Developmental Changes in Retrograde Messengers Involved in Depolarization-Induced Suppression of Excitation at Parallel Fiber-Purkinje Cell Synapses in Rodents

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INTRODUCTION

In the brain, retrograde signaling allows fast control of presynaptic neurotransmitter release by postsynaptic cells. The first example of such retrograde signaling, i.e., depolarization-induced suppression of inhibition (DSI), consists of a transient reduction of GABA release at inhibitory synapses impinging onto hippocampal pyramidal cells and onto cerebellar Purkinje cells (PCs), respectively (Llano et al. 1991a; Pitler and Alger 1992, 1994; Vincent et al. 1992) when these neurons are briefly depolarized (Glitsch et al. 2000; Pitler and Alger 1992; Wang and Zucker 2001; Wilson and Nicoll 2001). Although glutamate (Glu) was the first candidate to be proposed as retrograde messenger (Glitsch et al. 1996; Morishita et al. 1998), it was later established that DSI is due to retrograde release of endocannabinoids, which, by activating presynaptic Type 1 Cannabinoid (CB1) receptors, transiently reduces GABA release at inhibitory synapses in the hippocampus (Ohno-Shosaku et al. 2001; Wilson and Nicoll 2001; Wilson et al. 2001) and in the cerebellum (Diana et al. 2002; Kreitzer and Regehr 2001a).

At excitatory synapses impinging onto PCs, a similar mechanism exists and is termed depolarization-induced suppression of excitation (DSE) (Kreitzer and Regehr 2001b). Here again, brief depolarization of PCs transiently depresses excitatory inputs to these cells through retrograde release of endocannabinoids acting on presynaptic CB1 receptors located on parallel fibers (PFs) and on climbing fibers (Brenowitz and Regehr 2003; Kreitzer and Regehr 2001b; Safo and Regehr 2005).

Activation of postsynaptic mGluR1 metabotropic Glu receptors by PCs by selective agonists (Galante and Diana 2004; Levenes et al. 2001; Maejima et al. 2001) or by sustained PF stimulation (Brown et al. 2003; Maejima et al. 2001; Neale et al. 2001) also inhibits 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). As for DSE, most studies favored endocannabinoids as being the retrograde messenger involved (Galante and Diana 2004; Maejima et al. 2001). However, Levenes et al. (2001) suggested instead that retrograde release of Glu by PCs was responsible for the observed depression of PF-EPSCs, and this through activation of presynaptic ionotropic Glu receptors borne by PFs.

Because most studies on the role of endocannabinoids in retrograde signaling at PF-PC synapses were 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 might be explained by such developmental differences. Therefore the aim of the present experiments was to investigate the possibility that retrograde signaling at PF-PC synapses changes throughout development. Because DSE involves less possible indirect effects than suppression of PF-EPSCs by mGluR1 agonists, the study was mainly focused on retrograde messengers involved in DSE at parallel fiber (PF) to Purkinje cell (PC) synapses, depolarization-induced suppression of excitation (DSE) and suppression of PF-episynaptic currents (EPSCs) by activation of postsynaptic mGluR1 glutamate (Glu) receptors involve retrograde release of endocannabinoids. However, Levenes et al. suggested instead that Glu was the retrograde messenger in this latter case. Because the study by Levenes et al. was performed in nearly mature rats, whereas most others were performed in juvenile animals, DSE was re-investigated in juvenile versus nearly mature rats and mice. Indeed, DSE was partly indirect effects in this latter case. In 10- to 12-day-old rats, DSE of PF-EPSCs was entirely mediated through retrograde release of endocannabinoids. In 18- to 22-day-old rats, DSE was partly resistant to CB1 cannabinoid receptor antagonists. The remaining component was potentiated by the Glu uptake inhibitor D-threo-beta-benzylxoyaspartate (D-TBOA) and blocked by the desensitizing kainate (KA) receptor agonist (2S,4R)-4-methylglutamic acid (SYM 2081). This SYM-2081-sensitive component of DSE was accompanied by a paired-pulse facilitation increase that was also potentiated by D-TBOA and blocked by SYM 2081. In nearly mature wild-type mice, results fully confirmed the presence of an endocannabinoid-independent component of DSE that involves retrograde release of Glu and activation of presynaptic KA receptors including GluR6 receptor subunits. Therefore retrograde release of Glu by PCs participates to DSE at PF-PC synapses in nearly mature rodents but not in juvenile ones, and Glu probably operates through activation of presynaptic KA receptors that include GluR6 receptor subunits.


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PF-PC synapses in juvenile versus nearly mature rats as well as in wild-type and knock-out (KO) mice for the Glu receptor 6 (GluR6) subunit of kainate (KA) receptors.

METHODS

Experiments complied with guidelines of the French Animal Care Committee. They were performed on 10- to 12-day-old and on 18- to 22-day-old male rats (Sprague-Dawley) as well as on 18- to 24-day-old C56BL/6 and GluR6−/− mice. In all cases, animals were stunned before decapitation and parasagittal slices, 250 μm thick, were cut in ice-cold saline solution (see following text) from the cerebellar vermis with a vibroslicer. Slices were incubated at room temperature in saline solution bubbled with 95% O2/5% CO2 for ≥1 h. The recording chamber was perfused at a rate of 2 ml/min with oxygenated saline solution containing (in mM) 124 NaCl, 3 KCl, 24 NaHCO3, 1.15 KH2PO4, 1.15 MgSO4, 2 CaCl2, 10 glucose plus the GABA_A antagonist bicuculline methochloride (10 μM, Sigma Aldrich, St. Quentin Fallavier, France) with osmolarity 320 mOsm and final pH 7.35 at 24–25°C except when otherwise specified. PCs were directly visualized with Nomarski optics through the ×40 water-immersion objective of an upright microscope (Zeiss).

Drugs were added to the superfusate, d-threo-benzoyloxyaspartate (d-TBOA), (RS)-a-methylserine-O-phosphate (MSOP), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), (2S,4R)-4-methylglutamic acid (SYM 2081), and N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM-251) were purchased from Tocris (Illkirch, France). The CB1 cannabinoid receptor antagonist N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazol-3-carboxamide hydrochloride (SR141716-A) (see Levenes et al. 1998) was provided by Sanofi-Recherche (Montpellier, France). Stock solutions of drugs (dissolved in water and/or DMSO depending on the manufacturer recommendations) were added to oxygenated saline solution at the desired concentration.

Electrophysiology

Recordings using the patch-clamp technique were performed at a somatic level, using an Axopatch-200A amplifier (Axon instruments). Stimulating electrodes consisted in saline filled monopolar electrodes. PF stimulations were performed at 0.5 Hz, except otherwise specified. In experiments on DSE, patch pipettes (2–4 MΩ) were filled either with a solution containing (in mM) 140 Cs-glucocan, 6 KCl, 10 HEPES, 0.2 EGTA, 1 MgCl2, 4 Na2-ATP, 0.4 Na-GTP, and 20 TEA or with the same Cs-glucocan-based solution where TEA was omitted (pH and osmolarity adjusted accordingly). In experiments that included postsynaptic calcium buffer, the same Cs-glucocan-based solution without TEA as before was used, except that 40 mM Cs-glucocan was replaced by 40 mM Cs-BAPTA. Finally, in experiments on unitary synaptic events (uEPSCs; see following text), the internal solution contained (in mM): 140 K-glucocan, 6 KCl, 10 HEPES, 0.75 EGTA, 1 MgCl2, 4 Na2-ATP, and 0.4 Na-GTP; pH: 7.35 with KOH; 300 mOsm. 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. (1991b). Cells were held at a membrane potential of −70 mV and PF-EPSCs were associated with 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 amplitude were applied to the cell with an inter-stimulus interval of 30 ms, and ratio of the amplitude of the second PF-EPSC over the first one was calculated on-line. Mean PPF values of group data (see RESULTS) were obtained by two methods. The first one consisted of averaging PPFs in individual traces during the 2 min preceding the depolarizing step and between 2 and 5 min after this step, i.e., during control period and DSE, respectively, to obtain mean trace group PPF for each cell studied in a given condition. Because this conventional method can produce spurious results (Kim and Alger 2001), the alternative method proposed by these authors was also used that consisted in calculating for the same trace groups as above mean trace group PPF = mean amplitude of the second EPSC/mean amplitude of the first one. In both cases, these mean PPF values of trace group were then averaged for all cells recorded in a given experimental condition to obtain mean PPF values of group data during control period and during DSE. Because PPF increases associated to DSE could be small in certain experimental conditions (see RESULTS) and obscured by variability of basal PPF across cells, mean PPFs determined in trace groups by both methods were further normalized in these experiments by determining for each cell mean normalized PPF = 100 × (mean PPF/PF1), where PPF1 was the individual PPF value of the last trace preceding the depolarizing step. For the same reason, PPF plots in Figs. 5 and 6 were also constructed by using normalized PPF values.

Synaptic currents were usually filtered at 5 kHz and digitized on-line at 20 kHz, and PF-EPSCs were analyzed on- and off-line by using the Acqis1 computer program (Biologic). For analysis of unitary uEPSCs, currents were further filtered at 2 kHz and analyzed off-line using Axograph 4.6 software (Axon Instruments). The detection of synaptic events was automatically performed using a typical event as a template function (Clements and Bekkers 1997) and event detection was optimized using a signal-to-noise ratio with a threshold value equal to three times the SD noise. Events were retained for further analysis on the basis of windows for allowed minimum/maximum amplitudes, rise-times, half-widths, and instantaneous frequencies. These parameters were adjusted so that most visible synaptic events were detected and, on the contrary, no detection occurred in current traces recorded from the same cells in the presence of 20 μM CNQX added in the bath at the end of the recording session. Thus threshold amplitude for retained events was usually set at 5 or 10 pA depending on cells. For each cell, analysis was performed on a few hundred of such selected spontaneous or evoked synaptic events as described in the following text. Posttetanic uEPSCs were detected during the first 1 or 2 s after each train of PF-EPSCs (see RESULTS), whereas the 1 or 2 s preceding each train was considered as a control period. For each cell and for each experimental condition (see RESULTS), the relative frequency of evoked uEPSCs was calculated by dividing the total number of posttetanic uEPSCs evoked during 20–40 successive trials by that of corresponding spontaneous uEPSCs in the same sequences.

As in previous experiments by Daniel et al. (1999), epifluorescence microscopy (upright Zeiss microscope, ×60 water-immersion objective) was used to detect intracellular free calcium concentration changes from an area of 90 × 90 μm centered on dendrites of the recorded cells. In these experiments, the low-affinity calcium indicator Fluo-4FF impermeant (100 μM, Molecular Probes) was added to the same Cs-glucocan-based solution with or without TEA as used in experiments on DSE (see preceding text). The recording session started 30–45 min after whole cell break in to allow diffusion of the dye in the dendrites. Fluo4FF filter set was 485DF22 nm for excitation, DM505 nm dichroic and 530DF30 nm emission. Fluorometric signals were collected with a photometer and analyzed on- and off-line using the Acquis1 computer program. The fluorescence data were expressed as changes in ΔF/Δt, where F was the baseline fluorescence intensity, and ΔF the change induced by depolarizing pulses applied to PC soma.
RESULTS

DSE entirely depends on retrograde release of endocannabinoids in juvenile rats but not in nearly mature rats

In 10- to 12-day-old rats and when using a Cs-based intra-pipette solution with TEA and 2 mM QX-314 as in previous experiments by Brenowitz and Reggehr (2003), depolarization of PCs from −70 to 0 mV for 1 s, followed by their hyperpolarization to −90 mV for 200 ms, induced a large and fast DSE in eight of the nine tested cells that was generally reliably elicited two to three times for each of these cells. In these eight PCs, the decrease in PF-EPSC amplitude reached 69.35 ± 5.37% within the few seconds following the voltage step and PF-EPSCs completely recovered their initial amplitude within less than one minute on average (Fig. 1A1, 1 and 2). Furthermore and as expected for DSE, suppression of EPSCs was accompanied by a large and significant (P < 0.002) increase in PPF, from 1.31 ± 0.09 to 2.44 ± 0.22%. In the ninth cell tested, the depolarizing pulse failed to induce such fast DSE. In marked contrast, DSE was completely abolished when the CB1 receptor antagonist AM-251 was applied to the slice at a saturating concentration of 2 μM (Gatley et al. 1996) since ≥30 min (n = 7; Fig. 1A2). In four other cells, DSE was also completely abolished by bath application for 20–30 min of the other potent CB1 receptor antagonist SR141716-A (references in Levenes et al. 1998), at a saturating concentration of 1 μM (Petitet et al. 1996) (Fig. 1A2). Therefore these results fully confirm that DSE entirely depends on retrograde release of endocannabinoids in juvenile animals (Brenowitz and Reggehr 2003; Kreitzer and Reggehr 2001b; Safo and Reggehr 2005).

In 18- to 22-day-old rats, DSE induced in the same experimental conditions as before now consisted of an initial fast component followed by slower one (see following text) that could be also repeatedly and reliably elicited over the duration of an experiment. As in juvenile rats, the initial fast component lasted <1 min (Fig. 1B1, 1 and 2) and was again accompanied by a significant increase in PPF from 1.37 ± 0.08 to 1.79 ± 0.05% (n = 8; P < 0.01). However, the mean decrease in PF-EPSC amplitude was insignificantly smaller (P < 0.01) than in juvenile rats because it reached only 35.58 ± 6.64% within the few seconds following the voltage step. The slow component exhibited a much smaller amplitude (mean decrease = 13.67 ± 3.77%) and a much longer duration, i.e., ≤7 min (Fig. 1B2 and 3). Nearly identical results were obtained when QX-314 was omitted in the TEA-containing medium and when the depolarizing pulse was no longer followed by a 200 ms hyperpolarizing pulse to −90 mV (n = 6; Fig. 1B1, 2 and 3).

Moreover, whereas the initial fast component of DSE was still abolished by bath application of 1 μM SR141716-A or 2 μM AM-251 (n = 5 and 4, respectively), its slow component remained unaffected (peak amplitude = 14.92 ± 1.33%; Fig. 1B2, 2 and 3; see also Figs. 2C and 3C). Noteworthy, this CB1 receptor-independent component of DSE was very similar in amplitude and time course to DSE observed in experiments performed with a Cs-based intra-pipette solution without TEA, whatever CB1 receptor antagonist was present or not in the bath (see following text).

Differential dependence on calcium of components of DSE in nearly mature rats

From now on, electrophysiological experiments were always performed with the Cs-based intra-pipette solution without TEA. Moreover, whereas PF-EPSCs were still evoked at 0.5 Hz, their individual amplitudes were averaged off-line every three stimulations for clarity of plots extending on several minutes (Figs. 2C and 3C). In most PCs recorded in such conditions in 18- to 22-day-old rats, stepping the voltage of the
postsynaptic PC from −70 to 0 mV for 1 s induced a DSE of PF-EPSCs of small amplitude and prolonged duration, whatever CB1 receptor antagonist was present or not in the bath. In control bathing medium, this was the case for 8 of 11 recorded cells (Fig. 2C) with a mean decrease in PF-EPSC amplitude at the peak of DSE of 14.91 ± 2.54%. In the three remaining PCs, a fast initial component of DSE (see preceding text) was also present but with a small amplitude of only 20–26% depending on cells (not illustrated). In the presence of 1 µM SR141716-A, only the slow component of DSE could be observed in the 17 tested cells, with a mean peak amplitude of 12.14 ± 1.43% (Figs. 2C and 3, A1 and C).

Retrograde release of endocannabinoids responsible for DSE at PF-PC synapses is known to depend on free calcium concentration increase in depolarized PCs (Brenowitz and Regehr 2003). Therefore comparison of results obtained in 18- to 22-day-old rats with and without TEA in the internal solution and with or without SR141716-A in the bathing medium suggests that the endocannabinoid-independent component of DSE requires a larger free calcium increase during depolarization than that needed for the endocannabinoid-independent one, provided that the latter is also calcium dependent (see following text). Here, and as in experiments by Brenowitz and Regehr (2003) and by Maejima et al. (2001), the large free calcium concentration increase needed to release endocannabinoids in 18- to 22-day-old rats might have been achieved only when TEA was added to the internal solution to block a fraction of K channels of PCs that were not blocked by Cs alone (McKay and Turner 2004). This hypothesis was tested directly by measuring free calcium concentration increase elicited in PC dendrites by the same voltage steps applied to the soma as before in cells that were recorded with Cs-glutamate-based solutions with or without TEA. Significant differences (P < 0.01) in calcium transients were indeed observed since mean peak ∆F/ΔF induced by 1-s depolarizing pulses from −70 to 0 mV was 0.56 ± 11% (n = 4) when TEA was present in the intra pipette solution, whereas it was only 0.07 ± 0.02% (n = 4) when TEA was omitted (Fig. 2, A and B).

In the same SR141716-A-containing bathing medium as before, the calcium dependence of the endocannabinoid-independent component of DSE was tested directly in six PCs dialyzed for ≥30–40 min with an internal solution containing 40 mM BAPTA. In such conditions, DSE was significantly (P < 0.001) inhibited because its mean peak amplitude now amounted to only 6.71 ± 0.91% (Fig. 2C). This residual DSE was not accompanied by significant changes in PPF (Fig. 2C) thus in marked contrast with CB1 receptor-independent component of DSE recorded in control condition (see next sections). Therefore these results strongly suggest that in nearly mature rats, DSE at PF-PC synapses not only depends on retrograde release of endocannabinoids but also partly depends on another calcium-dependent mechanism (see Discussion). Furthermore, they also support the assumption that the endocannabinoid-dependent component of DSE requires a larger free calcium increase during depolarization than that needed for the cannabimoid-independent one.

Sensitivity of the CB1 receptor-independent component of DSE to Glu uptake blocker and to desensitizing KA receptor agonist in nearly mature rats

The study by Levenes et al. (2001) suggests that in nearly mature rats, retrograde release of Glu by PCs after activation of postsynaptic mGluR1 receptors decreases PF-EPSCs through activation of presynaptic ionotropic Glu receptors borne by PFs. If the endocannabinoid-independent component of DSE observed here in the presence of SR141716-A in 18- to 22-day-old rats is also due to retrograde release of Glu from depolarized PCs, it should be enhanced in the presence of the Glu uptake inhibitor d-TBOA (references in Angulo et al. 2004) and suppressed by ionotropic Glu receptor antagonists (see following text).

In the presence of 1 µM SR141716-A in the bath and as expected for a Glu uptake inhibitor, application of 100 µM d-TBOA induced an increase in amplitude of PF-EPSCs ranging between 15 and 65% depending on cells with a significant mean increase of 33.82 ± 5.80% (n = 14; P < 0.01; Fig. 3B). The rather large variability of potentiating effects of d-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. 3A, 1 and 2), probably due to slower clearance of Glu from synaptic cleft. In such conditions, the mean decrease in amplitude of PF-EPSCs 2 min after the depolarizing step to 0 mV was 17.04 ± 1.31% of control (n = 14; Fig. 3, A2 and C) against only 12.14 ± 1.43% as mentioned before in the presence of SR141716-A alone (Fig. 3, A1 and C). This 42% increase in amplitude of DSE in the presence of d-TBOA was highly significant (P < 0.001) when calculated on pooled values between 1.5 and 2.5 min after the depolarizing pulse. Therefore these results suggests that the cannabinoid-independent component of DSE observed in the presence
of CB1 receptor antagonists is at least in part due to retrograde release of Glu by PCs.

Now if one assumes that the CB1 receptor-independent component of DSE involves activation of presynaptic ionotropic Glu receptors borne by PCs (see preceding text), it should be blocked by ionotropic Glu receptor antagonists. Among possible candidates for such presynaptic receptors, two have been already identified, i.e., NMDA and KA receptors (Casado et al. 2000; Delaney and Jahr 2002). Kainate receptors are highly sensitive to SYM 2081, a potent ligand that, at micromolar concentrations, selectively blocks KA-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 and independently of whether 100 µM D-TBOA was present or not in the bath, superfusion of the slices for ≥30 min with 10 µM SYM 2081 markedly inhibited a late phase of the CB1 receptor-independent component of DSE while leaving its initial phase unaffected (n = 7 and n = 8 respectively; Fig. 3C). Differences with results obtained in the absence of SYM 2081 (see before) were quantified by determining for each cell a value \( \delta = 100(1 - \text{amplitude of normalized EPSC for each point of EPSC plots}) \) between 1 and 6 min after the depolarizing step, i.e., during a period of time corresponding to the SYM-sensitive component of DSE (see Figs. 3C and 5A, 1 and 2) and by averaging these values for all cells recorded in a given condition. For SYM 2081 experiments, \( \delta \) values were determined between 1 min and the time after the depolarizing step at which DSE was eventually replaced by a small and transient potentiation of PF-EPSCs (Fig. 3C). Thus in the absence of D-TBOA, mean \( \delta \) was only 195.07 ± 37.43 in the presence of SYM 2081 against 475.62 ± 60.19 in its absence, the difference being significant (P < 0.005). In the presence of D-TBOA, the difference was even more pronounced and significant (P = 0.001) since mean \( \delta \) calculated in the same conditions were now respectively 201.54 ± 67.52 and 613.80 ± 50.72, suggesting in turn that D-TBOA increases the amplitude of the SYM-sensitive component of DSE (see also Fig. 5A, 1 and 2). Finally, in the presence of SR141716-A in the bath, superfusion of 10 µM SYM 2081 + 50 µM D-APV inhibited the late phase of DSE to an extent that could not be distinguished from that obtained with SYM 2081 alone while also leaving the initial decrease of PF-EPSCs unaffected (n = 6; Fig. 3C). Altogether these results therefore suggest that a late phase of CB1 receptor-independent component of DSE depends on retrograde release of Glu and suggest moreover that Glu probably acts through activation of KA receptors. These receptors will be shown to be borne by PFs in next sections. The possible origin of the initial depression of PF-EPSCs, i.e., insensitive to SYM 2081, will be also discussed in next sections.

**Late phase of the endocannabinoid-independent component of DSE is also absent in GluR6 -/− mice**

Because SYM 2081 is a desensitizing KA receptor agonist rather than a genuine KA receptor antagonist, it was important to test the involvement of KA receptors in the late phase of the CB1 receptor-independent component of DSE in a more specific manner. Presynaptic KA receptors borne by PFs are likely to be heteromeric constructions including GluR6 and KA2 receptor subunits, and their activation up or down regulate Glu release depending on agonist concentration (Delaney and Jahr 2002; Petralia et al. 1994; see also ref. in Lerma et al. 2001). DSE was therefore studied in nearly mature GluR6 -/− mice, as compared with wild-type mice of the same strain (see METHODS), with the assumption that invalidating GluR6 receptor subunits renders presynaptic 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 presynaptic NMDA receptors and in the presence of 1 µM SYM 14176-A (n = 16)
or of 2 μM AM-251 (n = 8) to focus on the endocannabinoid-independent component of DSE. For comparison and except otherwise specified, all experiments in wild-type mice were also performed in the presence of 50 μM D-APV and of 1 μM SR141716-A.

In 18- to 24-day-old wild-type mice and as in nearly mature rats, stepping the voltage of the postsynaptic PC from −70 to 0 mV for 1 s induced an endocannabinoid-independent DSE of small amplitude and ≤8–10 min in duration, with a mean peak amplitude of 13.14 ± 1.19% (n = 11; Fig. 4B). Results non significantly different were obtained in six other cells when Δ-APV was omitted (not illustrated), thus confirming that presynaptic NMDA receptors are not involved in DSE in nearly mature animals.

In 18- to 24-day-old GluR6 −/− mice (n = 6), no gross alteration in PF to PC synaptic transmission could be evidenced (Fig. 4A) except for basal PPF, which amounted to 1.69 ± 0.04% (n = 22) against only 1.46 ± 0.07% in control mice (n = 10), the difference being highly significant (P < 0.001). Such an increase in basal PPF was also observed in SYM 2081 experiments, with a mean value of 1.81 ± 0.03% (n = 8). Now, and as in SYM 2081 experiments, a late phase of the CB1 receptor-independent component of DSE was strongly inhibited in 19 of these 22 PCs, whereas initial decrease of PF-EPSCs remained unaffected (Fig. 4B). Fourteen of these 19 PCs were recorded in the 4 GluR6 −/− mice studied on postnatal days 22–24, whereas the 5 others were recorded on postnatal day 18 in the two remaining mice (see following text). Very similar results were obtained when the concentration of SR141716-A was raised up to 5 μM (n = 5; not illustrated), rendering unlikely that the initial decrease of PF-EPSCs during DSE was due to an incomplete blockade of CB1 receptors. In contrast, suppression of PF-EPSCs lasted >15 and ≤40 min in the three remaining cells that were all recorded in the two GluR6 −/− mice studied on postnatal day 18 (see Fig. 6B). Interestingly, these two mice originated from two different litters, did not show any visible abnormality, and, moreover, exhibited typical DSE in five other recorded PCs (see preceding text). Therefore these prolonged suppression of PF-EPSCs were not discarded but were analyzed separately because one cannot preclude that they might have resulted from some developmental changes in these two constitutive GluR6 −/− mice. On the whole and except for the latter puzzling results, data in 18- to 24-day-old GluR6 −/− mice fully confirm that the late phase of the endocannabinoid-independent component of DSE depends on retrograde release of Glu and suggest moreover that Glu acts through activation of KA receptors that include GluR6 subunits.

**Fig. 4.** Endocannabinoid-independent component of DSE in nearly mature wild-type and GluR6 −/− mice. A: examples of PF-EPSCs recorded in the presence of 1 μM SR141716-A and 50 μM D-APV in a 24-day-old GluR6 −/− mouse. The superimposed sweeps were obtained prior to a depolarizing step at 0 mV for 1 s (1), at the peak of DSE (2), and after recovery (3). As in Figs. 1 and 3, 1–3 correspond to numbers indicated in plots in B. B: superimposed plots of mean normalized amplitudes of PF-EPSCs recorded in the presence of 1 μM SR141716-A and of 50 μM D-APV in 18- to 24-day-old wild-type and GluR6 −/− mice (● and □, respectively; see RESULTS). Amplitudes of PF-EPSCs were normalized against their respective control values and the depolarizing pulse at 0 mV for 1 s was applied at time 0. Note that a late component of DSE was abolished in GluR6 −/− mice, like in the plot in Fig. 3C in the presence of SYM 2081.

**Time course of the KA receptor-dependent component of DSE in nearly mature rats and mice**

The entire time course of the KA receptor-dependent component of DSE was estimated by subtracting mean DSE in SYM 2081 treated rats or in GluR6 −/− mice from mean DSE in control animals, respectively. In both cases, the KA receptor-dependent component of DSE developed slowly after the depolarizing pulse and then decayed equally slowly over several minutes (Figs. 5A1 and 6A; see also DISCUSSION). However, the fact that its estimated amplitude was initially small as compared with that of the KA receptor-independent component of DSE that peaked within the same period precluded reliable determination of its onset with such a crude method (Figs. 5, A1 and 6A; see DISCUSSION).

CB1 receptor-independent component of DSE is accompanied by PPF increase that is sensitive to Glu uptake blocker and to desensitizing KA receptor agonist in nearly mature rats

In nearly mature rats, if the CB1 receptor-independent component of DSE involves activation of presynaptic KA receptors borne by PFS, one expects that it will be accompanied by a correlative PPF increase. Moreover, this PPF increase should be potentiated by Glu uptake blockers and inhibited by SYM 2081.

In the 17 PCs studied in the presence of 1 μM SR141716-A alone (see preceding text), the endocannabinoid-independent component of DSE was accompanied by a small but highly significant (P < 0.001) increase in mean normalized PPF, from 100.28 ± 0.35% during control period to 104.53 ± 1.83% during DSE when individual PPF values within trace groups were averaged (Fig. 5A; see METHODS). By using the method of Kim and Alger (2001; see METHODS), very similar results were obtained because mean normalized PPF significantly (P <
0.001) increased from 100.22 ± 0.36% during control period to 104.39 ± 1.40% during DSE. Therefore and except otherwise specified, mean normalized PPFs were now calculated by simply averaging individual PPF values. In these conditions, mean normalized PPF peaked at 110.59 ± 3.46% of control when only considering the eight (of 17) cells that exhibited the most clear-cut PPF increase during DSE. For these cells as well as for the total sample of 17 tested cells, the ratio \( R = \text{mean normalized PPF increase during DSE}/\text{mean amplitude of the component of DSE sensitive to SYM 2081} \) was near 0.5 on average (Fig. 5A1). Indeed, individual values of normalized PPF increases and of amplitudes of the component of DSE sensitive to SYM 2081 (i.e., amplitudes of the endocannabinoid-independent component of DSE measured between 2 and 3 min after the depolarizing pulse; see Figs. 3C and 5A1) were highly correlated, with a correlation coefficient = 0.79 (Fig. 5B1). Finally, the time course of PPF changes well matched that of the SYM-sensitive component of DSE (Fig. 5A1; see preceding text).

No significant change in individual PPF values was observed after bath application of 100 \( \mu M \) t-TBOA (Fig. 3B), thus suggesting that t-TBOA did not sizably affect the presynaptic machinery by itself. In contrast, mean normalized PPF increased from 99.83 ± 0.80% during the control period to 107.39 ± 2.08% (n = 12) during the endocannabinoid-independent component of DSE, an increase that was significantly larger (\( P < 0.05 \)) than that observed in the presence of SR141716-A alone when calculated on pooled values between 2 and 5 min after the depolarizing pulse (Fig. 5A2). Nearly identical values were obtained by using the method of Kim and Alger (2001) because mean normalized PPF amounted to 107.18 ± 1.34% during the endocannabinoid-independent component of DSE against 100.26 ± 0.65% during the control period. Here again, the time course of PPF changes matched that of the SYM-sensitive component of DSE, with a ratio \( R \) still close to 0.5 (Fig. 5A2). However, individual values of normalized PPF increase and of amplitudes of the component of DSE sensitive to SYM 2081 were less consistently correlated probably due to the smaller sample of cells (Fig. 5B2).

Finally and as expected, the inhibition of the late phase of the endocannabinoid-independent component of DSE by SYM 2081 was accompanied by a correlative inhibition of PPF changes, whereas the initial phase of DSE, i.e., insensitive to SYM 2081, was still accompanied by a sizeable PPF increase and this, whatever t-TBOA was present or not in the bath (n = 6 and 7, respectively; Fig. 5A, 1 and 2). Therefore these results strongly suggest that KA receptors involved in the Glu-dependent component of DSE in nearly mature rats are borne by PFs and that presynaptic mechanisms are also likely to operate in the initial phase of the endocannabinoid- and Glu-independent component of DSE. Additional experiments aiming to determine these early presynaptic mechanisms (see supplementary results and supplementary figure S1) were focused on other presynaptic receptors known to be present on PFs, i.e., GABA\(_B\), adenosine \( A_1 \) and mGluR\(_4\) receptors (ref. in Levenes et al. 2001). Results showed that, in the presence of SR141716-A and SYM 2081 in the bath, blocking separately each of these receptors only marginally inhibited the endocannabinoid- and Glu-independent component of DSE and also let associated PPF increase unaffected. Blocking simultaneously these receptors did not induced further significant changes in DSE amplitude but, rather unexpectedly, now suppressed the associated PPF increase. This puzzling effect appeared genuine since the endocannabinoid-independent component of DSE (i.e., elicited in the absence of SYM 2081) and the associated mean normalized PPF increase were not significantly affected by bath application of the cocktail of GABA\(_B\), adenosine \( A_1 \), and mGluR\(_4\) receptor antagonists. Therefore these supplementary results suggest possible involvement of both pre- and postsynaptic mechanisms in the initial phase of the endocannabinoid- and Glu-independent component of DSE (see discussion). Finally, peak amplitude of this residual DSE was close to that of DSE recorded in the presence of 40 mM BAPTA in the intra pipette solution (see preceding text and Fig. S1).

CB1 receptor-independent component of DSE is accompanied by a GluR6-sensitive PPF increase in nearly mature mice

In nearly mature mice, if the CB1 receptor-independent component of DSE also involves activation of presynaptic KA receptors borne by PFs, one expects that it will be still accompanied by an increase in PPF that should be no longer present in GluR6 \(-/-\) mice. Indeed, in wild-type PCs, the CB1 receptor-independent component of DSE was accompanied by a small but significant (\( P < 0.05 \)) increase in mean normalized PPF, from 99.63 ± 0.66% during control period to 105.34 ± 1.07% during DSE (n = 9; Fig. 6A). Moreover and here again, the time course of mean PPF changes matched that of the GluR6-dependent component of DSE (Fig. 6A). As before, nearly identical results were obtained by using the method of Kim and Alger (2001) because mean normalized PPF significantly (\( P < 0.01 \)) increased from 99.98 ± 0.68% during control period to 104.97 ± 0.96% during DSE.

In 18- to 24-day-old GluR6 \(-/-\) mice, the inhibition of the late phase of the endocannabinoid-independent component of DSE observed in 19 of the 22 PCs studied (see preceding text) was accompanied by a correlative inhibition of PPF changes (Fig. 6A). In contrast—and as already observed in rat PCs in the presence of SYM 2081—the remaining initial component of DSE was still accompanied by a small but significant PPF increase (\( P < 0.002 \)), from 99.96 ± 0.79% prior to the depolarizing pulse to 105.17 ± 1.68% at the peak of PPF increase (compare Figs. 5A1 and 6A; see also discussion).

Finally, the long-lasting suppression of PF-EPSCs observed in three PCs in 18-day-old GluR6 \(-/-\) mice (see preceding text) was accompanied by a similarly long-lasting and significant (\( P < 0.01 \)) increase in PPF (Fig. 6B), thus suggesting again a presynaptic origin. This long-lasting suppression might therefore rely, for instance, on mechanisms similar to those operating in presynaptic long-term depression (LTD) that depends on endocannabinoids (Robbe et al. 2002). However, if endocannabinoids were also the retrograde messenger in this case, they should have to activate non-CB1 presynaptic receptors instead of the CB1 receptors that dominate at these synapses in control mice (Kawamura et al. 2006) because the latter were blocked by SR141716-A in the present experiments. Further studies are required to unravel the nature of retrograde messengers and presynaptic receptors involved in this unusually prolonged suppression of PF-EPSCs in 18-day-old GluR6 \(-/-\) mice.
FIG. 5. CB1 receptor-independent component of DSE is accompanied by PPF increase that is sensitive to d-TBOA and to SYM 2081 in nearly mature rats. A. 1 and 2, bottom: plots (□) of the difference between the plots of mean normalized amplitudes of PF-EPSCs during DSE induced in the presence or in the absence of SYM 2081 and without or with d-TBOA, respectively, as illustrated in left and right plots in Fig. 3C. For clarity, these 2 plots (□ and ■, respectively) have been also superimposed without SE. In all cases, 1 μM SR141716-A was present in the bathing medium. A. 1 and 2, top: mean normalized PPF of PF-EPSCs recorded in the presence of 1 μM SR141716-A alone and in the presence of 1 μM SR141716-A +100 μM d-TBOA, respectively, from the same PCs as in bottom panels (□). In A. 1 and 2, □ represent the plot of mean normalized PPF when SYM 2081 was added to the bath (SE omitted for clarity; see RESULTS). B1: plot of normalized PPF increase against normalized amplitude of the SYM-sensitive component of DSE in 17 PCs recorded in the presence of SR141716-A alone (□; see RESULTS). B2: plot of the normalized PPF increase against mean normalized amplitude of DSE in 20 PCs recorded in the presence of SR141716-A+d-TBOA (□). CC is the correlation coefficient and m is the slope of the linear regression.

Altogether, the results obtained in 18- to 24-day-old GluR6−/− mice strongly suggest that presynaptic KA receptors involved in the Glu-dependent component of DSE include GluR6 subunits. They also validate results obtained with the desensitizing KA receptor agonist SYM 2081 in rats.

Glu transporters and lack of KA-dependent component of DSE in juvenile rats

The so-called depolarization-induced potentiation of inhibition (DPI) that also 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 KA-dependent component of DSE in 10- to 12-day-old rats might simply result from an unbalance between Glu release and Glu uptake in favor of the latter at this early developmental stage. Supplementary results and supplementary figure S2 show that blocking Glu transporters in 10- to 12-day-old rats by d-TBOA unmasked a KA-dependent-like component of DSE in only two of the eight cells tested. This suggests that if the absence of KA-dependent component of DSE in juvenile rats can be partly explained by such an unbalance between Glu release and Glu uptake at this developmental stage, it also probably results from other causes such as, for instance, incomplete maturation of presynaptic KA receptors in these immature rats. Further studies are required to elucidate this point.

Effects of Glu uptake inhibitors and SYM 2081 on posttetanic quantal events after PF tetani in nearly mature rats

In the previous study by Levenes et al. (2001), it was shown that a prolonged discharge of uEPSCs (see METHODS) follows trains of PF-EPSCs induced by short tetanic stimulation of PFs at 100 Hz. These posttetanic uEPSCs were interpreted as probably resulting from activation of presynaptic ionotropic Glu receptors through retrograde release of Glu by PCs (Levenes et al. 2001), thus in keeping with the present results on DSE in nearly mature animals. However, one cannot preclude that they might have been also be due to residual calcium responsible for delayed release of Glu from PFs (Atluri and Regehr 1998). To distinguish between these two possibilities, the effects of d-TBOA and SYM 2081 on posttetanic uEPSC were analyzed with the assumption that blocking Glu uptake by d-TBOA would potentiate the prolonged discharge of posttetanic uEPSC, whereas blocking presynaptic KA receptors would inhibit this discharge if the retrograde release of Glu hypothesis is correct.

The present experiments were performed at 27–28°C. As described previously (Levenes et al. 2001), no significant change in relative frequency of evoked uEPSCs was observed with single PF stimulations, whereas maximal increase of frequency was observed with eight pulses (Fig. 7A). Thus the mean relative frequency of posttetanic uEPSCs after a tetanus of eight pulses at 100 Hz was 137.71 ± 9.68% (n = 13), against only 112.49 ± 3.63% for one pulse, the difference being significant (P < 0.01; Fig. 7C). The presence of 100 μM d-TBOA in the bath did not significantly alter the relative frequency of uEPSCs evoked by single PF stimulations but, unexpectedly, abolished the effects of PF trains at 100 Hz because the mean relative frequency of posttetanic uEPSCs was now only 101.01 ± 8.68% (n = 9), this value being significantly smaller (P < 0.01) than that obtained for eight pulses in control medium (Fig. 7C). Such unexpected results could hardly be due to a lower detection rate of unitary events in the presence of d-TBOA because a slight increase in the amplitude of spontaneous and evoked uEPSCs was induced in all cells by this compound (Fig. 7B).

A possible explanation might be that by potentiating PF-EPSCs (see Fig. 3A, 1 and 2), blockade of Glu uptake increases retrograde release of endocannabinoids by PCs and also increases activation of molecular layer inhibitory interneurons, thereby possibly potentiating their inhibitory effects on Glu release by PFs through activation of presynaptic CB1 as well as...
GABA_B receptors (Dittman and Regehr 1997). Therefore the same tetanic stimulations as before were applied in the presence of 1 μM SR141716-A alone or of 1 μM SR141716-A + 300 nM of the GABA_B Receptor antagonist CGP55845-A in the bath and this with or without superfusion with 100 μM D-TBOA. In the absence of D-TBOA, these treatments did not significantly alter the mean relative frequencies of uEPSCs evoked by one or by eight stimuli at 100 Hz (n = 19 and 15, respectively; Fig. 7C). In the presence of D-TBOA, whereas SR141716-A alone still failed to restore the effect of PF tetani on posttetanic uEPSCs (n = 15), the bathing medium with both SR141716-A and CGP55845-A not only restored the effect of PF tetani on posttetanic uEPSCs but now led to an increase in relative frequency of posttetanic uEPSCs that was significantly larger (P < 0.05) than that obtained under SR141716-A + CGP55845-A alone (154.29 ± 8.09 and 138.85 ± 4.43%, respectively; Fig. 7C).

Finally in the presence of 1 μM SR141716-A in the bath, the discharge of uEPSCs evoked by eight stimuli of PFs at 100 Hz was significantly (P < 0.05) inhibited by bath application of 10 μM SYM 2081 because the mean relative frequency of posttetanic uEPSCs evoked by a tetanus of eight pulses at 100 Hz was only 117.39 ± 5.35% (n = 8) against 139.99 ± 5.76% (n = 19) in the presence of 1 μM SR141716-A alone and of 50 μM t-APV prior to and after applying a depolarizing pulse at 0 mV for 1 s at time 0 in the 3 PCs that exhibited long-lasting suppression of PF-EPSCs in 18 day-old GluR6 −/− mice (B). Top: corresponding changes in mean normalized PPF of PF-EPSCs (c).

**DISCUSSION**

The present data confirm that in juvenile rats, DSE of PF-EPSCs is entirely mediated through retrograde release of Glu. In the absence of D-TBOA, whereas SR141716-A and CGP55845-A not only restored the effect of PF tetani on posttetanic uEPSCs but now led to an increase in relative frequency of posttetanic uEPSCs that was significantly larger (P < 0.05) than that obtained under SR141716-A + CGP55845-A alone (154.29 ± 8.09 and 138.85 ± 4.43%, respectively; Fig. 7C).

**FIG. 6.** CB1 receptor-independent component of DSE is accompanied by a GluR6-sensitive PPF increase in nearly mature mice. A, bottom: plot (□) of the difference between the plots of mean normalized amplitudes of PF-EPSCs during the endocannabinoid-independent component of DSE in 18- to 24-day-old wild-type and GluR6 −/− mice, as illustrated in Fig. 4B. For clarity, these 2 plots (● and ○, respectively) have been also superimposed without SE. The depolarizing pulse at 0 mV for 1 s was applied at time 0. A, top: mean normalized PPF of PF-EPSCs recorded in the presence of 1 μM SR141716-A from the same PCs as above in wild-type and in GluR6 −/− mice (● and ○, respectively). B, bottom: plot of mean normalized amplitudes of PF-EPSCs recorded in the presence of 1 μM SR141716-A and of 50 μM t-APV prior to and after applying a depolarizing pulse at 0 mV for 1 s at time 0 in the 3 PCs that exhibited long-lasting suppression of PF-EPSCs in 18 day-old GluR6 −/− mice (○). B, top: corresponding changes in mean normalized PPF of PF-EPSCs (○).
endocannabinoids (Brenowitz and Regehr 2003; Kreitzer and Regehr 2001b; Safo and Regehr 2005). In contrast, in nearly mature rats, DSE at PF-PC synapses not only depends on retrograde release of endocannabinoids but is also likely to partly depend on retrograde release of Glu from PCs and activation of presynaptic KA receptors borne by PFs, in keeping with the study by Levenes et al. (2001). However, one cannot totally preclude a more indirect origin of released Glu, such as for instance through liberation by depolarized PCs of a yet unidentified messenger that would in turn trigger Glu release from surrounding glial cells. Further studies will have to clarify this point. The present results in nearly mature wild-type and GluR6−/− mice strongly support the conclusion that Glu acts as a retrograde messenger and further suggest that presynaptic KA receptors involved in DSE include GluR6 receptor subunits. Mechanisms by which activation of presynaptic KA receptors lead to depression of PF-EPSCs (Levenes et al. 2001) have not been further investigated in the present experiments.

**Endocannabinoid-dependent component of DSE at PF-PC synapses in nearly mature rats**

Two points concerning the endocannabinoid-dependent component of DSE of PF-EPSCs in 18- to 22-day-old rats deserve comments. First its mean amplitude was only about half of that found in 11- to 12-day-old rats in experiments with TEA included into the internal medium. This difference is unlikely to result from experimental protocols because they were the same in both cases, and very similar to those used previously by Brenowitz and Regehr (2003). The difference is also unlikely to result from a lower density of presynaptic CB1 receptor or from a lower ability of PCs to release endocannabinoids in nearly mature rats than in juvenile ones. Indeed, Rancz and Haussler (2006) recently showed that bursts of synaptically activated calcium spikes in PC dendrites from 18- to 25-day-old rats are potent triggers of endocannabinoid release and can induce DSE as large as that induced in immature rats. In the present study, the smaller amplitude of the endocannabinoid-dependent component of DSE in nearly mature rats than in immature ones might therefore simply result from less efficient propagation of depolarizing pulses applied to the soma into fully developed dendrites than in still immature ones, thus probably leading to smaller dendritic calcium transients. On the other hand, comparison of the present results with those of Rancz and Haussler (2006) suggests that calcium concentration increase necessary to trigger release of endocannabinoids in nearly mature rats is more easily achieved by bursts of synaptically activated dendritic calcium spikes than by applying depolarizing voltage steps to the soma of voltage-clamped PCs and this even in the presence of TEA in the internal solution.

Second, in experiments without TEA in the internal medium, the CB1 receptor-dependent component of DSE was seldom observed, probably because sufficient calcium concentration increase into dendrites could not be achieved with depolarization applied to the soma in such conditions, due to still poorer voltage control of dendrites. Indeed fluorometric experiments confirmed that calcium transients induced in PC dendrites by somatic depolarization were significantly larger with TEA containing internal solution than without (see results) in keeping with the fact that Brenowitz and Regehr (2003) also used TEA containing internal solutions in their experiments on DSE of PF-EPSCs.

**Calcium dependence of the endocannabinoid-independent component of DSE at PF-PC synapses in nearly mature rats**

The significant inhibition of the CB1 receptor-independent component of DSE in cells loaded with BAPTA (see results) suggests that this component is also at least partly calcium dependent. However, inhibition was incomplete because DSE was only reduced by ~50%, which is difficult to interpret solely on the basis on incomplete buffering of calcium. As discussed in next sections, the CB1 receptor-independent component of DSE is primarily expressed at presynaptic level, but transient ionic unbalance after large depolarizing steps used to induce DSE in the present experiments might also contribute to its early phase. The presence of an early component of postsynaptic origin in DSE elicited in the presence of BAPTA is supported by the absence of any significant associated changes in PPF (see results) as well as by the fact that peak amplitude of this residual DSE was close to that of the SR141716-A and SYM 2081 resistant component of DSE recorded in the presence of GABA_B, adenosine A1 and mGluR4 receptor antagonists, that was also probably of postsynaptic origin (see following text).

Finally, the fact that the CB1 receptor-independent component of DSE was still present in experiments without TEA in the internal medium, whereas the endocannabinoid-dependent one was generally no longer observed suggests that retrograde release of endocannabinoids by PCs requires a larger free-calcium increase in dendrites during depolarization induced at a somatic level than that needed to release Glu from these cells. This is well in keeping with recent data showing that endocannabinoid-dependent DSE induced by short bursts of PF-EPSPs occurs when postsynaptic mGluR1 of PCs are activated, but is absent when otherwise very similar EPSPs are not accompanied by such mGluR1 activation (Marcaggi and Attwell 2005).

**Glu-dependent component of DSE at PF-PC synapses in nearly mature rats and mice**

In nearly mature rats, potentiation of the endocannabinoid-independent component of DSE and in particular of its late phase by the Glu uptake inhibitor d-TBOA (see results and Fig. 5A, 1 and 2), as well as inhibition of this late phase by the desensitizing KA receptor agonist SYM 2081 strongly suggests that a late component of DSE depends on retrograde release of Glu from PCs and subsequent activation of KA receptors. The partial blockade of the endocannabinoid-independent component of DSE by BAPTA, including that of its late component (see Fig. 2C), further suggests that Glu release is induced by postsynaptic calcium concentration increase, like retrograde release of Glu after activation of postsynaptic mGluR1 receptors (Levenes et al. 2001). Comparison of results obtained in 18- to 24-day-old wild-type and GluR6−/− mice fully confirms that a late phase of the endocannabinoid-independent component of DSE depends on retrograde release of Glu and further suggests that the KA receptors involved include GluR6.
subunits, thus in keeping with the histological and ultrastructural localization of these receptor subunits on PFs (Petraila et al. 1994). Moreover, in nearly mature rats and mice, the presynaptic location of these KA receptors is supported by the fact that the GluR6- and SYM 2081-sensitive components of DSE were accompanied by correlative increases in PPF (see Results).

Finally, the slow building up and decay over several minutes of the Glu-dependent component of DSE in nearly mature rats and wild-type mice (see Results) are very similar to those observed for DPI that also operates through Glu release from depolarized PCs and, in this case, activation of presynaptic NMDA receptors (Duguid and Smart 2004). As for DPI, the prolonged duration of the Glu-dependent component of DSE might therefore involve a slowly building up and slowly decaying calcium release from presynaptic ryanodine-sensitive calcium stores after an initial short-lived activation of presynaptic KA receptors that might be hardly detectable in experimental conditions used. In keeping with results of a previous study (Levenes et al. 2001), this might in turn lead to a sustained increased rate of spontaneous quantal EPSCs and to a sustained decreased rate of evoked ones through depletion of readily releasable pool of synaptic vesicles. Further studies will have to evaluate the role of presynaptic calcium stores in Glu-dependent component of DSE. The fact that the developmental pattern of DPI seems to differ from that of Glu-dependent component of DSE might partly or even entirely result from different dendritic locations and maturation processes of excitatory versus inhibitory synapses. Moreover, 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).

Mechanisms responsible for the initial phase of the endocannabinoid-independent component of DSE

Presynaptic mechanisms are also likely to be involved in the initial phase of the endocannabinoid-independent component of DSE because this initial phase was accompanied by small but significant PPF increase in nearly mature rats and mice (see Results). The relative prolonged duration of this initial phase as compared with that of the endocannabinoid-dependent component of DSE, together with the fact that it was not abolished by raising concentration of SR141716-A up to 5 μM in GluR6−/− mice (see Results), does not support the view that it might simply result from an incomplete blockade of CB1 receptors. On the contrary, supplementary results suggest that activation of GABAB, adenosine A1, and mGluR4 receptors known to be present on PFs (ref. in Levenes et al. 2001) may contribute to some extent to this early phase of DSE because their simultaneous blockade totally suppressed associated PPF increase in nearly mature rats. However, the fact that DSE amplitude was only marginally affected in such conditions remains puzzling and somehow weakens this conclusion even though GABA release from pyramidal cell dendrites has been recently documented (Zilberter et al. 1999). In any case, the respective contributions of GABAB, adenosine A1, and mGluR4 receptors to these presynaptic mechanisms would remain to be established.

On the other hand, the fact that the initial phase of the endocannabinoid-independent component of DSE recorded in the presence of GABAβ1, adenosine A1, and mGluR4 receptor antagonists was no longer accompanied by PPF increase suggests, with however the same word of caution as before, that this early phase also involves a component of postsynaptic origin, thus in keeping with results obtained in PCs dialyzed with BAPTA. Accordingly, the relative contribution of pre- and postsynaptic mechanisms to the initial phase of the endocannabinoid-independent component of DSE might explain why early PPF increases after depolarizing pulses varied in amplitude in different experimental conditions, depending to the relative amplitudes of the various pre- and postsynaptic components involved (see Figs. 5A, 1 and 2, and 6A).

Glutamate dependence of the increase in frequency of unitary EPSCs evoked by tetanic stimulation of PFs in nearly mature rats

Short tetanic stimulation of PFs is known to activate postsynaptic mGluR1s at PF-PC synapses (Batchelor et al. 1994; Takechi et al. 1998) and can also increase calcium concentration in distal PC dendrites and spines when mGluRs are blocked (see Eilers et al. 1995). Interestingly, the previous study by Levenes et al. (2001) showed that such short tetanic stimulation of PFs elicits a prolonged discharge of uEPSCs in PCs that was interpreted as resulting from retrograde release of Glu from these cells and activation of presynaptic ionotropic Glu receptors borne by PFs. The present results, by showing that the discharge of posttetanic EPSCs was indeed potentiated by d-TBOA in the presence of CB1 and GABAB receptor antagonists and was inhibited by SYM 2081, fully support this interpretation, thus in good agreement with results on DSE in nearly mature rats (see preceding text).

Functional consequences of retrograde release of endocannabinoids and glutamate in nearly mature rats

A first functional relevance of the present study follows the observation that in nearly mature rats, retrograde release of endocannabinoids by PCs probably requires a larger free calcium increase during depolarization than that needed to release of Glu from these cells (see Results). Accordingly, the possibility exists that retrograde release of endocannabinoids occurs on excessive activation of PCs, such as occurs when bursts of synaptically activated dendritic calcium spikes are induced by strong tetanic stimulation of PFs (Rancz and Hausser 2006), whereas retrograde release of Glu is involved in a less massive manner to exert subtle retrograde modulation of synaptic transmission at PF-PC synapses. This is again well in keeping with the recent results by Marcaggi and Attwell (2005), who showed that retrograde release of endocannabinoids by PCs requires short tetanic activation of nearby PF-PC synapses, as occurs with direct stimulation of PFs but is absent when more dispersed synapses are similarly activated as probably occur in more physiological conditions.

Second, if sustained depolarization of PFs through activation of presynaptic KA receptors during DSE or during the Glu-dependent discharge of uEPSCs after a short train of PF inputs to PCs is sufficient to trigger action potentials in
these fibers as previously suggested (Levenes et al. 2001), such long-lasting excitation might spread along these fibers to other synapses located in the same beam of PFs, thus introducing a form of local communication among PCs sharing the same PF input.

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REFERENCES


