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Purkinje Cell Survival and Axonal Regeneration Are Age Dependent: An *In Vitro* Study

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Purkinje cells are among the most resistant neurons to axotomy and the most refractory to axonal regeneration. By using organotypic cultures, we have studied age- and environment-related factors implicated in Purkinje cell survival and axonal regeneration. Most Purkinje cells taken from 1- to 5-d-old rats, the period in which these neurons are engaged in intense synaptogenesis and dendritic remodeling, die 1 week after plating, whereas if cultured before or after this period, Purkinje cells survive, even in the absence of deep nuclear neurons, their postsynaptic targets. Cerebellar slices taken from 10-d-old rats and kept *in vitro* for 1 week acquire a cellular composition resembling mature cerebellum. Their Purkinje cells are resistant to axotomy, but even when confronted with permissive environments (sciatic nerves or fetal cerebellar slices), their axons do not regenerate. In contrast, fetal rat and mouse Purkinje cells are able to regenerate their axons on mature cerebellar slices. This regeneration is massive, and the regrowing axons invade all cerebellar regions of the apposed mature slices, including white matter. These results show that Purkinje cell survival and axonal regeneration are age-related and independent from environmental constraints. Moreover, our observations suggest strongly that the onset of synaptogenesis of Purkinje cell axons could provide a signal to turn off their growth program and that, thereafter, permissive microenvironment alone is unable to reestablish such a program.

Key words: axonal regeneration; neuronal survival; cerebellum; Purkinje cell maturation; cerebellar organotypic cultures; axonal growth

Most adult neurons survive axotomy if the lesion occurs far from their cell bodies, whereas perinatal neurons often die even after far-away axotomy (for review, see Schwab and Bartholdi, 1996). Frequently, this perinatal cell death can be counteracted by neurotrophic factors or by embryonic tissue grafting (for review, see Schwab and Bartholdi, 1996). In addition, immature CNS neurons have a higher potential to regenerate their axons than adult CNS neurons (for review, see Schwab and Bartholdi, 1996). Nevertheless, if allowed to regrow into transplanted peripheral nerves, most adult CNS cut axons regenerate their lesioned processes (David and Aguayo, 1981; Aguayo, 1985). Furthermore, the application of blocking antibodies against CNS myelin inhibitory proteins favors regeneration of axotomized corticospinal axons after spinal cord injury (Schnell and Schwab, 1990; Schwab et al., 1993). These observations strongly support the hypothesis that environmental factors rather than the intrinsic inability of adult CNS axons to regenerate are the limiting factor in the failure of axonal regeneration.

Adult Purkinje cells are among the most resistant neurons to axotomy (Dusart and Sotelo, 1994) and the most refractory to axonal regeneration: they do not regenerate their axons even in the presence of their embryonic targets (Rossi et al., 1995). These cells in addition are one of the rare categories of neurons whose axons have never been observed to grow into grafted peripheral nerves (Dooley and Aguayo, 1982). Thus, it is likely that in the case of Purkinje cells, environmental modifications, even if necessary, are not sufficient to activate a regeneration program.

Recently, studies with organotypic cultures have approached the problem of axonal regeneration, particularly in the hippocampal and retinotectal systems. In these two systems, identified neurons survive the axotomy caused by organotypic culture processing and are able to regenerate axonal processes *in vitro* and to reestablish their correct pathway and target specificity (Li et al., 1994; Chen et al., 1995; Linke et al., 1995). Moreover, the ability to regenerate is age-dependent, because the regeneration fails in cultures taken from older animals (Chen et al., 1995; Li et al., 1995). Organotypic cultures could therefore mimic the *in vivo* situation, and they seem particularly suitable for studying Purkinje cell survival and regeneration. Indeed, it is possible to maintain in the same preparation Purkinje cells and their targets, deep cerebellar nuclei neurons, with functional contacts (Audinat et al., 1990; Mouginot and Gähwiler, 1995) and therefore to carry out axotomy *in vitro*.

Using organotypic cultures, we have developed an *in vitro* setup to study age- and environment-related factors potentially implicated in Purkinje cell survival and axonal regeneration. By reexamining the problem of the failure of Purkinje cell axonal regeneration in mature rat cerebellum (Dusart and Sotelo, 1994; Rossi et al., 1995), we have validated this *in vitro* system, which closely resembles the *in vivo* conditions. The results reported in this study provide evidence that during the period of active synaptogenesis between Purkinje cells and their target neurons [postnatal day (P) 1–5], the period during which these neurons follow a complete

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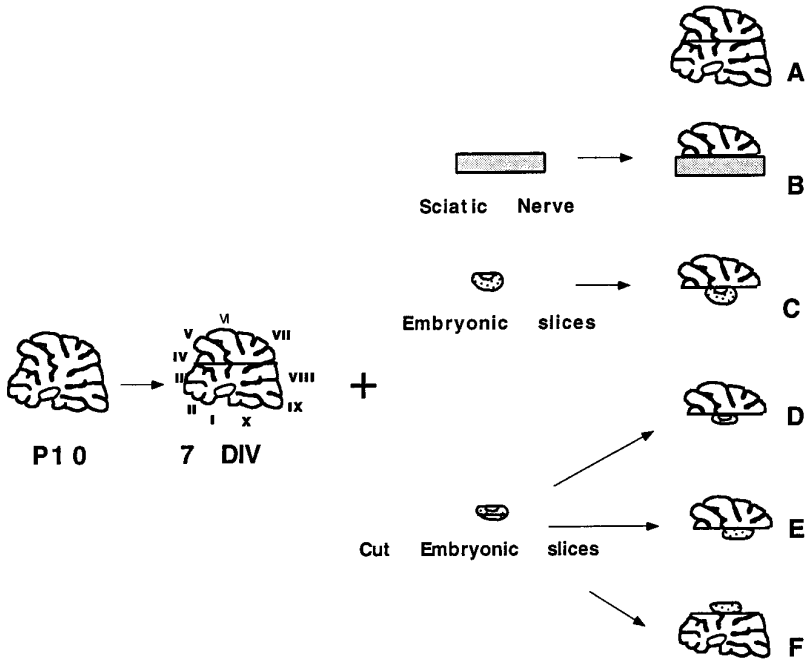

Figure 1. Schematic illustration of the manipulation of cerebellar slices. P10 cerebellar slices were plated, and after at least 7 DIV they were cut with a glass knife passing through lobules III/IV and VII/VIII. After separation of the two parts, *A*, they were reapposed and kept for 10 more DIV. *B*, The dorsal region of the cut cerebellar slice was cocultured with a slice of adult sciatic nerve. *C*, The dorsal region of the cut cerebellar slice was cocultured with an intact embryonic cerebellar slice. *D*, The dorsal region of the cut cerebellar slice was cocultured with the ventral region containing the deep nuclear region (in *white*) of a split fetal cerebellar slice. *E*, The dorsal region of the cut cerebellar slice was cocultured with the dorsal region containing the cortex (*dotted area*) of fetal cerebellar slice. *F*, The ventral region of the cut cerebellar slice was cocultured with the cortical region (*dotted area*) of a split fetal cerebellar slice.

regressive remodeling of their dendritic arbors (Armengol and Sotelo, 1991), the Purkinje cells are extremely susceptible to *in vitro* conditions and degenerate when cultured. Furthermore, Purkinje cell axonal regeneration is possible before their synaptogenic period, even in a mature cerebellar environment, and thus depends more on the stage of maturation of these neurons than on the environment of their severed axons.

MATERIALS AND METHODS

Slice cultures

In this study, fetuses from embryonic day (E) 17–E21 and P0, P1, P3, P5, P7, P10, and P15 Wistar rats (IFFA Credo, Arbresle, France) were used. Fetuses were obtained by cesarean section from pregnant rats anesthetized with chloral hydrate (350 mg/kg, i.p.). For all the animals, after decapitation brains were dissected out into cold Gey's balanced salt solution with 5 mg/ml glucose (GBSS-Glu), and meninges were removed. Cerebellar parasagittal slices (250 or 500 μm thick for the postnatal animals and 200 or 250 μm thick for fetuses) were cut on a McIlwain tissue chopper and separated gently into cold GBSS-Glu. The slices were cultured on the membrane of a 30 mm Millipore culture insert (Millicell, Millipore, Bedford, MA; pore size 0.4 μm) in 10 cm culture dishes containing 3 ml of medium composed of 50% basal medium with Earle's salts (Life Technologies, Gaithersburg, MD), 25% HBSS (Life Technologies), 25% horse serum (Life Technologies), L-glutamine (1 mM), and 5 mg/ml glucose at 35°C in an atmosphere of humidified 5% CO_2 (Gähwiler, 1981, 1988; Stoppini et al., 1991). After 1 week in culture, the culture medium contained 15% horse serum instead of 25%. For each type of staining or experiment, at least three rats were used. A total of 857 rat slices or co-cultures have been analyzed.

Because it is not possible to make organotypic cultures from rats older than 15 d (see Results), for mature-like rat cerebellar slices we cultured P10 vermal sections containing deep nucleus (three to four sections per cerebellum). The slices were left in culture for at least 1 more week before further manipulations. The axotomy was then performed in these organotypic cultures equivalent to P17 cerebellum (at this age the cerebellum is mature with respect to most of the characteristics we are interested in). Furthermore, during the days after culturing, many cells died, numerous macrophages were activated, and the cultures recovered after only 1 week. Thus, a wait of 7 d *in vitro* (7 DIV) fits our requirements. Thereafter, the cultures were transected with a glass knife passing through lobules III and VIII (Fig. 1), under a dissecting microscope. The two parts were separated gently to ensure a complete axotomy, and then apposed and kept for 10 more days *in vitro* (Fig. 1*A*). In some other cases, the dorsal regions of the amputated cultures were cocultured with either an apposed slice of adult sciatic nerve (see below) or fetal cerebellum (Fig. 1). The fetal slices (E17–P0) either were directly apposed to the transected older cerebellum or because from E19 it was possible to visualize the cortical and deep nuclear regions, we separated both with small pliers, and the resulting two parts were used in the cocultures in combinations detailed in Figure 1*C–F*, during 7 more days in culture.

Slices of sciatic nerve. Sciatic nerves were dissected out from adult female Wistar rats overdosed with chloral hydrate anesthesia (500 mg/kg). The sciatic nerves were embedded in Tissue Teck O.C.T. compound (Miles, Elkhart, IN), frozen in isopentane at −40°C, and stored at −80°C until cut on a cryostat in the longitudinal plane at a thickness of 50, 100, or 200 μm. Sections were collected in cold GBSS-Glu before being laid in front of the axotomized P10 cerebellar organotypic culture and kept for 10 more days in culture (Fig. 1*B*).

Some of the P7 cerebellar slices were axotomized at the time of the culture and kept for 10 more days *in vitro*.

Calbindin (CaBP)-knockout mice

Because CaBP immunostaining (see below) was commonly used to study Purkinje cells, we have used CaBP-knockout mice (CaBP−/−; Airaksinen et al., 1997) as donors for apposed mature-like cerebellar slices to analyze more precisely the fate of the regenerating axons of OF1 fetal Purkinje cells. In these mice, Purkinje cell responses to axotomy *in vivo* were similar to those encountered in wild-type mice, validating our *in vitro* approach (our unpublished results). Cerebella from 10-d-old CaBP−/− mice were cut into parasagittal 350-μm-thick slices. After 1 week in culture, axotomies were performed with a glass knife, and 200-μm-thick slices of OF1 mouse fetuses (from E16 to E18) were apposed to amputated CaBP−/− cerebellar organotypic cultures in combinations detailed previously for rat cerebellum (Fig. 1). The confronted

slices were left in culture for 7 more days. Thirty-three murine cocultures were performed.

Antibodies

Rabbit polyclonal antibody against CaBP (diluted 1:10,000, gift of Dr. E. M. Lawson; Spencer et al., 1976) and mouse monoclonal antibody against CaBP (diluted 1:10,000, gift of Dr. W. Hunziker; Pinol et al., 1990) were used to study the survival and morphological changes of Purkinje cells, particularly their axons.

Mouse monoclonal antibody SMI32, which recognizes a nonphosphorylated epitope in neurofilament H (200–210 kDa, diluted 1:4000; Sternberger Monoclonal Incorporated, Baltimore, MD; Sternberger and Sternberger, 1983), was used to visualize deep nuclear neurons in particular.

Mouse monoclonal antibody against parvalbumin (diluted 1:10,000; Sigma, St. Louis, MO) was used to visualize Purkinje cells and interneurons of the molecular layer in CaBP-knockout mice (Celio, 1990).

Rabbit polyclonal antibody against glial fibrillary acidic protein (GFAP) (diluted 1:4000; Dakopatts, Copenhagen, Denmark) was used to visualize astrocytes.

Mouse monoclonal antibody ED1 (diluted 1:500; Serotec, Oxford, UK) was applied to identify activated microglial cells and macrophages. ED1 is found in most rat macrophage subpopulations as well as in monocytes in peripheral blood (Dijkstra et al., 1985), and it also has been shown to label activated microglia (Graeber et al., 1990).

Mouse monoclonal antibody against myelin basic protein (MBP) (diluted 1:500; Boehringer Mannheim, Mannheim, Germany) was used to reveal the state of myelination of the organotypic cultures.

Mouse monoclonal antibody 192-IgG (diluted 1:2; gift of Dr. C. Henderson, INSERM U182, Marseille, France) directed against p75, the low-affinity nerve growth factor receptor (LNGFR) that binds all neurotrophins with a similar low affinity (Hantzopoulos et al., 1994), was used to visualize Schwann cells (Taniuchi et al., 1988). Purkinje cells have also been demonstrated to be immunoreactive for p75 during development (Yan and Johnson, 1988; Cohen-Cory et al., 1989), and some of them are still immunoreactive in the adult (Dusart et al., 1994).

Staining procedures

The cultures and cocultures were fixed in 4% paraformaldehyde in phosphate buffer (0.1 M), pH 7.4, for 1 hr at room temperature. After they were washed in PBS, the slices were taken off the Millicell and processed for immunocytochemistry. In all cases except for MBP-immunocytochemistry, the slices were incubated for 1 hr in 0.12 M phosphate buffer, pH 7.4, containing 0.9% NaCl, 0.25% Triton X-100, 0.1% gelatin, 0.1% sodium azide (PBSGTA), and lysine (0.1 M) before the first antibodies were applied at the dilutions indicated above in PBSGTA overnight. Antibodies against CaBP (both monoclonal and polyclonal) were revealed with fluorescein isothiocyanate (FITC) immunofluorescence; in cases of double-labeling, the other first antibody was revealed with Texas Red immunofluorescence (we always combined a mouse monoclonal with a rabbit polyclonal). Rabbit antibodies against CaBP were visualized with the secondary anti-rabbit antibodies coupled to FITC (1:200; Silenus Laboratories, Hawthorne, Australia); mouse monoclonal antibody against CaBP was visualized with secondary anti-mouse antibodies coupled to FITC (1:50; Amersham, Arlington Heights, IL). The other primary antibodies were visualized with either biotinylated anti-rabbit antibodies or biotinylated anti-mouse antibodies (1:200; Vector Laboratories, Burlingame, CA), and then the complex streptavidin–biotin–Texas Red (1:200; Life Technologies) was applied.

When antibodies against MBP were used, the sections were first immersed in Clark's solution (95% ethanol/5% acetic acid) for 25 min at 4°C to extract some of the lipids and thus make the MBP antigens accessible for the antibodies. The sections were rehydrated and washed several times in 0.12 M phosphate buffer, pH 7.4, and then the staining procedure was performed as described above but the solutions were made without Triton X-100. The slices were mounted in Mowiol (Calbiochem, La Jolla, CA) or in PBS-glycerol and analyzed using a Zeiss Axiophot microscope.

Crude evaluation of Purkinje cell and deep nuclear neuron survival

The survival of Purkinje cells and deep nuclear neurons has been evaluated from E19 to P10. For E19, E20, E21, P0, P1, P3, and P5, 250- and 500-μm-thick slices were made, and all the slices from each animal were kept. For P7 and P10, 250- and 500-μm-thick slices were made, and either all the slices or part of them were kept. The number of animals (N) and slices (n) are indicated for each age in Table 1. All of these slices were kept in culture for 7 d and immunostained with both rabbit anti-CaBP and SMI32, to visualize mainly Purkinje cells and deep nuclear neurons, markers that are suitable for our purpose. Nevertheless, the possibility that they could be downregulated in a minor fraction of Purkinje and deep nuclear cell populations cannot be excluded.

Table 1. Evaluation of Purkinje cell and deep nuclear neuron survival

			Purkinje cells			Deep nuclear neurons	
Age	*N*	*n*	I (%)	II (%)	III (%)	− (%)	+ (%)
E19	7	67	7	50	43	34	66
E20	4	51	2	63	35	30	70
E21	7	69	22	74	4	55	45
P0	6	70	61	39	0	63	37
P1	7	63	59	41	0	54	46
P3	7	63	86	14	0	87	13
P5	7	73	85	15	0	100	0
P7	3	31	39	61	0	71	29
P10	6	44	0	48	52	32	68

Purkinje cell survival: cultures of the first group (group I) were those with very few Purkinje cells and were included in this group when single or small clusters of fewer than 20 CaBP-positive cells were spread throughout the cortex of the cerebellar culture. In group II, considered as intermediate between groups I and III, all of the cultures were classified, including those with at least one Purkinje cell cluster containing >20 cells and those with large numbers of CaBP-positive cells distributed almost evenly throughtout the cortex, but containing at least three discontinuities (regions devoid of CaBP-positive cells). Group III included cultures with the highest rate of Purkinje cell survival. They were defined as cultures in which CaBP-positive cells filled the Purkinje cell layer, with a maximum of two discontinuities. For each age, the percentage of slices included in groups I, II, and III are given. Deep nuclear neuron survival: we have defined only two categories of slices: slices without deep nuclear neurons (−) and slices with at least one deep nuclear neuron (+). For each age, the percentage of slices in these two groups is given. *N*, Number of animals; *n*, number of slices.

Purkinje cell survival. We have defined three groups of slices. The first group (denoted I) included the slices with few and dispersed Purkinje cells, i.e., those with no compact group of >20 Purkinje cells. The third group (denoted III) included the slices containing a compacted layer of Purkinje cells all along the lobules and where a maximum of two discontinuities in the CaBP-immunostaining (regions devoid of Purkinje cells) occurred. Finally, the second group (denoted II) included all the slices that did not satisfy the criteria of groups I or III. For each age, we calculated the percentage of slices included in groups I, II, and III.

Deep nuclear neuron survival. We have defined only two categories of slices: slices without deep nuclear neurons and slices with at least one deep nuclear neuron. For each age we calculated the percentage of slices in these two groups.

RESULTS

In organotypic cultures, Purkinje cell survival is age-related

The survival of Purkinje cells in our culture conditions is *sine qua non* for the further analysis of the effect of axotomy on survival and axonal regeneration of these neurons. Because the stage of neuronal maturation can influence the response of a neuron to the culture conditions, our first aim in this study has been to correlate biological ages of Purkinje cells with their ability to survive *in vitro*. This correlation has been established for fetuses and newborn rats aged E19–P15, whose cerebellar slices were kept untouched, 7 DIV (see Materials and Methods). A large part of all the slices ($n = 69$) taken from P15 rats was necrotic and therefore deprived of Purkinje cells, demonstrating the impossibility of cultivating almost mature cerebellar slices.

The results that we obtained, illustrated in Figure 2, were extremely consistent and somewhat unexpected: the survival of Purkinje cells was not linearly related to their age. Thus, most Purkinje cells in E19–P0 cerebellar slices (Fig. 2*A,B*) survived, whereas most of those in P1–P5 cerebellar slices (Fig. 2*C,D*) did not. Finally, Purkinje cells in P7–P10 (Fig. 2*E,F*) had a good survival rate. Because of the difficulties in performing accurate cell counts in the cerebellar slices, in particular in fetuses and at P7 and P10 cerebellar slices (Fig. 2*A,F*), and the need to obtain quantitative data to support the qualitative results reported above, we have used easily identifiable parameters. As detailed in Materials and Methods, our analysis was based on the distribution of CaBP-immunoreactive Purkinje cells. Five hundred thirty-one double-immunostained organotypic cultures, taken from the cerebellum of rats of nine different ages, were used to establish an age/survival correlation and classified into three groups (Table 1). Cultures included in the first group (group I) were those with very few Purkinje cells, i.e., those with single or small clusters of fewer, <20, CaBP-positive cells spread throughout the cortex of the cerebellar culture (Fig. 2*C*). The vast majority of the organotypic cultures taken from P0–P5 rat cerebellum belonged to this group, whereas they were very rare in those cultures taken from E19 and E20 fetal cerebellum, and nonexistent in cultures taken from P10 rats (Table 1). Group II, considered as intermediate between groups I and III, is by definition the more heterogeneous and less reliable for quantification purposes. In this group were classified all cultures ranging from those with at least one Purkinje cell cluster containing >20 cells (Fig. 2*D*) to those with large numbers of CaBP-positive cells almost evenly distributed throughout the cortex, but containing at least three discontinuities (regions devoid of CaBP-positive cells) (Fig. 2*B*). Finally, group III included cultures with the highest rates of Purkinje cell survival. They were defined as cultures in which CaBP-positive cells filled the Purkinje cell layer, with a maximum of two discontinuities (Fig. 2*A,F*). None of the cultures taken from P0–P7 rats were included in this group, whereas 52% of the cultures taken from P10 rats were included (Table 1). Despite the lack of precision in the definition of group II, it is highly significant that cultures taken from P0–P5 were those with the lowest percentage in groups II and III (Table 1), which demonstrates not only that in these cultures Purkinje cell survival was the lowest but that the difference in survival rates between this age range and the others was extremely large. Thus, the analysis of the percentage of cultures per age group within each of the categories defined above has corroborated and enlarged our qualitative results: Purkinje cells in cerebellar slices taken from E19 and E20 had high survival rates, matched only by those taken from P10 rats (Table 1). Fewer Purkinje cells survived in cultures taken from E21 fetuses, and survival rates were extremely poor in slices taken from P0–P5 rats (Table 1). This drop in survival rate is reversed in slices taken from P7 rats and reached its maximum in those taken from P10 rats (Table 1).

Because the neurotrophic hypothesis postulates that neuronal survival depends on the occurrence of postsynaptic target cells (Levi-Montalcini, 1987), we performed a study of survival of deep nuclear neurons in the 531 organotypic cultures reported above. Our aim was to correlate Purkinje cell and deep nuclear neuron survival to determine whether the age-related differences in Purkinje cell survival could be attributed to the presence or absence of their main postsynaptic targets. The lack of specific immunomarkers to identify deep nuclear neurons was overcome by the use of the monoclonal antibody (mAb) SMI32, which although not specific for this neuronal population allowed the partial visualization of the neuronal cytoskeleton, thus revealing neuronal typology. Neurons immunolabeled with the mAb SMI32 and located in the central white matter where Purkinje cell axons, when present, become confluent were the only ones considered in this study to be deep nuclear neurons. These neurons exhibited a stellate shape and commonly had multiple long dendrites emerging from the cell bodies (Figs. 3*A*, 4*E,F*). Both the cell bodies and the dendrites were ensheathed by networks of CaBP-positive Purkinje cell axons (Figs. 3*A,B*, 4*F,G*). Occasionally, the pericellular networks were very dense, as if these neurons were hyperinnervated (Fig. 3*A,B*). Deep nuclear neurons as just described were absent or few in number in our cultures (from 1 to 23). Thus, we have classified the culture slices into two groups: those with one or more deep nuclear neurons, and those lacking this neuronal population. As indicated in Table 1, the age-dependency of deep nuclear neuron survival was parallel to that of Purkinje cell survival, i.e., the majority of organotypic cultures taken from P0, P1, P3, and P5 rats was devoid of deep nuclear neurons, whereas the majority of those taken from E19, E20, and P10 rats contained this neuronal population.

Organization of P10 cerebellar slices in organotypic cultures

One of the limitations of the *in vitro* systems is that they cannot be used with mature CNS (cerebellum of P15 rats in our case). Thus, although it is well established that embryonic and newborn organotypic cultures are excellent tools for studying cerebellar development (see references in Seil, 1972; Tanaka et al., 1994), the cellular composition and the degree of maturation attained by P10 cerebellar cultures after 7 or 17 DIV remain unknown. Awareness of the important role played by the microenvironment of severed axons for their regeneration made it essential to determine precisely the degree of maturation of the cerebellar slices at the moment of axotomy (biological age P17) and at the end of the postaxotomy survival period (biological age P27). Because no qualitative morphological differences were observed between the cultures maintained 7 or 17 DIV, the results were pooled in the following description.

After they were plated, the cultures flattened to ~50 μm in thickness, leading to a nearly two-dimensional system. Despite the almost complete loss of one dimension, many characteristics of the cytoarchitecture of the cerebellum were retained. Thus, a distinct cortical lobulation and a cortical/subcortical differentiation occurred (Figs. 2*F*, 3*D*). The characteristic three-layered organization of the cerebellar cortex was often preserved: a molecular layer with few neuronal cell bodies, a Purkinje cell layer with large neuronal somata, and a granular layer with small densely packed neuronal cell bodies (Fig. 3*C*) were present. Unlike the *in vivo* situation, these three layers were intermingled in some instances.

Purkinje cells morphology

The Purkinje cell layer was often multicellular, with up to five rows of Purkinje cell bodies (Figs. 2*F*, 3*D*). The dendritic arbors arose from one-stem dendrite at the apical pole of the cell body and branched extensively in the molecular layer, giving rise to distal spiny branchlets (Figs. 3*D*, 4*A*). In contrast to the *in vivo* situation, both proximal and distal branches were studded with spine-like processes (Fig. 4*B*), similarly to *in vivo* Purkinje cells devoid of their climbing fiber innervation (Sotelo and Arsenio-Nunez, 1976). When Purkinje cells remained isolated and/or were ectopic, two- or three-stem dendrites emerged from the cell body.

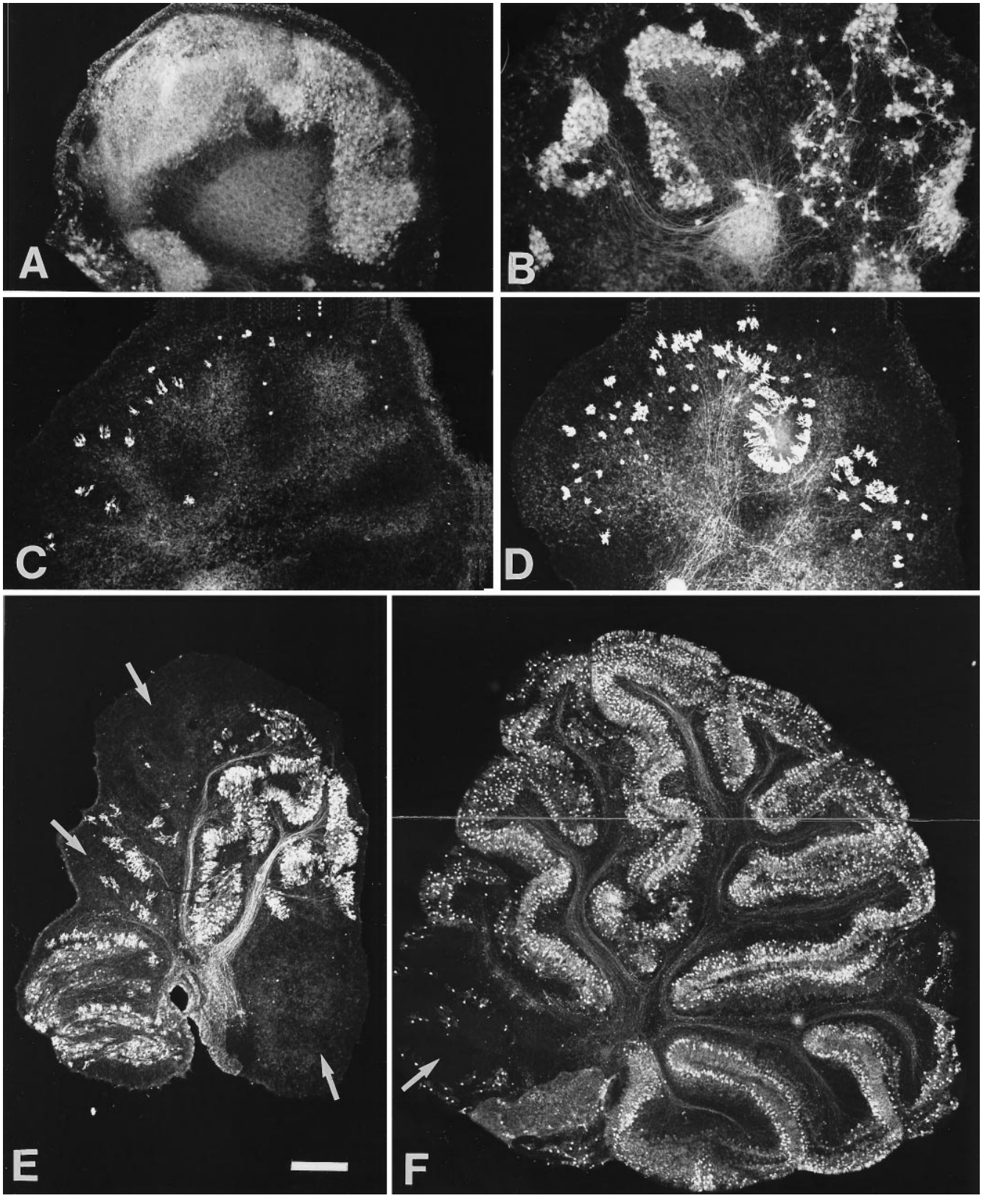

Figure 2. Purkinje cell survival. Photomicrographs of cerebellar slices maintained 7 DIV and immunostained with anti-CaBP antibodies. The slices were taken from E19 rats in *A* and *B*, P3 in *C* and *D*, P7 in *E*, and P10 in *F*. *A*, Purkinje cells are present all over the cortex; therefore this slice was included in group III (see Materials and Methods). *B*, Purkinje cells, although numerous, are not present all over the cortex and more than two discontinuities occur; however, clusters of >20 Purkinje cells are present (group II). *C*, Purkinje cells are few and dispersed (group I). *D*, Despite the scarceness of Purkinje cells, there is a group of >20 Purkinje cells (group II). *E*, Purkinje cells are present all over the cortex but three discontinuities occur (*arrows*, group II). *F*, Purkinje cells are present all over the cortex with the exception of one discontinuity (*arrow*, group III). Scale bar (shown in *E*): *A–D*, 320 μm; *E, F*, 480 μm.

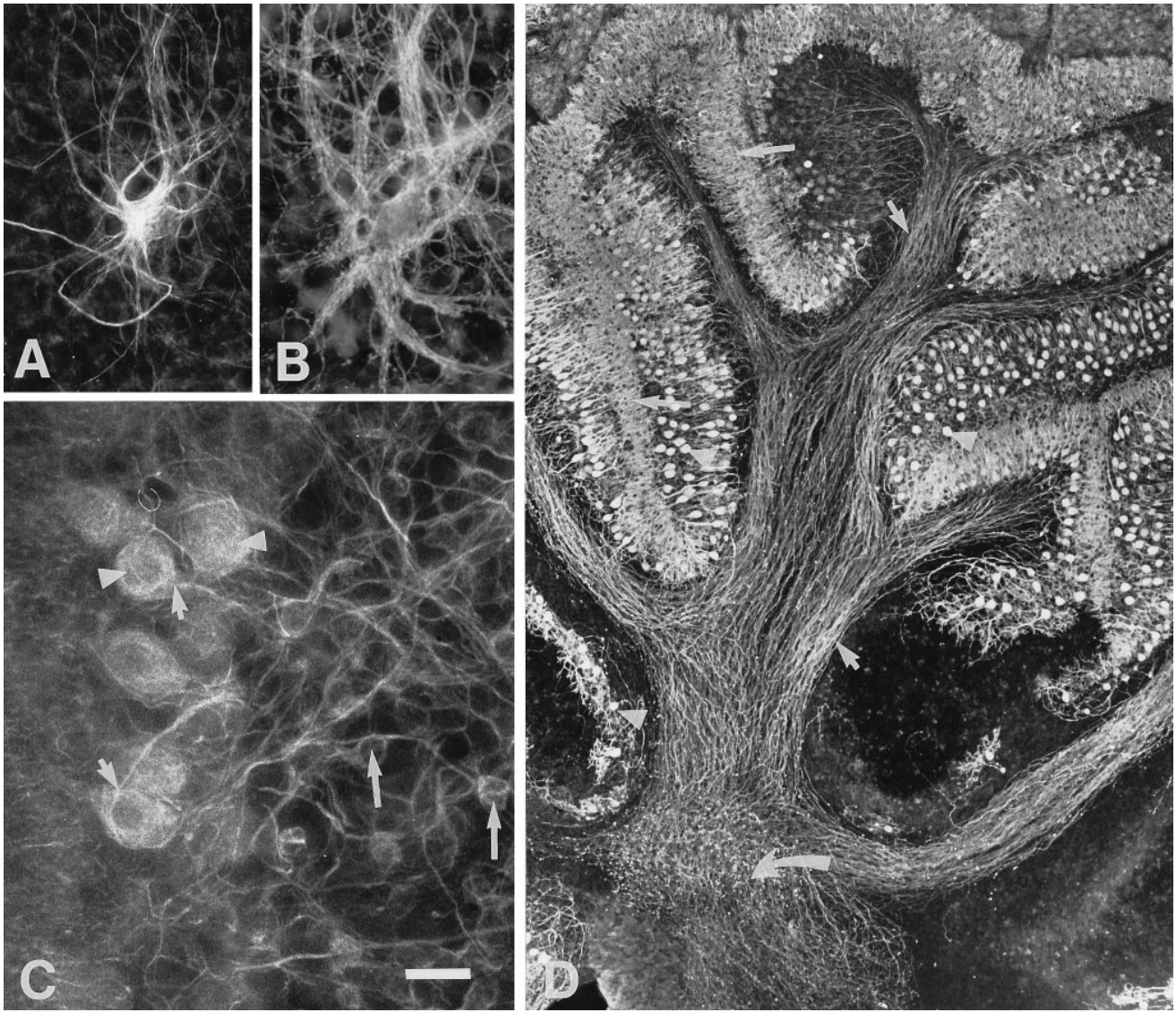

Figure 3. SMI32 and CaBP immunostaining. *A*, *B*, Photomicrographs of a P3 + 7 DIV cerebellar slice, double-immunostained with SMI32 (*A*) and anti-CaBP (*B*). The soma and dendrites of the deep nuclear neuron (*A*) are totally ensheathed by Purkinje cell axons (*B*). *C*, Photomicrograph taken from a P10 cerebellar slice kept for 17 DIV and immunostained with SMI32. The granular layer (*right part* of the micrograph) contains small rounded cells, with a thin perinuclear ring of labeled neurofilaments (*long arrows*). They have been identified as belonging to granule cells. At the interface between granular and molecular layers there is a row of labeled Purkinje cells (*arrowheads*). Notice basket cell axons forming pericellular nests on Purkinje cell bodies (*short arrows*). *D*, Photomicrograph of a P10 cerebellar slice + 7 DIV immunostained with an anti-CaBP antibody. Purkinje cells: their soma (*arrowhead*), dendrites (*long arrows*), and axons (*short arrows*) are labeled. Notice the deep nuclei area (*curved arrow*) at the confluence of Purkinje cell axons. Scale bar (shown in *C*): *A*, 40 μm; *B*, 20 μm; *C, D*, 33 μm; *E*, 175 μm.

The axon arose from the basal pole of the cell body, almost in a straight line with the stem dendrite, and proceeded through the granular layer toward the white matter, where it bent and entered parallel to the main axis of the folium (Figs. 3*D*, 4*C*). In the granular layer, the axon gave rise usually to one or two collaterals that could be hypertrophic and sometimes bore one or two axonal varicosities (the torpedoes; Fig. 4*C*). A few arciform axons were also observed (Fig. 4*C*), i.e., Purkinje cells exhibiting the disappearance of the distal segment of the axon and hypertrophy of recurrent collaterals. In the white matter, the axons were smooth (Fig. 3*D*) or could bear small varicosities (Fig. 4*C*). As in the *in vivo* adult cerebellum, the CaBP-immunoreactive Purkinje cell axons, in the central axes of the folia, ran parallel to one another in a loosely apposed manner, with relatively large intervening spaces. Thus, in these cerebellar cultures Purkinje cell axons remained defasciculated, as in *in vivo* adult cerebellum. The CaBP-immunoreactive Purkinje cell axons terminated in the central white matter, presumably in the region containing the deep nuclear neurons (Fig. 3*D*; see below). In this region, the axons branched repeatedly, forming a very dense meshwork (Fig. 4*D*), had few enlargements, and usually ended with a somewhat rounded varicosity (Fig. 4*D*).

Presence of deep nuclei and the corticonuclear projection in slice cultures

As described above, few deep nuclear neurons immunoreactive for SMI32 occurred (Fig. 4*E*). Their axons followed an unusual route, entering in the overlying gray matter and often ending in

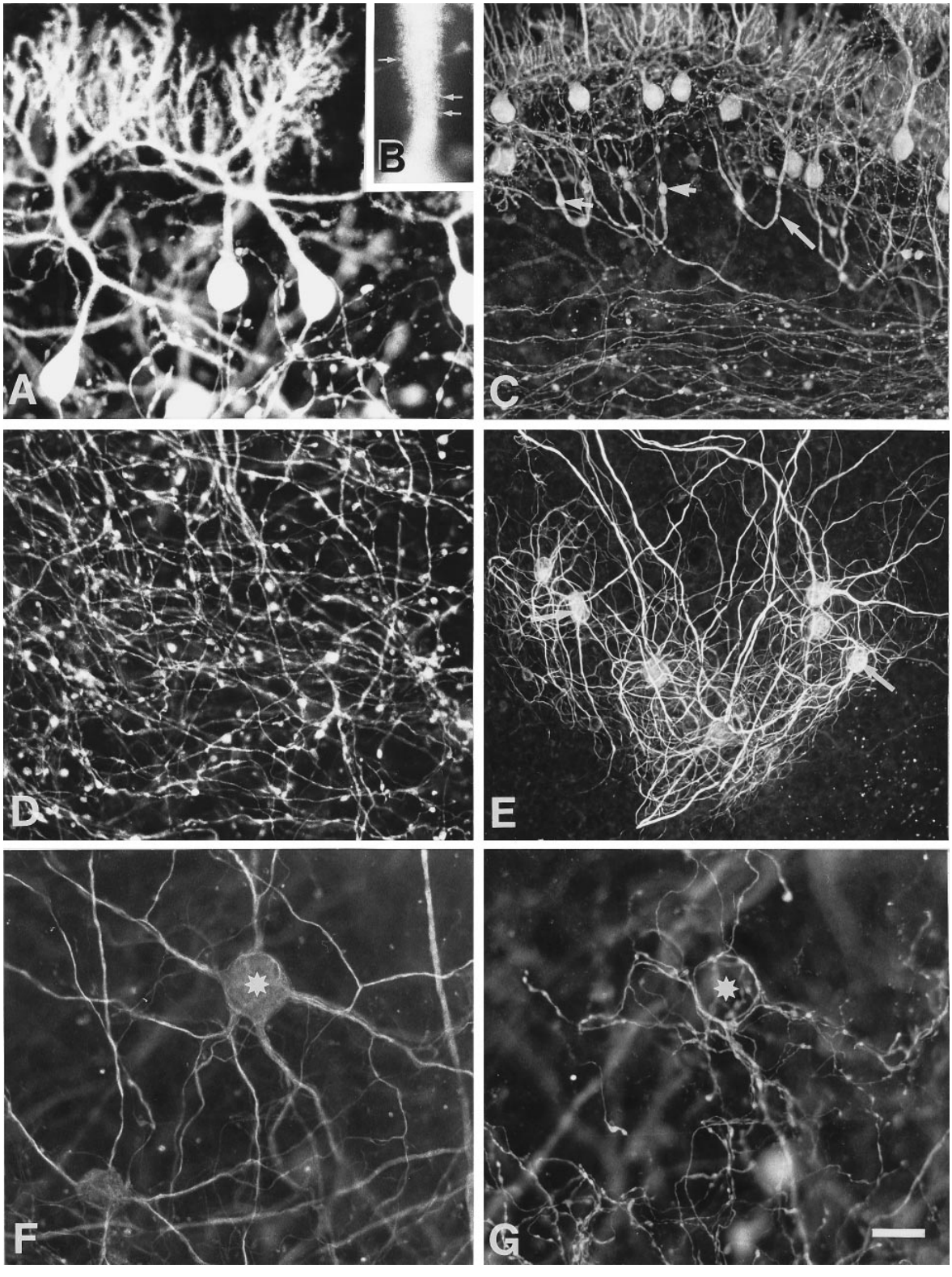

Figure 4. Morphology of Purkinje cells and deep nuclear neurons in "mature cerebellar culture." Photomicrographs taken from P10 cerebellar slices kept 7 DIV. *A*, Dendritic arbors of CaBP-immunoreactive Purkinje cells arise from one-stem dendrite at the apical pole of the cell bodies. The stem dendrite branches extensively in the molecular layer, giving rise to secondary and distal branches. *B*, CaBP-immunoreactive Purkinje cell stem dendrite studded with spine-like processes (*arrows*). *C*, In the granular layer, the CaBP-immunoreactive Purkinje cell axons usually give rise to one or two collaterals, some of them hypertrophic and bearing one or two axonal varicosities (*short arrows*). Arciform axons are also observed (*long arrow*). *D*, CaBP-immunoreactive Purkinje cell axons bearing abundant thin varicosities and branching repeatedly in the area containing deep nuclear neurons. *E*, Deep nuclear neurons immunoreactive for SMI32. They have large cell bodies (*arrows*) with abundant, long dendrites. In double-immunofluorescence preparations (*F, G*), CaBP-immunoreactive Purkinje cell axons (*G*) terminate over the soma (*asterisk*) and stem dendrites of a deep nuclear neuron immunoreactive for SMI32 (*F*). Scale bar (shown in *G*): *B*, 8 μm; *D*, 20 μm; *A, F, G*, 25 μm; *C*, 50 μm; *E*, 60 μm.

the Purkinje cell layer. In double-immunofluorescence preparations, few Purkinje cell axons terminated near the somata and stem dendrites of deep nuclear neurons (Fig. 4*F,G*). The large majority of the Purkinje cell axons did not seem to terminate on neurons, although they formed abundant thin varicosities in the region containing deep nuclear neurons. Either they contacted thin terminal dendritic branches, unstained with the SMI32 antibody, or more probably, because of the low number of neurons in these regions, most of these axons did not contact a neuronal target. Thus, even if a part of the corticonuclear projection was preserved in our organotypic slices, most Purkinje cells were devoid of their main target, the deep nuclear neurons.

Glial environment

Numerous GFAP-immunoreactive astrocytes were present in all the cerebellar slices (Fig. 5*A*), and most of them were star-shaped. In the molecular layer, however, GFAP-immunoreactive processes appeared thin, long, and perpendicularly orientated to the lobule lamination (Fig. 5*A*), as Bergmann fibers are *in vivo*. There were numerous ED1-positive cells all over the cerebellar slices (Fig. 5*B*), although no ED1-positive cells are found in mature intact cerebellum (Dusart and Sotelo, 1994). Most of them were globular, i.e., characterized by a large rounded soma; a few were pseudopodic, with a large soma from which a few thick processes emerged (Fig. 5*B*). These criteria identify them as highly activated microglial cells. Numerous MBP-immunoreactive fibers were present in the white matter, fewer in the granular layer, and none in the molecular layer (Fig. 5*C*), revealing the presence of functional oligodendrocytes. By double-immunostaining (against MBP and CaBP), we determined that most Purkinje cell axons were myelinated just after their initial segment. Their recurrent collaterals also were often myelinated (Fig. 5*D*). A few Purkinje cell axons devoid of myelin were also observed, suggesting that myelination was not complete.

In conclusion, these organotypic cultures exhibited most of the features of mature cerebellum, particularly the presence of myelinated axons, an essential feature to consider this *in vitro* system a good replication of the *in vivo* conditions for the study of the effects of axotomy.

In organotypic cultures, Purkinje cells from P10 + 7 DIV rats survive axotomy and do not regenerate their axons

The knife cut separated the cerebellar slice into two parts, a ventral and a dorsal part (Figs. 1, 6*A*). At the time of fixation (after 10 more days *in vitro*), three possible outcomes were observed: the two parts had fused and there was full continuity between them, or the two parts remained separated by a large cavity, or the two parts were almost fused with one or more small cavities occurring between them (Fig. 6*A*). In this third case the limit of the lesion was the straight line that passed through the cavities. To study the survival of Purkinje cells after axotomy, all the cultures were considered, but for the study of the fate of Purkinje cell axons after axotomy we have considered only those (34 of 60) with partial fusion, permitting regeneration, and with at least two small cavities, the minimum number necessary to determine the lesion line.

Mature Purkinje cells survive axotomy but do not regenerate their axons spontaneously

In the vast majority of the axotomized slices no apparent loss of Purkinje cells was noticed (Fig. 6*A*). In some axotomized slices, a variable loss of these cells occurred in both dorsal and ventral parts. Because of the position of the Purkinje cells and the route of their axons, only those in the dorsal part were axotomized (Fig. 6*A*); therefore, the observed Purkinje cell loss was not correlated with axotomy. In the slices, numerous CaBP-immunostained axons were present in the white matter axes of the cerebellar lobules dorsal to the lesion (Fig. 6*C*). These axons bore small enlargements and terminated in large rounded varicosities (terminal bulbs) close to the dorsal aspect of the knife cut (Fig. 6*C*). These transected axons were unable to cross the lesion line and formed an almost continuous front of variable thickness of axonal terminal bulbs along the dorsal aspect of this line (Fig. 6*C*). Nevertheless, in a few slices (6 of 34) one or two axons terminated away from the axonal front, crossing the lesion line (Fig. 6*B*). In these cases, the maximum length between the terminal varicosity and the lesion line was ~225 μm, too short to be considered a true regeneration. Thus, mature Purkinje cell axons *in vitro*, as is the case *in vivo*, do not regenerate after axotomy.

Mature-like Purkinje cells do not regenerate on sciatic nerve

In this set of experiments, double-labeling was achieved with the rabbit polyclonal antibody against CaBP to reveal the Purkinje cells and their severed axons, and the mouse monoclonal antibody against p75 to visualize surviving Schwann cells and to delineate the interface between the cerebellum and the sciatic nerve. Indeed, the p75-immunolabeling gave rise to a diffuse staining all over the cerebellar culture slices, allowing their identification (Fig. 7*B,D*). When slices of sciatic nerve were presented at the time of the axotomy in front of lesioned Purkinje cell axons, although in most cases (34 of 38) they remained apposed to cerebellar slices, regeneration was practically nonexistent. In 22 of the 34 cases, none of the Purkinje cell axons regenerated, and their terminal varicosities remained and formed the above-described axonal front. In one third of the cases (12 of 34), one or two Purkinje cell axons per cerebellar slice entered the apposed sciatic nerve (Fig. 7*A,C*). The maximum length of regrowth of axotomized Purkinje cell axons on sciatic nerve that we have observed was ~400 μm. These axons grew more or less in a straight line, either without giving off collaterals and ending on a terminal varicosity, which eventually was elongated (Fig. 7*C*), or giving off numerous collaterals, which made a bouquet-like arborization, each thin fiber ending on a small varicosity (Fig. 7*A,C*). To examine the possible role of Schwann cells in the very few attempts at regeneration, we searched for a correlation between the occurrence of regenerative axons and Schwann cells. In most cases (23 of 34), immunoreactivity for p75 was never observed on the sciatic nerve, suggesting that most Schwann cells died during experimental procedures (Fig. 7*B*; the survival of Schwann cells was better with 200- than with 50-μm-thick sciatic nerve slices). In 11 cases, p75-immunoreactive bands were observed (Fig. 7*D*). They were thin, parallel to each other, and followed the long nerve axis (Fig. 7*D*). Regenerative attempts occurred in both the presence (4 of 11; Fig. 7*C,D*) and absence (8 of 23; Fig. 7*A,B*) of p75-immunoreactive Schwann cells on the apposed sciatic nerve slices. Furthermore, when regenerative attempts occurred in the presence of immunoreactive Schwann cells, the Purkinje cell axons did not follow the bands of p75-immunoreactive Schwann cells, but on the contrary they crossed perpendicularly these bands without changing their direction of growth (Fig. 7*C,D*). Thus, the Schwann cells did not support the few attempts of regeneration observed, corroborating previous results *in vivo* with sciatic nerve transplants in cerebellum (Dooley and Aguayo, 1982).

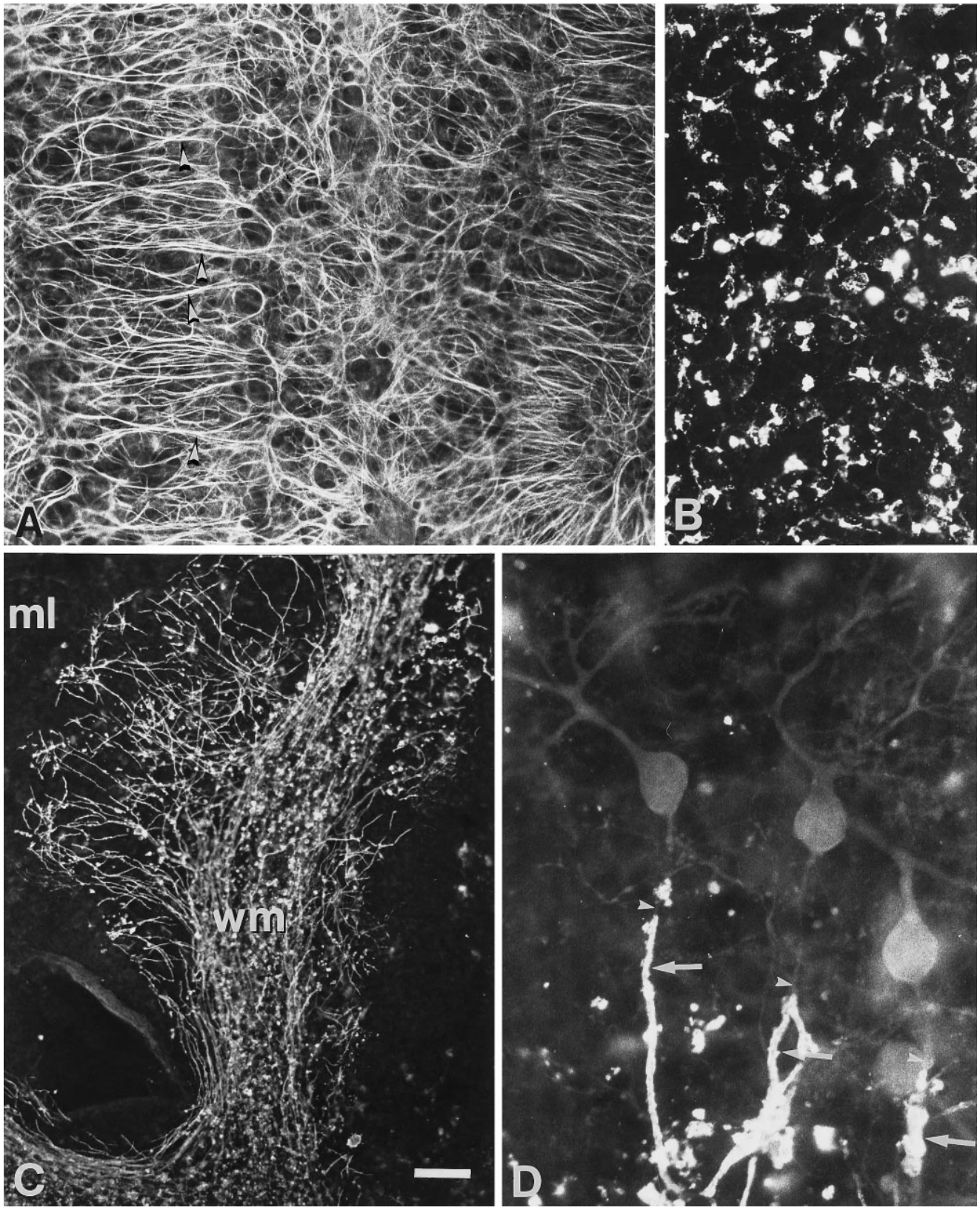

Figure 5. Glial environment in "mature cerebellar cultures." *A*, Astrocytes immunostained with an antibody against GFAP. Bergmann fiber-like processes span the molecular layer (*arrowheads*). Notice the astrocytic plexuses in the granular layer and white matter at the central region of the folium. *B*, ED1-immunoreactive cells are present in large numbers all over the slice culture. *C*, Myelinated fibers, immunostained with an antibody against MBP, are present in white matter (*wm*) and in the granular cell layer but not in the molecular layer (*ml*). *D*, The CaBP-immunoreactive initial segments of Purkinje cell axons (*arrowheads*) enter the MBP-immunoreactive myelin sheaths (*arrows*), showing that the myelinated fibers belong to Purkinje cell axons. Scale bar: *A, B*, 40 μm; *C*, 80 μm; *D*, 20 μm.

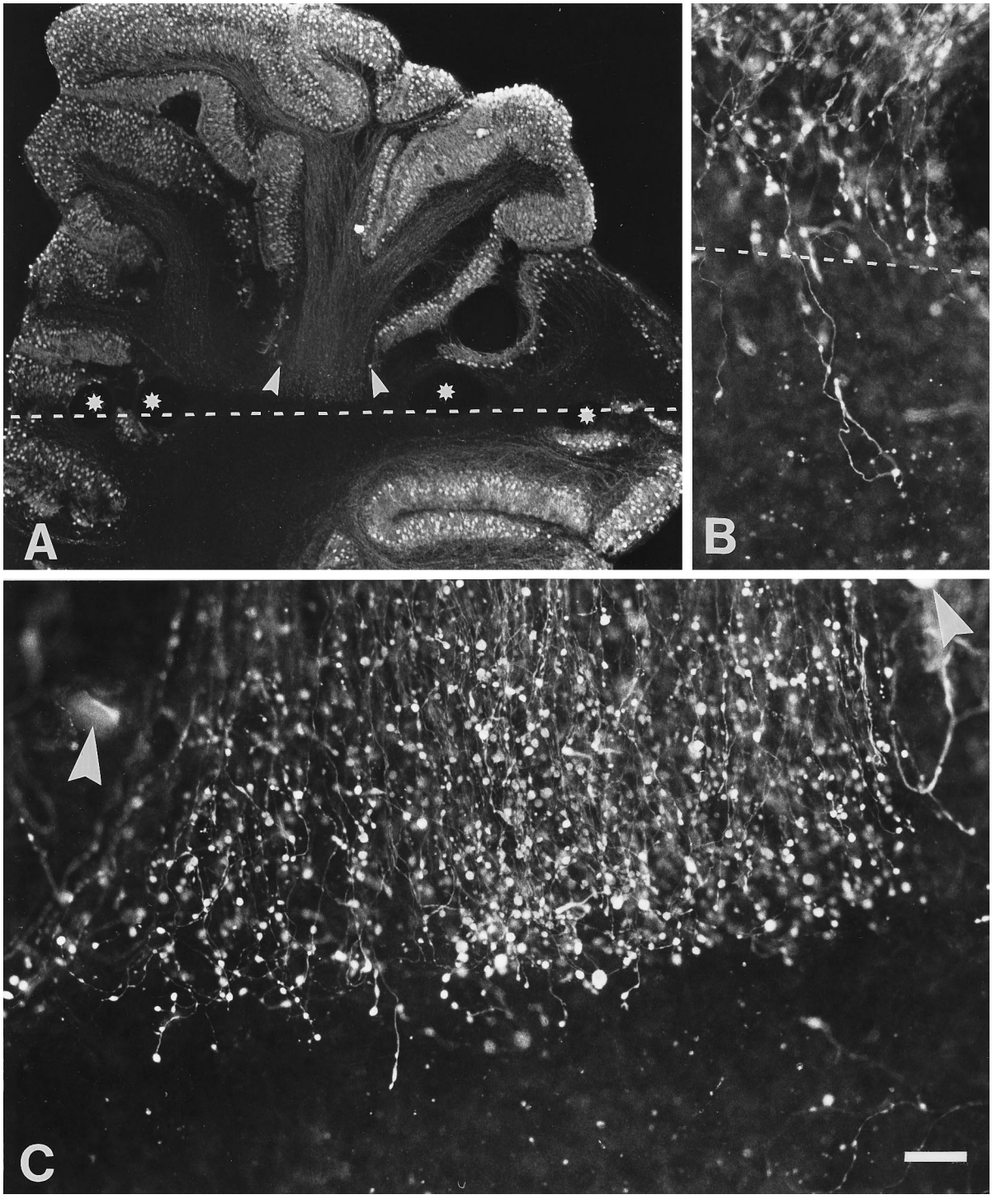

Figure 6. Purkinje cells do not regenerate spontaneously. *A*, Photomicrograph of a slice plated at P10, which has been cut in two parts after 7 DIV and then left for 10 more DIV, and immunostained with anti-CaBP antibodies. The lesion line (*dotted line*) passes through the four existing cavities (*stars*). Notice that Purkinje cell somata are present in all cerebellar lobules dorsal and ventral to the lesion and that there is no postaxotomy cell death. *C*, This micrograph illustrates an enlargement of the white matter region (*arrowheads* in *A* and *C* point to the same areas). Axons from Purkinje cells in the cerebellar lobules dorsal to the lesion are arrested at the lesion line. The amputated axons terminate in large rounded varicosities (terminal bulbs). *B*, One single axon crosses the lesion line (*dotted line*), but only for a short distance: 160 μm in this case. Scale bar: *A*, 400 μm; *B, C*, 40 μm.

Mature-like Purkinje cells do not regenerate on fetal cerebellum

We have also examined the behavior of axotomized Purkinje cells *in vitro* when confronted with an environment in which they grew axons during development, i.e., in fetal cerebellum. Just after the axotomy of the mature cerebellar slices (P10 + 7 DIV), the parts containing the bulk of axotomized Purkinje cell axons were apposed to one of the three classes of prepared fetal cerebellar slices: (1) the deep nuclei side of an entire slice (Fig. 1*C*); (2) the deep nuclei side of a cut slice (Fig. 1*D*); or (3) the side with the bulk of axotomized Purkinje cell axons (Fig. 1*E*). In all three

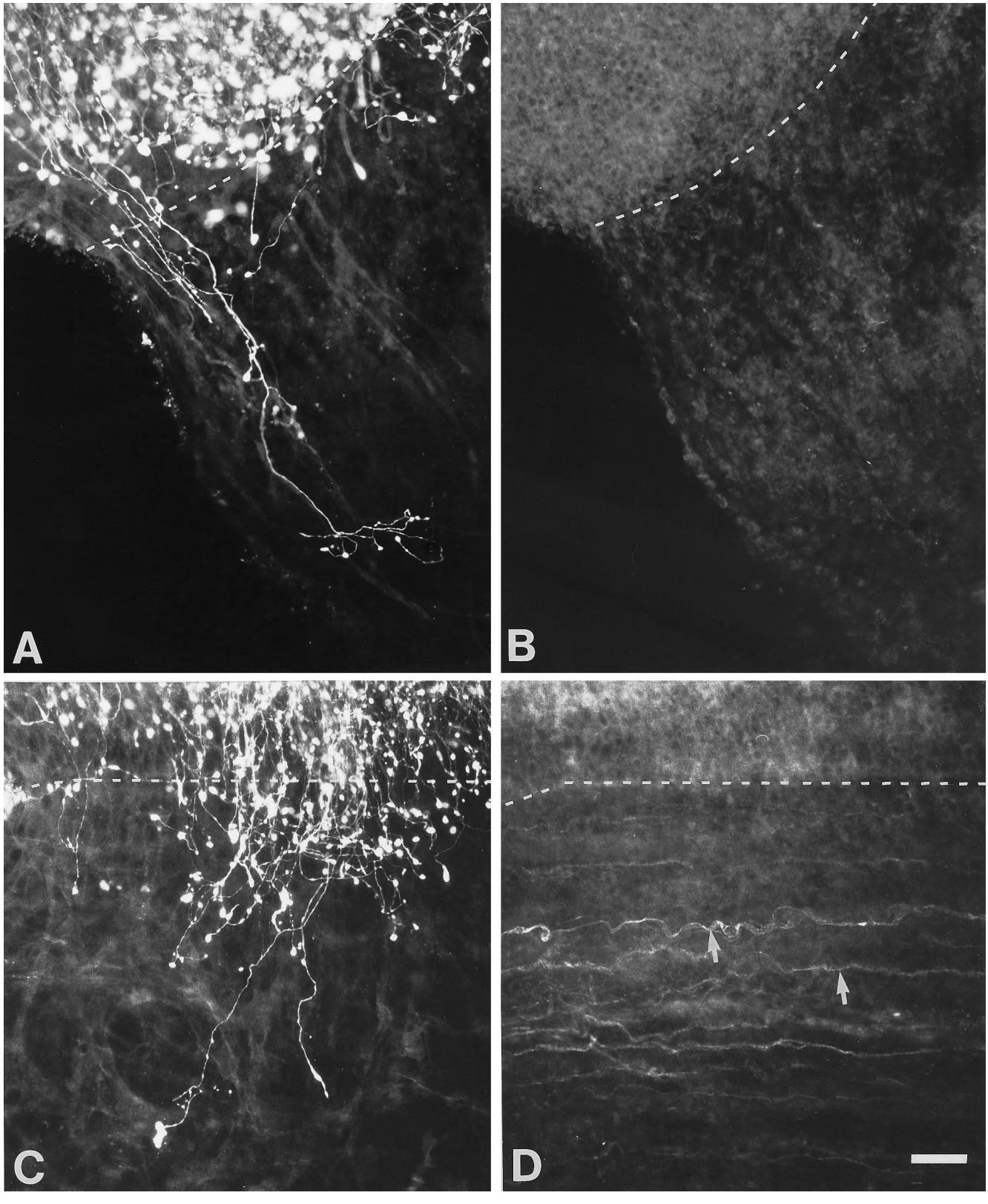

Figure 7. A few Purkinje cells regenerate axons on sciatic nerve for short distances. Two cocultures of cerebellar-sciatic nerve slices (*A–B, C–D*) were double-labeled with anti-CaBP (*A, C*) and anti-p75 (*B, D*) antibodies. A few CaBP-immunoreactive Purkinje cell axons enter and grow on sciatic nerve for short distances (*A, C*). The interface (*dotted line* in *A–D*) between the cerebellar slices and the sciatic nerve slices is determined by p75-staining (*B, D*). Indeed, the p75-immunolabeling gives rise to a diffuse staining all over cerebellar slices, allowing their identification. Purkinje cell axons can grow in the absence (*B*) or presence of Schwann cells (*arrows* in *D*). Note, however, that the Purkinje cell axons in *C* do not follow the bands of p75-immunoreactive Schwann cells visualized in *D*, but on the contrary they perpendicularly cross these bands without changing their direction of growth. Scale bar (shown in *D*): 50 μm.

cases, mature axotomized Purkinje cell axons did not regenerate, even when they were close to the area containing their immature targets. Moreover, in a very few cases, as was the case with sciatic nerve slices, axotomized Purkinje cell axons regrew for short distances on fetal cerebellum (Fig. 8*A,B*). The axons grew in a straight line and terminated with an elongated terminal varicosity. These results, together with those reported above, indicate that only a very low percentage of Purkinje cells seem able to respond to axotomy with regenerative attempts, even when the local environment of the amputated axons favors regeneration.

In organotypic cultures, fetal Purkinje cells grow and regenerate their axons on postnatal cerebellum

Fetal Purkinje cells regenerate their axons on postnatal cerebellum

As stated before, Purkinje cells survive well in intact cultures of prenatal rat cerebellum (E17–E21). To determine whether the prenatal Purkinje cells had the ability to grow axons into the mature cerebellar cultures, we performed coculture experiments in which E17–E21 Purkinje cells were apposed to P10 + 7 or more DIV amputated cerebellar cultures. Because it became possible to visualize the cortical and deep nuclear regions from E19, from this age on, two classes of fetal slices were used in the cocultures: (1) entire slices (Fig. 1*C*) and (2) dorsal regions of the slices in which the deep nuclei were eliminated (Fig. 1*E*). In the latter, Purkinje cell axons were axotomized, whereas in the former the only trauma suffered by Purkinje cells was that of the processing to obtain slices. The main difficulty with these cocultures was following and characterizing the axons of fetal Purkinje cells, because both fetal and mature Purkinje cells were CaBP-immunoreactive. Although difficult, the characterization was partially possible, because the two categories of axons were distinguishable: fetal axons were thinner and less densely immunostained than mature ones (Fig. 8*A,B*).

Axotomized and/or intact fetal Purkinje cell axons, facing either the ventral (axotomized Purkinje cells) or the dorsal (deafferented deep nuclei) (Fig. 1*F*) parts of the severed mature cerebellar cultures, were able to grow axons. In all instances, a large amount of fetal axons crossed the coculture interface and entered the mature cultures (Fig. 8*A,B*), whereas the axons of mature axotomized Purkinje remained close to this interface, forming the continuous front of axonal varicosities reported above (Fig. 8*A,B*). These results emphasize the different behavior of axotomized Purkinje cell axons according to age: mature axons do not regenerate (corroborating the above reported results), whereas immature axons do regenerate into more mature cerebellar slices, even in those lacking the deep nuclear region. Nevertheless, we were unable to follow the regenerating axons within the whole mature cerebellum, because they became almost unidentifiable once they reached regions containing axons, dendrites, or cell bodies of mature Purkinje cells.

Study of the regeneration of fetal Purkinje cell axons on postnatal CaBP-knockout mice

To solve the problem of axonal identification and to study more precisely the regeneration pattern of fetal Purkinje cells on postnatal cerebellum, we have confronted fetal wild-type mouse cerebellum with postnatal cerebellar cultures of CaBP-knockout mice. Thus, all the CaBP-immunoreactive axons observed in the cultures belonged to the fetal Purkinje cells. The combinations of cocultures were similar to those just reported for the rat.

Whatever the configuration of the cocultures, when the fetal slices were contiguous to the mature cerebellar slices their Purkinje cell axons grew or regenerated, invading the well organized P10 + 7 DIV CaBP−/− cerebellar slices. The extent, route, and mode of fasciculation of the regenerating axons varied from coculture to coculture, but in most instances—independent of the age of the donor embryo—the axonal growth was massive (Fig. 8*C*). Bundles of CaBP-positive axons often crossed the interface between cultures and spread throughout the mature cerebellum up to its most dorsal aspect, where these fascicles bent, following the surface of the cerebellar folia (Fig. 8*C*). In a few instances, the fascicles were thin and rare (Fig. 9*A*), and most of the regenerating axons spread within the mature cerebellar parenchyma as single fibers that also reached the dorsal aspect of the receiving cerebellum. To better appraise the route and sites of termination of regenerating axons, some cocultures were double-labeled with anti-CaBP and anti-parvalbumin antibodies. The latter, by immunostaining CaBP−/− Purkinje cells as well as basket and stellate cells, permitted the identification of the three cortical layers and underlying white matter (Fig. 9*D*). On such preparations it was possible to conclude that the fascicles of regenerating axons grew in all directions, crossing and/or following the white matter as well as the three cortical layers (Fig. 9*C,D*). These bundles, after various distances, began to give off single fibers up until their termination, where they split and formed small clusters of three or more thin fibers (Fig. 9*B,C*). The single fibers also branched, and together with the terminal bouquets formed dense plexuses all over the cerebellar structure, without apparent affinity for the molecular and granular layers, or the white matter; however, some of these plexuses terminated on the layer containing the several rows of Purkinje cell bodies, establishing pericellular nests (Fig. 9*E*), and also within the area containing the deep nuclei (Fig. 9*C*). The fasciculated fibers had regular diameters and were smooth. The terminal branches, on the contrary, exhibited a beaded appearance bearing small varicosities similar to boutons *en passant*.

These results show that axons of fetal Purkinje cells, axotomized or not, were able to elongate for long distances, invading the mature cerebellar slice without any apparent orientation. Thus, the growth or regrowth of these axons does not seem to be elicited by the presence of denervated specific postsynaptic targets. In this respect, it is of interest to recall that in nonaxotomized embryonic slices, Purkinje cell axons could cross the ventral region containing deep cerebellar neurons and pursue their course into the mature cerebellar slice for long distances (Fig. 9*F*).

As soon as they gain the ability to survive, postnatal Purkinje cells lose the ability to regenerate

Between P0 and P5, the rate of Purkinje cell survival was low (Table 1), and therefore we did not attempt to study the regenerative capability of Purkinje cell axons. The survival rate increased from P7 onward. To check whether P7 Purkinje cells *in vitro* resist axotomy and are able to regenerate their axons, some of the P7 cerebellar slices were axotomized at the time of culturing, and the dorsal and ventral portions of the slices were kept in apposition for 7–10 DIV. In these cultures, although Purkinje cell survival was good, their axons did not regenerate and formed an almost continuous front of axonal terminal bulbs in the dorsal aspect of the lesion, in a manner similar to P10 slices. Thus, after P7, Purkinje cells survive axotomy but do not regenerate their axons.

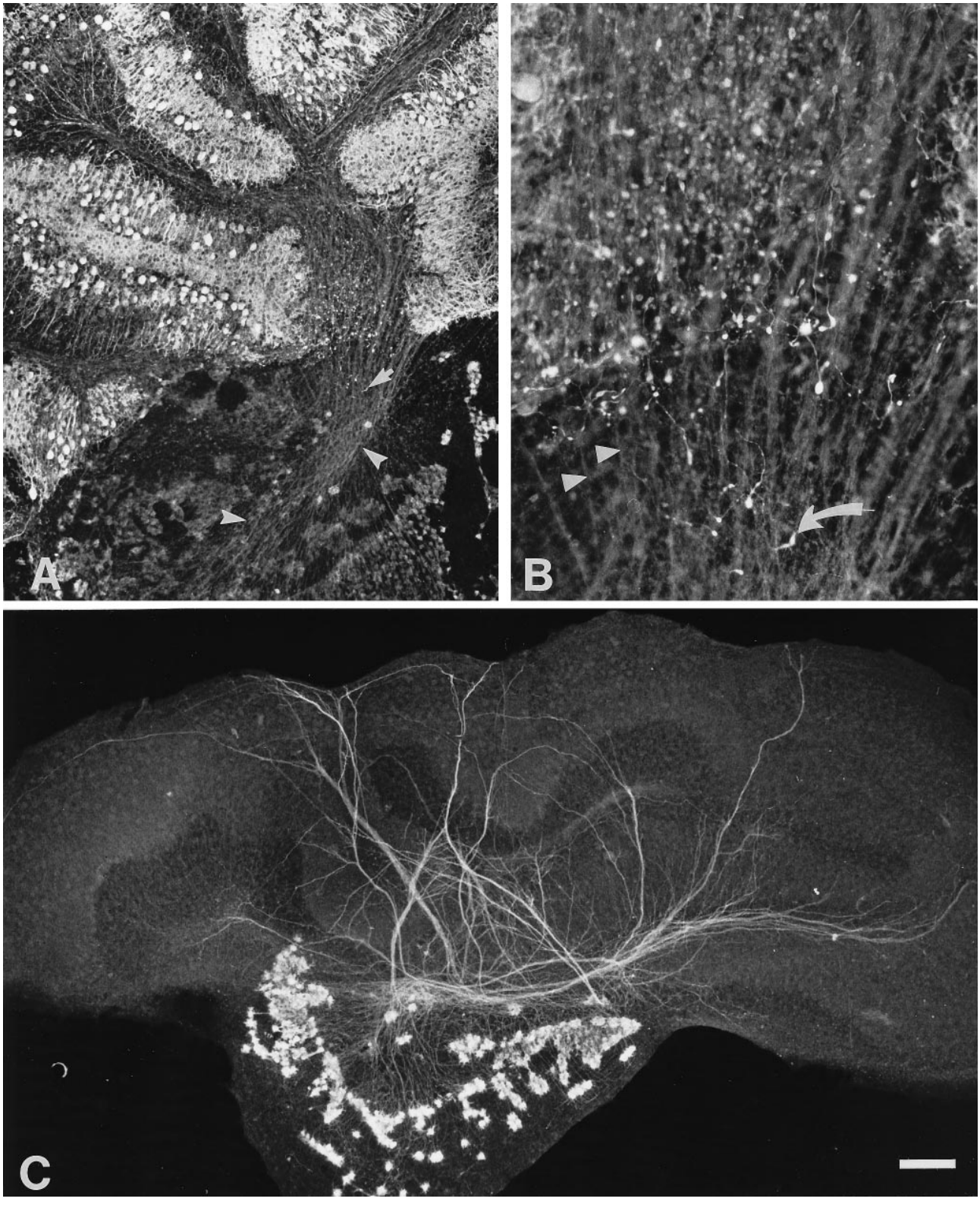

Figure 8. P10 + at least 7 DIV Purkinje cell axons do not regenerate on fetal cerebellar slices, whereas immature Purkinje cell axons regenerate on "mature cerebellar cultures." *A*, Coculture of P10 + 7 DIV dorsal region of a split cerebellar slice with an intact E20 fetal cerebellar slice. Mature and immature Purkinje cells are CaBP-immunoreactive. *B* is an enlargement of *A*. The *curved arrow* in *B* points to the same axonal terminal bulb as the one indicated by the *short arrow* in *A*. Embryonic axons (*arrowheads* in *A* and *B*), which are thinner and less densely immunostained than mature ones, enter into the mature slices and form small fascicles (*B*) but are rapidly hidden by the stronger immunoreactivity of the mature axons (*A*). Thus, fetal axons regenerate on mature cerebellar cultures, whereas mature axons do not regenerate on fetal slices. *C*, Coculture of the dorsal region of split P10 + 7 DIV cerebellar slices taken from a CaBP null mutant mouse with a wild-type E15 mouse cerebellar slice. In this case, only embryonic Purkinje cells are CaBP-immunoreactive. Note that the fetal axonal growth is massive. Bundles of CaBP-positive axons spread throughout the knockout cerebellum up to its most dorsal aspect, where these fascicles bend, following the surface of the cerebellar folia. Scale bar (shown in *C*): *A, C*, 160 μm; *B*, 40 μm.

DISCUSSION

The present *in vitro* study has revealed that Purkinje cell survival depends on maturational parameters: most Purkinje cells taken from P0 and P5 rats die in organotypic culture, whereas they survive when cultured before or after these ages. The regenerative failure observed in mature Purkinje cells is also the result of maturational changes intrinsic to the neurons themselves rather than of extrinsic environmental factors. Indeed, mature Purkinje cell axons regenerate neither spontaneously nor after confrontation with permissive environments (cocultured sciatic nerve, fetal cerebellum), whereas fetal Purkinje cell axons regenerate and massively invade the cocultured mature cerebellum.

Purkinje cell survival is highly impaired during the synaptogenic period

Our experiments show that, *in vitro*, Purkinje cell survival is age-dependent; however, this correlation is not a direct one. Although Purkinje cells in organotypic cultures taken from E19–E21 fetuses or from P7–P10 pups have excellent survival rates, those in cultures taken from P1–P5 pups die. Thus, Purkinje cell survival is not simply better the younger the neuron is, as predicted for other neuronal populations (see references in Schwab and Bartholdi, 1996).

During rat ontogenesis, Purkinje cells proliferate between E13 and E16 (Altman and Bayer, 1978) and then migrate and differentiate. From E17 onward, axonogenesis and oriented axonal growth are the main features of this differentiation (Wassef et al., 1985). The axons enter the deep nuclei domains by E18, and by E21–P0, terminal swellings appear to be associated with cell bodies (Eisenman et al., 1991). Thus, Purkinje cells in slices taken from E19–P0 rats are immature postmigratory neurons, involved mainly in axonal elongation for searching their postsynaptic targets and receiving their early presynaptic inputs from olivocerebellar afferents (Chédotal and Sotelo, 1992, 1993). Functional synapses between Purkinje cell axons and deep nuclear neurons are formed between P2 and P6, when they have acquired adult-like patterns (Gardette et al., 1985). It is during this same period that Purkinje cells follow a complete regressive remodeling of their dendritic arbors (Armengol and Sotelo, 1991). Thus, Purkinje cells in slices taken from P1–P6 rats are in a critical phase of synaptogenesis and dendritic remodeling. Because they do not survive to our *in vitro* conditions, we propose that during this critical period Purkinje cells are either dependent for their survival on cellular interactions missing in the slices or extremely susceptible to the mechanical injuries that occur during slice preparations.

Cerebellar slices as an *in vitro* system to study Purkinje cell axonal regeneration: advantages and limitations

An *in vitro* system able to mimic the conditions of the adult CNS must not only reproduce the local environment of the latter, but in addition the axons must have finished their developmental growth to allow distinction between new axonal growth and true regeneration. The difficulty is that CNS slices do not survive *in vitro* when taken after the end of the developmental period (P15 in our material). The present study extends previous reports on the high degree of organization and cellular differentiation of perinatal organotypic cultures (Gähwiler, 1988; Seil, 1972) to more mature cultures. Nevertheless, for Purkinje cells some differences with the *in vivo* organization occur. (1) The multilayering of their cell bodies results from the thinning of the slices *in vitro*, because by P10, Purkinje cells are already arranged in a monolayer (Altman and Winfree, 1977); (2) the hypertrophy of recurrent collaterals of Purkinje cell axons and the appearance of arciform axons (Ramón y Cajal, 1928) and torpedoes are considered to be nonspecific axonal responses to injury (Pioro and Cuello, 1988, 1990; Sotelo, 1990; Dusart and Sotelo, 1994; Rossi et al., 1994); and (3) although cortico-nuclear synaptic activity has been recorded *in vitro* (Audinat et al., 1990; Mouginot and Gähwiler, 1995), the scarceness of deep nuclear neurons could explain the atrophy of the terminal fields of Purkinje cell axons (McCrea et al., 1976).

The microenvironment of severed axons in mature cerebellar slices can be considered to be closely related to the fate of these axons (Aguayo, 1985; Schwab et al., 1993). Therefore, the comparative study of this microenvironment is of special interest to validate our *in vitro* system. *In vitro* as *in vivo*, myelination starts during the first postnatal week (Seil, 1972; Reynolds and Wilkin, 1988; Notterpeck et al., 1993). Thus, in our mature-like slices, as expected, most Purkinje cell axons are myelinated, mimicking the conditions of adult cerebellum. The increased density in GFAP-positive cells and the immunostaining of microglial cells and macrophages with the ED1 antibody indicates that both astrocytes and microglial cells are activated (Reier, 1986; Graeber et al., 1990). Therefore, the cellular environments of Purkinje cell axons *in vitro* and in adult cerebellum after axotomy are rather similar, because the axotomy activates both classes of glial cells *in vivo* (Dusart and Sotelo, 1994). From now on, we shall call these cultures "mature cerebellar cultures," to differentiate them from those taken earlier, i.e., fetal or early postnatal (first week) cerebella.

Reaction of "mature-like" Purkinje cells to axotomy: survival and absence of regeneration even when confronted with permissive microenvironments

Our study shows that the responses of mature Purkinje cells to axotomy are almost identical *in vitro* and *in vivo*: they are resistant to axotomy and lack spontaneous regeneration (Dusart and Sotelo, 1994); however, although *in vitro* continuity exists between the dorsal and ventral parts of the cut slices, the *in vivo* lesions involve the formation of cavities that prevent the axonal regeneration. The lack of spontaneous axonal regeneration *in vitro* indicates strongly that the cavity is not the only obstacle to axonal regeneration.

One of the great advantages of an *in vitro* system is the facility to modify the cellular milieu offered to amputated axons. Using a coculture approach, we have offered the axons two cellular milieux known for promoting axonal regeneration: sciatic nerve and embryonic cerebellar slices (see references in Schwab and Bartholdi, 1996). In none of these cocultures do severed Purkinje cell axons regenerate. These negative results confirmed a previous conclusion obtained with an *in vivo* approach (Rossi et al., 1995): permissive environmental cues and absence of inhibitory factors are necessary (Schwab et al., 1993), but not sufficient, to promote regeneration of adult Purkinje cell axons. Thus, the concept that only the local milieu in adult CNS prevents regeneration cannot be generalized.

Another interesting and new observation is the occurrence of a few regenerative attempts of the severed mature Purkinje cell axons. Their existence suggests that an extremely low percentage of adult Purkinje cells possess some ability to regenerate. Thus, Purkinje cells can react to axotomy like other central neurons. Indeed, only a very small percentage (1–5%) of axotomized neu-

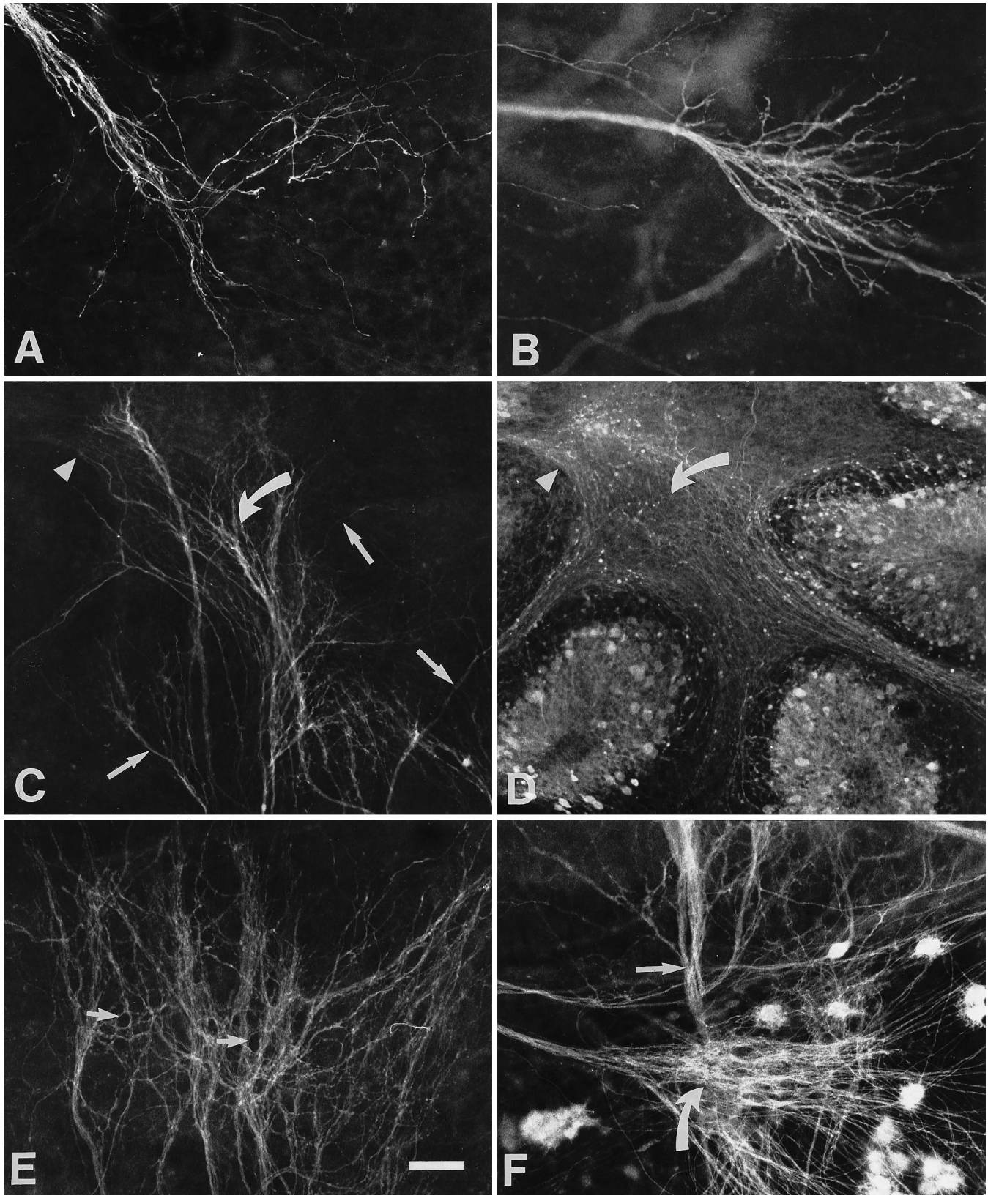

Figure 9. Regeneration and growth of murine fetal Purkinje cell axons on "mature cerebellar cultures" from CaBP knockout mice. Coculture of cerebellar slices of CaBP null mutant mice with a wild-type E16 cerebellar slice in *E*, and with dorsal regions of cut E17 in *A, C*, and *D*, and an E18 fetal cerebellar slice in *B*. *A*, Bundle of CaBP-immunoreactive embryonic Purkinje cell axons that give off single fibers near their end. *B*, Bundle of CaBP-immunoreactive fetal Purkinje cells that splits and forms small clusters of three or more thin fibers. *C, D*, Coculture double-labeled with anti-CaBP (*C*) and anti-parvalbumin (*D*) antibodies. In *D*, Purkinje as well as basket and stellate cells are parvalbumin-immunoreactive and therefore permit the identification of the three cortical layers and the underlying white matter. Although the majority of regenerating axons occupy (*Figure legend continues*)

rons, when confronted with modified environments, are able to regenerate (David and Aguayo, 1981; Vidal-Sanz et al., 1987; Villegas-Perez et al., 1988, Schwartz et al., 1991; Schnell and Schwab, 1990).

Purkinje cell axonal growth and regeneration is age-dependent

The simultaneous maturation of neurons and their glial environment has been advanced as the cause for the impairment of axonal regeneration of developing central neurons (Aguayo, 1985; Schwab and Bartholdi, 1996). Neurons pass through a regeneration-permissive critical period, the duration of which is determined by intrinsic and environmental conditions. To determine the presumptive occurrence and the end of the Purkinje cell regeneration-permissive critical period, we used a murine coculture setup, in which immature wild-type Purkinje cell axons of different ages were confronted with mature cultures taken from a CaBP−/− null mutant (Airaksinen et al., 1997). In these cocultures, intact and axotomized axons of immature Purkinje cells exhibit similar behavior: they invade extensively the mature slices, without any apparent selectivity for potential chemoattractants (supposedly their postsynaptic targets) or permissive environments. The inability of postsynaptic targets to stop the axonal outgrowth could be apparent only because (1) the amount of surviving deep nuclear neurons in the intact embryonic slices is very small and appears to be rapidly saturated with afferent inputs and (2) some of the embryonic Purkinje cell axons defasciculate and terminate in their target areas. In any case, Purkinje cells in these *in vitro* conditions differ from other classes of neurons that are able to terminate on their specific targets (Li et al., 1994).

The ubiquitous invasion of the growing axons of immature Purkinje cells in the cocultures contrasts with previous results using transplants of embryonic cerebellar precursors to adult mutant cerebellum (Keep et al., 1992). Although in both situations the regenerative capacity appears similar, in the transplants the axons grow in the host molecular layer without entering the granular layer. In the cocultures, this layer is not an obstacle, probably because being much younger than in the transplants, it has not yet became nonpermissive for Purkinje cell axons. Finally, the immature axons can grow along myelinated axons. This capacity, also shared by axons of grafted neurons in adult brain (Sotelo and Alvarado-Mallart, 1986; Wictorin et al., 1990, 1992; Keep et al., 1992, Li and Raisman, 1993), could result from the lack in immature neurons of receptors and/or transduction mechanisms needed to identify the inhibitory molecules blocking regeneration in adult CNS (see references in Schwab and Bartholdi, 1996).

This study of axonal regeneration and neuronal survival after axotomy *in vitro* has shown that during their development, Purkinje cells pass through three sequential stages: (1) an early one of active axonal elongation in which these neurons are resistant to axotomy and in addition are able to massively regenerate their amputated axons; (2) an intermediate stage of intense synaptogenesis with their target neurons and dendritic remodeling in which Purkinje cells are extremely vulnerable and do not survive the *in vitro* conditions; and (3) a final stage of neuronal maturation in which the Purkinje cells already behave like adult Purkinje cells, i.e., they are resistant to axotomy but lack axonal regeneration. The timing of these sequential stages reveals that the end of the developmental axonal growth and regeneration-permissive critical period coincides with the initiation of Purkinje cell efferent synaptogenesis. It can be suggested that this synaptogenesis may turn off the genetic program for axonal elongation (Tetzlaff et al., 1991; Chen et al., 1995), impairing the growth of regenerating Purkinje cell axons.

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a white matter location, some of the axons (*arrows* in *C*) grow in all directions, crossing and/or following the white matter as well as the three cortical layers (*arrowheads* in *C* and *D* point to approximately the same point). Note that fibers defasciculate in the area of deep nuclear neurons (*curved arrow*). In *E*, regenerating fetal Purkinje cell axons branch and form dense plexuses over the layer containing several rows of Purkinje cell bodies (*arrows*), establishing pericellular nests (*arrows*). In *F*, some CaBP-immunoreactive fetal Purkinje cell axons (*arrow*) cross the ventral region containing deep cerebellar neurons (*curved arrow*) and pursue their course into the mature cerebellar culture. Scale bar (shown in *E*): *A*, 60 μm; *B*, 35 μm; *C, D*, 100 μm; *E*, 40 μm; *F*, 25 μm.

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