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Impaired motor coordination and Purkinje cell excitability in mice lacking calretinin

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ABSTRACT **In the cerebellum, the parallel fiber-Purkinje cell synapse can undergo long-term synaptic plasticity suggested to underlie motor learning and resulting from variations in intracellular calcium concentration ($[Ca^{2+}]_i$). Ca^{2+} binding proteins are enriched in the cerebellum, but their role in information processing is not clear. Here, we show that mice deficient in calretinin ($Cr^{-/-}$) are impaired in tests of motor coordination. An impairment in Ca^{2+} homeostasis in $Cr^{-/-}$ Purkinje cells was supported by the high Ca^{2+}-saturation of calbindin-D28k in these cells. The firing behavior of Purkinje cells is severely affected in $Cr^{-/-}$ alert mice, with alterations of simple spike firing rate, complex spike duration, and simple spike pause. In contrast, in slices, transmission at parallel fiber- or climbing fiber-Purkinje cell synapses is unaltered, indicating that marked modifications of the firing behavior *in vivo* can be undetectable in slice. Thus, these results show that calretinin plays a major role at the network level in cerebellar physiology.**

The involvement of the cerebellum in motor control has long been recognized, but its exact role is poorly understood. The idea that the cerebellum is a primary site of motor learning is a largely supported but still controversial hypothesis (1–3). Purkinje cells, which are the only output neurons in the cerebellar cortex, have a final integrating position and show two types of firing activity. Complex and simple spikes are driven by inputs from olivary neurons through climbing fibers and from cerebellar granule cells through parallel fibers, respectively. The parallel fiber-Purkinje cell synapse can undergo long-term modifications in synaptic strength that have been suggested to underlie motor learning (3–5). Variations in intracellular calcium concentration ($[Ca^{2+}]_i$) (6) play a crucial role in information processing in Purkinje cells because they modulate the efficacy of both excitatory and inhibitory inputs (7, 8) and are involved in the induction of synaptic plasticity (4, 5, 8). $[Ca^{2+}]_i$ regulates a large variety of neuronal functions, including neurotransmitter release, ion channel permeability, enzyme activity, and gene transcription. The control of $[Ca^{2+}]_i$ is crucial for the neuron and is performed by pumps, exchangers, organelle sequestration, and cytoplasmic Ca^{2+} binding proteins (9). Among these, calbindin-D28k and calretinin, which are highly conserved across mammalian species (10, 11), are both particularly enriched in the cerebellum, calbindin-D28k in Purkinje cells and calretinin in granule, unipolar brush, and Lugaro cells (12). Calbindin-D28k controls Ca^{2+} homeostasis by buffering Ca^{2+} (13) and affects the time course and spatial spreading of intracellular Ca^{2+} transients (14, 15). Accordingly, marked changes in synaptically evoked Ca^{2+} transients have been observed in Purkinje cells of calbindin-D28k-deficient mice (16). Calbindin-D28k and calretinin also have been implicated in synaptic plasticity because calbindin-D28k overexpression in cultured hippocampal pyramidal neurons suppressed post-tetanic potentiation (17), reduced expression of calbindin-D28k in mice affected the induction of CA1 long-term potentiation (18), and calretinin gene inactivation in mice abolished long-term potentiation induction in the dentate gyrus (19). Alterations in Ca^{2+} homeostasis in cerebellar neurons would be expected to dramatically affect information processing in the cerebellum and to give new insights in the understanding of its physiology. Because calretinin is highly expressed in granule cells and the parallel fiber-Purkinje cell synapse is central to cerebellar physiology, calretinin gene inactivation likely would perturb cerebellar function. We therefore investigated the effects of a null mutation of the calretinin gene (19) on the cerebellar physiology in mice. We found that the absence of calretinin in the cerebellum resulted in impaired motor coordination, in the detection of an unexpected calretinin-like immunoreactivity in Purkinje cells and in marked abnormalities in the Purkinje cell firing *in vivo* with alterations of both simple and complex spikes.

MATERIALS AND METHODS

Mice. Sex- and age-matched calretinin-deficient ($Cr^{-/-}$) and calbindin-D28k-deficient ($Cb^{-/-}$) mice and calretinin and calbindin-D28k ($Cr^{-/-}Cb^{-/-}$) double knockout mice, generated on a mixed 129 × C57BL/6 genetic background (16, 19), were used in all experiments.

Motor Coordination Analysis. The runway and stationary horizontal thin rod tests were performed as described (16). In the wheel-running test, mice were put inside a rotating drum (14 cm in diameter, 7 cm wide). The test was performed both in unperturbed conditions and in the presence of a sensory perturbation produced by a plastic tape around the shank of the right hindlimb. Locomotion was analyzed during two successive periods of 60 s by using the optoelectronic ELITE System (BTS, Milan) analyzing in the sagittal plane the trajectory of a retroreflective marker placed on the right hindlimb foot. To maintain a similar level of alertness, a short air puff was given on the back part of animal every 5 s.

Northern and Western Blot Analysis and Two-Dimensional Gel Electrophoretic Analysis. RNA and protein from cerebella

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Abbreviations: Cb, calbindin-D28k; Cr, calretinin.
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of $Cr^{+/+}$ and $Cr^{-/-}$ mice were analyzed as described (19). Rabbit anti-calbindin-D28k (1:1,000; catalog no. CB38, Swant, Bellinzona, Switzerland) and anti-calretinin (1:1,000; catalog no. 7696, Swant) antisera served as antibodies. Two-dimensional gel electrophoretic analyses were done as described (20).

Immunoprecipitation. Cerebellum protein supernatants were iodinated by using the chloramine T technique and were incubated for 1 h at 4°C with 5 μl of either normal rabbit serum or rabbit anti-calretinin antiserum. The antibody–antigen reaction was incubated with protein A-Sepharose (100 μl, 50% vol/vol) for an additional h at 4°C. After 5 washes in 150 mM NaCl, 50 mM Tris (pH 8), 0.1% SDS, and 1% NP40, the pellet was resuspended, was heated to 85°C, and was loaded onto a 12% polyacrylamide gel.

Immunoblot. Immunoblots were prepared as described (21). In brief, recombinant rat calbindin-D28k (2 μg; Swant, Switzerland) was incubated in either 3 mM calcium and 1 mM EGTA or only 1 mM EGTA for 30 min at 22°C. Formaldehyde was added to samples to give a 4% final concentration. After 30 min, samples were diluted and applied onto nitrocellulose filter by using a dot-blot apparatus. Blots then were processed with the rabbit anti-calretinin antiserum.

Histology, Immunocytochemistry, and *in Situ* Hybridization Histochemistry. Standard procedures were used as described (19) with rabbit anti-calretinin (1/1,000), anti-calbindin-D28k (1/10,000), anti-calmodulin (1/1000), anti-S-100 protein (1/1,000), or anti-parvalbumin (1/1,000) antisera (Swant, Bellinzona, Switzerland) and calretinin and calbindin-D28k oligoprobes, respectively.

Electron Microscopy. Sagittal blocks of the vermis were fixed in 4% glutaraldehyde, were postfixed in 1% osmium tetroxide, and were dehydrated and embedded in LX 112. Ultrathin sections were examined, and the number of synapses on the dendritic spines was counted on microphotographs (15,000×) ($Cr^{+/+}$, $n = 12$; $Cr^{-/-}$, $n = 15$) in the molecular layer at the half depth from the pial side.

Cerebellar Slice Electrophysiology. Standard procedures were used (22). Extracellular voltage recordings or whole-cell patch-clamp recordings were made, at 35°C, from P19-P51 Purkinje cells voltage-clamped at −70 mV by using an internal solution containing, in mM, 140 K·D-gluconate, 6 KCl, 10 Hepes, 1 EGTA, 0.1 $CaCl_2$, 5 $MgCl_2$, 4 Na_2ATP, and 0.4 NaGTP. Inhibitory synaptic currents were blocked with 50 μM SR-95531, except during extracellular recording of Purkinje cell spike frequencies or current-clamp recording of climbing fiber potentials. Parallel fibers or climbing fibers were focally stimulated by applying square pulses of 0.1 ms and 0.01–10 mA. Data were acquired with PCLAMP 6 (Axon Instruments, Foster City, CA) and were analyzed with IGOR Pro (WaveMetrics, Lake Oswego, OR).

***In Vivo* Electrophysiology.** Mice were prepared for chronic recording of cerebellar neuronal activity in the vermis and were allowed to recover for 3 days before the recording sessions. The extracellular activity of the recorded neurons explored with glass micropipette (1.5–5 megaohms impedance) was amplified (bandwidth 0.1–10,000 Hz) and discriminated (Digitimer). Purkinje cells were identified by the presence of complex spikes, and the micropipette was positioned optimally to record simultaneously simple spikes and complex spikes of opposite polarities. For measurement of the transient pause in simple spike discharge induced by the complex spike, this latter was used as a trigger for the averaging of the simple spike discharge (transformed in rectangular pulses of 1 ms in duration).

Statistics. Results were expressed as mean ± SD, and comparisons among groups were made by using one-way ANOVA and Bonferroni's post hoc test.

RESULTS

Impaired Motor Coordination in $Cr^{-/-}$ Mice. The general behavior of ≈6-week-old calretinin-deficient ($Cr^{-/-}$) and wild-type ($Cr^{+/+}$) mice previously analyzed in a series of tests was not different (19). Motor coordination was tested on the runway, the horizontal stationary rod, and the wheel running test. Neither of the first two tests revealed significant differences between 4- to 6-week-old $Cr^{+/+}$ and $Cr^{-/-}$ mice (Fig. 1 *A* and *B*). In contrast, in the wheel running test, $Cr^{+/+}$ mice performed a well reproduced cyclic movement (Fig. 1*E*) whereas $Cr^{-/-}$ mice made only few small displacements (Fig. 1*F*). The number of steps per minute and the total horizontal displacement of the hindlimb foot were 122.2 ± 58.5 and 4.50 ± 2.80 m, respectively for $Cr^{+/+}$ mice and 32.4 ± 23.8 ($P < 0.003$) and 0.38 ± 0.24 m ($P < 0.0002$) for $Cr^{-/-}$ mice. In the presence of a sensory perturbation (see *Materials and Methods*), locomotion of $Cr^{+/+}$ mice was not significantly modified, with 104.4 ± 76.1 steps/min ($P > 0.5$) and 3.78 ± 3.2 m of horizontal displacement ($P > 0.5$) (Fig. 1*G*), and locomotion of $Cr^{-/-}$ mice remained very poor, with 31.4 ± 14.8 steps/min and 0.35 ± 0.22 m. However, in $Cr^{-/-}$ mice, elevations of the hindfoot was induced (Fig. 1 *D* and *H*) and

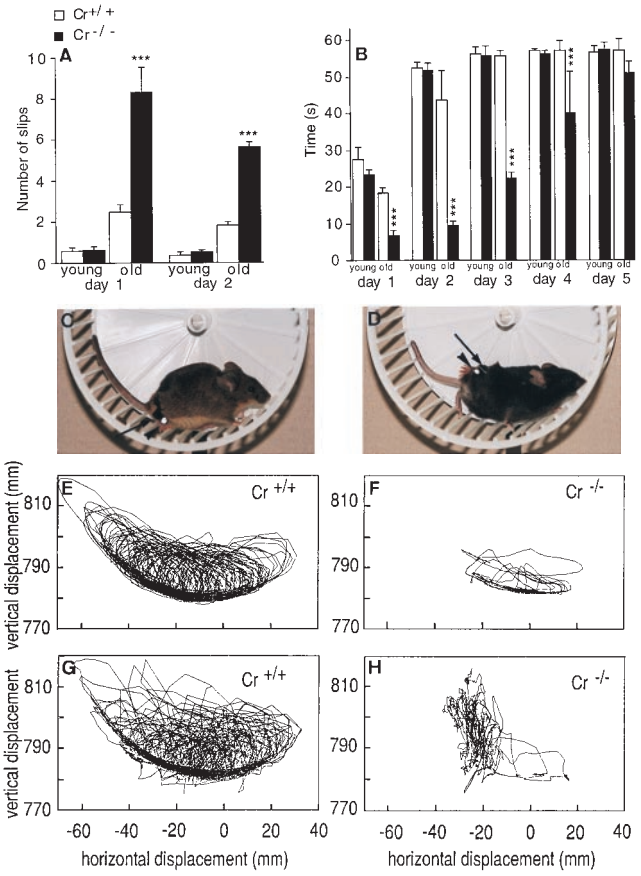

FIG. 1. Calretinin-deficient mice show motor coordination deficits. Young (≈6-week-old) and aged (18- to 24-month-old) $Cr^{+/+}$ and $Cr^{-/-}$ mice ($n = 4$–7 per group) were subjected to the runway test (*A*) and the stationary horizontal thin rod test (*B*). The number of slips was counted (*A*), and the retention time with a maximum of 60 s per trial was measured (*B*). Values are mean ± SD. ***, $P < 0.005$. Locomotion of young $Cr^{+/+}$ (*C*, *E*, and *G*) and $Cr^{-/-}$ mice (*D*, *F*, and *H*) ($n = 5$ per group) also was analyzed in the wheel running test (*C–H*). Locomotion of $Cr^{-/-}$ mice (*F* and *H*) was reduced both in an unperturbed condition (*F*) and in the presence of a sensory perturbation (*H*), which induced an oscillatory elevation of the hindlimb foot (*D* and *H*). Arrows and arrowheads point to plastic tapes used as sensory disturbers and retroreflective markers, respectively.

their number per minute was 0.6 ± 0.96 and 10.45 ± 6.1 for $Cr^{+/+}$ and $Cr^{-/-}$ mice, respectively ($P < 0.01$).

The motor coordination of aged (18- to 24-months old) $Cr^{+/+}$ and $Cr^{-/-}$ mice also was examined because the motor discoordination of calbindin-D28k-deficient mice has been shown to dramatically worsen with age (16). The feet of aged mutants slipped significantly more often than those of the wild-type in the runway test (Fig. 1*A*), and the time spent on the rod was significantly lower for aged $Cr^{-/-}$ mice in the horizontal stationary rod test (Fig. 1*B*). In the wheel-running test, aged $Cr^{-/-}$ mice presented the same deficit as young animals in the unperturbed condition and frequently fell after hindfoot elevations in the presence of the sensory perturbation.

Paradoxical Calretinin-Immunoreactivity in Cerebellum of $Cr^{-/-}$ Mice. Histological analysis showed that Purkinje cell bodies and granule cells have normal morphologies, densities, and locations within the different layers of the $Cr^{-/-}$ cerebellum (Fig. 2 *A* and *B*). Electron microscopy revealed that synaptic contacts on dendritic spines in the molecular layer appeared morphologically normal and in a normal density, with 11.03 ± 3.35 and 8.99 ± 2.38 synapses per 100 μm^2 for $Cr^{+/+}$ and $Cr^{-/-}$ mice, respectively ($P > 0.05$). Immunocytochemistry revealed that no calretinin immunoreactivity was observed in the brain of $Cr^{-/-}$ mice, as expected for the creation of a null mutation, with the exception of the cerebellum, in which Purkinje cell bodies and dendritic trees, as well as axons crossing the granule cell layer and terminals in deep cerebellar and vestibular nuclei, were strongly calretinin-immunoreactive (Fig. 2 *D*, *F*, and *H*). In granule cells of $Cr^{-/-}$ mice, no calretinin immunoreactivity was seen, in contrast to $Cr^{+/+}$ mice in which these cells and parallel fibers in the molecular layer are calretinin-immunoreactive (Fig. 2 *C*, *E*, and *G*). It is noteworthy that calretinin is not expressed in Purkinje cells of adult $Cr^{+/+}$ mice. Preincubation of the anticalretinin antiserum with purified calretinin eliminated immunoreactivity in both $Cr^{+/+}$ and $Cr^{-/-}$ brains whereas preincubation with calbindin-D28k did not decrease immunoreactivity in either $Cr^{-/-}$ Purkinje cells or $Cr^{+/+}$ brain. The cerebellar localization of calbindin-D28k and other related proteins, such as calmodulin, S-100 protein, and parvalbumin (data not shown) were similar in $Cr^{+/+}$ and $Cr^{-/-}$ mice. The calretinin immunoreactivity still was detected in Purkinje cells of 18- to 24-month-old $Cr^{-/-}$ mice, and no apparent change in its level was observed whereas the number of Purkinje cells had similarly decreased in both genotypes.

Calbindin-D28k Is Recognized by Anticalretinin Antiserum in Purkinje Cells of $Cr^{-/-}$ Mice. The nature of this calretinin immunoreactivity was investigated further. The corresponding mRNA was absent because no signal was detected on $Cr^{-/-}$ cerebellum RNA (data not shown) or in $Cr^{-/-}$ Purkinje cells on sections subjected to *in situ* hybridization (Fig. 3). However, when cerebellar proteins were analyzed by Western blotting with the anticalretinin antiserum, a faint signal was detected in $Cr^{-/-}$ mice (Fig. 4*A*). This calretinin-immunoreactive protein weighed ≈27,000 Da, lower than in $Cr^{+/+}$ mice (≈30,000 Da), and comigrated with calbindin-D28k. Furthermore, when iodinated proteins from $Cr^{+/+}$ and $Cr^{-/-}$ cerebella were immunoprecipitated with this anticalretinin antiserum, the expected band at 30,000 Da was detected in $Cr^{+/+}$ mice whereas a 27,000-Da protein was detected in $Cr^{-/-}$ mice (Fig. 4*B*). Two-dimensional gel electrophoresis confirmed in $Cr^{-/-}$ cerebellum the detection of proteins of same molecular weight and pI with either anti-calbindin-D28k or anticalretinin antisera (data not shown). These results suggested that, in $Cr^{-/-}$ cerebellum, calbindin-D28k is recognized by the anticalretinin antiserum. Confirming this hypothesis, no immunocytochem-

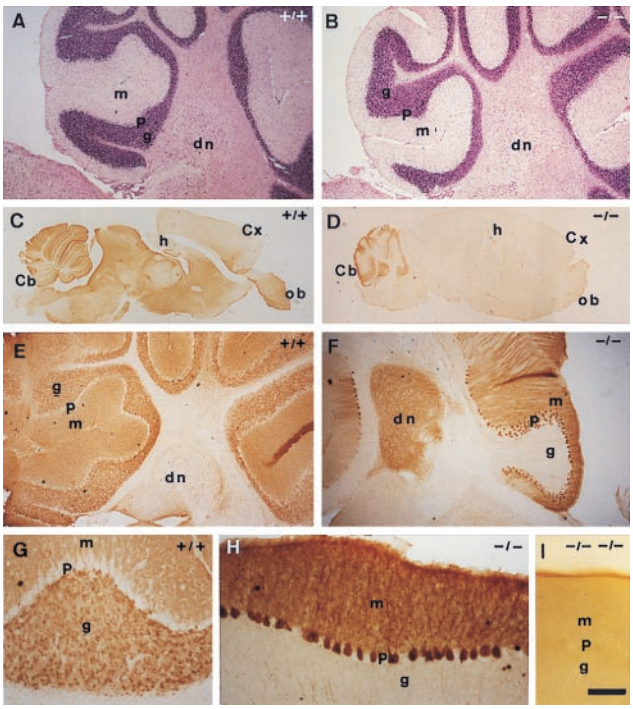

FIG. 2. Detection of calretinin-like immunoreactivity in $Cr^{-/-}$ Purkinje cells. Hematoxylin-eosin-stained sections of $Cr^{+/+}$ (*A*) and $Cr^{-/-}$ (*B*) cerebellum show no altered morphology. Shown is calretinin immunohistochemistry of $Cr^{+/+}$ (*C*, *E*, and *G*), $Cr^{-/-}$ (*D*, *F*, and *H*), and $Cr^{-/-}Cb^{-/-}$ (*I*) brain sections. Calretinin-immunoreactivity is distributed throughout the brain in $Cr^{+/+}$ mice (*C*), including in cerebellar granule cells and parallel fibers in the molecular layer (*E* and *G*). No labeling was observed in the brain of $Cr^{-/-}$ mice except in the cerebellum (*D*), in which Purkinje cell bodies, dendritic trees, axons, and terminals in deep cerebellar nuclei were strongly calretinin-immunoreactive (*F* and *H*). No labeling was detected in the cerebellum of $Cr^{-/-}Cb^{-/-}$ mice (*I*). [Bar = 300 μm (*A*, *B*, *E*, and *F*), = 2 mm (*C* and *D*), and = 150 μm (*G–I*). Cb, cerebellum; Cx, cerebral cortex; dn, deep cerebellar nuclei; g, granule cell layer; h, hippocampus; m, molecular layer; ob, olfactory tubercle; p, Purkinje cell layer.

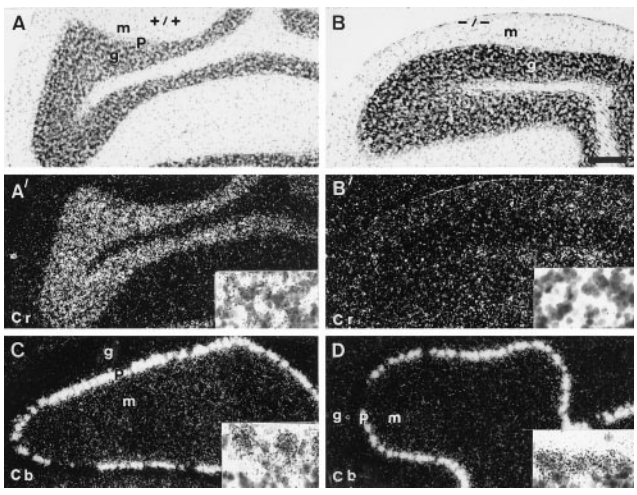

FIG. 3. Absence of calretinin mRNA expression and normal calbindin-D28k mRNA expression in cerebellum of mutant mice. Shown is calretinin (*A*, *A′*, *B*, and *B′*) and calbindin-D28k (*C* and *D*) mRNA detection on $Cr^{+/+}$ (*A*, *A′*, and *C*) and $Cr^{-/-}$ (*B*, *B′*, and *D*) cerebellar sections. Calretinin mRNA was detected in granule cells of $Cr^{+/+}$ mice and was undetected in $Cr^{-/-}$ cerebellum whereas the expression of calbindin-D28k mRNA was similar in both genotypes. *Insets* show granule cells (in *A′* and *B′*) and Purkinje cells (in *C* and *D*) at high magnification. [Bar = 150 μm (*A–D*) and = 20 μm (*Insets*). Bright- (*A*, *B*, and *Insets*) and darkfield (*A′*, *B′*, *C*, and *D*) illumination was used. Cb, calbindin-D28k mRNA; Cr, calretinin mRNA; g, granule cell layer; m, molecular layer; P, Purkinje cell layer.

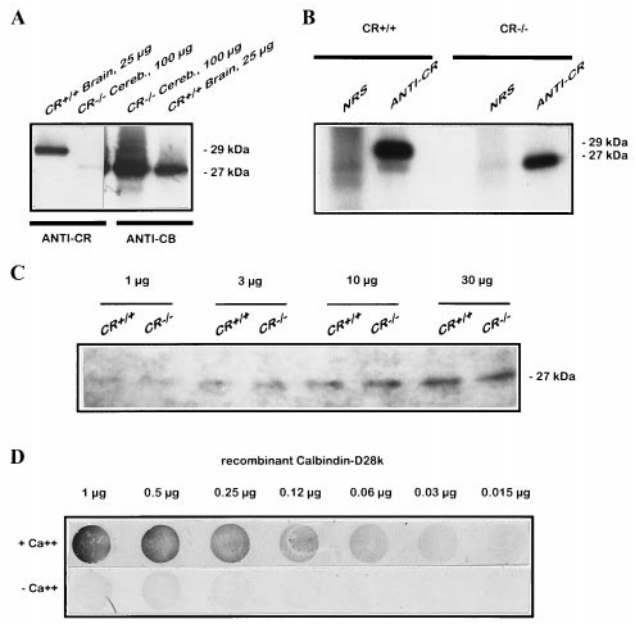

FIG. 4. Anti-calretinin antiserum recognizes calbindin-D28k in calretinin-deficient mice and recombinant calbindin-D28k when Ca^{2+}-saturated. (*A*) Western blot analysis. Proteins isolated from brain and cerebellum of $Cr^{+/+}$ and $Cr^{-/-}$ mice and transferred onto the same filter were probed either with anticalretinin or anti-calbindin-D28k antibodies. (*B*) Immunoprecipitation of a protein at the expected size of 30,000 Da from $Cr^{+/+}$ cerebellum and of a 27,000-Da protein from $Cr^{-/-}$ cerebellum. NRS, normal rabbit serum. (*C*) Western blot analysis using increased amounts of proteins isolated from cerebellum probed with anti-calbindin-D28k antibody shows no difference in calbindin-D28k-immunoreactivity. (*D*) Serial dilutions of purified recombinant calbindin-D28k preincubated in presence or absence of 3 mM calcium were immunoblotted by using the anticalretinin antiserum. Only calbindin-D28k saturated in Ca^{2+} could be detected.

ical signal was detected in Purkinje cells from $Cr^{-/-}Cb^{-/-}$ double knockout mice (Fig. 2*I*), which have a normal cerebellar cytoarchitecture. Together, these results support the idea that, in $Cr^{-/-}$ Purkinje cells, anticalretinin antiserum recognizes the closely related Ca^{2+} binding protein calbindin-D28k.

No obvious differences in calbindin-D28k-immunoreactivity or calbindin-D28k mRNA content were noted when using Western (Fig. 4*C*) and Northern (data not shown) blot analysis or immunocytochemistry (data not shown) and *in situ* hybridization (Fig. 3), demonstrating that the detection of calbindin-D28k by the anticalretinin antiserum in $Cr^{-/-}$ Purkinje cells was not related to an increase of calbindin-D28k expression. Alternatively, this detection could be explained by the induction of a qualitative modification of calbindin-D28k. Indeed, several anti-Ca^{2+} binding protein antibodies preferentially recognize peculiar calcium-induced protein conformations in a calcium-concentration-dependent manner (21). When serial dilutions of calbindin-D28k preincubated in presence or absence of 3 mM Ca^{2+} were immunoblotted by using the anticalretinin antiserum, it could be easily detected when saturated in Ca^{2+}, even for the highest dilutions (15 ng) whereas it was not or barely detectable even at the lowest dilution (1,000 ng) when preincubated in the absence of Ca^{2+} (Fig. 4*D*). This anticalretinin antiserum therefore recognizes calbindin-D28k only in a calcium-induced conformation and leads to the hypothesis that, in Purkinje cells of $Cr^{-/-}$ mice, calbindin-D28k binds more Ca^{2+} ions as compared with $Cr^{+/+}$ mice.

The Purkinje Cell Firing Behavior Is Altered in Alert $Cr^{-/-}$ Mice. The firing behaviors of 226 identified Purkinje cells were analyzed in 2- to 4-month-old and 2- to 2.5-year-old $Cr^{+/+}$ and $Cr^{-/-}$ alert mice. The spontaneous simple spike firing rate was significantly enhanced in both young and aged $Cr^{-/-}$ mice when compared with age-matched $Cr^{+/+}$ mice (Table 1). In contrast, the mean spontaneous firing rate of complex spike was not statistically different in $Cr^{-/-}$ and $Cr^{+/+}$ mice (Table 1). The duration of the complex spike was significantly reduced in both young and aged calretinin-deficient mice (Fig. 5 and Table 1). For all 226 Purkinje cells, a transient pause (complete suppression) in simple spike firing followed a spontaneous complex spike. To record this pause, we averaged the simple spike during a 100-ms epoch (with a pretrigger time of 10 ms) triggered by the spontaneous occurrence of a complex spike (Fig. 5). In $Cr^{+/+}$ mice, the Purkinje cell was well characterized by a complete suppression of the simple spike discharge after the complex spike (Fig. 5). In contrast, the duration of this pause was dramatically and significantly reduced in both young and aged $Cr^{-/-}$ mice (Fig. 5 and Table 1).

Excitatory Synaptic Transmission and Short-Term Plasticity Are Normal in $Cr^{-/-}$ Cerebellar Slices. To determine whether the electrophysiological differences seen *in vivo* resulted from direct modifications of the Purkinje cells or their afferent synapses, recordings were made from Purkinje cells in slices. Resting potential and input resistance were not different, with means of -50.9 ± 8.7 and -49.2 ± 12.2 mV ($P = 0.95$) and 217.2 ± 189.6 and 181.7 ± 127.8 megaohms ($P = 0.52$) in $Cr^{+/+}$ ($n = 15$ cells) and $Cr^{-/-}$ ($n = 21$ cells) mice, respectively. The simple spike frequency was not statistically different in $Cr^{+/+}$ and $Cr^{-/-}$ Purkinje cells, with a mean of 71.76 ± 4.76 ($n = 95$ cells) and 80.06 ± 5.03 spikes/min ($n = 73$ cells), respectively ($P = 0.24$). Although calretinin is normally expressed in presynaptic parallel fiber terminals, parallel fiber excitatory postsynaptic currents appeared unchanged in $Cr^{-/-}$ mice. Magnitude or interval-dependence (from 10 to 350 ms) of paired-pulse facilitation of the parallel fiber current were not different in $Cr^{+/+}$ and $Cr^{-/-}$ mice with ratios of 1.52 ± 0.05 and 1.47 ± 0.02, respectively, at a 50-ms interval ($P = 0.6$). The increase in size of the parallel fiber excitatory postsynaptic currents in response to increasing stimulation intensity was similar in $Cr^{+/+}$ and $Cr^{-/-}$ mice, with a slope of 34.37 ± 4.27 fC/mA ($n = 33$ cells) and 37.62 ± 5.39 fC/mA ($n = 29$ cells) ($P = 0.64$), indicating no detectable difference in parallel fiber recruitment. The duration of the complex spike elicited by climbing fiber stimulation and the number of peaks in this spike were not significantly different, 39.06 ± 2.89 and 44.88 ± 3.44 ms ($P = 0.2$) and 3.55 ± 0.17 and 3.48 ± 0.15 peaks ($P = 0.79$) in $Cr^{+/+}$ and $Cr^{-/-}$ mice,

Table 1. Purkinje cell firing behavior in alert $Cr^{+/+}$ and $Cr^{-/-}$ mice

Parameter	Young $Cr^{+/+}$, $n = 81$ cells	Young $Cr^{-/-}$, $n = 58$ cells	Aged $Cr^{+/+}$, $n = 31$ cells	Aged $Cr^{-/-}$, $n = 56$ cells
SSf, Hz	66.2 ± 24.5	$124.0 \pm 45.5^{\dagger}$	42.9 ± 19.6	$102.4 \pm 38.8^{\dagger}$
CSf, Hz	0.61 ± 0.3	0.54 ± 0.24	0.52 ± 0.23	0.59 ± 0.23
CSd, ms	9.4 ± 2.3	$6.4 \pm 1.8^{\dagger}$	12.5 ± 3.2	$6.3 \pm 2.1^{\dagger}$
Pause, ms	24.9 ± 6.6	$7.9 \pm 5.6^{*}$	31.4 ± 14.2	$5.4 \pm 6.1^{*}$

CSd, complex spike duration; CSf, complex spike firing rate; SSf, simple spike firing rate.
$^{*}P < 0.001$.
$^{\dagger}P < 0.0001$ as compared to age-matched, wild-type animals; one-way ANOVA.

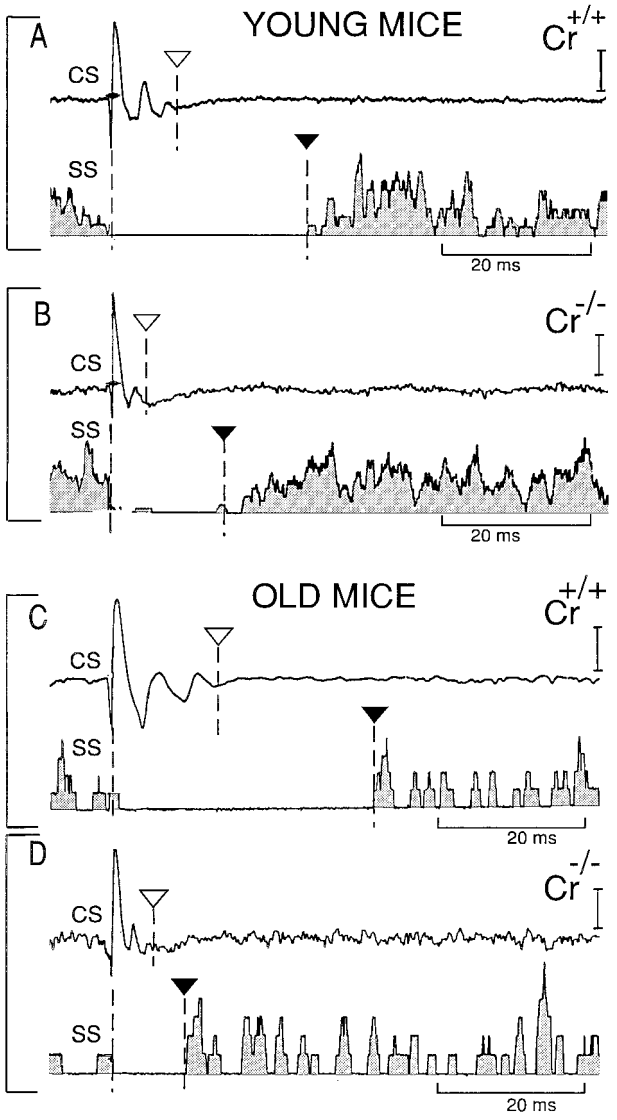

FIG. 5. The complex spike and simple spike pause durations are reduced in Purkinje cells of alert $Cr^{-/-}$ mice. The extracellular activity of Purkinje cells was explored *in vivo* in 2- to 4-month-old (*A* and *B*) and 2- to 2.5-year-old (n = 3–4 per group) (*C* and *D*) mice. The complex spike duration and simple spike pause duration were reduced in $Cr^{-/-}$ mice. Representative recording traces for $Cr^{+/+}$ (*A* and *C*) and $Cr^{-/-}$ (*B* and *D*) mice show start (vertical dashed line) and end (empty arrowheads) of the complex spike as well as end of the simple spike pause (filled arrowheads). For the determination of the simple spike pause duration, the complex spike was used as a trigger for the averaging of the simple spike discharge (transformed in rectangular pulses of 1 ms in duration) (lower rows). CS, complex spike; SS, simple spike. [Bars = 0.5 mV (*A* and *B*), = 1 mV (*C*), and = 0.3 mV (*D*).

respectively. This stimulation evoked all-or-none excitatory postsynaptic currents in both genotypes. Finally, the magnitude or interval-dependence (from 10 to 3,000 ms) of the climbing fiber current paired-pulse depression were not different in $Cr^{+/+}$ and $Cr^{-/-}$ mice with ratios of 0.68 ± 0.08 and 0.72 ± 0.07, respectively, at a 40-ms interval ($P > 0.05$). These results strongly suggest that the alterations in Purkinje cell activity observed in alert $Cr^{-/-}$ mice cannot be attributed to a difference in Purkinje cell excitability or abnormal function of its afferent synapses.

DISCUSSION

In this report, we have shown that the absence of calretinin expression results in impaired motor coordination, in the detection of an unexpected calretinin-like immunoreactivity in Purkinje cells, and in marked abnormalities in Purkinje cell firing with alterations of both simple and complex spikes. The cerebellar cytoarchitecture of $Cr^{-/-}$ mice was normal. Purkinje cell morphology, the formation of Purkinje cell synapses, and the localization or level of expression of Ca^{2+} binding proteins examined so far were unaltered.

Using biochemical techniques, we found strong evidence that the calretinin-like immunoreactivity detected in $Cr^{-/-}$ Purkinje cells corresponded to calbindin-D28k. This hypothesis was definitely demonstrated by the absence of immunoreactivity in Purkinje cells of $Cr^{-/-}Cb^{-/-}$ double knockout mice. This paradoxical immunoreactivity does not reflect an increased expression of calbindin-D28k but results from the appearance of a specific Ca^{2+}-induced conformation of this protein because the anticalretinin antiserum recognized it only when highly saturated with Ca^{2+}. A better recognition of Ca^{2+}-saturated Ca^{2+} binding proteins by their specific antisera already has been reported (21). Because this Ca^{2+}-dependent effect could be observed within a physiological $[Ca^{2+}]_i$ range (21), this qualitative modification of calbindin-D28k observed in $Cr^{-/-}$ Purkinje cells is highly suggestive of an impairment of Ca^{2+} homeostasis in these cells. Moreover, as far as we know, *in vivo* detection of a Ca^{2+}-induced change in protein conformation has not been described yet. A higher resting and/or evoked $[Ca^{2+}]_i$ in $Cr^{-/-}$ Purkinje cells represents the most reasonable hypothesis to explain this higher saturation of calbindin-D28k and could result from the alterations in synaptic activity (see below) and a consequent overflow of the calcium buffering capacity of the cell.

The impairment of Ca^{2+} homeostasis in Purkinje cells as well as the absence of calretinin-mediated Ca^{2+} buffering in parallel fibers would predict modifications of the excitatory synaptic transmission in cerebellar slices. Unexpectedly, neither the simple spike frequency, the parallel fibers recruitment, nor the complex spike duration were altered in slices taken from $Cr^{-/-}$ mice up to 2 months of age, strongly suggesting that intrinsic properties of $Cr^{-/-}$ Purkinje cells are not notably affected. In contrast, in alert $Cr^{-/-}$ mice in which signaling through cerebellar afferents as well as inhibitory networks are preserved, severe impairment of Purkinje cell firing properties was observed, with a doubling of the spontaneous simple spike firing rate, a halving of the duration of complex spike, and a 70–80% reduction in the duration of the simple spike pause after the complex spike. These results demonstrate that marked modifications of the neuronal firing behavior undetectable in brain slices can be unmasked by *in vivo* recording. In the most likely scenario, the primary change occurs in granule cells, and the increase in simple spike firing rate could either result from an increased responsiveness to mossy fibers or from an increased release activity at the parallel fiber-Purkinje cell synapse because of the absence of calretinin either in granule cells bodies or terminals, respectively. Because calretinin also is expressed in subsets of mossy fibers, their principal afference, and in less frequent cerebellar cell types such as unipolar brush and Lugaro cells (12), we cannot claim that the observed changes are exclusively attributable to changes in granule cells.

The hypothesis of an increased release activity at the parallel fiber-Purkinje cell synapse is less likely because, in slices, the release capacity of this synapse appeared normal. Paired-pulse facilitation, a presynaptic phenomenon (23) believed to strongly depend on residual $[Ca^{2+}]_i$ in the presynaptic terminal, was also unaltered. It is surprising that the absence of calretinin did not affect paired-pulse facilitation whereas overexpression of calbindin-D28k in hippocampal neurons suppressed post-tetanic potentiation, a similar short-term plasticity (17). However, at the parallel fiber-Purkinje cell synapse, it seems that the residual $[Ca^{2+}]_i$ dynamic is not the only mechanism involved (24) and that it mainly depends on

calcium extrusion and not on binding to fast endogenous Ca^{2+} buffers (24). In addition, Ca^{2+} binding protein(s) other than calretinin might be sufficient to assure the fast endogenous Ca^{2+} buffering in parallel fibers.

How can we explain the modifications in the durations of complex spike and simple spike pause? The response of Purkinje cell dendrites to parallel fiber stimulation consists of a monosynaptic excitatory postsynaptic potential followed by a disynaptic inhibitory postsynaptic potential resulting from the activation of inhibitory interneurons such as stellate and basket cells. This γ-aminobutyric acid-mediated inhibitory postsynaptic potential decreases the amplitude and duration of the climbing fiber-evoked response (25–27) and strongly reduces the dendritic climbing fiber-induced $[Ca^{2+}]_i$ increase (27). Parallel fiber stimulation may, therefore, importantly regulate the complex spike duration (25). In $Cr^{-/-}$ mice, the large increase in simple spike firing rate could, therefore, induce the observed reduction in complex spike duration. This would result in a large decrease in Ca^{2+} influx and, as a consequence, an attenuated Ca^{2+}-dependent potassium channel activation, leading to the reduction in the transient pause in simple spike firing that normally follows the complex spike. Similar changes in complex spike and transient pause durations have been described after direct stimulation of stellate cells *in vitro* (26). Following the interpretations of this author (26), the presumed increased activation of inhibitory interneurons in $Cr^{-/-}$ mice would result in an increased excitability of Purkinje cells, supporting the idea that the reduction in complex spike duration may in turn have an effect on the simple spike firing rate in $Cr^{-/-}$ Purkinje cells. Consistent with this hypothesis, decreased Ca^{2+} influx (28) or abolition of complex spike generation (29) have been shown to increase simple spike firing by 80–100%. A similar 90–135% increase was observed in $Cr^{-/-}$ mice whereas the complex spike duration was only reduced by 30–50% (see Table 1). This suggests that the shortening of the complex spike could not account for the entire increase in simple spike firing.

Alternative or additional mechanisms leading to an increased simple spike firing rate in $Cr^{-/-}$ Purkinje cells could be related to a probable decrease in Ca^{2+}-induced long-lasting rebound potentiation of inhibitory postsynaptic potential, which normally follows the complex spike (8), or to a chronic Ca^{2+}-induced decrease in γ-aminobutyric acid-activated currents (30).

The cerebellum is important for the control and fine tuning of movements (1–3). Ataxia is present in several mutant mice (16, 31, 32). However, unlike mice deficient for protein kinase Cγ (31) or metabotropic glutamate receptor mGluR1 (32), $Cb^{-/-}$ (16) and $Cr^{-/-}$ mice are not ataxic in standard environments but display impaired coordination when challenged. The cerebellum is required for adaptation of automated compound movements to novel environments (2), suggesting, therefore, that this function is particularly disturbed in $Cr^{-/-}$ and $Cb^{-/-}$ mice. Climbing fibers are believed to play a crucial role in the refinement of the motor program for a compound movement (3, 4) because they organize movement in time, with a complex spike duration being critical for optimal motor coordination (1). Thus, the impairment of the climbing fiber-evoked complex spike detected in alert $Cr^{-/-}$ mice may represent one of the major cellular correlates to the behavioral data.

The motor deficit of $Cr^{-/-}$ mice in classical tests (runway and stationary rod tests) was detected only in aged animals, an observation reminiscent of the worsened motor discoordination of aged $Cb^{-/-}$ mice (16). In aged animals, a similar loss of Purkinje cells occurred in both genotypes and may render aged $Cr^{-/-}$ mice more sensitive to cerebellar dysfunction resulting in the increased motor deficit. A significant alteration was observed in young $Cr^{-/-}$ mice during the wheel running test, which subjects mice to a more complex situation for which they must produce self-generated locomotor activity against an unrestrained ground. In addition, a sensory perturbation induced an abnormal motor pattern in $Cr^{-/-}$ mice, although it did not alter the locomotor performance of wild-type animals. This pattern likely results from an alteration of sensory data processing in the cerebellum (33) and a failure of motor behavior adaptation (2, 3).

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