Role of the vesicular transporter VGLUT3 in retrograde release of glutamate by cerebellar Purkinje cells

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Crépel F, Galante M, Habbas S, McLean H, Daniel H. Role of the vesicular transporter VGLUT3 in retrograde release of glutamate by cerebellar Purkinje cells. J Neurophysiol 105: 1023–1032, 2011. First published December 22, 2010; doi:10.1152/jn.00736.2010.—In the cerebellum, retrograde release of glutamate (Glu) by Purkinje cells (PCs) participates in the control of presynaptic neurotransmitter release responsible for the late component of depolarization-induced suppression of excitation (DSE), as well as for depolarization-induced potentiation of inhibition (DPI). It might also participate in the depolarization-induced slow current (DISC) in PCs, although this contribution was later challenged. We also know that both DPI and DISC are soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-dependent processes, although the molecular nature of the vesicular transporter was not determined. In PCs, VGLUT3 is the only known vesicular glutamate transporter identified and is expressed during the same developmental frame as when DPI, DISC, and the Glu-dependent component of DSE are observed. We therefore tested the hypothesis that all these processes depend on the presence of VGLUT3 by comparing the Glu-dependent component of DSE, DPI, and DISC in nearly mature (2- to 3-wk-old) wild-type and VGLUT3 knockout mice. Our data demonstrate that, in nearly mature mice, the slow component of DSE occurs through vesicular release of Glu that involves VGLUT3. This Glu-dependent component of DSE is no longer present in fully mature mice. This study also establishes that, in nearly mature mice, DPI also depends on the presence of VGLUT3, whereas this is not the case for DISC. Finally, the unusually large basal paired-pulse facilitation observed in nearly mature VGLUT3−/− mice but not in adult ones suggests that some basal retrograde release of Glu occurs during development and contributes to basal concentrations of extracellular Glu.

depolarization; parallel fibers; postsynaptic currents

RETROGRADE SIGNALING THAT allows presynaptic control of neurotransmitter release by postsynaptic elements is now well established at both inhibitory and excitatory synapses. At inhibitory synapses impinging onto hippocampal pyramidal cells and onto cerebellar Purkinje cells (PCs), the so-called depolarization-induced suppression of inhibition (DSI; Gittis et al. 2000; Llano et al. 1991a; Pitler and Alger 1992; Pitler and Alger 1994; Vincent and Kimura 1992; Wang and Zucker 2001; Wilson et al. 2001) is due to retrograde release of endocannabinoids and activation of presynaptic type 1 cannabinoid (CB1) receptors (Diana et al. 2002; Kreitzer and Regehr 2001a; Ohno-Shosaku et al. 2001; Wilson et al. 2001). At immature excitatory synapses impinging onto PCs, depolarization-induced suppression of excitation (DSE; Kreitzer and Regehr 2001b) is also due to retrograde release of endocannabinoids acting on presynaptic CB1 receptors located on both parallel fibers (PFs) and climbing fibers (Brenowitz and Regehr 2003; Kreitzer and Regehr 2001b). In nearly mature rats and mice, however, a late component of DSE and of agonist-induced suppression of PF- excitatory postsynaptic currents (EPSCs) is due to retrograde release of Glu that depresses excitatory synaptic inputs by activating presynaptic kainate (KA) receptors located on PFs (Crépel 2007; Crépel and Daniel 2007).

Retrograde release of Glu is also involved in depolarization-induced potentiation of inhibition (DPI; Duguid and Smart 2004; Duguid et al. 2007), as well as in the depolarization-induced slow current (DISC) observed in PCs, that results from activation of postsynaptic type I metabotropic glutamate receptors (mGluR1) by Glu released from depolarized PCs (Duguid et al. 2007; Shin et al. 2008). Interestingly, it was further established for both DPI and DISC that retrograde release of Glu depends on vesicular glutamate transporters and on vesicular fusion, even though the molecular nature of the vesicular Glu transporter was not determined (Duguid et al. 2007; Shin et al. 2008). However, the origin of DISC was challenged by Kim et al. (2009), who presented evidence that this current rather results from autocrine activation of postsynaptic dopamine (DA) receptors by DA released by depolarized PCs.

In rodents, VGLUT3 is the only identified vesicular Glu transporter in PCs. It is well expressed during the first postnatal week but fades away later on and is no longer present in fully mature animals (Gras et al. 2005). As VGLUT3 expression in immature PCs clearly overlaps the period during which DPI, DISC, and the Glu-dependent component of DSE are observed (Crépel 2007; Duguid and Smart 2004; Duguid et al. 2007; Shin et al. 2008), it is possible that these three forms of plasticity all depend on the vesicular glutamate transporter VGLUT3. We therefore tested this hypothesis by comparing the Glu-dependent component of DSE, DPI, and DISC in immature (2- to 3-wk-old) wild-type and VGLUT3 knockout (KO) mice. We show that the Glu-dependent component of DSE as well as DPI depends on the presence of VGLUT3 but that DISC is still present in VGLUT3 KO mice. This later finding supports the idea that DISC does not involve retrograde release of Glu.

METHODS

Experimental procedures complied with guidelines of the French Animal Care Committee. Experiments were performed on nearly mature (17- to 20-day-old) and on adult (3- to 4-mo-old) 129Sv wild-type and KO mice for the vesicular glutamate transporter VGLUT3, hereafter referred to as VGLUT3−/− mice (Gras et al.

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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 vibrissa. Slices were incubated at room temperature in saline solution bubbled with 95% O2-5% CO2 for at least 1 h. For DSE and DISC experiments, the recording chamber was perfused at a rate of 2 ml/min with oxygenated saline solution containing the following (in mM): 124 NaCl, 3 KCl, 24 NaHCO3, 1.15 KH2PO4, 1.15 MgSO4, 2 CaCl2, and 10 glucose and the GABA(A) antagonist bicuculline methiodide (10 μM; Sigma Aldrich, St. Quentin Fullavier, France), with osmolarity of 320 mosM and final pH of 7.35 at 24-25°C except when otherwise specified. For experiments on DPL, the same bathing medium was used but bicuculline methiodide was omitted and replaced by 500 nM tetrodotoxin citrate (TTX) plus 10 μM 2,3-dixo-6-nitro-1,2,3,4-tetrahydrobenzoxo-(f)quinoxaline-7-sulfonamide (NBQX). PCs were directly visualized with Nomarski optics through the ×40 water-immersion objective of an upright microscope (Zeiss).

Drugs were added to the superfusate. TTX, NBQX, d-2-amino-5-phosphophentanolic acid (d-APV), (2S,4R)-4-methylglutamic acid (SYM 2081), and haloperidol were purchased from Tocris (Illkirch, France), whereas the CB1 cannabinoid receptor antagonist SR141716-A [N-(piperidin-1-yl)-5H-[1,4]-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazol-3-carboxamide hydrochloride; 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 with an Axopatch-200A amplifier (Axon instruments). In DSE and DISC experiments, patch pipettes (2–4 MΩ) were filled with a solution containing the following (in mM): 140 Cs-glucuronate, 6 KCl, 10 HEPES, 0.2 EGTA, 1 MgCl2, 4 Na2-ATP, 0.4 Na-GTP, and 20 TEA (pH and osmolarity adjusted accordingly). In DPL experiments, Cs-glucuronate in the internal solution was replaced by the same concentration of Cs-chloride. Internal solution components were purchased from Sigma (Sigma-Aldrich).

In 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 (junction potentials corrected), and brief 10-mV hyperpolarizing voltage steps that allowed monitoring of the passive electrical properties of the recorded cell were applied throughout the experiment at the beginning of each recording sequence (Llano et al. 1991b).

In DSE experiments, PF stimulations were performed at 0.5 Hz. Recorded currents were filtered at 5 kHz and digitized on line at 20 kHz, and PF-EPSCs were analyzed on- and off-line by using the Acquis1 computer program (Biologic). DSE was induced after a control period of at least 5 min by depolarizing PCs from −70 to 0 mV for 1 s (Crépel 2007). Paired-pulse facilitation (PPF) values were obtained by applying two stimulations of the same amplitude to the PC soma. The data corrected for background fluorescence were collected with a photometer and analyzed on- and off-line using the Acquis1 computer program. The data corrected for background fluorescence were expressed as changes in ΔFF, where ΔF was the baseline fluorescence intensity and ΔF was the change induced by depolarizing pulses applied to the PC soma.

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

RESULTS

VGLUT3 participates in DSE in nearly mature wild-type mice. In 18- to 20-day-old wild-type mice, PC depolarization from −70 to 0 mV for 1 s elicited a DSE that consisted of an initial fast component followed by a slower one (Fig. 1A/ and see below). Like in juvenile or nearly mature rodents in previous studies (Brenowitz and Regehr 2003; Crépel 2007; Kreitzer and Regehr 2001b; Safo and Regehr 2005), the initial
Fig. 1. Depolarization-induced suppression of excitation (DSE) in wild-type and VGLUT3−/− mice. A1: superimposed plots of the mean (±SE; same in Figs. 1–5 except where otherwise specified) normalized amplitudes of parallel fiber (PF)-excitatory postsynaptic currents (EPSCs) recorded from 18- to 20-day-old wild-type and VGLUT3−/− Purkinje cells [PCs; blue filled circles (n = 6) and red filled circles (n = 8), respectively]. In all cases, the depolarizing pulse (−70 to 0 mV for 1 s) was applied at time t = 0 (arrow). PF-EPSCs were normalized against responses immediately preceding the depolarizing pulse, and averages were calculated every 2 s. Inset: example of PF-EPSCs elicited in a typical control experiment before (dark blue) and 30 s after DSE induction (light blue) by 2 identical PF stimuli separated by 30 ms. A2: superimposed plots of mean paired-pulse facilitation (PPF) of PF-EPSCs in the same DSE experiments as shown in A1 (blue lozenges and red lozenges for wild-type and VGLUT3−/− mice, respectively). The plot of mean basal PPF observed in the presence of 10 μM (2S,4R)-4-methylglutamic acid (SYM 2081) in VGLUT3−/− mice (no depolarizing pulse; blue open lozenges; n = 6) has been also superimposed for comparison. Note the large PPF increase associated with the initial phase of DSE in wild-type and VGLUT3−/− mice. In contrast, note that the PPF increase associated with the late component of DSE in 18- to 20-day-old wild-type mice was absent in VGLUT3−/− mice. B1 and B2: plots of mean amplitude and normalized mean PPF of PF-EPSCs (n = 9) during DSE in VGLUT3−/− mice of the same age in the presence of 10 μM SYM 2081 have also been superimposed (red open circles and lozenges, respectively) for comparison. C1 and C2: same as in A1 and A2, respectively, in adult wild-type and VGLUT3−/− PCs. Note the absence of the Glu-dependent component of DSE in both adult wild-type and VGLUT3−/− PCs, signaled by the absence of PPF increase during the residual depression of PF-EPSCs (see RESULTS). Also note that the elevated level of basal PPF observed in nearly mature VGLUT3−/− mice was absent in adult ones (A2 and C2, respectively).

fast component decayed within 1 min (Fig. 1A1). At its peak, the mean decrease in PF-EPSC amplitude was 28.61 ± 6.48% (n = 6) and was accompanied by a highly significant (P < 0.001) increase in mean PPF from 1.26 ± 0.02 to 1.55 ± 0.07% (Fig. 1A2). This initial component was completely abolished by 20- to 30-min bath application of the potent CB1 receptor antagonist SR141716-A to block the endocannabinoid-dependent component of DSE (Crépel 2007) and that mean PPF was normalized (see METHODS). In B1 and B2, plots of mean amplitude and normalized mean PPF of PF-EPSCs (n = 9) during DSE in VGLUT3−/− mice of the same age in the presence of 10 μM SYM 2081 have also been superimposed (red open circles and lozenges, respectively) for comparison. C1 and C2: same as in A1 and A2, respectively, in adult wild-type and VGLUT3−/− PCs. Note the absence of the Glu-dependent component of DSE in both adult wild-type and VGLUT3−/− PCs, signaled by the absence of PPF increase during the residual depression of PF-EPSCs (see RESULTS). Also note that the elevated level of basal PPF observed in nearly mature VGLUT3−/− mice was absent in adult ones (A2 and C2, respectively).
VGLUT3\(^{+/+}\) mice (\(n = 8\); Fig. 1, A1 and A2). This suggests that endocannabinoid signaling was not affected by the VGLUT3 mutation. In marked contrast, the mean decrease in PF-EPSC amplitude during the slow component of DSE was significantly (\(P < 0.002\)) smaller than that of the Glu-dependent component of DSE in wild-type mice of the same age since it amounted to only 7.52 ± 1.22% in the presence of SR141716-A (\(n = 10\); Fig. 1B1). In the absence of selective KA receptor antagonists, we used SYM 2081 to preclude that this residual slow component of DSE could still be due to retrograde release of Glu from depolarized PCs but in an attenuated form resulting from some indirect effect of the absence of VGLUT3. SYM 2081 is 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; Epstein et al. 2005; Li et al. 1999; Zhou et al. 1997). As shown in Fig. 1B1, superfusing the slices with 10 \(\mu\)M SYM 2081 for at least 30 min in the presence of 1 \(\mu\)M SR141716-A failed to significantly attenuate (\(n = 9\)) the endocannabinoid-independent component of DSE in 18- to 24-day-old VGLUT3\(^{+/+}\) mice. Moreover, and except for its initial phase, this residual DSE was no longer accompanied by an increase in mean normalized PPF, a feature also observed in the absence of SYM 2081 (Fig. 1B2). The origin of this endocannabinoid- and Glu-independent component of DSE is unclear. As suggested in a previous study (Crépel 2007), it might partly result from the activation of other presynaptic receptors such as GABA\(_B\) and adenosine A\(_1\) receptors known to be present on PFs (refs. in Levenes et al. 2001). At a postsynaptic level, it might also partly result from the ionic unbalance that follows large depolarizing steps used to induce DSE.

Therefore, these data support our (Crépel 2007) previous finding that the slow component of DSE in nearly mature wild-type mice is due at least in part to retrograde release of Glu from depolarized PCs, which in turn depresses synaptic transmission through activation of presynaptic KA receptors located on PFs. The present data further establish that retrograde release of Glu during DSE involves the vesicular transporter VGLUT3.

Finally, on postnatal days 18 to 20, mean basal PPF was significantly (\(P < 0.001\)) larger in VGLUT3\(^{-/-}\) mice (1.51 ± 0.01%; \(n = 8\)) than in wild-type ones (1.26 ± 0.02%; \(n = 6\); Fig. 1A2). Again, this abnormally high basal PPF was very alike that observed in wild-type mice of the same age in the presence of 10 \(\mu\)M SYM 2081 (\(n = 6\); Fig. 1A2). Therefore, this suggests that in wild-type PCs under resting conditions some basal retrograde release of Glu occurs and contributes to basal levels of ambient Glu in extracellular medium that in turn contributes to tonic activation of PF releasing sites through activation of presynaptic KA receptors (Crépel 2007; Delaney and Jahr 2002).

VGLUT3 does not participate in DSE in adult wild-type mice. In adult wild-type mice, as in nearly mature ones, PC depolarization from −70 to 0 mV for one s elicited a DSE that consisted of an initial fast component followed by a slower one (Fig. 1C1). Although no attempt was made to further characterize the nature of the initial fast component, it was reminiscent of the endocannabinoid-dependent component of DSE seen in nearly mature wild-type mice (see above). However, its amplitude was significantly (\(P < 0.05\)) smaller, with a mean depression of only 17.98 ± 3.38% (\(n = 8\)). The amplitude of the slow component of DSE (mean decrease = 7.87 ± 2.21%) was also significantly (\(P < 0.01\)) smaller than that of the slow component of DSE observed in nearly mature wild-type mice (Fig. 1C1). Moreover, and in contrast to the Glu-dependent component of DSE in nearly mature wild-type mice, the slow component of DSE in adult wild-type mice was not accompanied by an increase in mean normalized PPF (Fig. 1C2). These results are in agreement with previously published data (Crépel 2009).

In adult VGLUT3\(^{-/-}\) mice, neither the mean time-course nor the mean amplitude (8.37 ± 1.28%; \(n = 8\)) of the slow component of DSE differed from those of DSE in adult wild-type mice (Fig. 1C1) nor was it accompanied by an increase in mean normalized PPF (Fig. 1C2). These results strongly suggest that the Glu-dependent component of DSE is lacking in adult wild-type mice, which in turn suggests that retrograde release of Glu no longer operates, in keeping with the absence of VGLUT3 in adult animals (Gras et al. 2005). These results are also in keeping with previously published data on the lack of the Glu-dependent component of DSE in adult wild-type and KO mice for the GluR6 subunit of presynaptic KA receptors (Crépel 2009).

Here again, the amplitude and time course of the slow component of DSE in adult wild-type mice were very similar to those of the residual endocannabinoid- and Glu-independent component of DSE seen in nearly mature rodents (Crépel 2007). Finally and interestingly enough, mean basal PPF in adult wild-type and VGLUT3\(^{-/-}\) mice was also no longer significantly different (Fig. 1C2). This suggests that in adult resting PCs, in contrast to nearly mature ones (see before), there is no retrograde release of Glu under resting conditions.

Exocytosis at PF-PC synapses in nearly mature wild-type and mutant mice. At central synapses, changes in mEPSCs frequency are generally thought to reflect modulation in spontaneous synaptic release. To ascertain that the abnormally high basal PPF observed in nearly mature VGLUT3\(^{-/-}\) mice (see above) was not indirectly linked to the constitutive invalidation of VGLUT3, we compared features of mEPSCs and uEPSCs in 17-day-old wild-type and mutant mice. As illustrated in Fig. 2, A and B1, amplitudes, kinetics and frequencies of mEPSCs were very alike in both groups of cells (\(n = 9\) and \(n = 8\), respectively). Thus mean amplitudes of mEPSCs were 15.2 ± 2.1 and 14.4 ± 1.3 pA, with mean frequencies of 0.91 ± 0.14 and 0.79 ± 0.10 mEPSC/s in VGLUT3\(^{+/+}\) and VGLUT3\(^{-/-}\) mice, respectively. These values were not significantly different. Very similar results were obtained with uEPSCs. Mean frequencies were 0.86 ± 0.16 and 0.76 ± 0.15 mEPSC/s in VGLUT3\(^{+/+}\) and VGLUT3\(^{-/-}\) mice, respectively (\(n = 8\) in both cases; Fig. 2B2).

Taken together, these results suggest that the machinery of activity-independent and activity-dependent vesicular release at PF-PC synapses is not grossly altered in nearly mature mutant mice and that, consequently, the abnormally high basal PPF observed in these KO mice genuinely results from a lower probability of evoked release at PF-PC synapses. If one cannot totally preclude that such a difference might be due to some undetected developmental differences between nearly mature wild-type and constitutive KO mice, a more attractive hypothesis is that the absence of basal retrograde release of Glu in the latter leads to a lower level of tonic activation of presynaptic...
KA receptors and, therefore, to a decrease of PF excitability. This hypothesis is strengthened by our observation that basal PPF in nearly mature wild-type mice was significantly increased in the presence of the desensitizing KA receptor agonist SYM 2081 (see above) and perfectly matched that observed in VGLUT3/−/− mice of the same age.

VGLUT3 participates in DPI in nearly mature wild-type mice. In 17-day-old wild-type mice and as previously established (Duguid and Smart 2004; Duguid et al. 2007), direct PC depolarization by a brief depolarizing burst (see METHODS) induced a clear-cut DSI: the mean mIPSC frequency decreased to 77.41 ± 14.47% of control value (n = 5; Fig. 3C). Dissipation of DSI after the stimulus (Δt = 1 min) was followed by a robust DPI that consisted of a large increase in the mean mIPSC frequency (190.02 ± 14.88%; Δt = 7 min), which was still present 15 min after the depolarizing burst (Fig. 3, A, B, and C).

In 17-day-old VGLUT3/−/− mice, DPI was undistinguishable from that induced in wild-type mice (Fig. 3C), suggesting again that endocannabinoid signaling was unaffected in these mice (see above). In marked contrast, DPI was almost totally absent since the mean mIPSC frequency at Δt = 7 min was only 126.44 ± 13.15 (n = 5). This residual DPI was no longer due to retrograde release of Glu from depolarized PCs acting on presynaptic NMDA receptors (Duguid and Smart 2004) since it was still present in the presence of 100 μM D-APV (the mIPSC frequency at Δt = 7 min was 118.32 ± 19.20; n = 6; Fig. 3C). This conclusion was confirmed by the fact that DPI elicited in 17-day-old wild-type mice in the presence of 100 μM n-APV (n = 7; Fig. 3C) was undistinguishable from that observed in KO mice of the same age in the presence or in the absence of n-APV. It is therefore plausible that such residual DPI simply results from a slightly better detection rate of mIPSCs during the rebound potentiation of inhibition that also followed the depolarizing burst (Duguid and Smart 2004; Fig. 3, A and B) during the same time window in both wild-type and VGLUT3/−/− mice. Indeed, mean mIPSC amplitudes at Δt = 7 min were 153.50 ± 17.21 and 145.93 ± 9.47% of control values for wild-type and VGLUT3/−/− mice, respectively. At Δt = 15 min, these amplitudes were still greater than control values: 133.36 ± 16.26 and 128.09 ± 10.63% for wild-type and VGLUT3/−/− mice, respectively (data not shown).

Therefore, these results are in keeping with previous observations showing that DPI depends on retrograde release of Glu from depolarized PCs through a N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-dependent process (Duguid and Smart 2004; Duguid et al. 2007) and further establish that this process involves the vesicular transporter VGLUT3.

VGLUT3 does not participate in DISC in nearly mature wild-type mice. In PCs located in the bank of lobule X facing lobule IX in 17-day-old wild-type mice (Fig. 4A) and as previously established (Duguid et al. 2007; Kim et al. 2009; Shin et al. 2008), depolarizing stimuli delivered 5 min after attaining the whole cell configuration evoked a biphasic inward current (Fig. 4B1), the earlier phase of which was quite variable and labile. According to Shin et al. (2008), this early inward current is likely to be mediated, at least in a large part, by a calcium-dependent chloride conductance (Llano et al. 1991a). In contrast, the later phase of the inward current, previously referred to as DISC (Shin et al. 2008), was elicited with greater reliability and relatively stable amplitude over the 20-min recording period (Fig. 4B2). In the present experiments, DISC usually peaked within 1 s, with a mean peak amplitude of 128.46 ± 38.49 pA (n = 9), and decayed over the next 2 to 3 s (Fig. 4, B1 and C).

In PCs located in the same bank of lobule X in 17-day-old VGLUT3/−/− mice, biphasic inward currents with very similar time courses to those observed in wild-type mice were also reliably elicited over the 20-min recording period (Fig. 4, B1, B2, and C). Surprisingly enough, the mean peak amplitude of DISC across cells 10 min after break-in was significantly (P < 0.001) larger than that observed in wild-type mice at the same Δt (362.90 ± 57.23 pA; n = 9; Fig. 4C), and the same was true for the initial inward current (Fig. 4, B1 and C). However, DISC was still nearly abolished by bath application of 100 μM of the broad-spectrum dopaminergic receptor antagonist haloperidol (n = 6; Fig. 4C), ruling out the possibility that its unusually large amplitude was due to the recruitment of additional nondopaminergic mechanisms.
Therefore, these results confirm that DISC is not due to autocrine activation of PC mGluR1 receptors by retrograde release of Glu from these cells but is rather due to autocrine activation of D₃ dopamine receptors of PCs by calcium-dependent release of vesicular postsynaptic dopamine (Kim et al. 2009). The fact that the amplitudes of the initial inward current and DISC were larger in VGLUT3⁻/⁻ mice than in control ones suggests in turn that calcium transients elicited by depolarizing bursts were also larger. We therefore tested this “calcium” hypothesis by directly comparing calcium transients induced in PCs located in this lobule by depolarizing pulses in nearly mature VGLUT3⁻/⁻ and in wild-type mice.

Effects of VGLUT3 invalidation on calcium transients and PC morphology in lobule X. As in the above-described electrophysiological experiments on DISC, changes in the intracellular free calcium concentration were elicited in PCs located in the bank of lobule X facing lobule IX by five 50-ms-long depolarizing voltage steps from −70 to 0 mV delivered at 10 Hz. In cells from both 17-day-old wild-type (n = 7) and VGLUT3⁻/⁻ mice (n = 7), calcium signals in PC dendrites peaked at the end of the fifth depolarization and relaxed with very similar kinetics within 1 s (Fig. 5A, blue and red lines, respectively). Differences between calcium transients obtained in PC dendrites from wild-type and VGLUT3⁻/⁻ mice were quantified for each cell by determining a value y that was the integral of ΔF/F values (bin size = 20 ms) measured during the five successive evoked calcium signals and by averaging these y values for the two groups of mice. In PCs from wild-type mice, the mean y value was 17.04 ± 4.97 compared with 49.76 ± 13.55 in

![Fig. 3. Depolarization-induced potentiation of inhibition (DPI) in 17-day-old wild-type and VGLUT3⁻/⁻ mice. A: successive sequences of miniature inhibitory postsynaptic currents (mIPSCs; 10 s in duration and recorded every 30 s; see METHODS) in control artificial cerebrospinal fluid before and after a train of stimuli (Depol; five 1,000-ms pulses to 0 mV, 10-ms interstimulus) that induced robust DPI and rebound potentiation in a representative wild-type PC. The depolarization-induced inward current was removed to improve clarity. Note that in this cell, initial depolarization-induced suppression of inhibition (DSI) was hardly detectable by simple eye inspection. B: high-resolution mIPSCs recorded in the same cell as in A during the control period and during DPI. Note also the increase in mIPSC amplitude, which signaled that a clear-cut rebound potentiation was also induced by the train of stimuli. C: normalized mean mIPSC frequencies before and after application of a 5-s stimulus (Depol) in control artificial cerebrospinal fluid in 17-day-old wild-type and VGLUT3⁻/⁻ mice (blue and red filled circles, respectively) and in the presence of 100 μM d-2-amino-5-phosphopentanoic acid (d-APV) in 17-day-old wild-type and VGLUT3⁻/⁻ mice (blue and red open circles, respectively). DSI was induced in all groups of mice, whereas DPI was impaired in VGLUT3⁻/⁻ mice to the same extent as in wild-type mice in the presence of d-APV.

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PCs from VGLUT3\(^{-/-}\) mice, the difference being significant (\(P < 0.05\)). These results show that the depolarization-mediated calcium changes evoked in PC dendrites by the same depolarizing voltage steps as those used for DISC induction are larger in VGLUT3\(^{-/-}\) mice than in wild-type mice. If one assumes that these results can be extended to depolarizing voltage steps used in DPI experiments, the fact that calcium-dependent DSI and rebound potentiation (Glitsch et al. 2000; Kano et al. 1992) were nearly identical in wild-type and VGLUT3\(^{-/-}\) mice (see before) suggests that the calcium signaling responsible for these processes was already saturated in wild-type animals in the present experiments.

To test whether these differences in calcium transients might be related to differences in PC morphology between VGLUT3\(^{+/+}\) and VGLUT3\(^{-/-}\) mice that render the latter more electrically compact, we loaded wild-type and mutant PCs located in the bank of lobule X facing lobule IX (\(n = 8\) and \(n = 10\), respectively) with lucifer yellow for at least 30 min. Visual examination of VGLUT3\(^{+/+}\) and VGLUT3\(^{-/-}\)-stained cells showed that their morphology was very similar, with dendritic arborizations extending through the molecular layer to the pial surface in both cases (Fig. 5, B1 and B2, respectively). Moreover, the mean area occupied by dendritic arborizations as well as the mean soma diameters of these PCs were not significantly different (Fig. 5C). Therefore, we cannot conclude that differences in calcium transients between wild-type and VGLUT3\(^{-/-}\) mice simply result from differences in cell morphology. In keeping with these results, mean input resistances of PCs in lobule X were also nearly identical in 17-day-old wild-type and VGLUT3\(^{-/-}\) mice since they amounted to 252.14 ± 37.35 M\(/\Omega\) (\(n = 8\)) and 255.62 ± 34.36 M\(/\Omega\) (\(n = 8\)), respectively.

**DISCUSSION**

In the present study, we analyzed the effects of genetic impairment of the Glu vesicular transporter VGLUT3 on DSE, DPI, and DISC in nearly mature and adult mice. The data fully confirm that, in nearly mature mice, the slow component of DSE is at least in part due to retrograde release of Glu from depolarized PCs. Furthermore, we show that this process occurs through vesicular release of Glu that involves VGLUT3. This Glu-dependent component of DSE is no longer present in fully mature mice, in keeping with the absence of VGLUT3 in adult animals. The present results also confirm that DPI depends on retrograde release of Glu from depolarized PCs through a SNARE-dependent process (Duguid and Smart 2004; Duguid et al. 2007) and further establish that this process also involves VGLUT3. In contrast, the fact that DISC was still present in VGLUT3\(^{-/-}\) mice and was nearly abolished by the broad-spectrum dopaminergic receptor antagonist haloperidol agree with previous observations showing that DISC is not due to autocrine activation of PC mGluR1 receptors by retrograde release of Glu from these cells but is rather due to autocrine activation of PC D3 dopamine receptors by calcium-dependent release of vesicular postsynaptic dopamine (Kim et al. 2009). Finally, the unusually large basal PF observed in nearly mature VGLUT3\(^{-/-}\) mice suggests that even under resting conditions, some basal retrograde release of Glu occurs in nearly wild-type animals that in turn contributes to tonic activation of PF-releasing sites through activation of presynaptic KA receptors (Crépel 2007; Delaney and Jahr 2002). In contrast, such basal retrograde release of Glu no longer occurs in fully mature animals and therefore no longer contributes to basal levels of ambient Glu.

**Vesicular-dependent release of Glu from PCs.** In immature PCs, previous studies (Duguid and Smart 2004; Duguid et al.
2007) on DPI have established that retrograde release of Glu requires a rapid rise in intracellular calcium concentration and is blocked by disrupting the SNARE-dependent vesicular release pathway. The latter study also discounted the possibility that Glu is released from other cellular structures present in the molecular layer and therefore unequivocally identified PCs as the source of Glu release. However, the precise molecular mechanisms that underlie somatodendritic Glu release remained unclear, although VGLUT3 was suspected to play a role in vesicle filling since the postnatal expression of VGLUT3 in PCs is entirely consistent with such a role (Boulolland et al. 2004; Gras et al. 2005). The present results on DPI and on the Glu-dependent component of DSE in nearly mature mice fully confirm this hypothesis by showing that both processes are impaired in VGLUT3 mice. This hypothesis is also supported by the fact that the Glu-dependent component of DSE is absent in fully mature mice (see RESULTS), in keeping with the disappearance of VGLUT3 in PCs at the end of the third postnatal week (Gras et al. 2005). Finally, the present results also comply with the observation that VGLUT3 play a role in vesicle filling in layer 2/3 neocortical pyramidal cells (Harkany et al. 2004; Zilberter 2000).

One might argue that the apparent lack of function of retrograde Glu signaling in immature VGLUT3 mice does not result from the absence of VGLUT3 but, rather, from altered postsynaptic calcium dynamics in KO mice (see Effects of VGLUT3 invalidation on calcium transients and PC morphology in lobule X) following, for instance, blanking of Glu stores or desensitization of a calcium-dependent mechanism. This hypothesis seems unlikely since the highly calcium-
dependent endocannabinoid component of DSE, DSI, and rebound potentiation (Brenowitz and Regehr 2003; Glitsch et al. 2000; Kano et al. 1992) was not significantly affected by the VGLUT3 mutation (see RESULTS). However, a rescue approach acutely expressing VGLUT3 in KO mice PCs might definitely eliminate this concern.

DISC, calcium transients, and morphology of PCs in nearly mature VGLUT3−/− mice. SNARE-dependent release of Glu from depolarized PCs was also proposed to be involved in generating DISC in these neurons through autocrine activation of postsynaptic mGluR1 receptors (Duguid et al. 2007; Shin et al. 2008). However, the origin of DISC current was later challenged by Kim et al. (2009), who presented evidence that DISC might rather be due to autocrine activation of postsynaptic DA receptors by DA released by depolarized PCs. Here, we show that the initial hypothesis is unlikely to be true since DISC was still present in nearly mature VGLUT3−/− mice and was nearly abolished by the broad-spectrum dopaminergic receptor antagonist haloperidol. Our results then tend to support Kim et al. (2009).

Unexpectedly, our results also show that in lobule X, DISC, as well as the initial calcium-dependent chloride current, was significantly larger in nearly mature VGLUT3−/− mice than in wild-type ones (see RESULTS). In keeping with the calcium dependence of these two currents, these results are therefore consistent with the fact that calcium transients elicited by depolarizing bursts were also larger in mutant mice than in wild-type ones. At the moment, the origin of such unusually large calcium transients in VGLUT3−/− mice remains obscure, even though one cannot exclude that it could result from indirect developmental consequences of the constitutive invalidation of VGLUT3 genes. However, we have shown that the unusually large calcium transients in VGLUT3−/− mice do not simply result from smaller dendritic arborizations or delayed maturation of PCs in these mice since their morphology appeared to be similar to wild-type PCs (see RESULTS).

Retrograde release of Glu and origin of basal extracellular Glu. Glial stores are generally considered as the main source of basal extracellular Glu through continuous nonvesicular release from the intracellular compartment of these cells (Jabaudon et al. 1999). In the striatum, this nonvesicular release results from the sustained activity of a cystine-glutamate antiporter (Baker et al. 2002). To protect neurons from excitotoxic injury and to ensure a high signal-to-noise ratio for glutamatergic synaptic transmission, the continuous release of Glu is normally compensated by its basal uptake through activity of Glu transporters (Bergles et al. 1997; Clark and Barbour 1997; Jabaudon et al. 1999). Here, we show for the first time that in the cerebellum nearly mature PCs probably constitute another significant source of extracellular Glu through basal release of this amino acid, which may in turn contribute to basal activation of presynaptic KA receptors. Since a weak activation of these receptors upregulates evoked Glu release at PF synapses (Delaney and Jahr 2002), this mechanism might favor information transfer in still immature cerebellar networks. In contrast, a larger increase of Glu release by depolarized PCs during DSE induction is likely to induce a slowly building up and slowly decaying calcium release from presynaptic ryanodine-sensitive calcium stores, following an initial short-lived activation of presynaptic KA receptors (Crépel 2007; Crépel and Daniel 2007). In keeping with results of a previous study (Levenes et al. 2001), this mechanism might well be responsible for the Glu-dependent component of DSE through an increased rate of spontaneous quantal PF-EPSCs and a decreased rate of evoked ones following depletion of the readily releasable pool of synaptic vesicles. On the other hand, such a sustained depolarization of PFs might also trigger action potentials in these fibers as previously suggested (Levenes et al. 2001). By spreading to other synapses located along the same beam of PFs, this propagated excitation might thus introduce a form of long-range communication among PCs sharing the same PF input.

Finally, retrograde release of Glu from depolarized PCs might participate in developmental processes such as establishment of synaptic circuits since the present results as well as previous studies on DSE and DPI (Crépel 2007; Duguid and Smart 2004; Duguid et al. 2007) indicate that this retrograde and autocrine signaling mechanism operates at least during the three first postnatal weeks in rats and mice. This hypothesis appears therefore plausible since, in particular, regression of the multiple innervation of PCs by climbing fibers that mainly occur during the second and third postnatal weeks in rodents (Crépel 1982) depends on sustained activation of NMDA receptors (Rabacchi et al. 1992). Even though the cellular localization of these receptors was not identified in this latter study, it is tempting to speculate that basal release of Glu from developing PCs is involved in this process. Further studies are required to evaluate whether or not regression of the multiple innervation of PCs by climbing fibers is impaired in VGLUT3−/− mice.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


