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DEVELOPMENT OF THE RODENT CEREBELLUM AND SYNAPTIC RE-FORMATION OF DONOR CLIMBING TERMINALS ON SPINES OF THE HOST PURKINJE DENDRITES AFTER CHEMICAL DEAFFERENTATION

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Summary

Reinnervation of host Purkinje cells by donor climbing fibers was observed in the following experiments. Medullary primordial tissue (from E14–E16) containing the inferior olive was grafted into a host rat cerebellum, in which the inferior olivary complex and climbing fibers had been destroyed by intraperitoneal injection of 3-acetylpyridine (3-AP). After 3 weeks, immature as well as mature types of climbing fiber terminals bearing packed round vesicles were found that had established synaptic contacts on dendritic spines of the host Purkinje cells. Quantitative analysis at the ultrastructural level has been carried out. The main results are as follows. (1) The number of preterminals that formed synaptic contacts with spines of the host Purkinje dendrites in the transplanted material increased by 3.4-fold compared to the control (3-AP-treated non-grafted material). (2) The number of mature climbing-type preterminals increased from 0.3–0.9 % to 5 % after grafting (cf. 22 % in normal brain tissue), and the number of immature climbing-type preterminals also increased from 2–10 % (control) to 20 % after grafting. These changes were statistically significant ($P<0.01$). (3) The number of parallel-type preterminals increased from 13 % (control) to 27 % after grafting, which was also statistically significant ($P<0.01$). Thus, it appears that the donor climbing fibers grow and develop to find unoccupied spines on the host Purkinje dendrites and establish synaptic contacts, and also that the host parallel fibers may generate axonal sprouts to search their new targets and ultimately to form synaptic contacts with unoccupied spines. In the process of re-modeling the brain, competition for targets is likely to occur between the two kinds of axonal processes, i.e. the donor climbing fibers and the host parallel fibers.

Introduction

The organization patterns of the mammal olivocerebellar projection have been shown to be precise (Groenewegen and Voogd, 1977; Groenewegen *et al.* 1979; Kawamura and Hashikawa, 1979; Brodal and Kawamura, 1980). Both cells of origin, the inferior olivary, and of termination, the Purkinje cells, arise from immature cell groups located in the alar plate of the developing medulla

Key words: inferior olive, climbing fiber, synaptic formation, Purkinje cell, transplantation.

oblongata. These initiate migration in the mid-embryonic days after the final mitosis of the neuroepithelial cells; one cell type moves in a dorsal direction through the parenchyme of the rhombic lip and the other moves ventrally through the medullary parenchyme, with a small number directed along the subpial region (Miale and Sidman, 1961; Altman and Bayer, 1978; Sotelo, 1978; Bourrat and Sotelo, 1988). Each group of neurons continues to develop at the terminal site of migration. At the perinatal period, each particular group of developing olivary axon terminals finds its own specific group of Purkinje cells as proper targets and makes synaptic contact with the membranes of these cells (Mason and Gregory, 1984; Mason, 1987; Landis and Weinstein, 1987) to establish the basic pattern of organization in the olivocerebellar projection, which is surprisingly strict. Whether such connectivity will be maintained after cerebellar injury is an intriguing topic. Since at present we are not at a stage to pursue this problem directly, current experiments have been designed to examine the biological behavior of immature olivary neurons when they are transplanted into the climbing-fiber-deprived cerebellum. The beginning of this article gives a brief survey of the generation of the climbing fiber system before going on to the synaptogenesis and plastic changes occurring in the host cerebellum. These issues are discussed on the basis of our experimental results and, in the light of an overwhelming number of unsolved problems, we consider how best we should proceed at present, and what should be the main thrust of future research in this field.

Organization of the olivocerebellar projection (Figs 1 and 2)

The olivocerebellar projection of carnivores and rodents has been shown to be precisely organized (see Brodal and Kawamura, 1980, for a review). The inferior olivary complex of rodents is formed in the mid-embryonic days (E12–E16) after the final settlement of migratory cells from the medullary alar plate region. Immature olivary cells move within two migratory streams: one superficial, under the pia mater (the marginal stream), and the other deeper in the medullary parenchyme (the submarginal stream), according to a recent axonal tracing study of Bourrat and Sotelo (1988), who used the *in vitro* slab technique. Conglomerates of these immature cells differentiate to form several separate groups that form the medial accessory olive (MAO), the dorsal accessory olive (DAO) and the principal olive (PO), as well as smaller cell groups; the dorsal cap, the nucleus β, the dorsolateral cell column and the ventrolateral outgrowth (Brodal, 1940). As for the projections of the larger nuclear groups, the caudal half of the MAO projects to the most medial vermal zone (A zone) and the rostral half to the intermediate zone (C2 zone) of the cerebellar cortex, while the caudal half of the DAO projects to the lateral vermal zone (B zone) and the rostral half to intermediate zones (zones C1 and C3). The dorsal and ventral lamellae of the PO project to the lateral parts (D1 and D2, respectively) of the hemisphere (Brodal and Kawamura, 1980). It is intriguing how this type of precise pattern can be

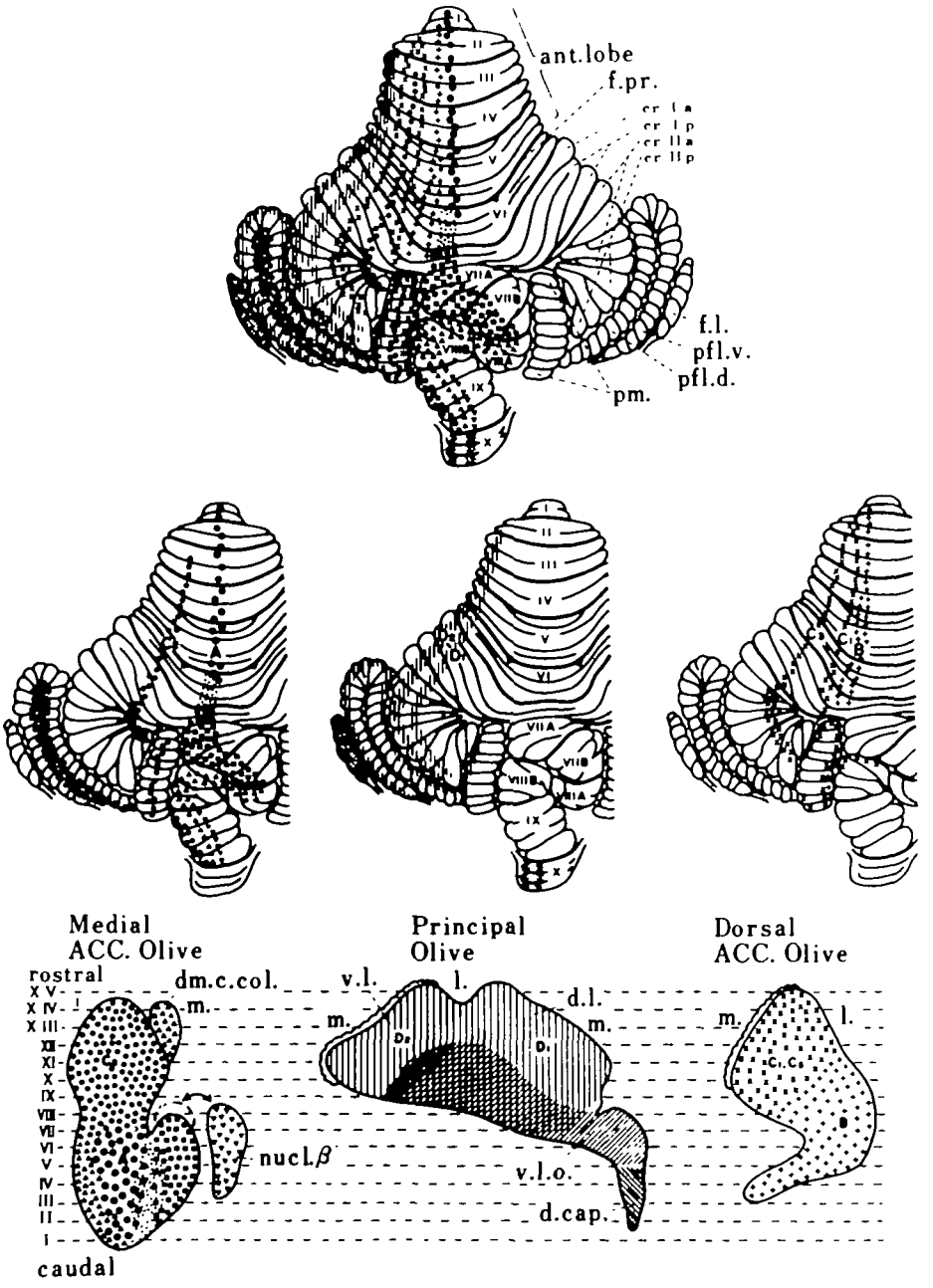
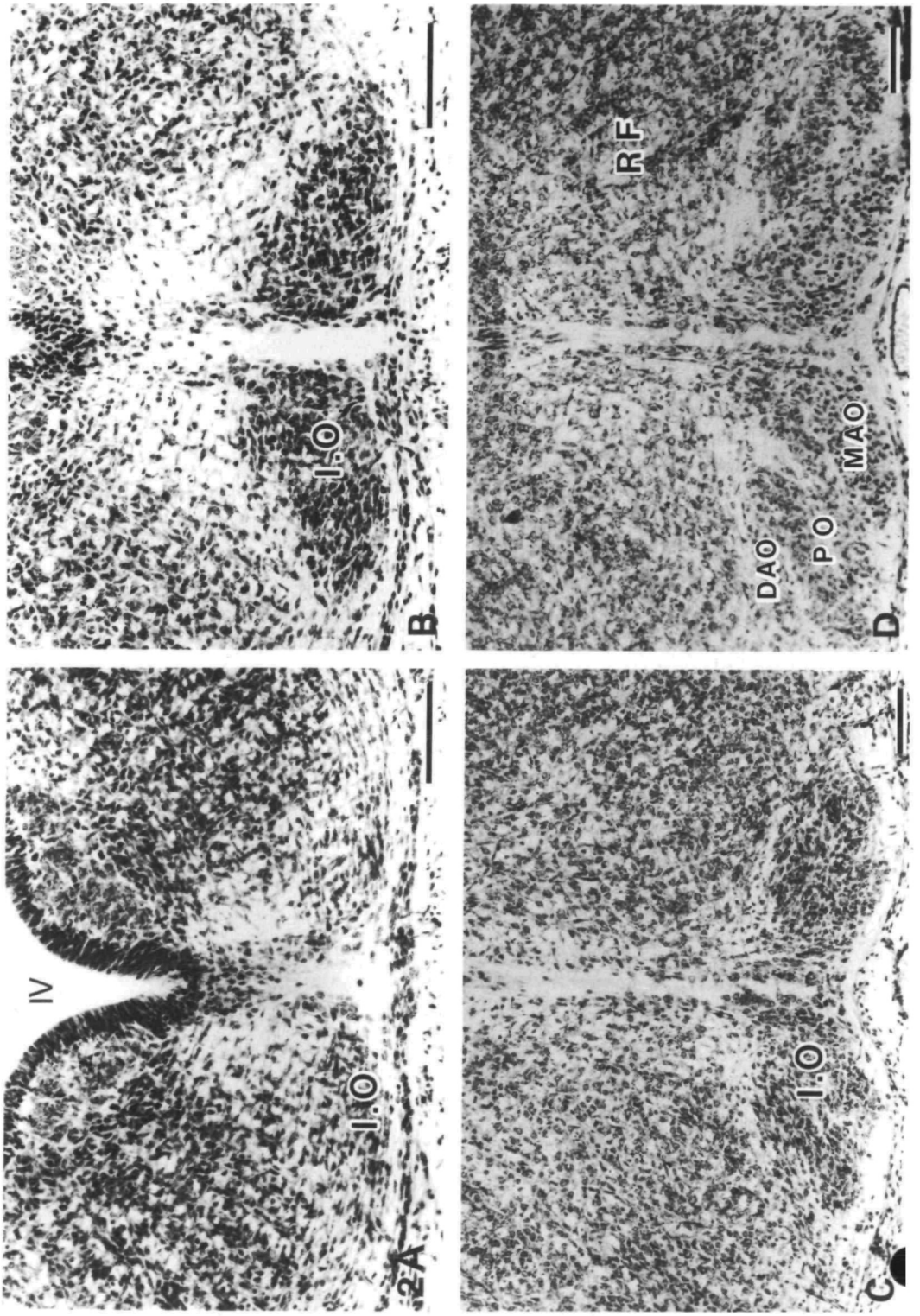


Fig. 1. Organization of the olivocerebellar projection as studied in the cat (Kawamura, 1980). Different symbols are used to designate sites of origin and terminations of this topographic projection. The organization in rodents is, in principle, the same. ant.lob., anterior lobe; f.pr., fissura prima; cr., crus; f.l., flocculus; pfl.v., paraflocculus ventralis; pfl.d., paraflocculus dorsalis; pm., lobulus paramedianus; ACC, accessory; dm.c.col., dorsomedial cell column; m., medial; nucl.β, nucleus beta; v.l., ventral lamella; l., lateral; d.l., dorsal lamella; v.l.o., ventrolateral outgrowth; d.cap., dorsal cap.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2A
I.O
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DAO
PO
MAO
D
I.O
C

Fig. 2. Photomicrographs to show the development of the mouse inferior olive. Pictures are taken from the middle part of the olivary complex at (A) embryonic day 12 (E12), (B) embryonic day 14 (E14), (C) late embryonic day 15 (E15) and (D) embryonic day 16 (E16). Nissl stain, 10 μm transverse sections. DAO, dorsal accessory olive; I.O, inferior olive; MAO, medial accessory olive; PO, principal olive; RF, reticular formation; IV, fourth ventricle. Scale bar, 100 μm.

formed, after seemingly independent development of the olive and the cerebellum.

Development of Purkinje cells and climbing fibers (Fig. 3)

Primordial Purkinje cells in rodents originate from the neuroepithelium of the fourth ventricle, from which they start to migrate radially towards the pial surface after their final cell division (E13–E15 in rats, Altman and Bayer, 1978; E11–E13 in mice, Miale and Sidman, 1961). It could be that immature Purkinje cells migrate using non-neuronal processes as possible guides. These are presumably immature types of astroglial processes. There is also an involvement of the extracellular matrix. In the vestibulocerebellum, a group of developing Purkinje cells rearrange to take up a final position in a single cell layer underneath the immature external granule cells. These cells at later stages also migrate downwards, while extending their axonal processes parallel to the pial surface. Primary and secondary dendrites of Purkinje cells develop during this postnatal period and their spines receive afferent axon terminals.

It has been shown that almost all climbing fibers originate from the inferior olive (Courville and Faraco-Cantin, 1978). They cross the midline of the medulla and enter the contralateral, inferior peduncle to reach the developing Purkinje cells around the perinatal period. Formation of synapses between Purkinje cells and climbing fibers occurs during the maturation of the target cells (Mason and Gregory, 1984; Landis and Weinstein, 1987).

Grafting of embryonic medulla after destruction of the host olivocerebellar projection (Fig. 4)

We transplanted embryonic medulla containing the olive into crus II of the adult rat cerebellum, whose inferior olive, apart from the most medial part of the caudal MAO, had been destroyed by intraperitoneal injection of 3-acetylpyridine (3-AP, 50 mg kg^{-1}). Harmaline (10 mg kg^{-1}) and niacinamide (300 mg kg^{-1}) were also injected, as previously reported (Desclin and Escubi, 1974; Sotelo *et al.* 1975). Crus II was selected as a site of olivary implantation since it is known that the caudal MAO does not project to this region (Brodal and Kawamura, 1980). The ventral part of the caudal medulla that contained olivary cells was grafted from E14–E16 rats 2 days after the injections. After 3 weeks, brain tissue was processed for electron microscopical observation.

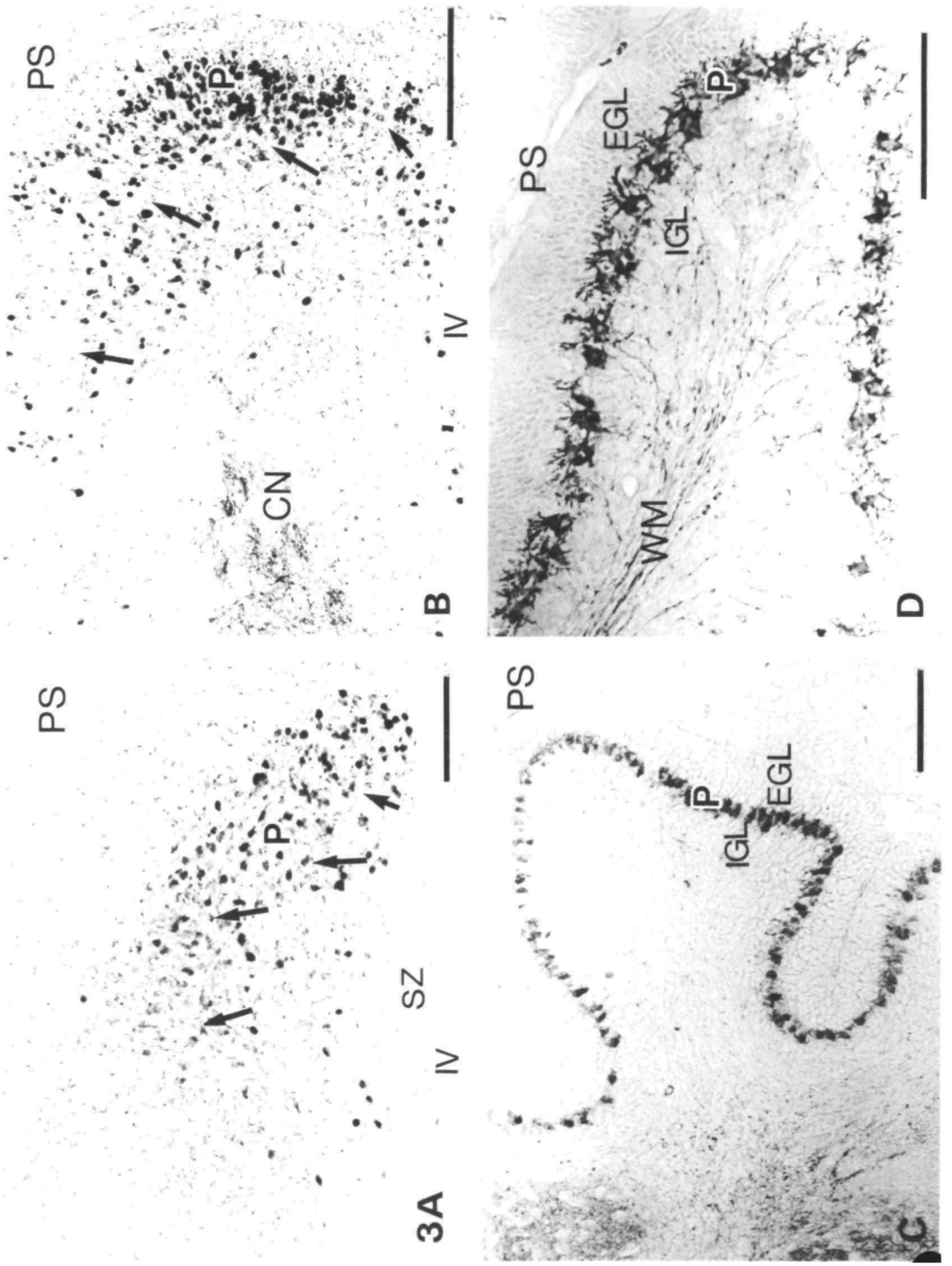
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Fig. 3. Photomicrographs to show the development of the mouse Purkinje cells in the vestibulo-cerebellum at (A) embryonic day 15 (E15); (B) embryonic day 18 (E18); (C) post-embryonic day 2 (P2); (D) post-embryonic day 7 (P7). After final mitosis the primordial Purkinje cells, immunostained with anti-spot-35 antibody (Takahashi-Iwanaga *et al.* 1986), are seen to migrate (A and B), settle (C) and grow their dendritic trees (D). Arrows in A and B indicate the direction of cell migration. 10 μm sections. CN, cerebellar nuclei; EGL, external granular layer; IGL, internal granular layer; P, Purkinje cell; PS, pial surface; SZ, subventricular zone; WM, white matter; IV, fourth ventricle. Scale bar, 100 μm.

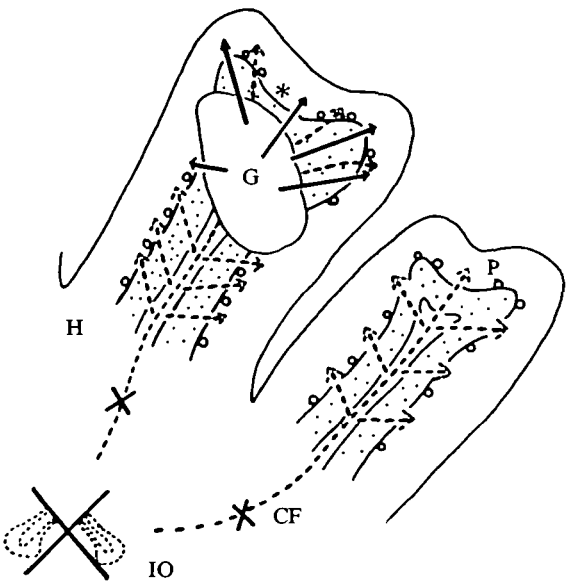

Fig. 4. Drawing of the experimental area showing the region (*) of the cerebellar cortex where electron microscopical analysis was performed after transplantation of the embryonic olive in 3-AP-treated material. New climbing fibers are expected to grow (unbroken arrows) from the graft (G) in the host (H) cerebellum where climbing fibers (CF) and inferior olive (IO) have been destroyed, as indicated by broken lines and crosses.

In the deep molecular layer of the 3-AP-treated material astroglial proliferation was revealed. This very often ensheathed free spines that had probably increased in number, as previously described (Sotelo *et al.* 1975; Bradley and Berry, 1976) (see Fig. 7 and Table 1). It appears that the number of spines emerging from secondary and tertiary Purkinje dendrites increased, while the level of spine density in the spiny branchlets was unchanged (see Sotelo, 1978). Thus, spine formation can be induced after deafferentation of climbing fibers (Bradley and Berry, 1976; Sotelo, 1978). Under these circumstances, when donor climbing fibers generate and invade this region, their growth cones can search for free spines as their proper targets.

Reorganization of the cerebellum as viewed from transplantation experiments in rodents

The deep one-third of the host molecular layer at the site of grafting was analyzed to answer the following questions. Do climbing fibers generate from the donor tissue? Do they reinnervate the host Purkinje dendritic spines whose own climbing terminals had previously been removed? Does competition occur between the donor climbing terminals and the host parallel fiber terminals?

In this region, mature as well as immature types of climbing terminals were found establishing synaptic contacts with dendritic spines of the host Purkinje cells. It is generally thought that spines in the outer molecular layer make contact mainly with parallel fibers while those in the deep layer make contact with climbing fibers (Palay and Chan-Palay, 1974). Profiles of these synapses are shown in Figs 5–8. Some terminals contacting dendritic spines harbored tightly packed, small, round vesicles, others contained aggregated but not closely packed vesicles, the third type had randomly distributed vesicles. Although we are at present unable to identify these different types of axon terminals immunohistochemically, they can be characterized into morphological groups based on their appearance under the electron microscope. The numbers of different types of axon terminals have been counted and are listed in Table 1. Terminals were categorized into three types, the typical climbing fiber terminal type (type A), the quasi-climbing type (type B) and the parallel fiber terminal type (type C) (see Fig. 5). Preterminals unattached to spines are classified as type D. Quantitative analyses were also carried out on control (3-AP-treated) material (Fig. 7) taken from a non-grafted region and on normal (not treated with 3-AP) material. These results are shown in

Table 1. *The number and occurrence of presynaptic terminals attached (types A, B and C) or unattached (type D) to dendritic spines of Purkinje cells in the deep molecular layer of crus II in normal (no. 1) and experimental (nos 2–4) brains*

	Animal number			
	1	2	3	4
Animals grafted	No	Yes	Yes	No
3-AP-treated	No	Yes	Yes	Yes
Area examined (crus II)	Normal	Control (other side)	Grafted site	Control (3-AP only)
Total number of synapses	428	493	476	469
Type A	94 (22%)	1 (0.3%)	25 (5%)	4 (0.9%)
Type B	42 (10%)	10 (2%)	94 (20%)	46 (10%)
Type C	172 (40%)	63 (13%)	128 (27%)	246 (52%)
Type D	120 (28%)	419 (85%)	229 (48%)	173 (37%)

All values within a single type in animals 1–3 are significantly different (chi-squared test).
The controls are taken from 3-AP-treated, grafted material (animal 2) and 3-AP-treated but non-grafted (animal 4) materials.

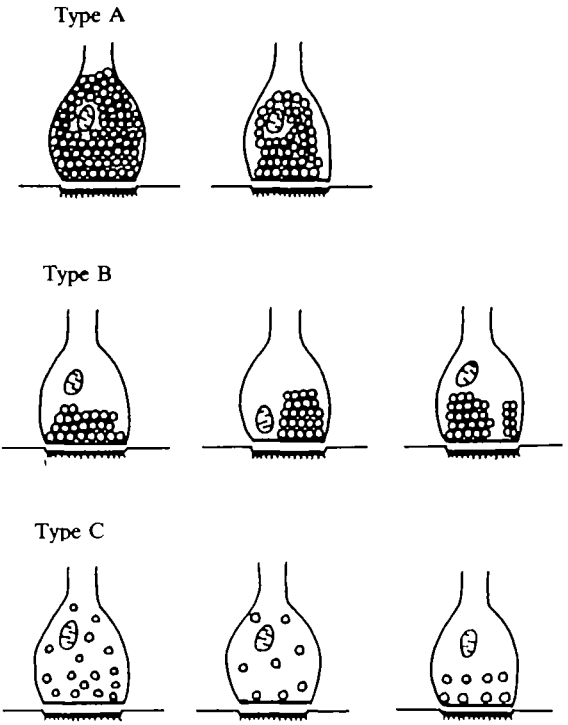

Fig. 5. Schematic drawings to show various features of preterminals either attached (types A, B and C) or unattached (type D, not shown) to dendritic spines of Purkinje cells. The categories A–C are based upon the features of the presynaptic terminals (see text). Spines that are separated by glia, thus being free from preterminals, are classified as type D (as unattached spines).

Table 1 and demonstrate the increase of statistically significant ($P<0.01$) numbers of climbing fiber terminals that made synapses on the host Purkinje dendritic spines. In counting the number of synapses, we always identified the presence of presynaptic vesicles and postsynaptic thickenings on spines of dendrites that contained a hypolemmal cisterna(e), this being characteristic of the Purkinje cell (Palay and Chan-Palay, 1974).

Our results can be summarized as follows. (1) The total number of preterminals that formed synaptic contacts with spines of the host Purkinje dendrites in the transplanted material increased 3.5-fold (from 15 to 52%) compared with the control group. This was statistically significant ($P<0.01$ using the chi-squared test). (2) Based upon the classification mentioned above, the number of type A terminals (mature climbing features) increased from 0.3% (in the control) to 5% after grafting (compared with 22% in normal tissue), and the number of type B terminals (immature climbing features) increased from 2% (in the control) to 20% after grafting (compared with 10% in normal tissue). These increases were statistically significant ($P<0.01$). (3) A decrease in the number of type C terminals (parallel fiber features) in the control area (down to 13% from 40%) after 3-AP

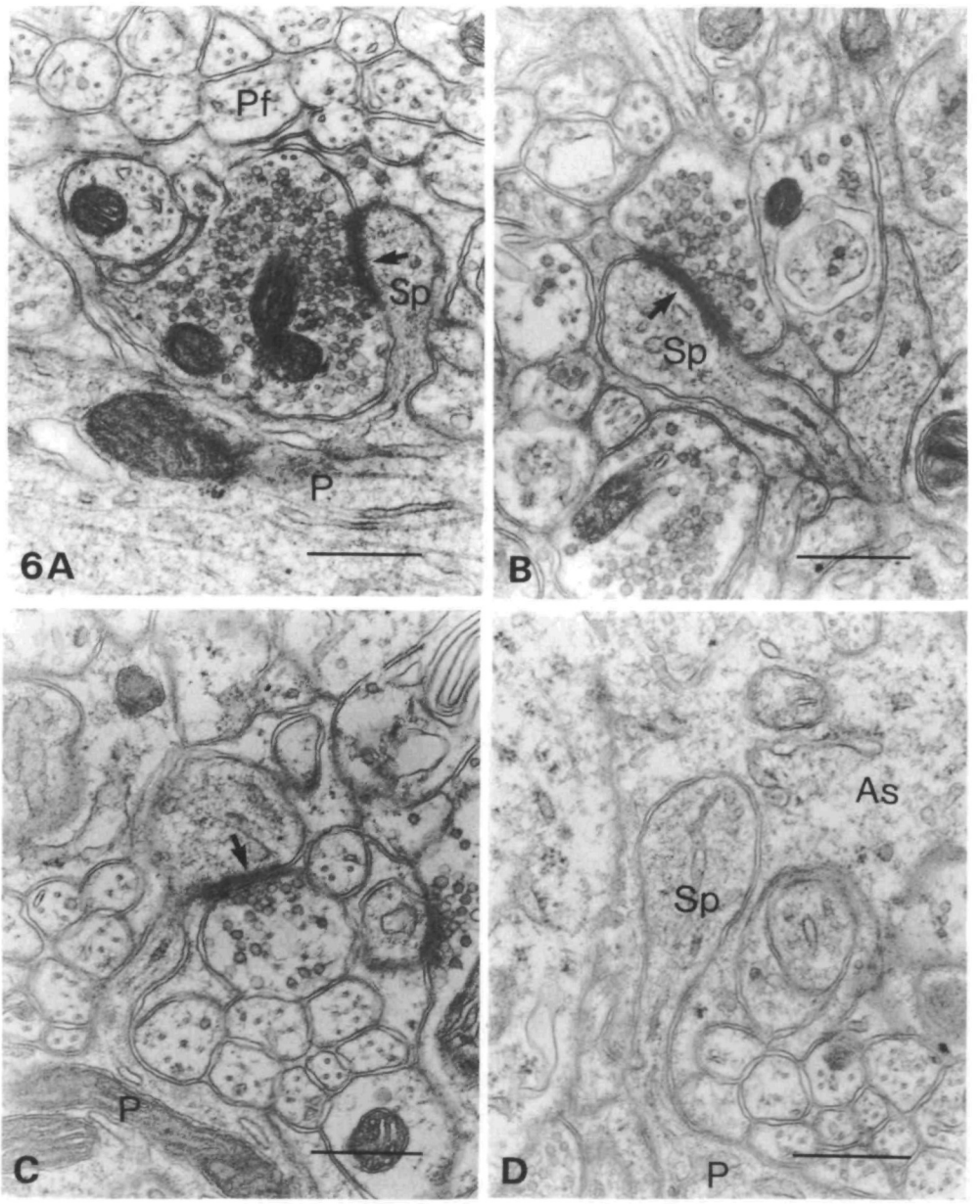

Fig. 6. Synaptic structures of dendritic spines of Purkinje cells appearing in the deep one-third molecular layer of the normal rat cerebellum. (A) A type A preterminal containing closely-packed small vesicles; (B) a type B preterminal with less densely packed vesicles; and (C) a type C preterminal containing sparsely distributed vesicles all making synaptic contact with spines belonging to Purkinje dendrites. (D) Unattached spine engulfed by astroglia. As, astrocyte; P, Purkinje cell; Pf, parallel fiber; Sp, spine; arrows indicate postsynaptic sites. Scale bar, 0.5 μm.

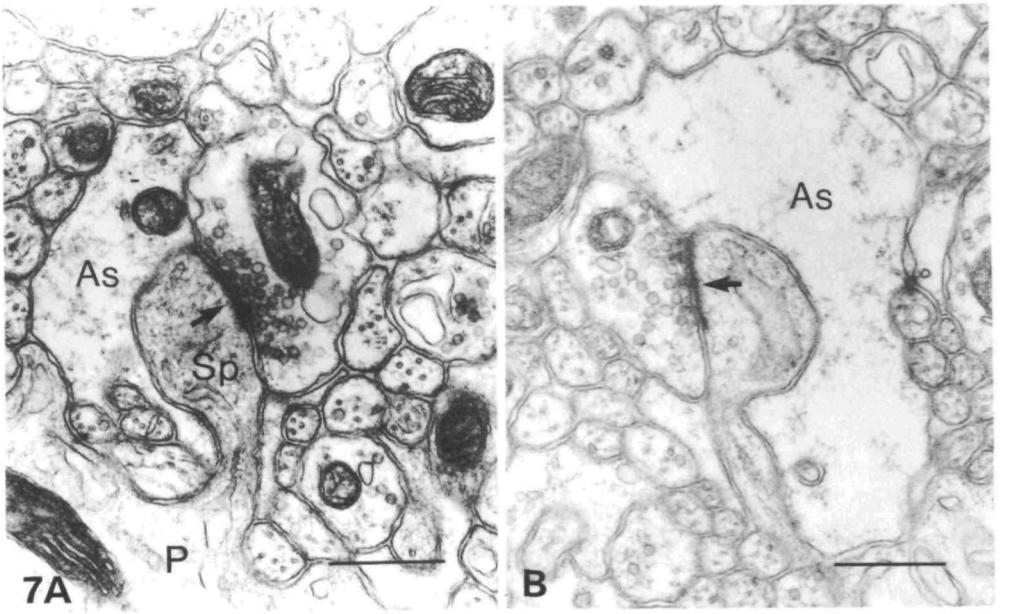

Fig. 7. Synaptic structures of dendritic spines of Purkinje cells in the corresponding area in the 3-AP-treated rat cerebellum. (A) A type B preterminal, (B) a type C preterminal. As, astrocyte; P, Purkinje cell; Sp, spine; arrows indicate postsynaptic sites. Scale bar, 0.5 μm.

treatment and an increase in the grafted area (up to 27 % from 13 %) were also noted. Both the decrease and the increase were statistically significant ($P<0.01$). This control material was taken from a non-grafted region of the graft-containing cerebellum of the 3-AP-treated animal (Table 1). The high proportion (85 %) of unattached spines in this deafferented material may be caused by the proliferation of reactive astrocytes following brain injury as a result of the grafting process (Takamiya *et al.* 1988). It could be argued, however, that the decrease of all three types of preterminals may not accurately reflect the influence of 3-AP administration. To resolve this problem, an analysis was carried out of material in which the same amount of chemical had been injected without inflicting the brain damage caused by the grafting procedure. As indicated in Table 1, the percentage of unattached Purkinje spines (type D) was quite low (37 % compared with 85 % in the brain-injured material). An increase of type C preterminals (from 40 % in the normal material to 52 %) was also noted as a result of this examination. This increase of type C preterminals, under conditions in which types A and B decrease, strongly suggests that there is sprouting of parallel fiber endings of the host.

It has been shown that the occurrence of two types of terminals changes after chemical treatment and/or grafting. It is likely that in areas close to the graft the donor climbing fibers grow and develop to find unoccupied spines on the host

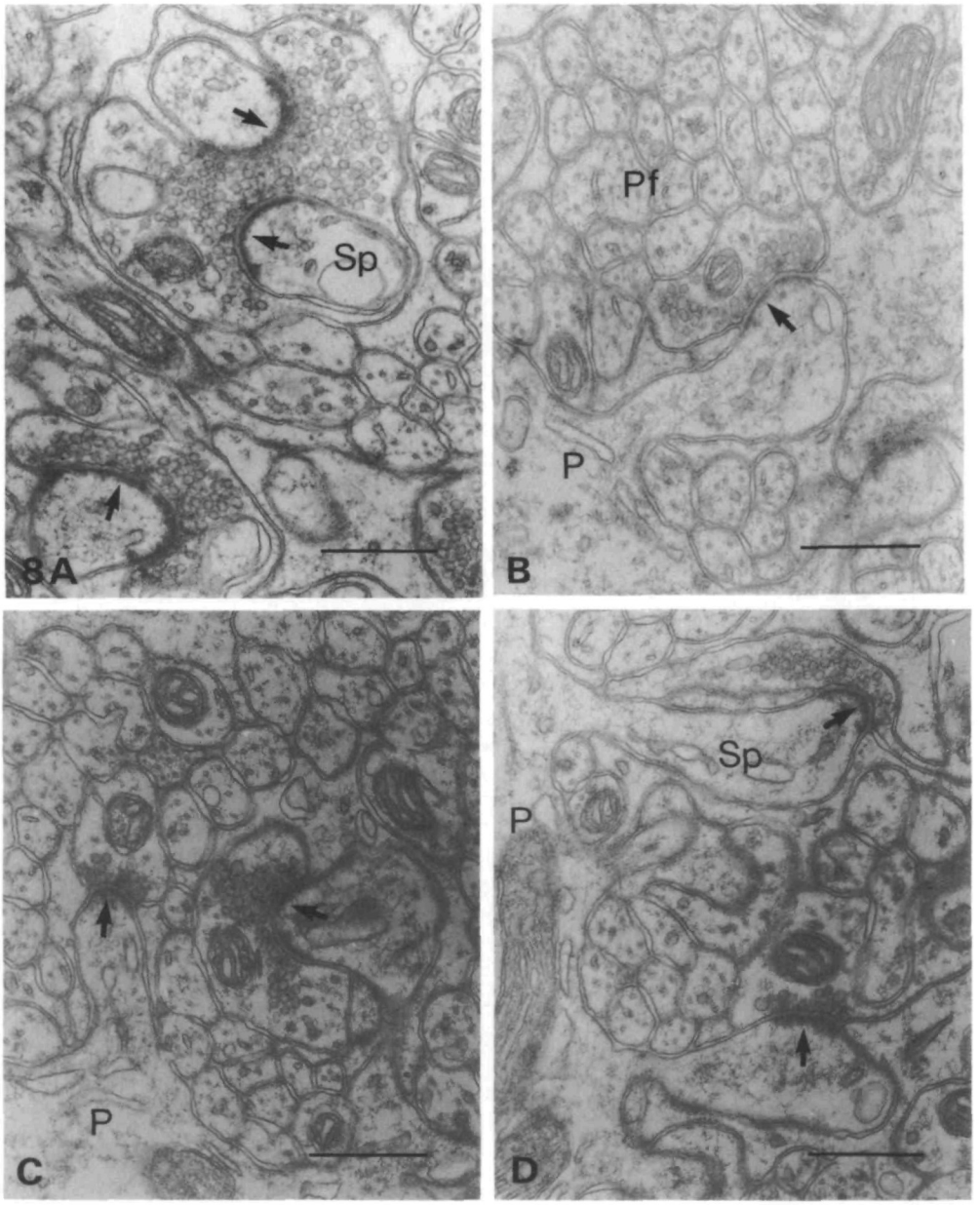

Fig. 8. Synaptic structures of dendritic spines of Purkinje cells in the corresponding area in the grafted brain of a rat after 3-AP treatment. (A) A type A terminal, (B) a type B terminal. C and D both contain type B and C terminals. P, Purkinje cell; Pf, parallel fiber; Sp, spine; arrows indicate postsynaptic sites. Scale bar, 0.5 μm.

Purkinje dendrites and that the host parallel fibers generate axonal sprouts to search their new targets, both struggling to form synaptic contacts with regrowing spines.

Consideration of future directions

In the present experimental situation, where developing olivary cells are implanted in the climbing-fiber-deprived cerebellum, new dendritic spines on the host Purkinje cells will regrow after their loss of climbing preterminals (Sotelo *et al.* 1975). The situation resembles that of the perinatal stage in cerebellar development, when mossy and climbing fibers enter from the brainstem (Mason and Gregory, 1984; Landis and Weinstein, 1987).

Numerous factors, or prerequisites, have to be considered for the achievement of favourable reorganization of synaptogenesis. An exploration of these factors that support, promote and guide axonal growth is the first problem. At the site of grafting, proliferation of reactive astroglia occurs (Miller *et al.* 1986). These reactive cells could have two possible roles; being both obstacles to synaptogenesis and yet having promoting activity for axonal growth. As shown in Figs 6D and 7, deafferented spines were surrounded by processes of astroglia that might have inhibited contact with new axonal ingrowth. In contrast, reactive astroglia synthesize and excrete a variety of extracellular matrix and cell adhesion molecules (Liesi *et al.* 1984, 1986; Kruse *et al.* 1985). Laminin is one of them, and it could play an important role as a promoter of axonal growth. *In vivo* and *in vitro* studies have unambiguously shown such activity (Davis *et al.* 1987; Hantaz-Ambroise *et al.* 1987). Production of laminin by reactive astroglia has been shown by Liesi *et al.* (1984) and laminin has also been detected immunohistochemically in the embryonic cerebellum (Liesi, 1985). Therefore, an immunohistochemical analysis of the expression of this extracellular matrix with respect to the synaptic organization after neural transplantation is needed. As for the astroglial lineage, type I astrocytes are considered to participate in scar formation in the neural tissue (Miller *et al.* 1986). Examination of astroglial subtypes that participate in the production of the extracellular matrix and the promotion of axonal growth is a challenging problem.

Guidance of growing and/or regrowing axons may also involve the glial factor. In the development of the optic nerve (Bovolenta and Mason, 1987) and vestibular axons (Morris *et al.* 1988), subpial axonal growth on the endfeet of radial glia has recently been described. By using a marker for embryonic radial glia (Ono *et al.* 1989), the participation of a radial glial component in the injured and regenerating brain is now being examined in our laboratory.

In the promotion of synaptic re-modeling, trophic factors of a humoral nature should also be considered. They may be produced from both embryonic grafts and injured host tissue. The sprouting of parallel fiber terminals observed in our experiments must be explained by considering the participation of such factors. A combination of deafferentation and injury (animal 2 in Table 1) induced the

reduction of parallel-type preterminals (type C, from 40 to 13 %) and grafting of the embryonic olive (animal 3 in Table 1) induced an increase of preterminal type C (from 13 to 27 %).

During both the development and the regeneration of the central nervous system, structural changes occur that, in principle, are based upon the expression of a series of genes involved in developing morphology both spatially and temporally. Recently, cDNA of growth-cone-associated protein, GAP-43, was introduced into fibroblasts and the extension of neuron-like processes was induced (Zuber *et al.* 1989). The detection of the expression of genes related to the development of axons and synapses is a target for future research. Moreover, retrovirus-mediated gene transfer has been applied to the grafted cells to confer new functions (Gage *et al.* 1987; Tsuda *et al.* 1990).

Many questions remain unanswered concerning synaptogenesis and stabilization of synaptic formation, both during normal development of the brain and during the reorganization of the damaged brain. The transplantation methods and results outlined here, as well as future application of genetically modified grafts, may provide the tools for investigating this regeneration.

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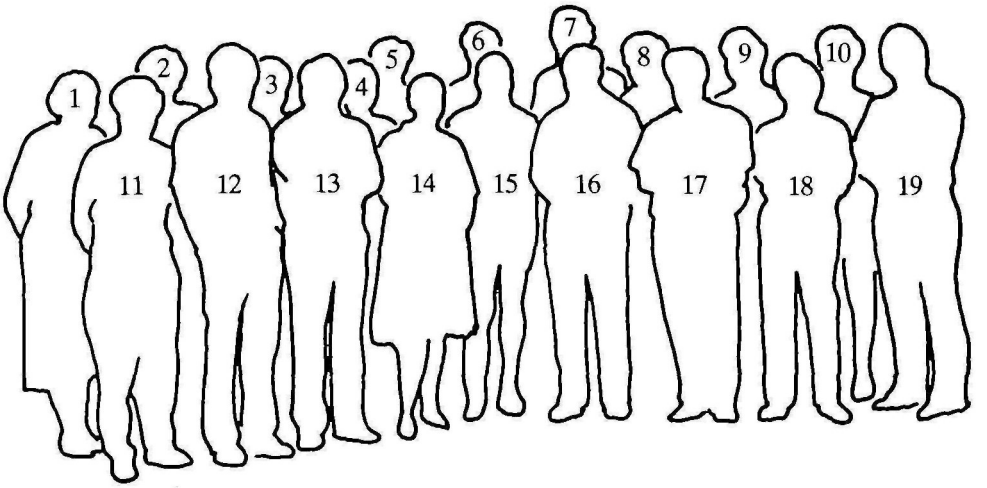
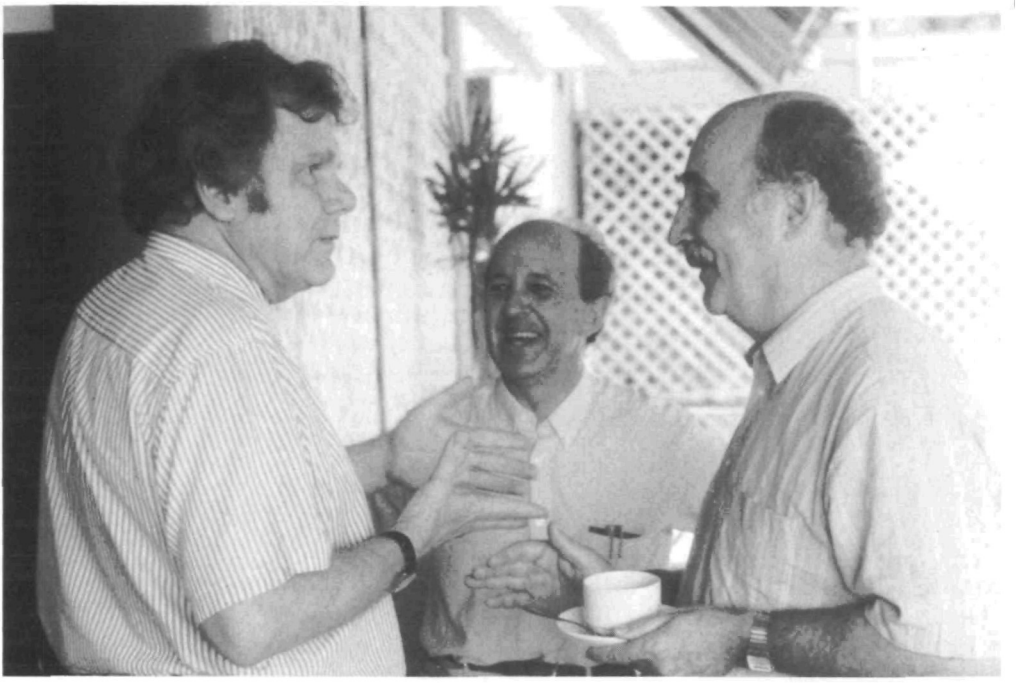
Photographs taken at the Discussion Meeting held on Batam Island, Indonesia, in March 1990



1. M. Clements
2. R. Skaer
3. O. Yi
4. U. McMahan
5. D. Trisler
6. W. Singer
7. S. Fraser
8. F. Bonhoeffer
9. H. Atwood
10. G. Raisman
11. K.-F. So
12. R. Kalil
13. J. Hamori
14. E. Howes
15. E. Frank
16. A. Aguayo
17. C. Sotelo
18. J. Nicholls
19. K. Kawamura



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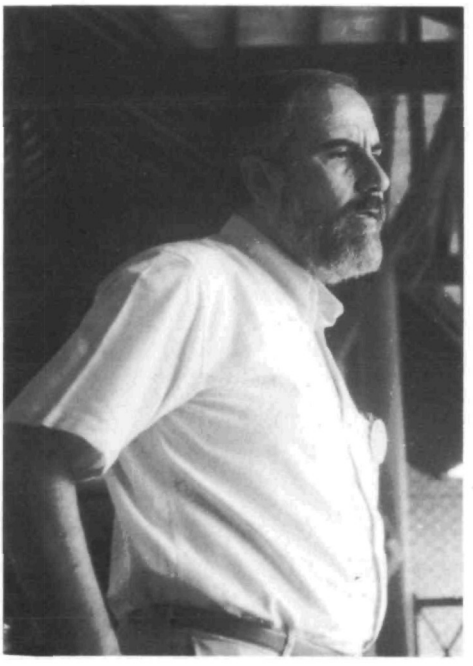
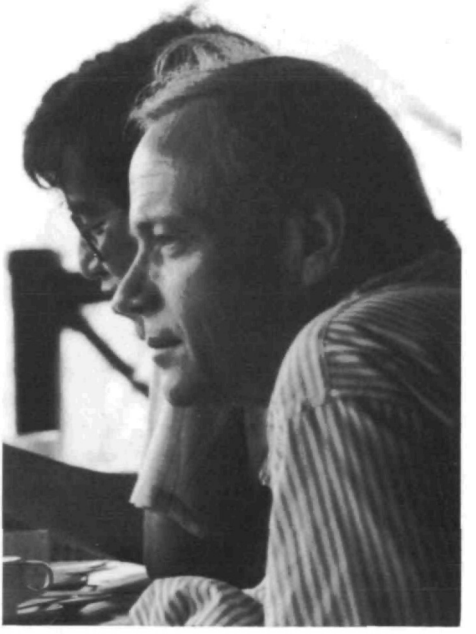


C. Sotelo



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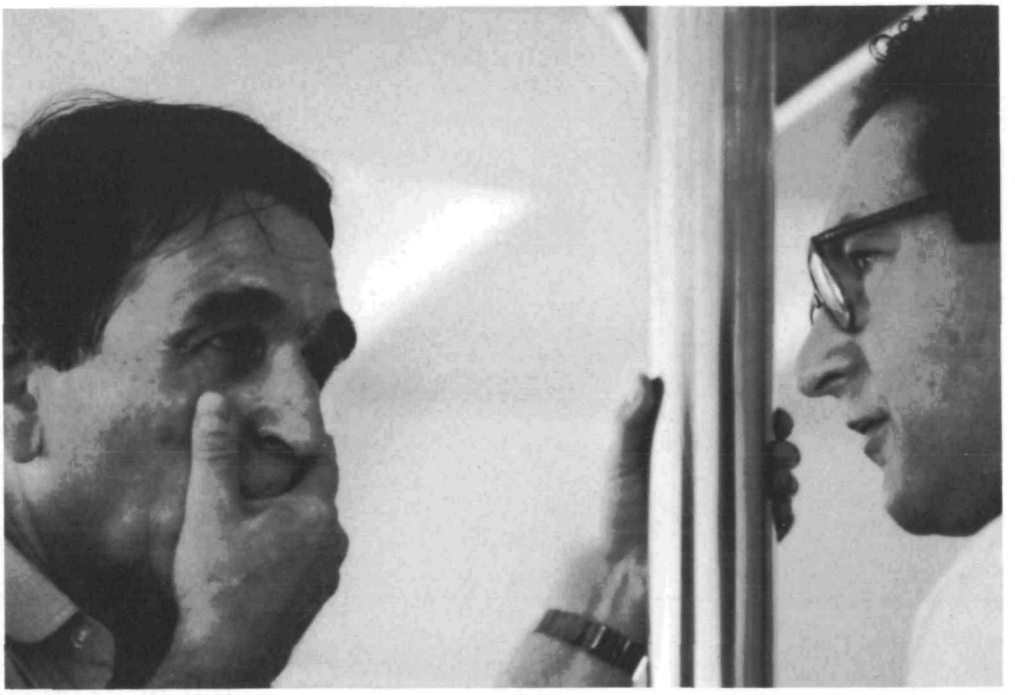

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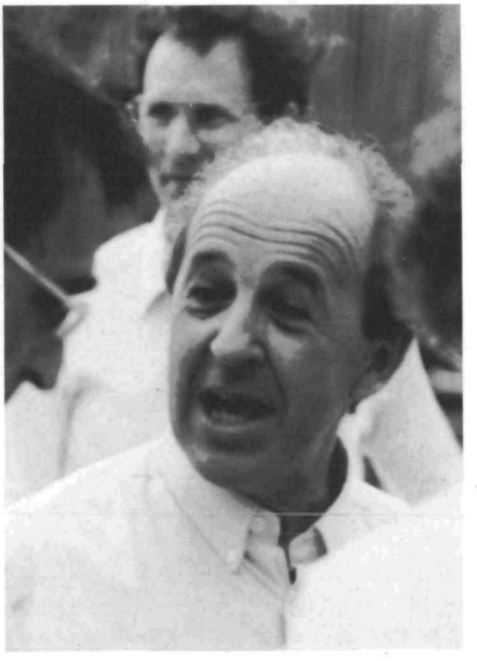
S. Fraser



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S. Fraser K. Kawamura M. Clements E. Howes

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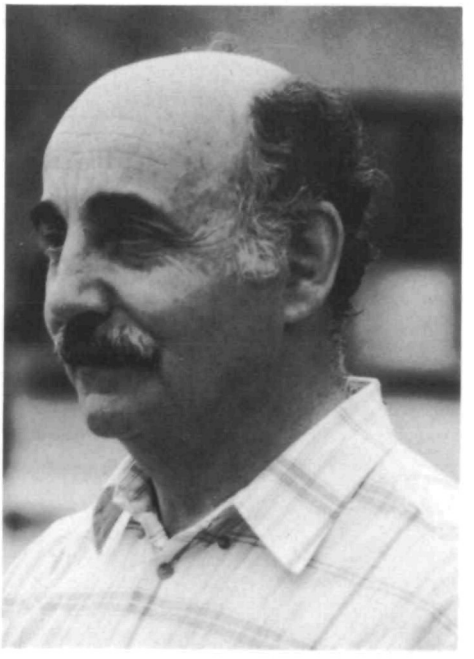
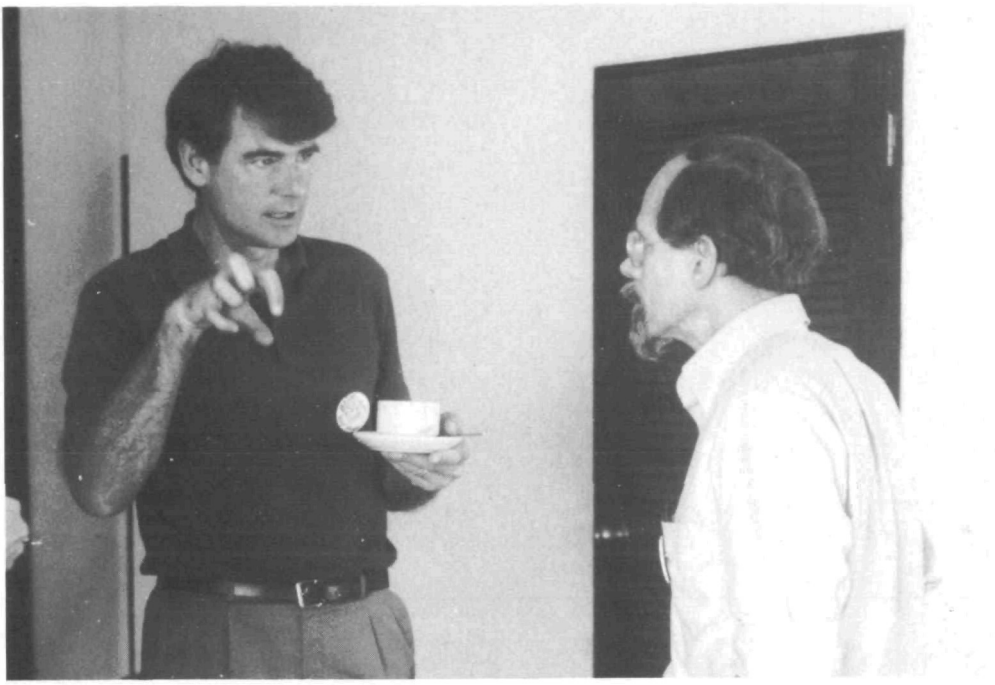
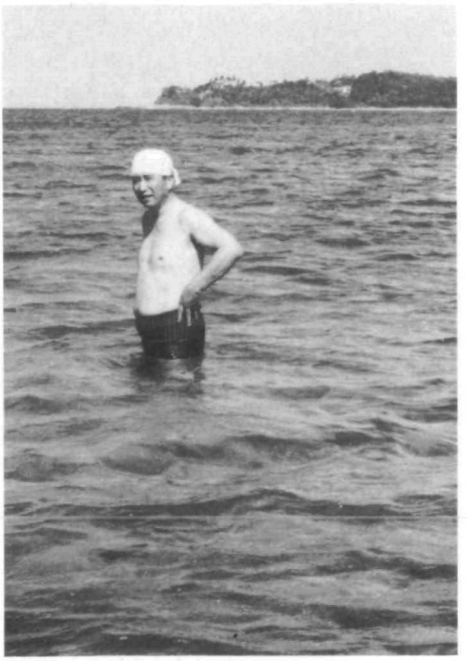


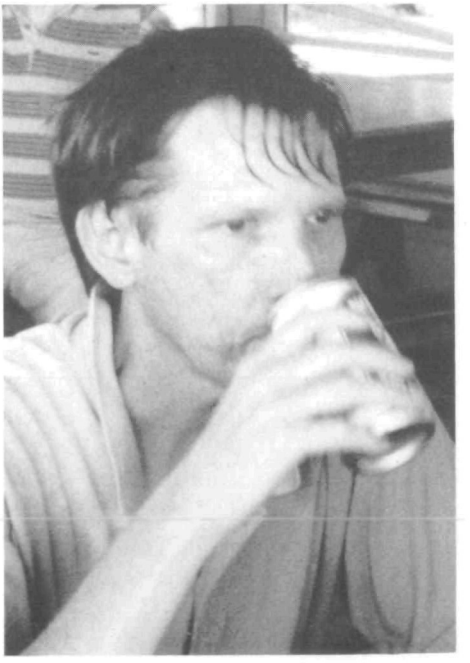
A. Aguayo



W. Singer E. Frank



K. Kawamura



D. Trisler