM 2081), 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX), CGP55845-A, N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM-251) and ryanodine were purchased from Tocris (Illkirch, France). The CB1 cannabinoid receptor antagonist SR141716-A (N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazol-3-carboxamide hydrochloride) was provided by Sanofi-Recherche (Montpellier, France). N⁵-nitro-L-arginine methyl ester (L-NAME) was purchased from Sigma Aldrich, St. Quentin Fallavier, France.
Stock solutions of drugs (dissolved in water or DMSO depending on manufacturer recommendations) were added to the oxygenated saline solution at the desired concentration.

**Electrophysiology**

Whole cell patch-clamp recordings were performed from PC somas, using an Axopatch-200A amplifier (Axon instruments). Stimulating electrodes consisted in saline filled monopolar electrodes. PF stimulations were performed at 0.33 Hz, except for studies on unitary quantal events and on DSE where stimulation rates were 0.1 Hz and 0.5 Hz respectively. Patch pipettes (2-4 MΩ) were filled with a solution containing (in mM): K-gluconate, 140; KCl, 6; HEPES, 10; EGTA, 0.75; MgCl2, 1; Na2-ATP, 4; Na-GTP, 0.4; pH: 7.35 with KOH; 300 mOsm. In experiments on DSE, patch pipettes (2-4 MΩ) were filled with a solution containing (in mM): Cs-gluconate, 140; KCl, 6; HEPES, 10; EGTA, 0.2; MgCl2, 1; Na2-ATP, 4; Na-GTP, 0.4 (pH and osmolarity adjusted accordingly). The components of internal solutions were purchased from Sigma (Sigma Aldrich, St. Quentin Fallavier, France).

In the cells retained for analysis, access resistance (usually 5-10 MΩ) was partially compensated (50-70%), according to the procedure described by Llano et al. (Llano et al., 1991b). Cells were held at a membrane potential of -70 mV and PF-EPSCs were subjected to 10 mV hyperpolarizing voltage steps that allowed monitoring of the passive electrical properties of the recorded cell throughout the experiment (Llano et al., 1991b).

For paired-pulse facilitation (PPF) experiments (Atluri and Regehr, 1996; McNaughton, 1982; Schultz et al., 1994), PF stimulations of the same intensity were applied to the cell with an inter-stimulus interval of 30 ms, and the ratio of the amplitude of the second PF-EPSC over the first one was calculated on line. Mean PPF values were obtained by averaging PPFs in individual traces for each cell studied in a given condition. Although this
conventional method can produce spurious results (Kim and Alger, 2001), no use was made of the alternative method proposed by these authors since both methods produced very similar results in a recent study on DSE at PF-PC synapses (Crepel, 2007). Since PPF increases associated to agonist-induced suppression of PF-EPSCs could be small in certain experimental conditions (see results) and obscured by variability of basal PPF across cells, mean PPFs were further normalized in these experiments by determining for each cell mean normalized PPF = 100 x (mean PPF/PPFi), where PPFi was the individual PPF value of the last trace preceding the depolarizing step. The contribution of pre-synaptic factors in the variation of synaptic responses was also examined by using the coefficient of variation (CV) (Kullmann, 1994; Martin, 1966), where CV is given by: \[ CV = \frac{s}{M} \times 100 \% \], in which \( s \) is the standard deviation of the amplitude distribution of EPSCs corrected for the background noise, and \( M \) is the mean amplitude of EPSCs during the same epoch. In the present experiments, CV were calculated on sets of 40 to 60 stable EPSCs according to the procedure previously described (Blond et al., 1997).

**Fluorometry**

Parallel fibers in coronal slices from nearly mature and juvenile rats (see results) were loaded by focal application of 100 µM of the low-affinity calcium sensitive dye Fluo-4FF-AM (Molecular Probes) as previously described (Levenes et al., 2001). After loading for at least 45 min, fluorescent signals from labeled PFs were recorded in a 20 X 50 µm window placed above the molecular layer, 500 - 800 µm away from the loading site and 100 µm above PC layer. The epifluorescence excitation light at 485 ± 22 nm was gated with an electromechanical shutter (Uniblitz, Rochester, NY, USA), and the emitted light was collected by a photometer through a barrier filter at 530 ± 30 nm. The rather selective and weakly desensitizing KA receptor agonist domoate (Lerma et al., 1993) was applied for one minute
and at a concentration of 20 µM by local superperfusion through a theta-tube (rate of 0.5 ml.min\(^{-1}\)) placed about 50µm above the surface of the slice, at the level of the molecular layer and parallel to labeled PFs. With such a protocol that minimized possible movements of the slice when switching superfusion through the theta-tube from standard saline solution to that with domoate included, this compound was unlikely to diffuse in significant concentration to nearby granule cells whereas it was probably still at near saturating concentrations for pre-synaptic KA receptors (Renard et al., 1995) and this, even after partial dilution during superfusion of the tissue. In these experiments, the 1-minute superfusion duration was chosen since, in pilot experiments, it was the shortest time that gave rise to reliable pre-synaptic calcium signals. Fluorescence signals corrected for dye bleaching and background fluorescence, were expressed as relative fluorescence changes ΔF/ΔF, where F was the baseline fluorescence intensity and ΔF the change induced by domoate.

In sagittal slices, epifluorescence microscopy was also used to detect variations in intracellular free calcium concentration changes from an area of 90 µm x 90 µm centered on dendrites of recorded PCs, as previously described (Crepel, 2007). In these experiments, the low affinity and impermeant calcium indicator Fluo-4FF (100 µM, Molecular probes) was added to the same Cs-gluconate-based solution as used in experiments on DSE (see above). The recording session started 30 to 45 min after whole-cell break in, to allow diffusion of the dye to the dendrites. Fluorescence data corrected for dye bleaching and background fluorescence were again expressed as changes in ΔF/ΔF, where F was the baseline fluorescence intensity, and ΔF the fluorescence change induced by depolarization of PC soma from -70 mV to 0 mV for one second.

In all cases, pre- and post-synaptic calcium signals were recorded while superfusing the slices with a cocktail of GABA\textsubscript{A}, GABA\textsubscript{B}, and adenosine A1 receptor antagonists, i.e. bicuculline methochloride (10 µM), CGP55845 (300nM) and DPCPX (100nM) respectively.
Fluorometric measurements were analysed on- and off-line using the Acquis1 computer program (Biologic).

Statistical significance was assessed by paired or unpaired t-tests, as appropriate, with $P < 0.05$ (two-tailed) considered to be significant. All error values given are mean ± SE.
RESULTS

Agonist-induced suppression of PF-EPSCs depends entirely on retrograde release of endocannabinoids in juvenile rodents but only partly in nearly mature ones.

In 10-12 day-old mice, as shown in the previous study by Maejima et al. (2001), 5 minute bath application of the selective mGluR1 and mGluR5 agonist DHPG, at an apparent saturating concentration of 100 μM (Canepari et al., 2004; Pin and Duvoisin, 1995; Schoepp et al., 1994; see below), induced a large and significant \((P < 0.001)\) decrease in the mean amplitude of PF-EPSCs to 54.17 ± 5.12 % of control \((n = 6; \text{Fig. 1A, B})\). This suppression was accompanied by a large and significant \((P < 0.002)\) increase in PPF, from 1.35 ± 0.06% in control to 1.86 ± 0.19% at the peak of the DHPG effect. This is in keeping with a pre-synaptic action of DHPG at PF-PC synapses through retrograde signaling in juvenile mice (Maejima et al., 2001). PF-EPSCs recovered their initial amplitude within less than 2 minutes of DHPG application and thereafter were potentiated for several minutes \((123.48 ± 8.64\% \text{ of control on average})\) (Fig. 1A, B). Very similar results were obtained in 10-12 day-old rats since bath application of 100 μM DHPG also induced a reversible decrease in the mean amplitude of PF-EPSCs to 68.22 ± 4.37 % of control that was also followed by a transient potentiation of PF-EPSCs \((n=6; \text{Fig. 1C})\). However, this potentiation was shorter than in 10-12 day-old mice and was later followed by a transient, albeit not significant, depression of PF-EPSCs that amounted to 8.98 ± 8.95% on average (Fig. 1C). In both mice and rats, the transient potentiation that followed the initial suppression of PF-EPSCs did not give rise to any significant variation in PPF (data not shown). It was therefore reminiscent of transient potentiations observed in older animals after blockade of endocannabinoid dependent- and endocannabinoid-independent components of DHPG-induced suppression of PF-EPSCs (see
next sections). Accordingly, these potentiating effects are likely to be induced at a post-synaptic level in immature as well as in nearly mature PCs (see discussion).

In 10-12 day-old mice and as in experiments by Maejima et al. (2001), DHPG-induced suppression of PF-EPSCs was nearly totally abolished by 30 minute bath application of the CB1 receptor antagonist AM-251 (n = 9; Fig. 1B) applied at saturating concentration of 2 μM (Gatley et al., 1996; see below). In 10-12 day-old rats, DHPG-induced suppression of PF-EPSCs was similarly abolished (n=6; Fig. 1C) in the presence of the CB1 receptor antagonist SR141716-A (Rinaldi-Carmona et al., 1994) at saturating concentration of 1 μM (Petitet et al., 1996; see below). In both cases, blockade of agonist-induced suppression of PF-EPSCs by either AM-251 or SR141716-A revealed that the transient potentiation induced by DHPG had an earlier onset than that seen in the absence of CB1 receptor antagonists (Fig. 1B, C). In 10 to 12 day-old rats, the amplitude and time course of this potentiation were also slightly, although not significantly, increased in the presence of SR141716-A (Fig. 1C). Altogether, these results confirm that the suppression of PF-EPSCs by DHPG in juvenile rodents depends entirely on retrograde release of endocannabinoids (Maejima et al., 2001).

In nearly mature (18 to 22 day-old) rats (n=10) and for all cells tested, 5 minute bath application of 100 μM DHPG induced a transient and significant (P < 0.001) decrease in the amplitude of PF-EPSCs that amounted to 43.23 ± 5.08 % of control (Fig. 2A, B1). Most importantly, this transient suppression of PF-EPSCs was always accompanied by a significant increase in PPF (P < 0.001) from 1.32 ± 0.08 in control conditions to 1.95 ± 0.22 at the peak of the DHPG effect (Fig. 2B2). This is in keeping with a pre-synaptic site of action of DHPG at PF-PC synapses through retrograde signaling in nearly mature rats (Levenes et al., 2001). However, in this earlier study, suppression of PF-EPSCs of similar amplitude as those reported here were obtained with (S)-DHPG concentrations of only 50 μM (compare Fig. 2 B1 of the present study with Fig. 2 B in Levenes et al., 2001). In pilot experiments,
suppression of PF-EPSCs induced by 50 μM (S)-DHPG applications was on average 1.5 times smaller than that achieved with the same concentration in this earlier study (n=5; supplementary figure S1). With 25 μM S-DHPG, the late (endocannabinoid-independent; see below) component of suppression of PF-EPSCs was nearly totally absent and the initial component was still further reduced in amplitude (n=5; supplementary figure S1). This suggests that, taking into account the present recording chamber’s exchange time, this nominal concentration was too low to fully saturate mGluR1 receptors during 5 minute bath applications. Therefore, concentrations of 100 μM (S)-DHPG that gave rise to more robust suppressions of PF-EPSCs were used throughout the present study in nearly mature animals. This was also the case in experiments on juvenile rats and mice (see above) to compare results with mature ones in the same experimental conditions.

In marked contrast with results obtained in juvenile animals, perfusing nearly mature slices for at least 30 minutes with 1 μM SR141716-A only partly inhibited the DHPG-induced suppression of PF-EPSCs. Indeed, mean amplitude decrease was only 22.11 ± 4.30 % (n=14), a value significantly smaller (P < 0.01) than that observed in control conditions (Fig. 2B1). The partial inhibitory effect of 1 μM SR141716-A on the DHPG-induced suppression of PF-EPSCs by was unlikely to result from an incomplete blockade of pre-synaptic CB1 receptors by SR141716-A since bath application of AM-251 at a saturating concentration of 2 μM did not further antagonize this inhibition of PF-EPSCs. Indeed, in the presence of 2 μM AM-251, the mean decrease in PF-EPSC amplitude in the presence of 100 μM DHPG was 26 ± 3.75 % (n=6; data not shown), a value very similar to that obtained in the presence of 1 μM SR141716-A (see above). Moreover and in agreement with our previous study (Levenes et al., 1998), these concentrations of SR141716-A and of AM-251 fully antagonized the depressant effect of 1 μM bath application of the selective CB1 receptor agonist WIN55,212-2 (Devane et al., 1988) on PF-EPSCs (n=3 in each case; data not shown),
Finally, and most importantly, in the presence of CB1 receptor antagonists, the remaining suppression of PF-EPSCs was still accompanied by a significant \( P < 0.01 \) increase in PPF that amounted to 17\%, i.e. from 1.29 ± 0.05 in control conditions to 1.51 ± 0.08 at the peak of the DHPG effect (Fig. 2B2). These latter results suggest that suppression of PF-EPSCs by DHPG in nearly mature rats not only involves activation of pre-synaptic CB1 receptors like in juvenile animals (see above and Maejima et al., 2001), but also involves at least one other pre-synaptic mechanism. In contrast, kinetics of PF-EPSCs was not significantly affected. Thus, mean values of the 10 to 90\% rise time were 2.06 ± 0.21 and 1.90 ± 0.20 ms in control conditions and at the peak of DHPG-induced suppression of PF-EPSCs respectively, and mean time constants of decay had values of 11.39 ± 0.75 and 10.90 ± 0.79 ms in the same conditions. Although kinetics are certainly severely biased in nearly mature PCs by dendritic filtering of synaptic currents, the present data does not suggest that post-synaptic AMPA receptors at PF-PC synapses are sizably affected during the endocannabinoid-independent component of DHPG-induced suppression of PF-EPSCs (see discussion).

**Sensitivity of the CB1 receptor-independent component of agonist-induced suppression of PF-EPSCs to a Glu uptake blocker and to a desensitizing KA receptor agonist in nearly mature rats.**

In nearly mature rats, retrograde release of Glu by PCs participates in DSE at PF-PC synapses through activation of pre-synaptic KA receptors that include GluR6 receptor subunits (Crepel, 2007). In keeping with this recent finding, the previous study by Levenes et al. (2001) already suggested that activation of post-synaptic mGluR1 decreases PF-EPSCs through retrograde release of Glu and activation of pre-synaptic ionotrophic Glu receptors borne by PFs. Accordingly, in 18 to 22 day-old rats, the endocannabinoid-independent
component of agonist-induced suppression of PF-EPSCs should be enhanced in the presence of the Glu uptake inhibitor DL-TBOA and suppressed by pharmacological blockade of pre-synaptic KA receptors (see below).

In the presence of 1 μM SR141716-A and as expected for a Glu uptake inhibitor, application of 100 μM DL-TBOA increased the amplitude of PF-EPSCs (Fig. 3A1). This increase ranged between 8% and 76% depending on cells, with a significant mean increase of 34.90 ± 9.79 % (n=9; P < 0.01). As seen in a previous study (Crepel, 2007), the rather large variability of potentiating effects of DL-TBOA on PF-EPSCs might result from the patterned expression of PC Glu transporters EAAT4 in rat cerebellar cortex (Wadiche and Jahr, 2005). In all cells, this effect was accompanied by marked changes in EPSC kinetics (Fig. 3A1), probably due to slower clearance of Glu from synaptic cleft. At the plateau of the DL-TBOA effect, application of 100 μM DHPG decreased the mean amplitude of PF-EPSCs that amounted to 39.78 ± 4.63 % of control amplitude prior to DHPG application (Fig. 3A2, B1). This decrease in amplitude was significantly larger (P < 0.01) than that observed in the presence of SR141716-A alone (Fig. 3B1), and was accompanied by a significantly larger increase in PPF (P < 0.02) since PPF increased from 1.24 ± 0.17 in SR141716-A + DL-TBOA containing medium to 1.77 ± 0.15 at the peak of the DHPG effect (Fig. 3B2). Although slower Glu clearance in the presence of DL-TBOA may complicate the interpretation of this difference, the fact that DL-TBOA alone did not induce any significant change in PPF (Crepel, 2007) suggests that DL-TBOA did not affect the presynaptic machinery to such an extent as to sizably affect the level of suppression of PF-EPSCs by DHPG. Therefore, the present results are consistent with the assumption that retrograde release of Glu participates, together with retrograde release of endocannabinoids, to the depressant effect of DHPG on PF-EPSCs in nearly mature rats. However, it should be noted
that group 1 mGluRs are not restricted to PCs but are also found, for instance, on glial cells (Angulo et al., 2004; Karakossian and Otis, 2004; Parpura et al., 1994; see below).

In keeping with the previous study by Crepel (2007), involvement of pre-synaptic KA receptors in agonist-induced suppression of PF-EPSCs in 18 to 22 day-old rats was tested by using SYM 2081, a potent ligand that, at micromolar concentrations, selectively blocks kainate-induced currents through a process of agonist-induced desensitization (Cho et al., 2003; Cossart et al., 2002; DeVries, 2000; Epsztein et al., 2005; Li et al., 1999; Zhou et al., 1997). In the presence of 1 μM SR141716-A, superfusing the slices with 10 μM SYM 2081 did not lead to large changes in PF to PC synaptic transmission, except for a significant ($P < 0.001$) increase in basal PPF which amounted to $1.53 \pm 0.07\%$ ($n = 11$), compared to $1.29 \pm 0.05$ in control conditions (see above). In marked contrast, SYM 2081 totally inhibited the late phase of the endocannabinoid-independent suppression of PF-EPSCs induced by bath application of 100 μM DHPG, and the same was true for the associated increase in mean normalized PPF ($n$=11; Fig. 4 A, B). Nearly identical results were obtained in 6 other cells with 10 μM SYM 2081 + 100 μM D-APV (data not shown). These results strongly suggest that, like for DSE in nearly mature rats, a late phase of the endocannabinoid-independent suppression of PF-EPSCs induced by bath application of 100 μM DHPG involves activation of pre-synaptic KA receptors.

However, an early component of the DHPG-induced suppression of PF-EPSCs remained unaffected by SYM 2081 and was still accompanied by a significant ($P < 0.05$) increase in PPF (Fig. 4 A, B). This suggests that one or several other pre-synaptic components underlie the depressant effect of DHPG on PF-EPSCs. Accordingly, this early SYM 2081-insensitive component and associated PPF increase were partly inhibited by bath application of 300 nM of the GABA$_B$ receptor antagonist CGP55845-A ($n$ = 5; data not shown) and were almost totally blocked when 200 μM of the NO-synthase inhibitor L-NAME (Knowles et al.,
1989) was further added to the bath (n=8; Fig. 4 A, B). This suggests that the early component of the endocannabinoid-independent suppression of PF-EPSCs is due to activation of pre-synaptic GABA<sub>B</sub> receptors (Dittman and Regehr, 1997) by GABA released by molecular layer inhibitory interneurons, as well as to release of nitric oxide (NO) from these same cells (Bredt et al., 1990; Shin and Linden, 2005; Vincent and Kimura, 1992). Indeed, one knows that molecular layer inhibitory interneurons bear group I mGluRs (Baude et al., 1993; Karakossian and Otis, 2004) and are therefore likely to be stimulated by DHPG, thus leading to release of GABA and of NO, which in turn transiently depress PF-EPSCs by pre-synaptic mechanisms (Blond et al., 1997). This pre-synaptic mGluR1 receptor/NO cascade is reminiscent of the pre-synaptic N-methyl-d-aspartate (NMDA) receptor/NO cascade found in molecular layer inhibitory interneurons and involved in the induction of cerebellar long-term depression (LTD) through cGMP-dependent inhibition of postsynaptic protein phosphatases (Shin and Linden, 2005). Therefore, NO released by molecular layer inhibitory interneurons is likely to be involved in both short-term pre-synaptic and long-term post-synaptic modulation of PF-PC synaptic transmission, depending on additional mechanisms involved, such as activation of post-synaptic mGluR1, protein kinase Calpha, and phosphorylation of ser-880 on the AMPA receptor subunit GluR2 for cerebellar LTD (references in Shin and Linden, 2005). Finally, blockade of the early component of the endocannabinoid-independent suppression of PF-EPSCs in the 8 PCs mentioned above unmasked a short-term potentiation of PF-EPSCs in 5 of them, whereas no such potentiation was seen in the other 3. On average, this potentiation amounted to 130.13 ± 10.11% of control, and was accompanied by a slight although non-significant mean normalized PPF decrease (Fig. 4 A, B). Altogether, these results suggest that, like for DSE (Crepel, 2007), a late phase of agonist-dependent suppression of PF-EPSCs in nearly mature rats depends on retrograde release of Glu by PCs and activation of pre-synaptic KA receptors located on PFs.
Sensitivity of the CB1 receptor-independent component of suppression of PF-EPSCs by DHPG to non saturating concentrations of CNQX and GYKI in nearly mature rats.

Because SYM 2081 is a desensitizing KA receptor agonist rather than a genuine KA receptor antagonist, it was important to confirm the involvement of pre-synaptic KA receptors in the late phase of agonist-induced suppression of PF-EPSCs. We reasoned that the concentration of Glu achieved at the level of pre-synaptic KA receptors involved in the CB1 receptor-independent component of agonist-induced suppression of PF-EPSCs is likely to be much lower than that seen by post-synaptic AMPA receptors during PF-PC EPSCs. If so, it is possible that a non saturating concentration of the competitive AMPA/KA antagonist CNQX (Honoré et al., 1988) that only partly block PF-EPSCs is sufficient to fully antagonize pre-synaptic KA receptors and thus inhibit agonist-induced suppression of PF-EPSCs.

Indeed, bath application of 1 μM CNQX in the presence 1 μM SR141716-A reduced the amplitude of PF-EPSCs to 36.33 ± 3.76% of control value (n=11). Unexpectedly, this effect was accompanied by a large and highly significant (P < 0.001) PPF increase, from 1.30 ± 0.08 to 1.75 ± 0.14 (Fig. 5 A1) that was reminiscent of that observed for basal PPF in the presence of SYM 2081 in nearly mature rats and in GluR6 -/- mice (see above and Crepel, 2007). In keeping with this latter result that suggests a pre-synaptic site of action of CNQX in addition to its well established post-synaptic effect, mean CV of PF-EPSCs also significantly (P < 0.001) increased, from to 0.045 ± 0.006 in control to 0.081 ± 0.012 at the steady state of the depressant effect of CNQX (n=9; Fig. 5 B).

Like for SYM 2081, CNQX also markedly inhibited the late phase of the CB1 receptor-independent suppression of PF-EPSCs induced by bath application of 100 μM DHPG (Fig. 5 A2). Moreover, its initial phase was also significantly (P < 0.01) inhibited since the mean decrease in peak amplitude of PF-EPSCs was only 11.09 ± 2.25 % (n=11) compared to 22.11
± 4.30 % (n=14) in the presence of SR141716-A alone (Fig. 5 A2). This near 50% inhibition of the initial phase of the agonist-induced suppression of PF-EPSCs by CNQX was accompanied by a similar near 50% inhibition of associated PPF increase. As such, the remaining 9% PPF increase, from 1.75 ± 0.14 in the presence of CNQX alone to 1.91 ± 0.15 at the peak of the residual depressant effect of DHPG, was no longer significant (Fig. 5 A1).

To preclude that the strong reduction in PF-EPSC amplitude by CNQX was not solely responsible for the lack of suppression of PF-EPSCs by DHPG in the experiments reported above, we also studied the effect of non saturing concentrations of GYKI, a non competitive and more selective AMPA receptor antagonist (Bureau et al., 1999, Renard et al., 1995; Wilding and Huettner, 1995). On average, bath application of 20 µM GYKI reduced the amplitude of PF-EPSCs to 42.63 ± 3.92% of control values (n=11), a decrease non-significantly different from that obtained with 1 µM CNQX (see above). Like for experiments with CNQX, this decrease in EPSC amplitude was also accompanied by a significant (P < 0.01) PPF increase, although about only half of that obtained in the presence of CNQX (Fig. 5 A1). However, and in marked contrast with results obtained with CNQX, mean CV of PF-EPSCs did not significantly increase during the depressant effect of GYKI on PF-EPSCs, since mean CV values during the control period and at the steady state of the effect of GYKI were 0.046 ± 0.005 and 0.051 ± 0.006 respectively (Fig. 5 B).

In 8 out of the 11 tested cells, GYKI did not significantly inhibit the CB1 receptor-independent suppression of PF-EPSCs induced by bath application of 100 µM DHPG since the mean decrease in peak amplitude of PF-EPSCs was 28.48 ± 7.07 % compared to 22.11 ± 4.30 % (n=14) in the presence of SR141716-A alone (Fig. 5 A2). Moreover, the time course of PF-EPSC suppression was unchanged (compare Fig. 2 B1 and 5 A2). In the remaining 3 cells, GYKI totally abolished the CB1 receptor-independent suppression of PF-EPSCs induced by bath application of 100 µM DHPG, which unmasked a short-term potentiation of
PF-EPSCs (Fig. 5A2). Since there was no apparent difference between these 2 groups of cells with respect to the effect of GYKI on PF-EPSC amplitude and on basal PPF, DHPG results were pooled, leading to a mean suppressing effect of this compound on peak amplitude of PF-EPSCs that amounted to 20.83 ± 5.76 %. This value was very close to that obtained in the presence of SR141716-A alone. Accordingly, DHPG suppression of PF-EPSCs was still accompanied by a significant (P < 0.05) and near 20 % increase in mean PPF, from 1.54 ± 0.08 in the presence of SR141716-A + GYKI to 1.82 ± 0.18 at the peak of DHPG effect (Fig. 5A1).

Taken together, CNQX and GYKI results fully confirm that pre-synaptic KA receptors are likely to be involved in CB1 receptor-independent suppression of PF-EPSCs by mGluR1 agonists (see discussion).

Finally, since PF-EPSC amplitude did not reach a steady state during DHPG application (Fig. 2B1, 5A2), no attempt was made to apply the CV method here. In contrast, such a near steady state was achieved during the endocannabinoid-independent component of DSE (Crepel, 2007), and was accompanied by a significant increase in CV (see supplementary results and supplementary figure S2), thus confirming involvement of pre-synaptic mechanisms in the CB1 receptor-independent component of DSE (Crepel, 2007).

**DHPG sensitivity of PF-EPSCs in wild type and GluR6 -/- mice.**

Pre-synaptic KA receptors located on PFs are likely to be heteromeric constructions that include GluR6 and KA2 receptor subunits (Petralia et al., 1994; Lerma and al., 2001). Their activation up or down regulates Glu release depending on agonist concentration (Delaney and Jahr, 2002). Agonist-induced suppression of PF-EPSCs was studied in nearly mature GluR6 -/- mice and compared to that of wild type mice of the same strain (see methods), with the assumption that invalidating GluR6 receptor subunits renders pre-synaptic KA receptors non
functional (Ruiz et al., 2005). All experiments in GluR6 -/- mice were performed in the presence of 50 μM D-APV to minimize possible developmental compensations by pre-synaptic NMDA receptors, and in the presence of 1 μM SR141716-A to focus on the endocannabinoid-independent component of agonist-induced suppression of PF-EPSCs. For comparison, all experiments in wild type mice were also performed in the presence of 50 μM D-APV and of 1 μM SR141716-A.

In 22 to 24 day-old wild type mice, 5 minute bath application of 100 μM DHPG induced an endocannabinoid-independent suppression of PF-EPSCs of similar amplitude and duration as in nearly mature rats, with a mean peak amplitude decrease of 17.62 ± 2.76 % (n = 6; Fig. 6, A). This suppression was accompanied by a significant (P < 0.001) increase in mean normalized PPF, from 100.39 ± 3.66 % in control conditions, to 116.27 ± 3.24 % at the peak of the DHPG effect (Fig. 6, B).

In 22 to 24 day-old GluR6 -/- mice and in keeping with a previous study (Crepel, 2007), no major alteration in PF to PC synaptic transmission was evidenced, except for basal PPF which amounted to 1.54 ± 0.01 % (n = 6) compared to 1.31 ± 0.05 % in control mice, the difference being highly significant (P < 0.001). In contrast, the late phase of the endocannabinoid-independent suppression of PF-EPSCs by DHPG and of its associated increase in PPF was strongly inhibited in all cells while the initial phase remained largely unaffected (Fig. 6 A, B). Therefore, these results are very similar to those obtained in experiments performed in the presence of SYM 2081 and of CNQX, and fully confirm that the late phase of the endocannabinoid-independent suppression of PF-EPSCs by DHPG depends on retrograde release of Glu. Moreover, they suggest that Glu acts through activation of KA receptors that include GluR6 subunits.
GDP-ßS Sensitivity of the CB1 receptor-independent component of agonist-induced suppression of PF-EPSCs in nearly mature rats.

As mentioned before, group 1 mGluRs are not restricted to PCs but are also found on various cell types including granule cells, molecular layer interneurons and glial cells (Angulo et al., 2004; Baude et al., 1993; Parpura et al., 1994). As such, glutamate release from these cells might participate in DHPG-induced suppression of PF-EPSCs. In an attempt to exclude this possibility, we selectively blocked G-protein activity in the post-synaptic compartment by dialyzing PCs through the patch pipette for at least 30 minutes after break-in with a conventional K-gluconate internal solution with 4 mM GDP-ßS added (Galante and Diana, 2004); GDP-ßS is a nonhydrolyzable GTP-analog that inhibits G-protein activity. Experiments were performed in the presence of 1 μM SR141716-A to focus on the endocannabinoid-independent suppression of PF-EPSCs. In 5 out of the 6 cells tested, the late phase of the endocannabinoid-independent suppression of PF-EPSCs induced by bath application of 100 μM DHPG was abolished and replaced by a short-term potentiation of PF-EPSCs whereas its initial phase was much less affected (Fig. 7A). In these 5 cells, the late phase of mean normalized PPF increase associated with the endocannabinoid-independent suppression of PF-EPSCs was also inhibited and replaced by a non significant 7.72 ± 2.83 % decrease of the mean normalized PPF (Fig. 7B). In the remaining PC, the late phase of the endocannabinoid-independent suppression of PF-EPSCs was only partly abolished (data not shown). In contrast, in 5 other PCs dialyzed during the same period of time after break-in with a conventional K-gluconate internal solution without GDP-ßS, a clear-cut endocannabinoid-independent suppression of PF-EPSCs and associated PPF increase were still induced by bath application of 100 μM DHPG (Fig. 7A, B).
These results strongly suggest that the Glu release responsible for the late phase of mGluR1-induced suppression PF-EPSCs originates mainly from the recorded PC themselves, with only minor (if any) contribution of spillover arising from neighboring cells.

**Ryanodine sensitivity of the CB1 receptor-independent component of agonist-induced suppression of PF-EPSCs and of DSE in nearly mature rats.**

Depolarization-induced potentiation of inhibition (DPI) also operates through Glu release from depolarized PCs. In this case, Glu activates pre-synaptic NMDA receptors, resulting in a slow build-up and decay (over several minutes) of calcium release from pre-synaptic ryanodine-sensitive calcium stores (Duguid and Smart, 2004). As for DPI, the KA-dependent components of agonist-induced suppression of PF-EPSCs and of DSE at PF-PC synapses might therefore involve such a mechanism in nearly mature rats, as suggested previously (Crepel, 2007, but see also Carter et al., 2002 and discussion). This hypothesis was tested by the following experiments.

In the presence of 1 μM SR141716-A and of 100 μM ryanodine, the CB1 receptor-independent suppression of PF-EPSCs by bath application of 100 μM DHPG was strongly inhibited and replaced by a transient potentiation of PF-EPSCs, 112.84 ± 9.43 % on average (n=6; Fig. 8 A1). In these cells, PPF increase associated with this CB1 receptor-independent suppression of PF-EPSCs was also strongly inhibited (Fig. 8 A2). Interestingly, ryanodine also markedly reduced basal PPF since its mean value was only 1.09 ± 0.06 (n = 6; Fig. 8 A2), thus suggesting that pre-synaptic ryanodine-sensitive calcium stores also contribute to basal PPF at PF-PC synapses in nearly mature rats.

In the same bathing medium, the late phase of the CB1 receptor-independent suppression of PF-EPSCs induced by PC depolarization (from -70 mV to 0 mV for one second) was also strongly inhibited as well as the associated mean normalized PPF increase
Here again, bath application of 100 μM ryanodine also markedly reduced basal PPF since the mean paired-pulse ratio was only 0.90 ± 0.04 in these experiments (n = 6). The fact that the early component of DSE that was partly resistant to bath application of ryanodine was not accompanied by significant changes in paired-pulse ratio (Fig. 8 B1, B2) suggests in turn that it might be partly post-synaptic in origin, i.e. due to a transient ionic unbalance following large depolarizing steps in post-synaptic cells (Crepel, 2007).

Altogether, the marked effects of ryanodine on agonist-induced suppression of PF-EPSCs and on DSE at PF-PC synapses suggests that, as for DPI, their prolonged duration involves long-lasting calcium release from pre-synaptic ryanodine-sensitive calcium stores (but see below), following an initial and more short-lived activation of pre-synaptic KA receptors. Moreover, the fact that ryanodine also partly inhibited the early phase of the CB1 receptor-independent suppression of PF-EPSCs by DHPG, i.e. the portion of the response that was resistant to bath application of SYM 2081 (compare fig. 4A and 8B1), suggests in turn that ryanodine also inhibits the pre-synaptic GABA_B- and NO- dependent components of agonist-induced suppression of PF-EPSCS (see above).

In the present experiments, inhibition of the Glu-dependent component of the agonist-induced suppression of PF-EPSCs by ryanodine might also result from an inhibition of calcium release from post-synaptic ryanodine-sensitive calcium stores (Carter et al., 2002; Isokawa and Alger, 2006) that would in turn inhibit retrograde release of Glu from PCs. To compensate, at least partly, for these post-synaptic effects, we used 2 second rather than 1 second depolarizing pulses from -70 mV to 0 mV in another series of DSE experiments in the presence of ryanodine. Indeed, this duration was likely to give rise to post-synaptic calcium transients of similar amplitude to those observed with 1 second depolarizing pulses in the absence of ryanodine (see next section and Fig. 9 B1). As illustrated in figure 8 B1, the Glu-
dependent component of DSE (Crepel, 2007) was still significantly inhibited (n=9; P < 0.01), whereas the early residual DSE was not significantly different from that induced in the same conditions with 1 second depolarizing pulses (Fig. 8 B1).

Fluorometric experiments were also performed to distinguish between pre- and postsynaptic ryanodine effects on DSE, to avoid as much as possible slow time resolution inherent to conventional bath application of mGluR1 agonists. More specifically, we tested the effects of 100 μM bath applied ryanodine on the amplitude and time course of calcium transients induced in PCs by a depolarizing voltage step from -70 to 0 mV for one second on the one hand, and induced in PFs by focal application (see methods) of the rather selective and weakly desensitizing KA receptor agonist domoate (Lerma et al., 1993) on the other hand. The latter protocol was directly derived from that used by Duguid and Smart (2004) to demonstrate involvement of pre-synaptic calcium-induced calcium release in DPI.

Sensitivity to ryanodine of pre- and post-synaptic calcium signaling involved in the CB1 receptor-independent component of DSE in nearly mature rats.

PFs loaded with Fluo-4FF-AM were subjected to focal superfusion of 20 μM domoate for 1 minute. This elicited a transient increase in the cytosolic calcium signal in these fibers that peaked shortly after the end of domoate application and relaxed slowly thereafter in less than 30 minutes (n=6; Fig. 9A1, A2). 100 μM ryanodine present in the bath for at least 30 minutes markedly reduced the duration of domoate-induced calcium transients, while the peak calcium transient was only marginally affected (n=5; Fig. 9A1, A2). Indeed, peak ΔF/F in control conditions and in the presence of ryanodine were 3.58 ± 0.10 % and 3.42 ± 0.94 % respectively; these values are not significantly different. To further quantify differences in calcium transients recorded in control conditions and in the presence of ryanodine, we determined, for each cell \( \partial = \sum (\Delta F/F \text{ for each point of } \Delta F/F \text{ plots}) \) between the beginning of
domoate application and up to 30 minutes thereafter. We then averaged these values for all cells recorded in a given condition (see Crepel, 2007). Mean Δ was significantly lower in the presence of ryanodine, since it amounted to only 0.61 ± 0.14 compared to 1.52 ± 0.30 in its absence ($P<0.05$). These results strongly suggest that prolonged calcium release from pre-synaptic ryanodine-sensitive stores occurs in PFs following brief activation of pre-synaptic KA receptors.

In control bathing medium, calcium signals induced in PC dendrites by the above described DSE protocol (depolarizing voltage steps from -70 to 0 mV for 1 second) peaked to $\Delta F/F = 14.10 \pm 2.85$ % at the end of the depolarizing steps (Fig. 9B1) and then rapidly decayed over the course of 10 to 15 seconds ($n=6$; Fig. 9B2). Superfusion of the slices for 30 minutes with 100 μM ryanodine significantly ($P<0.05$) inhibited these depolarization-induced calcium transients since mean $\Delta F/F$ was now only 7.77 ± 1.63 % ($n=6$; Fig. 9B1, B2). Differences between control and ryanodine results were further quantified by determining again, for each cell, mean $\bar{F}$ values calculated from the start of the depolarizing step until 4 seconds after, i.e the period of time during which $\Delta F/F$ plots in control conditions and in the presence of ryanodine appeared clearly different (Fig. 9B2). The mean $\bar{F}$ value was significantly lower ($P<0.05$) with ryanodine (4.56 ± 1.17) than in its absence (8.52 ± 1.27). Here again and in keeping with previous results by Carter et al. (2002), this suggests that calcium release from intracellular ryanodine-sensitive stores participates in calcium transients induced in PC dendrites by short depolarizing voltage steps identical as those used to induce DSE (see above and Crepel, 2007). However, these calcium transients were much shorter than those induced in PFs by domoate application, and moreover, they were also much shorter than the duration of the KA-sensitive component of DSE in nearly mature rats (Crepel, 2007). Therefore, electrophysiological and fluorometric experiments with ryanodine clearly suggest that prolonged calcium release from pre-synaptic ryanodine-sensitive stores is responsible for
the prolonged duration of DSE and of agonist-induced suppression of PF-EPSCs in nearly mature rats.

**Origin of the lack of the Glu-dependent component of agonist-induced suppression of PF-EPSCs in immature rats.**

Depolarization-induced potentiation of inhibition that operates through Glu release from depolarized PCs is strictly dependent on the activity of surrounding Glu transporters (Duguid and Smart, 2004). Therefore, the lack of a Glu-dependent component of agonist-induced suppression of PF-EPSCs in juvenile animals might simply result from an unbalance between Glu release and Glu uptake in favor of the latter at early developmental stages. In 10-12 day-old rats and in the presence of 1 μM SR141716-A, bath application of the Glu uptake inhibitor DL-TBOA (100 μM) did not unmask any sizeable Glu-dependent component of agonist-induced depression of PF-EPSCs (n=5; Fig. 10A). However, the transient potentiation elicited by DHPG was significantly (P < 0.001) inhibited as compared to that observed in the presence of SR141716-A alone (Fig. 10A), suggesting that it was counterbalanced by a DHPG-induced suppression of PF-EPSCs of similar amplitude and time course. Thus, and as shown for DSE (Crepel, 2007), the absence of a Glu-dependent component of agonist-induced depression of PF-EPSCs in juvenile rats may be partly explained by the activity of surrounding Glu transporters. However, this absence might also be due to other factors such as incomplete maturation of pre-synaptic KA receptors or of pre-synaptic calcium-induced calcium release, since DL-TBOA failed to reveal any fully developed KA-dependent component of agonist-induced suppression of PF-EPSCs in these animals. Therefore, fluorometric experiments were also performed to test these hypotheses.

In 12 day-old rats and like in experiments in nearly mature rats, PFs loaded with Fluo-4FF-AM were subjected to focal superfusion of 20 μM domoate for 1 minute. Here again,
this elicited a transient increase in cytosolic calcium signals in these fibers, which peaked shortly after the end of domoate application (n=5; Fig. 10B1). However, these calcium transients were significantly ($P < 0.001$) smaller than in nearly mature rats in the same conditions (see above) since the mean peak $\Delta F/F$ was only $1.82 \pm 0.21\%$. Moreover, calcium transients relaxed within less than 2 minutes instead of within 30 minutes in nearly mature rats (compare Fig. 9A2 and 10B1). Therefore, these results are in agreement with the lack of a Glu-dependent component of agonist-induced suppression of PF-EPSCs in immature rats (see above) and suggest that pre-synaptic KA receptors and/or calcium release from internal stores are not yet fully developed at this early developmental stage.

In contrast, when domoate application was performed in the presence of 100 $\mu$M DL-TBOA, cytosolic calcium signals were significantly ($P < 0.001$) larger ($\Delta F/F= 4.74 \pm 0.53\%$) and now decayed over 15 minutes (n=5; Fig. 10B1). Moreover, this decay was markedly shortened when 100 $\mu$M ryanodine was also present in the bath (n=5; Fig. 10B1), so that the mean $\hat{\delta}$ value was significantly lower ($P<0.05$) in the presence of ryanodine ($0.96 \pm 0.30$) than in its absence ($2.65 \pm 0.47$). These results therefore suggest that at least some calcium induced calcium release may be triggered in immature PFs.

However, the prominent effect of DL-TBOA on cytosolic calcium signals was puzzling. Indeed, and to the best of our knowledge, significant uptake of this agonist is unlikely. However, domoate is known to induce release of excitatory amino acids from cultured cerebellar granule cells through reversal of the Glu transporter (Berman and Murray, 1997). Therefore, the possibility exists that, in the present experiments, domoate not only acts through activation of pre-synaptic KA receptors, but also activates other pre-synaptic Glu receptors following release of Glu from the superfused molecular layer. We therefore tested this possibility by studying the effect of bath applied D-APV (50 $\mu$M) on calcium transients elicited in PF by focal superfusion of the slices for one minute with 20 $\mu$M domoate in the
presence of DL-TBOA. We performed these experiments because NMDA receptors are characterized by their high affinity for Glu and are probably present and functional on PFs (Casado et al., 2000). However, this conclusion has been challenged recently (Shin and Linden, 2005) and, to date, it still has not been established by electron microscopic analysis that NMDA receptors exist on PFs. As illustrated in figure 10B2, D-APV shortened calcium transients induced by domoate in the presence of DL-TBOA (n=6), to nearly the same extent as that observed with ryanodine since mean $\bar{\delta}$ values were not significantly different, i.e. 0.96 ± 0.18 and 0.96 ± 0.30 respectively. This latter result indicates that activation of pre-synaptic NMDA receptors probably contributes to calcium transients induced by domoate in the presence of Glu uptake blockers, thus corroborating the aforementioned hypothesis, and also supports the previous finding that NMDA receptors are present on PFs (Casado et al., 2000). Furthermore, the present results also strongly suggest that activation of NMDA receptors is necessary to trigger calcium-induced calcium release in immature PFs. In contrast, D-APV did not significantly affect calcium transients induced by domoate in control bathing medium in 20 to 22 day-old rats (n=4; data not shown), suggesting that pre-synaptic NMDA receptors are not recruited in this case.

In 12 day-old rats, the mean $\bar{\delta}$ values of calcium transients elicited in the presence of DL-TBOA and D-APV or in the presence of DL-TBOA and ryanodine were still significantly ($P<0.05$) higher (0.96 ± 0.18 and 0.96 ± 0.30 respectively) than in control medium (0.10 ± 0.02; Fig. 10B2). It is therefore likely that an additional mechanism participates to calcium transients induced by domoate in PFs in the presence of DL-TBOA. One hypothesis is that Glu uptake blockers lead to a progressive accumulation of Glu within slices that tonically activates granule cells. Such activity might therefore increase basal calcium concentration within PFs and facilitate induction of calcium-induced calcium release upon activation of pre-synaptic KA receptors. If this were so, prolonged calcium transients induced by domoate in
the presence of DL-TBOA and D-APV should be no longer observed in the presence of TTX. However, in 6 experiments performed in the presence of 1 μM tetrodotoxin, calcium transients induced by focal application of 20 μM domoate in the presence of both 100 μM DL-TBOA and 50 μM D-APV was not significantly altered as compared to that induced when TTX was omitted (Fig. 10B3). At the moment, we have no other plausible explanation to explain that calcium transients elicited in the presence of DL-TBOA and D-APV or in the presence of DL-TBOA and ryanodine were still significantly larger and of longer duration than in control medium.

**DISCUSSION**

In keeping with the study by Levenes et al. (2001) and Crepel (2007), the present data strongly suggest that, in nearly mature rats, retrograde release of Glu is involved in the suppression of synaptic transmission at PF-PC synapses following activation of post-synaptic mGluR1. Moreover, results with GDP-βS are consistent with the view that Glu mainly originates from the recorded PC themselves, with only minor (if any) contribution of spillover arising from neighboring cells. As for the KA-dependent component of DSE at PF-PC synapses in nearly mature rats (Crepel, 2007), Glu is likely to operate through activation of pre-synaptic KA receptors located on PFs and prolonged calcium release from pre-synaptic ryanodine-sensitive calcium stores. Finally, results in nearly mature wild type and GluR6 -/- mice further suggest that, as for DSE (Crepel, 2007), pre-synaptic KA receptors involved in agonist-induced suppression of PF-EPSCs include GluR6 receptor subunits.

The present results also demonstrate that retrograde release of endocannabinoids is another major component in agonist-induced suppression of excitation at these nearly mature PF-PC synapses. In contrast, in juvenile rats and mice, suppression of synaptic transmission at PF-PC synapses by activation of post-synaptic mGluR1 appears to be entirely mediated
through retrograde release of endocannabinoids by PCs and activation of pre-synaptic CB1 receptors. Our results are in agreement with those previously obtained in juvenile animals for agonist-induced suppression of PF-EPSCs (Maejima et al., 2001) and for DSE (Brenowitz and Regehr, 2003; Crepel, 2007; Kreitzer and Regehr, 2001b).

**Suppression of PF-EPSCs by DHPG in juvenile rats and mice depends entirely on retrograde release of endocannabinoids.**

In juvenile rats and mice, DHPG-induced suppression of PF-EPSCs exclusively involved retrograde release of endocannabinoids by PCs and activation of pre-synaptic CB1 receptors. This is in complete agreement with previously published results in juvenile mice by Maejima et al. (2001). The lack of any detectable Glu-, GABA$_B$-, or NO-dependent components of suppression of PF-EPSCs by DHPG in juvenile animals suggests in turn that these pathways are likely to mature later than endocannabinoid signaling. However, the present results (see above) also strongly suggest that the absence of endocannabinoid-independent suppression of PF-EPSCs by DHPG in juvenile rats is partly due to an unbalance between Glu release and Glu uptake in favor of the latter at early developmental stages, coupled with either immature pre-synaptic KA receptors and/or not fully developed calcium release from ryanodine sensitive internal stores.

In the present study as well as in that of Maejima et al. (2001) in juvenile animals, a transient potentiating effect of DHPG on PF-EPSC was observed, in particular in the presence of CB1 receptor antagonists. This was also true in nearly mature synapses when all pathways of the DHPG suppressive effect on PF-EPSCs were blocked (see results). This potentiation might be of postsynaptic origin since it was not accompanied by any significant change in PPF, in keeping with the potentiating effect of mGluR1 agonists on PC responsiveness to
ionophoretic application of Glu in their dendritic field (Levenes et al., 2001). Further studies will have to unravel underlying mechanisms of this transient potentiation of PF-EPSCs.

Therefore, sequential development of mechanisms underlying short-term plasticity at PF-PC synapses may at least partly explain apparent contradictions between data by Levenes et al. (2001) and by Maejima et al. (2001).

DHPG suppression of PF-EPSCs in nearly mature rats depends partly on retrograde release of glutamate and activation of pre-synaptic KA receptors.

In nearly mature rats, suppression of PF-EPSCs by DHPG was only partly blocked by CB1 receptor antagonists. Moreover, the remaining component was potentiated by Glu uptake inhibitors and markedly inhibited by the desensitizing KA receptor agonist SYM 2081 as well as by non saturating concentrations of the competitive AMPA/KA receptor antagonist CNQX (Honoré et al., 1988). However, this inhibitory effect was not reproduced by non saturating concentrations of the more selective AMPA receptor antagonist GYKI (Bureau et al., 1999, Renard et al., 1995; Wilding and Huettner, 1995). These results strongly suggest that the depressant effect of DHPG on PF-EPSCs not only involves retrograde release of endocannabinoids as in immature rats and mice, but also involves retrograde release of Glu by PCs and subsequent activation of KA receptors. Moreover, and as mentioned above, results in wild type and GluR6 -/- mice further suggest that these KA receptors include GluR6 subunits. Finally, the nearly complete blockade of endocannabinoid-independent suppression of PF-EPSCs with GDP-ßS makes unlikely that Glu responsible for this suppression might be spilling over in significant concentrations from neighboring cellular elements such as glial cells (Angulo et al., 2004; Parpura et al., 1994; see below).

The fact that the endocannabinoid-independent suppression of PF-EPSCs by mGluR1 agonists was still accompanied by an increase in PPF that itself was potentiated by Glu uptake
inhibitors and markedly inhibited by SYM 2081 as well as by non saturing concentrations of CNQX further suggests that, like for DSE in nearly mature rats (Crepel, 2007), KA receptors involved in agonist-induced suppression of PF-EPSCs are located on PFs. In contrast, the absence of significant modification of PF-EPSC kinetics during the endocannabinoid-independent suppression of PF-EPSCs (see results) suggests that released Glu does not reach post-synaptic AMPA receptors in significant concentrations, probably due to their lower sensitivity to glutamate than KA receptors (Lerma et al., 2001) and retarded transmitter diffusion around synaptic spines (Barbour et al., 1994).

Finally, DHPG suppression of PF-EPSCs in nearly mature rats is also likely to involve activation of pre-synaptic GABA\textsubscript{B} receptors and release of NO from molecular layer inhibitory interneurons (see results). Such a complexity of effects pertaining to DHPG may well have led to oversight of the CB1 receptor-dependent component of the PF-EPSC suppression induced by mGluR1 agonists in the previous study by Levenes et al. (2001).

**Effect of non saturing concentrations of CNQX and of GYKI on PPF and CV.**

In a previous study, Delaney and Jahr (2002) showed that weak activation of pre-synaptic KA receptors borne by PFs up-regulate Glu release by these fibers. This fits well with present results showing that non saturing concentrations of CNQX increase basal PPF and CV. Indeed, if one assumes that the basal extracellular Glu concentration is sufficient to induce weak tonic activation of pre-synaptic KA receptors that in turn increase release probability at PF-PC synapses, reversal of such an effect by CNQX will effectively lead to the observed increases in basal PPF and CV. Similarly, Delaney and Jahr (2002) showed that larger activation of pre-synaptic KA receptors borne by PFs down-regulates Glu release by these fibers. As such, the larger Glu concentration achieved through retrograde Glu release during DSE or during agonist-induced suppression of PF-EPSCs is now likely to down-
regulate Glu release at these synapses, perhaps through depletion of the readily releasable pool of synaptic vesicles (Crepel, 2007; Levenes et al., 2001), explaining the associated PPF increase.

In the present study, the increase in basal PPF induced by non saturating concentrations of GYKI raises a question, in particular because it was not accompanied by a corresponding increase in mean CV, at least when considering the first EPSCs within PF-EPSC pairs in PPF experiments. Accordingly, and even though this PPF increase was only half of that induced by non saturating concentrations of CNQX causing similar reduction in PF-EPSC amplitude (see results), it remains difficult to interpret these results solely on the basis of a partial antagonist effect of GYKI on pre-synaptic KA receptors that contain GluR6 subunits (Bureau et al., 1999). Indeed, this PPF increase was observed in all tested cells whereas the strong inhibition by GYKI of the effect of DHPG on PF-EPSCs only concerned less than a third of them (see results). Among other possible explanations for such a puzzling effect of GYKI, one can point to potassium efflux that occurs through post-synaptic AMPA receptors during PF-EPSCs. In PPF experiments, if one assumes that clearance of potassium released during pairs of EPSCs is incomplete when the next pair is elicited (PF stimulations at 0.33 Hz), such a residual increase in extracellular potassium concentration might contribute to steady depolarization of active zones, thereby leading to a decrease of basal PPF. Reversal of this effect by strong blockade of post-synaptic AMPA receptors by GYKI would therefore reduce release probability of Glu during EPSCs and thus explain the observed 16% basal PPF increase in these experiments. In keeping with this hypothesis, we nearly always noticed a progressive increase in PF-EPSC amplitude and a correlative progressive decrease of associated PPF during the first minutes of PF stimulations, prior to reaching a quasi steady-state (see Fig. 5A). Along the same line, increasing the frequency of PF stimulation from 0.1 to 0.33 Hz decreased within a few minutes basal PPF from $1.94 \pm 0.18$ to $1.42 \pm 0.20$ in the 4 tested cells
and this effect was accompanied by a correlative increase in amplitude of PF-EPSCs. Noteworthy, CV appeared more reliable than PPF in establishing that GYKI suppresses PF-EPSCs by selectively blocking post-synaptic AMPA receptors in these experiments since this depressant effect was not accompanied by any significant correlative increase in CV (see results). Following this interpretation, only about half of the basal PPF increase accompanying inhibition of PF-EPSCs by non saturating concentrations of CNQX would be genuinely due to its blocking effect on pre-synaptic KA receptors, in agreement with the correlative increase in CV.

Finally, it must be emphasized that the ratio $r = \text{mean basal PPF increase} / \text{mean EPSC amplitude decrease}$ during superfusion of the slices with GYKI was only 0.29 compared to 0.97 and 0.76 respectively for the ratio $R = \text{mean PPF increase} / \text{mean EPSC amplitude decrease}$ during DHPG application in the presence of SR141716-A + GYKI and in the presence of SR141716-A alone (see results). Therefore, the mechanism proposed above to explain the unexpected effects on PPF of blocking post-synaptic AMPA receptors by GYKI can hardly explain the bulk of the PPF increase accompanying suppression of PF-EPSCs by mGluR1 agonists. The latter remains therefore most likely due to activation of pre-synaptic KA receptors by Glu released by PCs. Concerning the KA-dependent component of DSE, the ratio $R$ was close to 0.5 on average (Crepel, 2007), so that it was no longer possible, by simply comparing $r$ values, to exclude that a significant contribution of prolonged clearance of potassium following PF-EPSCs participates in the observed PPF increase. However, the KA-dependent component of DSE was also accompanied by a significant increase in CV and, moreover, its prolonged time-course closely matched that of pre-synaptic ryanodine sensitive calcium transients induced by short domoate application to PFs (see results). Therefore, here again, PPF and CV increases accompanying DSE of PF-EPSCs are most likely primarily due to activation of pre-synaptic KA receptors by Glu released by PCs. On the whole, the present
data are also consistent with earlier reports on dendritic release of Glu from neocortical neurons (Ali et al., 2001; Harkany et al., 2004).

**Contribution of pre- and post-synaptic ryanodine-sensitive calcium stores to the KA-dependent components of DSE and to agonist-induced suppression of PF-EPSCs in nearly mature rats.**

Results of fluorometric experiments as well as those showing a nearly complete inhibition of the KA-dependent components of DSE and of agonist-induced suppression of PF-EPSCs by ryanodine suggest that their long duration is due to prolonged calcium release from pre-synaptic ryanodine-sensitive calcium stores, following initial activation of pre-synaptic KA receptors by Glu released by PCs. In particular, no prolonged ryanodine-sensitive calcium transient could be observed in PC dendrites following the same depolarizing pulses applied to PC soma as those used to induced DSE, whereas such prolonged ryanodine-sensitive calcium transients were readily elicited within PFs by focal application of low concentrations of the selective KA agonist domoate (see results). Due to technical constraints (see methods), we used focal applications of domoate lasting 60 seconds instead of only 4 seconds as in experiments by Duguid and Smart (2004). This may well explain why the duration of pre-synaptic ryanodine-sensitive calcium transients in the present experiments was about twice that of inhibitory synaptic noise elicited in PCs by brief activation of pre-synaptic NMDA receptors (Duguid and Smart; 2004) and also nearly 3 times longer than duration of the KA-dependent component of DSE (see Fig. 8B1; Crepel, 2007).

In addition, the present results also suggest that pre-synaptic ryanodine-sensitive calcium stores participate in the residual calcium increase responsible for basal PPF at PF-PC synapses since ryanodine also markedly reduced basal PPF values (see results). Finally, one cannot totally exclude that calcium release from post-synaptic ryanodine-sensitive calcium
stores (Carter et al., 2002, Isokawa and Alger, 2007) also participates to retrograde release of Glu from PCs. However, this contribution is unlikely to be important since, in DSE experiments in the presence of ryanodine, compensation for inhibition of post-synaptic calcium-induced calcium release by increasing post-synaptic stimulation failed to restore normal DSE (see results).

It must be acknowledged that these results appear to contradict previous observations by Carter et al. (2002), who concluded that pre-synaptic ryanodine-sensitive calcium stores do not contribute to synaptic transmission at PF-PC synapses in 10 to 22 day old rats. However, and interestingly enough, these authors suggested that internal calcium stores may well play a role in presynaptic function at later developmental stages as is the case for granule cells and their associated parallel fibers in the avian cerebellum where ryanodine receptors are only prominent in mature animals (Ouyang et al., 1997). Therefore, the possibility remains that subtle age differences of animals under study explain the apparent contradiction between the two set of results since, in particular, ages of rats in the present experiments correspond to the upper limit of those included in that of Carter et al. (2002).

Finally, the mechanisms described here could take place in physiological conditions and have functional consequences. Indeed, one knows that post-synaptic mGluR1 are activated by high frequency activity of PFs (Batchelor et al., 1994) and that the prolonged discharge of excitatory quantal events that follows short PF tetanus is sensitive to the mGluR1 antagonist AIDA as well as to the KA receptor blocker SYM 2081 (Crepel, 2007; Levenes et al., 2001). Thus, if one assumes that this Glu-dependent discharge of excitatory quantal events is sufficient to trigger action potentials along these fibers as previously suggested (Levenes et al., 2001), this would introduce a form of local communication among PCs sharing the same PF input. However, in these experiments, this enhanced discharge was terminated a few seconds after PF tetanus, whereas in the present study, the duration of the KA-sensitive
component of suppression of PF-EPSCs by S-DHPG outlasted its wash-out by several minutes (see results). Therefore, quantal events following short PF tetanus were likely to be due to the initial activation of pre-synaptic KA receptors by Glu released by PCs, i.e. the initial phase of the KA-dependent suppression of PF-EPSCs, rather than due to the subsequent prolonged ryanodine-sensitive calcium-induced calcium release seen here. One plausible hypothesis is that the initial activation of pre-synaptic KA receptors following short PF tetanus was insufficient to trigger such calcium-induced calcium release. Further studies will be required to determine physiological conditions in which fully developed endocannabinoid-independent suppression of PF-EPSCs can be induced.
AKNOWLEDGMENTS

We thank Alain Marty for precious advices and comments during the present experiments and on the manuscript, and Christophe Mulle for kindly providing GluR6 -/- mice as well as for helpful discussions. We also thank Gérard Sadoc for the Acquis1 software.
FIGURE LEGENDS

FIG. 1: Effects of DHPG on PF-EPSCs in control medium and in the presence of CB1 receptor antagonists in juvenile mice (A, B) and rats (C). A: Superimposed sweeps of PF-EPSCs elicited in one PC by 2 successive PF stimulations (interstimulus interval = 30 ms) in control solution (1), in the presence of 100 μM DHPG (2) and after washout (3) in a 11 day-old mouse. B: plot of mean (± SE; same in all figures except otherwise specified) normalized amplitudes of PF-EPSCs recorded from PCs over time in 10-12 day-old mice in control medium (black squares) and in the presence of 2 μM AM-251 (white squares). DHPG (100 μM) was added to the bath for 5 minutes, as indicated by the corresponding horizontal bar. Note that (1), (2) and (3) correspond to numbers indicated in A. C: same plots as in B in 10-12 day-old rats in control solution (black squares), and in the presence of 1 μM SR141716-A (white squares).

FIG. 2: Effects of DHPG on PF-EPSCs in control medium and in the presence of CB1 receptor antagonist in nearly mature rats. A: Superimposed sweeps of PF-EPSCs elicited in one PC by 2 successive PF stimulations (interstimulus interval = 30 ms) in control solution (1), in the presence of 100 μM DHPG (2) and after washout (3) in a 18 day-old rat. B1: plot of mean normalized amplitudes of PF-EPSCs recorded from PCs over time in 18-22 day-old rats in control medium (black squares) and in the presence of of 1 μM SR141716-A (white squares). Horizontal bar: duration of DHPG application as in Fig. 1. Note that (1) and (2) correspond to numbers indicated in A. B2: superimposed plots of mean PPF over control, 100 μM DHPG and washing periods in control medium (black lozenges) and in the presence of 1 μM SR141716-A (white lozenges) for the same PCs as in B1.
FIG. 3: Effect of DL-TBOA on the CB1 receptor-independent component of agonist-induced suppression of PF-EPSCs in nearly mature rats. A1: Superimposed sweeps of PF-EPSCs elicited by 2 successive PF stimulations in the presence of 1 μM SR141716-A and in the presence of 1 μM SR141716-A + 100 μM DL-TBOA. A2: idem as in A1 when 100 μM DHPG was added to the bath, and after wash out of DHPG. Note the marked effect of DL-TBOA on EPSC kinetics. B1: plots of mean normalized amplitudes of PF-EPSCs recorded from PCs over time in the presence of 1 μM SR141716-A alone (white squares) or of 1 μM SR141716-A + 100 μM DL-TBOA (grey squares). Horizontal bar: duration of DHPG application as in Fig. 1. PF-EPSC amplitudes in the presence of DL-TBOA were normalized with respect to values immediately prior to DHPG application. B2: plots of mean PPF of PF-EPSCs recorded in the same conditions and from the same PCs as in B1. White lozenges: 1 μM SR141716-A alone; grey lozenges: 1 μM SR141716-A + 100 μM DL-TBOA.

FIG. 4: Sensitivity to SYM 2081 of the CB1 receptor-independent component of agonist-induced suppression of PF-EPSCs in nearly mature rats. A: superimposed plots of mean normalized amplitudes of PF-EPSCs recorded from PCs over time in the presence of 1 μM SR141716-A alone (white squares), when 10 μM SYM 2081 was also present in the bath (grey squares), and when 300 nM CGP55845-A + 200 μM L-NAME were further added to the bath (black squares). Horizontal bar: duration of DHPG application as in Fig. 1. B: superimposed plots of mean normalized PPF of PF-EPSCs recorded in the same conditions and from the same PCs as in A. White lozenges: 1 μM SR141716-A alone; grey lozenges: 1 μM SR141716-A + 10 μM SYM 2081; black lozenges: 1 μM SR141716-A + 10 μM SYM 2081 + 300 nM CGP55845-A + 200 μM L-NAME.
FIG. 5: Sensitivity to CNQX and GYKI of the CB1 receptor-independent component of agonist-induced suppression of PF-EPSCs in nearly mature rats. A1: superimposed plots of mean PPF of PF-EPSCs recorded from PCs over time in the presence of 1 μM SR141716-A alone, when 1 μM CNQX or 20 μM GYKI (black and white lozenges respectively) were added to the superfusing medium, and when 100 μM DHPG was further added to the bath for 5 minutes as indicated by corresponding horizontal bars. A2: superimposed plots of mean normalized amplitude of PF-EPSCs recorded over time from the same PCs as in A1 in the presence of 1 μM SR141716-A + 1 μM CNQX (black squares) or in the presence of 1 μM SR141716-A + 20 μM GYKI (white and grey symbols; see below), and when 100 μM DHPG was further added to the bath for 5 minutes as indicated by the corresponding horizontal bar. Plots with grey and white squares correspond to the 3 and 8 cells where GYKI inhibited or not suppression of PF-EPSCs by DHPG respectively (see text). B: histogram of mean CV (+ SE) of PF-EPSC in PPF experiments. Bars labeled Con-1, CNQX-1, and GYKI-1 represent mean CV of first EPSCs within pairs recorded in the presence of 1 μM SR141716-A alone (white bar), of 1 μM SR141716-A + 1 μM CNQX (black bar) and of 1 μM SR141716-A + 20 μM GYKI (grey bar).

FIG. 6: Sensitivity of PF-EPSCs to DHPG in wild type and GluR6 -/- mice. A: superimposed plots of mean normalized amplitudes of PF-EPSCs recorded from PCs in the presence of 1 μM SR141716-A and when 100 μM DHPG was further added to the bath for 5 minutes (horizontal bar) in 22 to 24 day-old wild type (white squares) and in 22 to 24 day-old GluR6 -/- (black squares) mice. B: superimposed plots of mean normalized PPF of PF-EPSCs recorded in the same conditions and from the same PCs as in A. White lozenges: wild type mice; black lozenges: GluR6 -/- mice.
FIG. 7: Sensitivity to GDP-βS of the CB1 receptor-independent component of agonist-induced suppression of PF-EPSCs in nearly mature rats. 

A: superimposed plots of mean normalized amplitudes of PF-EPSCs recorded from PCs with a conventional K-gluconate internal solution (white squares) or with the same solution with 4mM GDP-βS added (black squares). In both cases, after at least 30 minutes dialysis of PCs, 100 μM DHPG was added to the bath at time 0 for 5 minutes as indicated by horizontal bar. In all experiments, 1 μM SR141716-A was present in the bathing medium throughout the recording period. 

B: superimposed plots of mean normalized PPF of PF-EPSCs recorded in the same conditions and from the same PCs as in A. White lozenges: conventional K-gluconate internal solution; black lozenges: same solution with 4mM GDP-βS added.

FIG. 8: Sensitivity to ryanodine of agonist-induced suppression of PF-EPSCs and of DSE in nearly mature rats. 

A1: superimposed plots of mean normalized amplitudes of PF-EPSCs recorded from PCs over time in the presence of 1 μM SR141716-A alone (white squares) or of 1 μM SR141716-A + 100 μM ryanodine (black squares) before, during and after 100 μM DHPG was further added to the bath, as indicated by the corresponding horizontal bar. 

A2: superimposed plots of mean PPF of PF-EPSCs recorded in the same conditions and from the same PCs as in A1. White lozenges: 1 μM SR141716-A alone; black lozenges: 1 μM SR141716-A + 100 μM ryanodine. 

B1: superimposed plots of mean normalized amplitudes of PF-EPSCs recorded from PCs over time in the presence of 1 μM SR141716-A alone (white squares; data taken from Crepel, 2007) or of 1 μM SR141716-A + 100 μM ryanodine before during and after a depolarizing pulse from -70 to 0 mV for 1 sec (black squares), applied at time 0 (arrow). Plot with grey squares; same as plot with black squares, except that depolarizing pulse duration was 2 sec. 

B2: superimposed plots of mean normalized PPF of
PF-EPSCs recorded in the same conditions and from the same PCs as in B1. White squares: 1 μM SR141716-A alone; black squares: 1 μM SR141716-A + 100 μM ryanodine.

FIG. 9: In 18 to 22 day-old rats, ryanodine inhibit cytosolic calcium transients elicited in PFs by local superfusion of domoate (A), and in PC dendrites by depolarizing voltage steps applied to the soma (B). A1: superimposed mean ΔF/F plots of the initial phase (5 minutes) of pre-synaptic calcium signals induced in PFs by focal application of domoate (see methods) for 1 minute (arrow at time 0) in control condition and in the presence of 100 μM ryanodine in the bath (black and white lozenges respectively). A2: same as in A1 over the entire recording period of 30 minutes. B1: superimposed mean ΔF/F plots of the initial phase (1.5 seconds) of pre-synaptic calcium signals induced in PC dendrites by depolarizing voltage steps of 1 second from -70 to 0 mV (lower trace) in control condition and in the presence of 100 μM ryanodine in the bath. B2: same as in B1 over the entire recording period of 20 seconds. Same symbols as in A1, A2.

FIG 10: Origin of the lack of KA-dependent component of agonist-induced suppression of PF-EPSCs in immature rats. A: plot of mean normalized amplitudes of PF-EPSCs recorded from PCs over time in 10-12 day-old rats in the presence of 1 μM SR141716-A (white squares), as well as in the presence of 1 μM SR141716-A + 100 μM DL-TBOA in the bath (grey squares). DHPG (100 μM) was added to the bath for 5 minutes, as indicated by the corresponding horizontal bar. B1, B2, B3: pre-synaptic calcium signals induced in PFs by focal application of domoate (see methods) for 1 minute at time 0 (horizontal bar) in 12 day-old rats. B1: superimposed mean ΔF/F plots in the presence of 1 μM SR141716-A alone (white lozenges), in the presence of 1 μM SR141716-A + 100 μM DL-TBOA (grey lozenges) and when 100 μM ryanodine was also present in the bath (black lozenges). B2: same as in B1
when ryanodine was replaced by 50 μM D-APV in the bath (black circles). B3: superimposed mean ΔF/F plots in the presence of 1 μM SR141716-A alone (white lozenges), in the presence of 1 μM SR141716-A + 100 μM DL-TBOA + 50 μM D-APV (black circles) and when 1 μM TTX was also present in the bath (grey circles).
REFERENCES


Baude A, Nusser Z, Roberts JDB, Mulvihill E, McIlhinney RAJ, Somogyi P. The metabotropic glutamate receptor (MGluR1a) is concentrated at perisynaptic membrane of neuronal subpopulations as detected by immunogold reaction. Neuron 11: 771-787, 1993.

Berman FW, Murray TF. Domoic acid neurotoxicity in cultured cerebellar granule neurons is mediated predominantly by NMDA receptors that are activated as a consequence of excitatory amino acid release. J Neurochem 69: 693-703, 1997.


Kim J, Alger BE. Random response fluctuations lead to spurious paired-pulse facilitation. J


Shin JH, Linden DJ. An NMDA receptor/nitric oxide cascade is involved in cerebellar LTD but is not localized to the parallel fiber terminal. *J Neurophysiol*: 4281-4289, 2005.


FIG. 1: Effects of DHPG on PF-EPSCs in control medium and in the presence of CB1 receptor antagonists in juvenile mice (A, B) and rats (C). A: Superimposed sweeps of PF-EPSCs elicited in one PC by 2 successive PF stimulations (interstimulus interval = 30 ms) in control solution (1), in the presence of 100 μM DHPG (2) and after washout (3) in a 11 day-old mouse. B: plot of mean (± SE; same in all figures except otherwise specified) normalized amplitudes of PF-EPSCs recorded from PCs over time in 10-12 day-old mice in control medium (black squares) and in the presence of 2 μM AM-251 (white squares). DHPG (100 μM) was added to the bath for 5 minutes, as indicated by the corresponding horizontal bar. Note that (1), (2) and (3) correspond to numbers indicated in A. C: same plots as in B in 10-12 day-old rats in control solution (black squares), and in the presence of 1 μM SR141716-A (white squares).
FIG. 2: Effects of DHPG on PF-EPSCs in control medium and in the presence of CB1 receptor antagonist in nearly mature rats. A: Superimposed sweeps of PF-EPSCs elicited in one PC by 2 successive PF stimulations (interstimulus interval = 30 ms) in control solution (1), in the presence of 100 μM DHPG (2) and after washout (3) in a 18 day-old rat. B1: plot of mean normalized amplitudes of PF-EPSCs recorded from PCs over time in 18-22 day-old rats in control medium (black squares) and in the presence of 1 μM SR141716-A (white squares). Horizontal bar: duration of DHPG application as in Fig. 1. Note that (1) and (2) correspond to numbers indicated in A. B2: superimposed plots of mean PPF over control, 100 μM DHPG and washing periods in control medium (black lozenges) and in the presence of 1 μM SR141716-A (white lozenges) for the same PCs as in B1.
FIG. 3: Effect of DL-TBOA on the CB1 receptor-independent component of agonist-induced suppression of PF-EPSCs in nearly mature rats. A1: Superimposed sweeps of PF-EPSCs elicited by 2 successive PF stimulations in the presence of 1 μM SR141716-A and in the presence of 1 μM SR141716-A + 100 μM DL-TBOA. A2: idem as in A1 when 100 μM DHPG was added to the bath, and after wash out of DHPG. Note the marked effect of DL-TBOA on EPSC kinetics. B1: plots of mean normalized amplitudes of PF-EPSCs recorded from PCs over time in the presence of 1 μM SR141716-A alone (white squares) or of 1 μM SR141716-A + 100 μM DL-TBOA (grey squares). Horizontal bar: duration of DHPG application as in Fig. 1. PF-EPSC amplitudes in the presence of DL-TBOA were normalized with respect to values immediately prior to DHPG application. B2: plots of mean PPF of PF-EPSCs recorded in the same conditions and from the same PCs as in B1. White lozenges: 1 μM SR141716-A alone; grey lozenges: 1 μM SR141716-A + 100 μM DL-TBOA.
FIG. 4: Sensitivity to SYM 2081 of the CB1 receptor-independent component of agonist-induced suppression of PF-EPSCs in nearly mature rats. A: superimposed plots of mean normalized amplitudes of PF-EPSCs recorded from PCs over time in the presence of 1 μM SR141716-A alone (white squares), when 10 μM SYM 2081 was also present in the bath (grey squares), and when 300 nM CGP55845-A + 200 μM L-NAME were further added to the bath (black squares). Horizontal bar: duration of DHPG application as in Fig. 1. B: superimposed plots of mean normalized PPF of PF-EPSCs recorded in the same conditions and from the same PCs as in A. White lozenges: 1 μM SR141716-A alone; grey lozenges: 1 μM SR141716-A + 10 μM SYM 2081; black lozenges: 1 μM SR141716-A + 10 μM SYM 2081 + 300 nM CGP55845-A + 200 μM L-NAME.
FIG. 5: Sensitivity to CNQX and GYKI of the CB1 receptor-independent component of agonist-induced suppression of PF-EPSCs in nearly mature rats. A1: superimposed plots of mean PPF of PF-EPSCs recorded from PCs over time in the presence of 1 μM SR141716-A alone, when 1 μM CNQX or 20 μM GYKI (black and white lozenges respectively) were added to the superfusing medium, and when 100 μM DHPG was further added to the bath for 5 minutes as indicated by corresponding horizontal bars. A2: superimposed plots of mean normalized amplitude of PF-EPSCs recorded over time from the same PCs as in A1 in the presence of 1 μM SR141716-A + 1 μM CNQX (black squares) or in the presence of 1 μM SR141716-A + 20 μM GYKI (white and grey symbols; see below), and when 100 μM DHPG was further added to the bath for 5 minutes as indicated by the corresponding horizontal bar. Plots with grey and white squares correspond to the 3 and 8 cells where GYKI inhibited or not suppression of PF-EPSCs by DHPG respectively (see text). B:
histogram of mean CV (+ SE) of PF-EPSC in PPF experiments. Bars labeled Con-1, CNQX-1, and GYKI-1 represent mean CV of first EPSCs within pairs recorded in the presence of 1 μM SR141716-A alone (white bar), of 1 μM SR141716-A + 1 μM CNQX (black bar) and of 1 μM SR141716-A + 20 μM GYKI (grey bar).
FIG. 6: Sensitivity of PF-EPSCs to DHPG in wild type and GluR6 -/- mice. A: superimposed plots of mean normalized amplitudes of PF-EPSCs recorded from PCs in the presence of 1 μM SR141716-A and when 100 μM DHPG was further added to the bath for 5 minutes (horizontal bar) in 22 to 24 day-old wild type (white squares) and in 22 to 24 day-old GluR6 -/- (black squares) mice. B: superimposed plots of mean normalized PPF of PF-EPSCs recorded from the same PCs and from the same conditions as in A. White lozenges: wild type mice; black lozenges: GluR6 -/- mice.
FIG. 7: Sensitivity to GDP-βS of the CB1 receptor-independent component of agonist-induced suppression of PF-EPSCs in nearly mature rats. A: superimposed plots of mean normalized amplitudes of PF-EPSCs recorded from PCs with a conventional K-gluconate internal solution (white squares) or with the same solution with 4mM GDP-βS added (black squares). In both cases, after at least 30 minutes dialysis of PCs, 100 μM DHPG was added to the bath at time 0 for 5 minutes as indicated by horizontal bar. In all experiments, 1 μM SR141716-A was present in the bathing medium throughout the recording period. B: superimposed plots of mean normalized PPF of PF-EPSCs recorded in the same conditions and from the same PCs as in A. White lozenges: conventional K-gluconate internal solution; black lozenges: same solution with 4mM GDP-βS added.
FIG. 8: Sensitivity to ryanodine of agonist-induced suppression of PF-EPSCs and of DSE in nearly mature rats. 

A1: superimposed plots of mean normalized amplitudes of PF-EPSCs recorded from PCs over time in the presence of 1 μM SR141716-A alone (white squares) or of 1 μM SR141716-A + 100 μM ryanodine (black squares) before, during and after 100 μM DHPG was further added to the bath, as indicated by the corresponding horizontal bar. 

A2: superimposed plots of mean PPF of PF-EPSCs recorded in the same conditions and from the same PCs as in A1. White lozenges: 1 μM SR141716-A alone; black lozenges: 1 μM SR141716-A + 100 μM ryanodine. 

B1: superimposed plots of mean normalized amplitudes of PF-EPSCs recorded from PCs over time in the presence of 1 μM SR141716-A alone (white squares; data taken from Crepel, 2007) or of 1 μM SR141716-A + 100 μM ryanodine before during and after a depolarizing pulse from -70 to 0 mV for 1 sec (black squares), applied at time 0 (arrow). Plot with grey squares; same as plot with black squares, except that depolarizing pulse duration was 2 sec. 

B2: superimposed plots of mean normalized PPF of PF-EPSCs recorded in the same conditions and from the same PCs as in B1. White squares: 1 μM SR141716-A alone; black squares: 1 μM SR141716-A + 100 μM ryanodine.

177x127mm (600 x 600 DPI)
FIG. 9: In 18 to 22 day-old rats, ryanodine inhibit cytosolic calcium transients elicited in PFs by local superfusion of domoate (A), and in PC dendrites by depolarizing voltage steps applied to the soma (B). A1: superimposed mean ΔF/F plots of the initial phase (5 minutes) of pre-synaptic calcium signals induced in PFs by focal application of domoate (see methods) for 1 minute (arrow at time 0) in control condition and in the presence of 100 μM ryanodine in the bath (black and white lozenges respectively). A2: same as in A1 over the entire recording period of 30 minutes. B1: superimposed mean ΔF/F plots of the initial phase (1.5 seconds) of pre-synaptic calcium signals induced in PC dendrites by depolarizing voltage steps of 1 second from -70 to 0 mV (lower trace) in control condition and in the presence of 100 μM ryanodine in the bath. B2: same as in B1 over the entire recording period of 20 seconds. Same symbols as in A1, A2.
FIG 10: Origin of the lack of KA-dependent component of agonist-induced suppression of PF-EPSCs in immature rats. A: plot of mean normalized amplitudes of PF-EPSCs recorded from PCs over time in 10-12 day-old rats in the presence of 1 μM SR141716-A (white squares), as well as in the presence of 1 μM SR141716-A + 100 μM DL-TBOA in the bath (grey squares). DHPG (100 μM) was added to the bath for 5 minutes, as indicated by the corresponding horizontal bar. B1, B2, B3: pre-synaptic calcium signals induced in PFs by focal application of domoate (see methods) for 1 minute at time 0 (horizontal bar) in 12 day-old rats. B1: superimposed mean ΔF/F plots in the presence of 1 μM SR141716-A alone (white lozenges), in the presence of 1 μM SR141716-A + 100 μM DL-TBOA (grey lozenges) and when 100 μM ryanodine was also present in the bath (black lozenges). B2: same as in B1 when ryanodine was replaced by 50 μM D-APV in the bath (black circles). B3: superimposed mean ΔF/F plots in the presence of 1 μM SR141716-A alone (white lozenges), in the presence of 1 μM SR141716-A + 100 μM DL-TBOA + 50 μM D-APV (black circles) and when 1 μM TTX was also present in the bath (grey circles).