Glutamate Receptor δ2 Subunit in Activity-Dependent Heterologous Synaptic Competition

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In the adult cerebellum, the glutamate receptor δ2 subunit (GluRδ2) is selectively targeted to the spines of the distal Purkinje cell dendrites, the spiny branchlets, that are innervated by the parallel fibers. Although GluRδ2 has no known channel function, it is presumed to be involved in the formation and stabilization of these synapses. After block of electrical activity by tetrodotoxin, GluRδ2s appear in the postsynaptic densities of the proximal dendritic spines, which then lose their contact with climbing fibers and become ectopically innervated by parallel fibers. This phenomenon suggests that climbing fiber activity prevents GluRδ2 targeting to proximal dendrites and that GluRδ2s admitted to the postsynaptic density of the spine cause withdrawal of the silent climbing fiber. To test this hypothesis, we studied the distribution of GluRδ2s in the rat cerebellum by immunoelectron microscopy during the recovery period that follows removal of the electrical block, and during the sprouting of climbing fibers that follows subtotal deletion of the parent inferior olivary neurons by administration of the drug 3-acetylpyridine. We found that after removal of the electrical block, the climbing fibers reinnervate proximal spines that bear GluRδ2s and these subunits are successively repressed. Similarly, after subtotal lesion of the inferior olive, reinnervation of denervated Purkinje cells occurs on spines bearing GluRδ2s. Thus, GluRδ2s are not responsible for displacing silent climbing fibers. We propose instead that GluRδ2s are associated with climbing fiber-to-Purkinje cell synapses, during development or at early stages of climbing fiber regeneration or sprouting, and are downregulated during the process of synapse maturation.

Key words: **glutamate receptor δ2 subunit; afferent fiber competition; activity-dependent competition; cerebellum; parallel fiber; climbing fiber**

Introduction

Glutamate receptor (GluR) channels mediate most of the fast excitatory synaptic transmission in the vertebrate CNS and play an essential role in various types of plasticity (Mayer and Westbrook, 1987; McDonald and Johnston, 1990). The δ2 subunit (GluRδ2) belongs to the family of glutamate receptor channels. It is selectively expressed in Purkinje cells and has no known channel function (Araki et al., 1993; Lomeli et al., 1993). During development, GluRδ2s are expressed in both parallel fiber- and climbing fiber-innervated spines. In contrast, at the end of the developmental period, these subunits remain exclusively or nearly exclusively confined to the parallel fiber synapses (Takayama et al., 1996; Landsend et al., 1997; Zhao et al., 1998). In the GluRδ2 knock-out mouse, nearly one-half of the branchlet spines are free of innervation, although the total spine density is normal (Kashiwabuchi et al., 1995; Kurihara et al., 1997). In addition, the climbing fiber terminal arbor in this mouse extends its territory of innervation distally to the parallel fiber dendritic domain (Ichikawa et al., 2002). These observations suggest that GluRδ2s play a role in the differential distribution and stabilization of the parallel fiber and climbing fiber synapses on the distal and proximal domains of the Purkinje dendritic arbor.

Experimental block of electrical activity by tetrodotoxin (TTX) has also shown that GluRδ2s are involved in the competition between parallel fibers and climbing fibers to acquire their postsynaptic domains on the Purkinje cells (Bravin et al., 1999; Morando et al., 2001). After block of electrical activity, climbing fibers lose their contacts with the spines of the proximal dendrites. However, before their withdrawal, GluRδ2s appear in these spines. This fact suggests that the active climbing fiber exerts a repressive action on the expression of GluRδ2, and that *de novo* targeting of this subunit to the spines innervated by the inactive climbing fibers is responsible for the withdrawal of the terminal afferents. Additionally, after block of electrical activity the proximal dendritic domain develops a large number of new spines that also bear a high density of GluRδ2s and are ectopically innervated by the parallel fibers. Therefore, a complementary view is that the active climbing fiber represses the number of spines of the proximal dendrites and that, in the absence of electrical activity, the parallel fibers gain a competitive advantage and displace the synapses of silent climbing fibers from GluRδ2-bearing spines.

To test these hypotheses, we decided to study the process of climbing fiber reinnervation of the proximal Purkinje cell dendrite that takes place on TTX removal and after 3-acetylpiridine (3-AP)-induced subtotal deletion of inferior olivary neurons, which are the source of climbing fibers. Specifically, we set out to ascertain whether during the recovery period the growing climbing fiber terminals are able to reinnervate spines bearing GluRδ2s or whether they form synapses only with spines devoid of them.

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Materials and Methods

Toxin delivery. We used adult Wistar albino rats (body weight, 150–250 gm; age, 1.5–3 months; Charles River, Calco, Italy). TTX (80 μM in 0.12 M phosphate buffer, pH 7.2; Sigma, St. Louis, MO) was infused for 7 d into lobule VII of the dorsal vermal cortex by means of an osmotic minipump (Alzet 2002; Alza, Cupertino, CA) (Bravin et al., 1999). Control animals were infused with vehicle. Ultrastructural analysis was performed on vehicle-infused rats ($n = 3$), TTX-infused rats killed after 7 d of treatment ($n = 3$), and TTX-infused rats that survived 45 d ($n = 3$) and 135 d ($n = 3$) after TTX removal. Because TTX infusion was not successful in every animal, only rats displaying cerebellar ataxia were selected for our study. All surgical procedures were performed under general anesthesia by a mixture of ketamine (100 mg/kg, Ketavet; Gellini Farmaceutici, Latina, Italy) and xylazine (5 mg/kg, Rompum; Bayer, Leverkusen, Germany). The experimental plan was designed according to the guidelines of Italian law for care and use of experimental animals (DL 116/92) and approved by the Italian Ministry of Health.

Inferior olive lesion. We obtained a subtotal lesion of the inferior olive in another set of adult rats (body weight, 120–170 gm; age, 35–40 d) by a single intraperitoneal injection of 3-AP (Fluka; Sigma; 65 mg/kg body weight). After survival periods of 6, 40, 90, 150, and 270 d, 3-AP-treated rats that were affected by severe ataxia were transcardially perfused with 500 ml of glutaraldehyde (0.1%), formaldehyde (4%), and picric acid (0.2%) in 0.12 M sodium phosphate buffer, pH 7.4. The extent of the inferior olive lesion was checked in 25-μm-thick serial sections of the medulla oblongata stained with the Nissl method (Hess et al., 1988). The numbers of surviving neurons were calculated relative to the control numbers as determined by Schild (1970). Only the cerebella of rats ($n = 3$ for each survival period) with a number of surviving olivary neurons of <12% were processed for additional study. Three untreated rats were used as controls.

Electron microscopy. For ultrastructural analysis, sagittal slices of the cerebellum obtained with a vibrating blade microtome (Leica, Wien, Austria) were embedded in Epon/Araldite resin (Fluka; Sigma). For serial reconstructions, 15 series consisting of 10–15 ultrathin sections were prepared and mounted on single-slot copper grids. After contrast enhancement with uranyl acetate and lead citrate, photographs were taken on an electron microscope operated at 80 kV (model 410; Philips, Eindhoven, The Netherlands) at a magnification of 4000× and printed at a final magnification of 16,000×. Proximal Purkinje cell dendrites were identified by their size and typical hypolemmal cisterns of smooth endoplasmic reticulum (Palay and Chan-Palay, 1974; Bravin et al., 1999). A proximal dendritic profile was selected in each ultrathin section series, and all spines in continuity with that profile were identified. Each spine or spine-like protrusion identified in a given section was followed until it disappeared downstream and upstream in the section series.

Freeze substitution and Lowicryl embedding. After perfusion with 500 ml of glutaraldehyde (0.1%), formaldehyde (4%), and picric acid (0.2%) in 0.12 M sodium phosphate buffer, pH 7.4, cerebellar blocks were sagittally cut (500 μm) on the vibrating blade microtome and slammed to a polished copper block cooled with liquid N_2 (MM80 E cryofixation apparatus; Reichert, Wien, Austria). The morphological analysis of vehicle- and TTX-treated cerebella was performed in folia on either side of lobule VII, which was the injection site of the minipump cannula. Slices were transferred to 0.5% uranyl acetate dissolved in anhydrous methanol (−90°C) in a freeze-substitution apparatus (CS Auto; Leica). The temperature was raised stepwise to −50°C. Samples were infiltrated with Lowicryl HM20 resin (Chemische Werke Lowi, Waldkraiburg, Germany) and polymerized by UV light.

Immunoincubation. Ultrathin sections (90–110 nm) of Lowicryl-embedded blocks were collected on uncoated nickel grids and processed for immunogold cytochemistry. The sections were etched with a saturated solution of NaOH in absolute ethanol for 2–3 sec, rinsed with double-distilled water, and incubated sequentially in the following solutions (at room temperature): (1) 0.1% sodium borohydride and 50 mM glycine in Tris buffer (5 mM) containing 0.9% NaCl and 0.1% Triton X-100 (TBST; 10 min); (2) 2% human serum albumin (HSA) in TBST (10 min); (3) primary antibody (rabbit anti-GluRδ2; kindly provided by M. Watanabe, Department of Anatomy, Hokkaido University, Sapporo, Japan; 2.5 μg/ml) in TBST containing 2% HSA (overnight); (4) TBST (several rinses) and 2% HSA in TBST (10 min); and (5) goat anti-rabbit Fab fragments coupled to 10 nm colloidal gold particles (GFAR10; British BioCell International, Cardiff, UK), diluted 1:20 in TBST with 2% HSA and 0.05% polyethyleneglycol (1 hr). The nickel grids were then rinsed several times in double-distilled water, counterstained with uranyl acetate and lead citrate, and examined in the electron microscope. The specificity of the GluRδ2 antiserum was confirmed by the complete lack of immunostaining in sections incubated only with TBST omitting the primary antibody.

Quantitative analysis. Quantitative analysis of immunogold particles representing GluRδ2 antiserum binding sites was performed on electron micrographs of climbing fiber and parallel fiber synapses identified by standard morphological criteria (Palay and Chan-Palay, 1974). Small clusters of vesicles abutting the synaptic contact characterize parallel fiber terminals. In contrast, climbing fiber boutons contain a high density of uniformly distributed vesicles and some dense core vesicles. It is possible that the regenerating climbing fibers have a different morphological profile. This is unlikely, because it has been demonstrated previously (Rossi et al., 1991a) that in 3-AP-treated rats the regenerating climbing fibers maintain their typical morphology. These synapses were sampled throughout the thickness of the cerebellar molecular layer. The length of the postsynaptic density was measured, and the number of gold particles was counted. Then the immunolabeling density was expressed as the number of gold particles per micrometer of postsynaptic density length. Statistical evaluation was performed using Student's *t* test. Quantitative analysis of GluRδ2 immunoreaction in the spines innervated by climbing fiber in both TTX- and vehicle-treated cerebella was performed on 10 areas selected from 10 grids for each experimental group. On each grid, composed of 300 squares, we selected, by randomly shifting the grid, only five squares (for a total area of 34,445 μm^2) entirely occupied by molecular layer. The average number of climbing fiber synapses was expressed per a unit area of 34,445 μm^2.

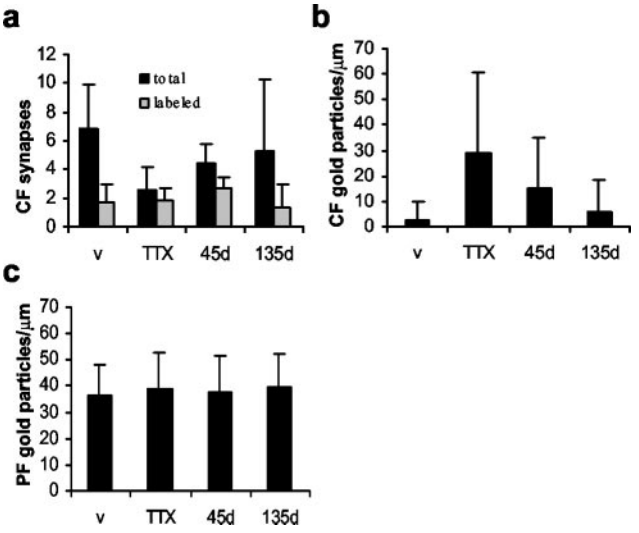

Figure 1. Quantitative evaluation of proximal and distal Purkinje cell dendritic spines innervated by climbing fiber (*CF*) terminals and by parallel fiber (*PF*) varicosities and their GluRδ2 expression in the TTX experimental group. Immunogold particle counts were done after 7 d of infusion with vehicle (*v*) and TTX and at 45 and 135 d after TTX removal. *a*, Average number ± SD of *CF* synapses per unit area of 34,445 μm^2. *b*, *c*, Gold particle density per micrometer of postsynaptic density length in *CF*- and *PF*-innervated spines. The total number of climbing fiber-innervated spines (*black columns*) drops under TTX and recovers at 45 and 135 d (*a*). The number of GluRδ2-labeled synapses (*gray columns*) is a small fraction in vehicle-treated animals (*a*), and they have a very small gold particle density (*b*), whereas under TTX they represent a high fraction (*a*) and have a high gold particle density (*b*). Parallel fiber synapses on the spiny branchlets maintain similar values of high GluRδ2 density in all conditions (*c*).

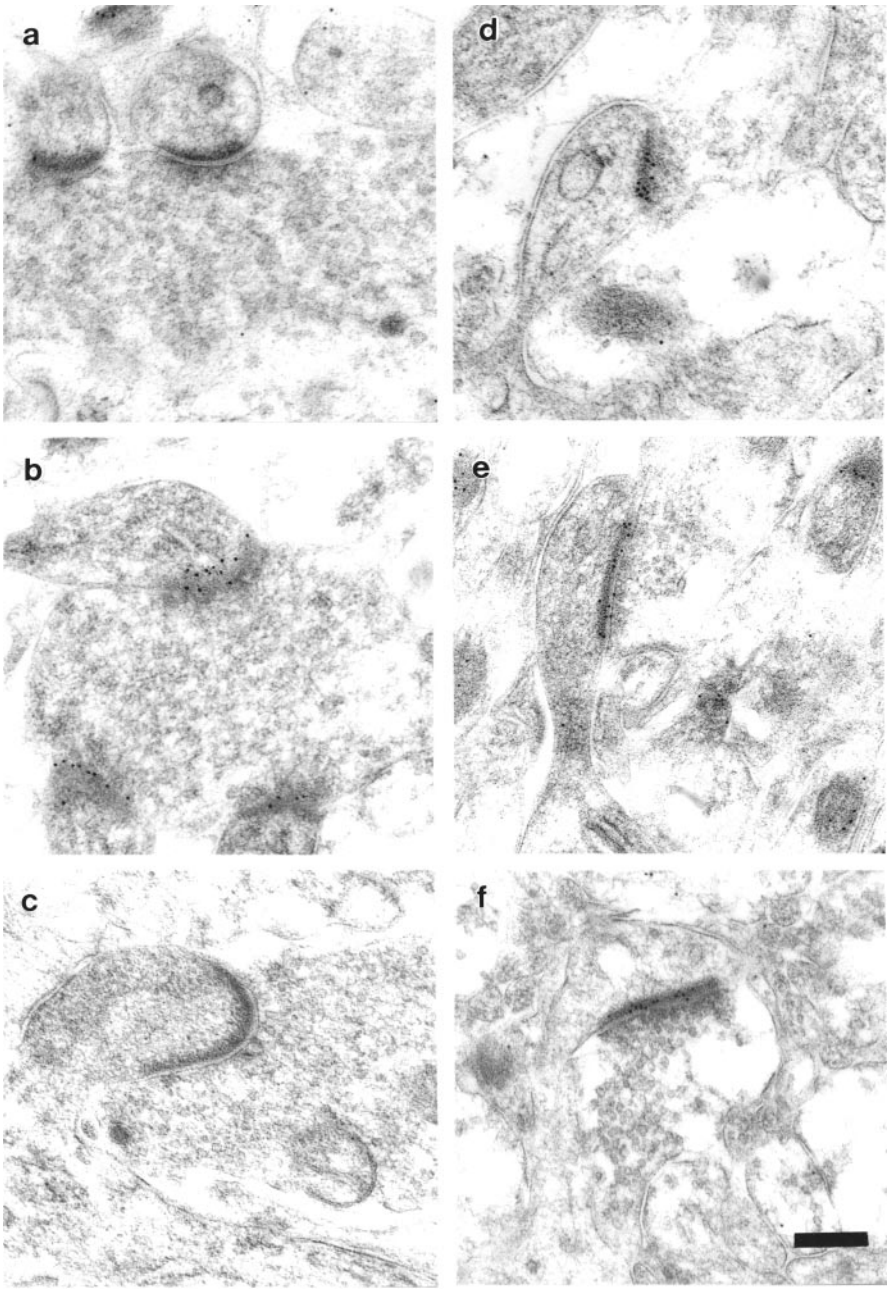

Figure 2. GluRδ2 expression in TTX-treated cerebella. *a–c*, Climbing fiber-innervated spines. GluRδ2 expression is virtually absent in vehicle-treated cerebella (*a*) and after 135 d of recovery (*c*), whereas it is highly expressed at 7 d of TTX treatment (*b*). *d–f*, Parallel fiber-innervated spines emerging from the branchlets. GluRδ2 expression is high in all three conditions. Scale bar, 0.23 μm.

Results

To elucidate the role of GluRδ2s in the competition between parallel fibers and climbing fibers, we used two different experimental protocols that produce reinnervation of Purkinje cells by the climbing fibers. In the first model, we topically blocked all electrical activity in the cerebellar cortex by TTX for 7 d. Under TTX block, the climbing fiber terminal arbor retracts from its target and becomes atrophic, the Purkinje cell proximal dendrites undergo a remarkable increase in spine density, and the parallel fiber input, which is normally limited to the distal dendritic domain, innervates the newly formed spines (Bravin et al., 1999; Morando et al., 2001). Four weeks after removal of the electrical activity block, the climbing fiber arbor reacquires its normal morphology. It expands its territory to reinnervate the proximal dendritic domain and, at the same time, it induces elimination of the competing parallel fiber synapses from the same territory, thus restoring the original pattern of low spine density typical of the proximal dendrites.

In the second model, we performed a subtotal lesion of the inferior olive to eliminate the majority of climbing fibers. A few days after the lesion, a large number of new spines appear on the Purkinje cell proximal dendrites, and parallel fibers sprout and contact the newly formed spines (Sotelo et al., 1975; Rossi et al., 1991a,b). At variance from the TTX experiments, the new spines develop in the presence of increased Purkinje cell activity (Benedetti et al., 1984; Savio and Tempia, 1985; Strata, 1985). In addition, collateral branches of climbing fibers of surviving inferior olivary neurons reinnervate many climbing fiber-deprived Purkinje cells. This process is especially prominent during the first 3 months after the lesion, although it lasts several months. During the climbing fiber regenerative process, the two excitatory inputs, climbing fiber and parallel fibers, coexist on the proximal Purkinje cell dendrite to segregate again when the new climbing fiber reaches its full maturation and displaces the parallel fiber synapses (Rossi et al., 1991a,b).

Recovery of climbing fibers after TTX block

Below, we present quantitative data on GluRδ2 in proximal dendritic spines in four experimental groups of subjects, each consisting of three rats, after: (1) vehicle infusion for 7 d (control group), (2) 7 d of TTX block, and (3) two time points (45 d and 135 d) of recovery from TTX block. In each experimental group, quantitative analysis was done on 10 randomly selected areas of the molecular layer measuring 34,445 μm^2 each (see Materials and Methods).

In the vehicle group, we counted the number of climbing fiber-innervated spines per unit area. All of these spines were counted regardless of whether or not they were seen emerging from a dendrite, because we never observed climbing fibers contacting spines of distal branchlets (see below). We found an average of 6.9 ± 3.0 SD climbing fiber-innervated spines (Figs. 1*a*, 2*a*). Of these spines, 1.7 ± 1.2 SD (24.6%) expressed GluRδ2 (Fig. 1*a*) and the gold particle density was 2.8 per micrometer (±7.2 SD) of postsynaptic density length (Fig. 1*b*).

In the 7 d TTX-block group, we made a similar evaluation and found that the number of climbing fiber-innervated spines per unit area was significantly ($p < 0.002$) lower, with an average value of 2.6 ± 1.6 SD (Fig. 1*a*). Among these spines, those bearing GluRδ2 had a value of 1.8 ± 0.9 SD (Figs. 1*a*, 2*b*), not significantly

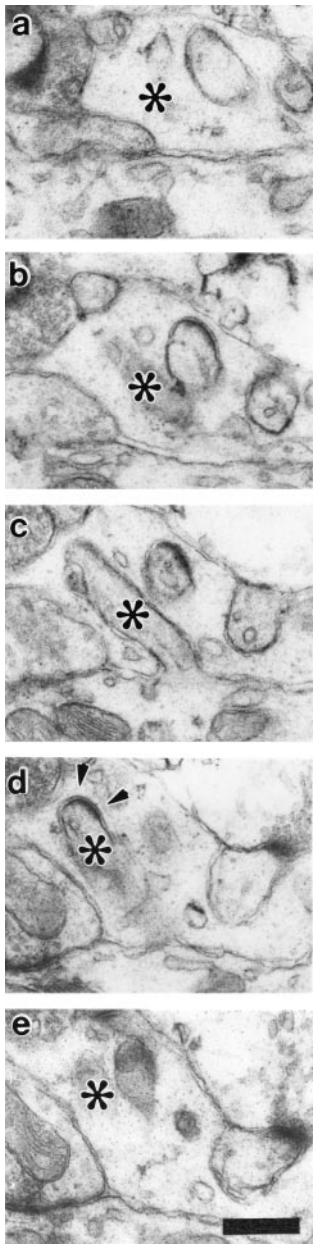

Figure 3. Serial reconstruction of a spine (*asterisk*) free of presynaptic terminal that emerges from the proximal dendrites of a Purkinje cell at 6 d from a subtotal lesion of the inferior olive. *a–e*, Consecutive sections showing the entire spine profile with no presynaptic terminals. Note that in *d* (*arrowheads*) there is a thickening of the postsynaptic membrane. Scale bar, 0.36 μm.

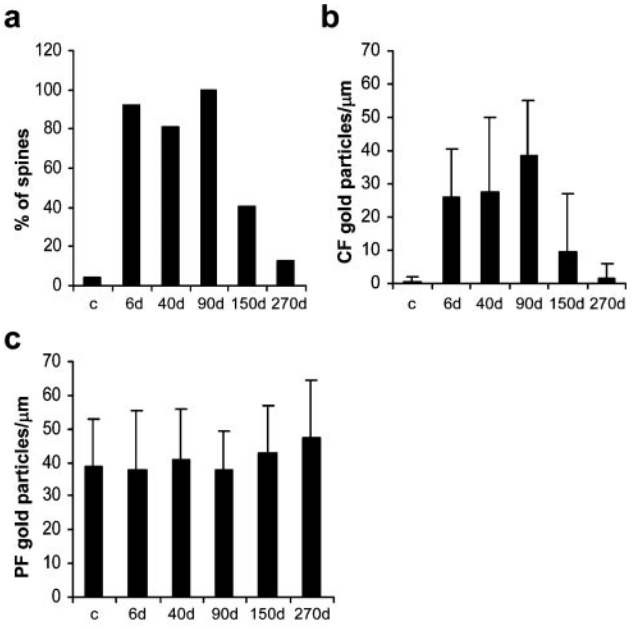

Figure 4. Quantitative evaluation of proximal dendritic spines innervated by climbing fiber (*CF*) and distal dendritic spines innervated by parallel fiber (*PF*) and their GluRδ2 expression in rats with inferior olive lesion at different periods in days. *a*, Percentage of climbing fiber-innervated spines bearing GluRδ2. The percentage value increases remarkably up to 90 d after the lesion and then recovers at 150 d and is similar to control (*c*) at 270 d. *b*, *c*, Number of gold particles per micrometer of postsynaptic density length in climbing fiber- and parallel fiber-innervated spines. The density in the climbing fiber-innervated spines is increased up to 90 d and then recovers at 150 d and is similar to control at 270 d, whereas the density in the parallel fiber-innervated spines is normal in all conditions.

($p > 0.8$) different from that of the vehicle group, but representing 69.2% of the total number of climbing fiber-innervated spines. In addition, the gold particle density had a significantly higher value of 28.9 per micrometer (±32.0 SD; $p = 3.49 \times 10^{-4}$) relative to the same value in the vehicle group (Fig. 1*b*). These results are in line with the description of a decrease in climbing fiber synapses consequent to the climbing fiber atrophy and an overexpression of GluRδ2 in the remaining synapses (Morando et al., 2001).

After removal of TTX block, the climbing fiber terminal arbor grows to acquire its original extent (Bravin et al., 1999). Therefore, we aimed to see whether the growing climbing fibers were able to innervate spines bearing GluRδ2. In the rats allowed to recover for 45 d, the average number of climbing fiber synapses per unit area recovered significantly ($p < 0.02$) with a value of 4.4 ± 1.4 SD (Fig. 1*a*) and an increase of 69.2% relative to the value in the 7 d TTX group. Also, the number of GluRδ2-labeled synapses increased significantly ($p < 0.025$) to a value of 2.7 ± 0.7 SD (Fig. 1*a*) with an increase of 50% relative to the same value in the 7 d TTX group. This means that during this recovery period there was a significant number of new GluRδ2-labeled spines innervated by climbing fibers, which represented 61.4% of the total number of climbing fiber synapses. In these synapses, the average density of gold particles was 14.9 per micrometer (±20.0 SD) (Fig. 1*b*). The significant increase in the climbing fiber GluRδ2-labeled synapses demonstrates that the climbing fibers are able to innervate spines bearing GluRδ2. Nevertheless, it is also possible that the initial synaptic contact between a newly sprouted climbing fiber terminal and a dendritic protrusion gives rise to a synapse that is devoid of GluRδ2s. Then the GluRδ2s would appear while the synapse is still immature and is unable to repress them. In either case, the expression of GluRδ2 clearly does not interfere with the formation of climbing fiber synapses.

In the rats examined after 135 d of recovery, the number per unit area of climbing fiber synapses was still significantly higher ($p < 0.02$) relative to the number found after 7 d of TTX treatment, with a value of 5.3 ± 5.0 SD (Figs. 1*a*, 2*c*), which is not significantly different ($p > 0.05$) from that of vehicle-treated animals. The number of GluRδ2-labeled spines per unit area was 1.3 ± 1.6 SD (Fig. 1*a*), which is not significantly different from the vehicle-treated and from the 7 d TTX-treated animals ($p > 0.05$). In addition, the gold particle density had a value of 5.7 per micrometer (±12.5 SD) (Fig. 1*b*), which is very similar ($p > 0.05$) to that of the vehicle-treated rats. These data demonstrate

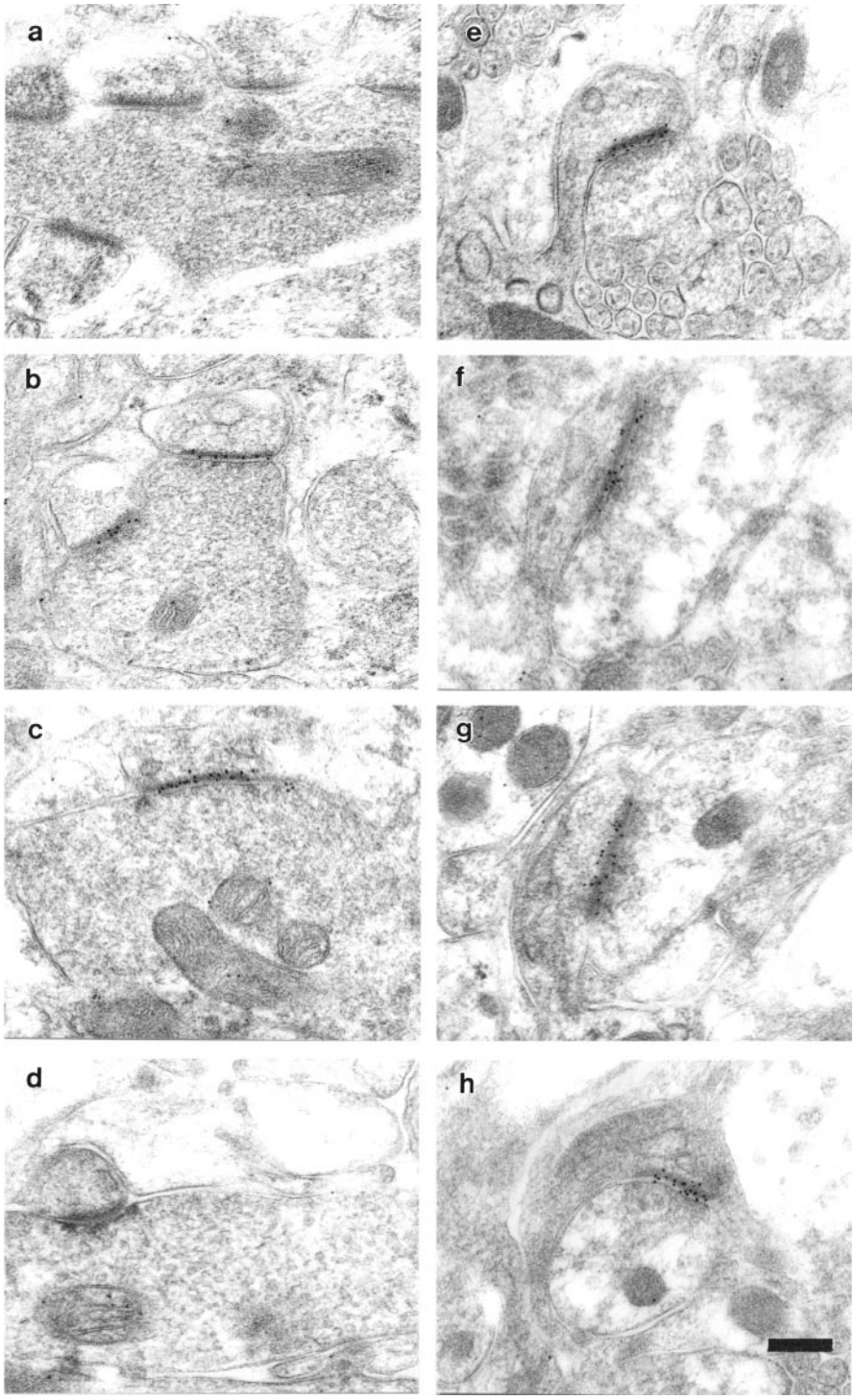

Figure 5. GluRδ2 expression after a subtotal lesion of the inferior olive. *a–d*, Climbing fiber-innervated spine in control (*a*) and at 6 (*b*), 90 (*c*), and 270 (*d*) d after the lesion. A high density of GluRδ2s is present in *b* and *c* and not in *a* and *d*. *e–h*, Parallel fiber-innervated spines emerging from the branchlets at the same periods after lesion are shown. A high density of GluRδ2s is visible in all conditions. Scale bar, 0.23 μm.

that at 135 d, the effects of the activity block had been significantly reversed.

In the same material, we aimed to see whether changes in GluRδ2 expression were limited to the proximal dendrites. Therefore, we checked the expression of this subunit in the parallel fiber-innervated spines of the branchlets. To unequivocally distinguish branchlet spines from those of the proximal dendrites, we selected only the spines encountered at their point of emergence from the stems of distal dendrites. All spines had GluRδ2. Moreover, the average density of immunogold particles was not significantly changed compared with findings after activity block and during the recovery period (all p values >0.13) (Fig. 2*d–f*). The gold particle density values per micrometer of postsynaptic density length were 36.2 ± 11.8 SD ($n = 60$) in the vehicle-treated cerebella, 38.7 ± 14.4 SD ($n = 64$) at 7 d after TTX infusion, and 37.7 ± 13.6 SD ($n = 56$) and 39.7 ± 12.5 SD ($n = 58$), respectively, in the rats examined at 45 and 135 d of recovery (Fig. 1*c*).

Reinnervation of Purkinje cells by climbing fibers

To further confirm that growing climbing fibers in the adult cerebellum can innervate spines bearing GluRδ2, we used a second experimental model that is represented by subtotal deletion of the inferior olivary neurons by intraperitoneal injection of 3-AP. Data were obtained from six groups (three subjects per group) consisting of normal rats and rats at 6, 40, 90, 150, and 270 d after 3-AP administration.

The 6 d group was extensively studied by routine and immunoelectron microscopy to establish a baseline. In these rats, we observed the presence of spines innervated by parallel fibers emanating from proximal Purkinje cell dendrites, as demonstrated previously (Sotelo et al., 1975; Rossi et al., 1991a,b). We prepared 15 sets of serial electron micrographs to count the spines emerging from proximal Purkinje cell dendrites and to determine: (1) to what extent they were innervated, and (2) whether their innervation was provided by other afferents besides the parallel fibers. Of 97 reconstructed spines, 23 (24%) were free of synaptic contacts (Fig. 3). Four of them had a postsynaptic density (Fig. 3*d*) that, however, was quite small, because it was evident only in one section (100 nm thick), whereas the postsynaptic density of the contacted spines extended in several sections. The small postsynaptic densities might represent either the remnants of previous postsynaptic densities innervated by climbing fibers or new postsynaptic densities formed before ectopic innervation by parallel fibers. Of the 74 spines provided with well defined postsynaptic densities, 59 (80%) were innervated by parallel fibers, 3 (4%) by climbing fibers, and 12 (16%) by large terminals synapsing both with the spine and with the dendritic shaft. Similar large terminals with double synaptic contacts were identified as being GABAergic by an anti-GABA antibody in a previous study (Morando et al., 2001).

Furthermore, we studied the distribution of GluRδ2s in the

spines in control rats and during the process of Purkinje cell reinnervation. In the controls, GluRδ2s were abundant in parallel fiber-innervated spines and were extremely rare or absent in climbing fiber-innervated spines (Landsend et al., 1997; Zhao et al., 1998; Morando et al., 2001). Spines innervated by parallel fibers had an average GluRδ2 immunogold particle density of 39.0 per micrometer (±14.1 SD; $n = 108$) (Figs. 4*c*, 5*e*). In contrast, in 25 spines innervated by climbing fibers (Fig. 5*a*), only one (4%) was labeled by gold particles, and the average density value was 0.34 per micrometer (±1.7 SD) (Fig. 4*a,b*). These data were in line with previous measurements (Landsend et al., 1997; Zhao et al., 1998).

Deletion of the inferior olive amounted to between 88 and 99% of its neurons and was followed within a period of 3 months by a massive growth of collaterals from the surviving climbing fibers that extended the territory of a single climbing fiber by 7–10 times, as shown previously (Benedetti et al., 1983; Rossi et al., 1991a,b). At 6, 40, and 90 d after the lesion, nearly all of the climbing fiber-innervated spines bore GluRδ2s (Figs. 4*a*, 5*b,c*). Specifically, values were 91.8, 81.3, and 99.3%, respectively. Given the strong olivary deletion, it is logical to assume that the number of spines innervated by newly sprouted climbing fiber collaterals largely surpassed that of spines innervated by climbing fibers unaffected by the lesion. Although we cannot exclude the possibility that GluRδ2s might also be expressed by the latter category of spines (see Discussion), these receptors must belong in large part to new synapses. It is relevant also that the density of GluRδ2 immunogold particles per micrometer of postsynaptic density in climbing fiber-innervated spines was very high at all three periods after the lesion, the values of gold particle density being, respectively, 26.2 ± 14.1 SD ($n = 61$), 27.6 ± 22.3 SD ($n = 77$), and 38.4 ± 16.6 SD ($n = 58$) (Fig. 4*b*). All of these values were significantly higher relative to controls (respectively, $p < 3.14 \times 10^{-21}$, $p < 6.88 \times 10^{-17}$, and $p < 4.26 \times 10^{-23}$). The incidence of climbing fiber-innervated spines bearing GluRδ2s dropped to 40.3% at 150 d (Fig. 4*a*) from the lesion, and the gold particle density also dropped to a value of 9.6 per micrometer (±17.5 SD; $n = 67$) (Fig. 4*b*), which was still significantly higher ($p < 5.46 \times 10^{-5}$) than controls. Only at 270 d from the lesion was the incidence of climbing fiber-innervated spines bearing GluRδ2 (13%) (Figs. 4*a*, 5*d*) very close to normal and the gold particle density reduced to a value of 1.3 per micrometer (±4.7 SD; $n = 46$) (Fig. 4*b*), which was not significantly different from controls ($p = 0.22$).

Next, we examined whether the inferior olive lesion affects GluRδ2 expression in branchlet spines, which maintained their parallel fiber innervation. As for the TTX experimental group, we analyzed only spines emerging from the branchlets. Figures 4*c* and 5*f–h* show that the GluRδ2 immunogold particle density in all groups of lesioned rats presented values similar to controls ($p > 0.6$). In control rats, the gold particle density value per micrometer of postsynaptic density length was 39.0 ± 14.1 SD ($n = 108$), and in the rats at 6, 40, 90, 150, and 270 d the values were, respectively, 37.6 ± 17.9 SD ($n = 103$), 40.8 ± 14.9 SD ($n = 91$), 37.8 ± 11.8 SD ($n = 54$), 42.9 ± 14.0 SD ($n = 63$), and 47.3 ± 17.1 SD ($n = 65$).

Finally, in one of three rats at 6 d after olivary lesion we found dark degenerating climbing fiber terminals in contact with spines, all of which had postsynaptic densities with abundant GluRδ2s (Fig. 6). The postsynaptic densities of these spines had an average gold particle density of 38.3 per micrometer (±14.7 SD; $n = 22$), which is significantly higher relative to the climbing fiber-innervated spines of control rats ($p < 4.8 \times 10^{-11}$). The expression of these receptors in the spines still attached to degenerating terminals is likely the consequence of the loss of climbing fiber activity, as described previously (Morando et al., 2001).

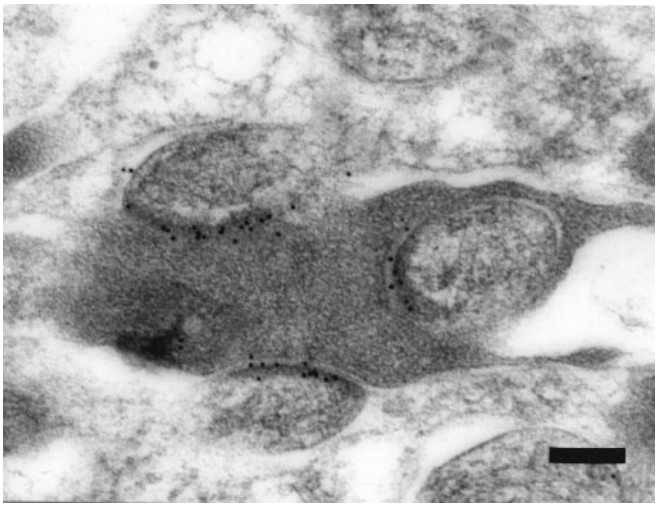

Figure 6. Dark degenerating climbing fiber terminal innervating three spines at 6 d after subtotal lesion of the inferior olive. Note the high expression of GluRδ2. Scale bar, 0.18 μm.

Discussion

Postsynaptic GluRδ2s are considered to represent important cues for the formation of synapses between the spines of the Purkinje cell dendrites and their afferents (Guastavino et al., 1990; Kurihara et al., 1997; Ichikawa et al., 2002). In the adult cerebellum, however, expression of GluRδ2s is restricted to the synapses between the parallel fibers and the spiny branchlets and is virtually lacking in the synapses between the climbing fibers and the sparse spines of the proximal dendrites (Landsend et al., 1997; Zhao et al., 1998; Morando et al., 2001; Strata, 2002). Yet GluRδ2s appear again in proximal dendritic spines after loss of the climbing fiber or in the absence of electrical activity before retraction of the climbing fiber from its target (Bravin et al., 1999; Morando et al., 2001). We wondered whether the GluRδ2s ectopically targeted to the postsynaptic density of spines innervated by the climbing fibers might be responsible for the climbing fiber retraction. Two experiments presented here indicate that this is not the case, and that regrowing climbing fibers are able to establish new synapses with spines bearing GluRδ2s, although these receptors are repressed at later stages. Evidence for this interpretation is provided by quantitative immunoelectron microscopic studies on the reinnervation of Purkinje cell dendrites by climbing fibers during a 135 d period after removal of an electrical activity block induced by topical infusion of TTX and during a 270 d period after subtotal deletion of inferior olivary neurons by 3-AP.

As shown previously (Bravin et al., 1999), at 7 d of TTX treatment there was a large drop in the number of climbing fiber-innervated spines, and most of the proximal dendritic spines contained GluRδ2s localized to the postsynaptic densities. However, at 45 d after removal of the electrical block, there was a significant increase in spines expressing GluRδ2s that were innervated by climbing fibers. This finding indicates that new climbing fiber terminals made contact with GluRδ2-bearing spines. At 45 d after removal of the TTX block, we found that the GluRδ2 density in climbing fiber-innervated spines was significantly decreased relative to the condition under block. A reasonable interpretation of this finding is that, along with the process of recruiting new spines, the growing climbing fiber exerts a repressive action on

the targeting of these receptors to the spines with which it establishes synaptic contacts (see also Morando et al., 2001). After a recovery period of 135 d, the entire picture is similar to the control. It should be noted that although a significant recovery of the climbing fiber arbors was demonstrated as early as 28 d (Bravin et al., 1999), a degree of recovery that includes normalization of the GluRδ2 pattern requires a much longer time and it is still incomplete at 45 d.

In the experimental model consisting of subtotal lesion of the inferior olive by 3-AP, we confirmed in serial sections that at 6 d after the lesion the proximal dendrites had spines innervated by parallel fibers (Sotelo et al., 1975; Rossi et al., 1991a). In addition, we demonstrated that a substantial number of spines were free of innervation. At this stage, the density of GluRδ2s in the parallel fiber–Purkinje cell synapses of the branchlets was similar to that of control rats. However, GluRδ2s were present at high density also in the climbing fiber-innervated spines at this early time point. The expression of GluRδ2s in the climbing fiber synapses might be attributable to an abnormal activity of the surviving inferior olive neurons. 3-AP is a toxic agent that interferes with the oxidative metabolism, and it is possible that the surviving neurons undergo a period of decreased or absent activity. During such a period, a likely release of the repressive action of climbing fibers on GluRδ2 expression may explain the significant increase in the receptor density. Support for this possibility comes from the observation that many dark degenerating climbing fiber terminals were found in one rat at 6 d after the lesion, and they had a high GluRδ2 density.

Because of the difficulty of distinguishing the original climbing fiber-innervated spines from those that have been formed as a result of collateral sprouting, we cannot conclude that new synapses have GluRδ2, although sprouting is already present 3 d after the lesion (Rossi et al., 1991a). However, the surviving climbing fibers undergo an extensive collateral sprouting that leads to the progressive extension of their target territory, reaching a peak at the end of the third month after the lesion (Benedetti et al., 1983; Rossi et al., 1991a,b; Strata and Rossi, 1998). Our experiments show that up to 90 d after the lesion the percentage of climbing fiber-innervated spines bearing GluRδ2 is between 81 and 99%. This fact strongly supports the results of the TTX experiments in which new climbing fiber-innervated spines were found to express the GluRδ2s. It was striking to find such a high incidence of GluRδ2s several months after inferior olivary lesion. We expected that as the sprouting climbing fibers extended their territory during the first 3 months, new climbing fiber-innervated synapses would lose their GluRδ2s. During collateral sprouting, the inferior olivary neurons present phenotypic alterations aimed at adapting to the burden of innervating a much larger target territory (Neppi-Modona et al., 1999). It is likely that full repression of the GluRδ2s in the reinnervated spines requires that inferior olivary neurons adapt to the new condition. This situation is reminiscent of cerebellar development, during which climbing fiber-innervated spines transiently express GluRδ2s. Nevertheless, it should be noted that the downregulation of GluRδ2s achieved at the end of the developmental period (Zhao et al., 1998) may occupy a time span shorter than that observed in our experimental situation.

Our data show that during the reinnervation process, new climbing fiber terminals make contact with GluRδ2-bearing spines. It is possible that early contact between a climbing fiber and a Purkinje cell spine takes place in the absence of GluRδ2s, and that these subunits emerged after the initial contact. In developing hippocampal neurons, the process of synapse formation *in vivo* occurs in <2 hr (Alsina et al., 2001), and presynaptic differentiation precedes postsynaptic differentiation (Friedman et al., 2000; Okabe et al., 2001). However, the addition of GluRδ2s to the postsynaptic densities of the spines was unable to displace the active climbing fiber. On the contrary, during maturation of the synapse, climbing fiber activity represses the GluRδ2s. One may argue that the climbing fiber, instead of repressing the GluRδ2 in the innervated spines, moves onto a new spine lacking this subunit, perhaps with the aid of rapid spine movements (Dunaevsky et al., 1999, 2001). However, the shift of a climbing fiber terminal onto a new spine lacking GluRδ2 under TTX treatment is unlikely. In fact, despite the significant loss of climbing fiber synapses, the number of those bearing GluRδ2s does not decrease relative to vehicle values. In addition, an upregulation of this subunit is detectable in these remaining synapses. Subsequently, after a recovery period of 45 d we observed an additional 50% increase in the number of climbing fiber GluRδ2-labeled synapses. Moreover, up to 90 d after 3-AP treatment, the percentage of climbing fiber-innervated spines bearing GluRδ2 was between 81 and 99%. Thus, if the climbing fibers moved onto new spines without GluRδ2s to stabilize their synapses, almost all of the climbing fiber branches formed by collateral sprouting would have abandoned the GluRδ2-bearing spines in search of spines lacking this subunit. Thus, we favor the view that climbing fibers can initially contact GluRδ2-bearing spines and then repress this subunit.

In conclusion, spinogenesis seems to be an intrinsic Purkinje cell property (Sotelo et al., 1975; Sotelo, 1978) that is activity independent (Bravin et al., 1999), although a role of a spontaneous release of glutamate from inactive terminals cannot be excluded. All spines have a constitutive presence of GluRδ2 (Morando et al., 2001) that is important for the maturation and stabilization of the parallel fiber synapses (Kashiwabuchi et al., 1995; Kurihara et al., 1997). Active climbing fibers are able to innervate GluRδ2-bearing spines, limited to their dendritic domain. The present data suggest that after achieving the mature state, the active climbing fibers downregulate the GluRδ2s in the innervated spines. In addition, they displace the competitor afferents, the parallel fibers, to the distal dendritic territory (Ichikawa et al., 2002). By this mechanism, the active climbing fiber established in its dendritic domain the characteristic synaptic profile that consists of sparse clusters of spine innervated by a single afferent axon. Such a profile is congruent with the peculiar function of the climbing fiber to elicit the large all-or-none synaptic current accompanied by a large increase in $[Ca^{2+}]$ (Strata et al., 2000).

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