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Review

Interactions between cerebellar Purkinje cells and their associated astrocytes

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Summary. Some neurons, including cerebellar Purkinje cells, are completely ensheathed by astrocytes. When granule cell neurons and functional glia were eliminated from newborn mouse cerebellar cultures by initial exposure to a DNA synthesis inhibitor, Purkinje cells lacked glial sheaths and there was a tremendous sprouting of Purkinje cell recurrent axon collaterals, terminals of which hyperinnervated Purkinje cell somata, including persistent somatic spines, and formed heterotypical synapses with Purkinje cell dendritic spines, sites usually occupied by parallel fiber (granule cell axon) terminals. Purkinje cells in such preparations failed to develop complex spikes when recorded from intracellularly, and their membrane input resistances were low, making them less sensitive to inhibitory input. If granule cells and oligodendrocytes were eliminated, but astrocytes were not compromised, sprouting of recurrent axon collaterals occurred and their terminals projected to Purkinje cell dendritic spines, but the Purkinje cells had astrocytic sheaths, their somata were not hyperinnervated, the somatic spines had disappeared, complex spike discharges predominated, and membrane input resistance was like that of Purkinje cells in untreated control cultures. When cerebellar cultures without granule cells and glia were transplanted with granule cells and/or glia from another source, a series of changes occurred that included stripping of excess Purkinje cell axosomatic synapses by astrocytic processes, reduction of heterotypical axospinous synapses in the presence of astrocytes, disappearance of Purkinje cell somatic spines with astrocytic ensheathment, and proliferation of Purkinje cell dendritic spines after the introduction of astrocytes. Dendritic spine proliferation was followed by formation of homotypical axospinous synapses when granule cells were present or persistence as unattached spines in the absence of granule cells. The results of these studies indicate that astrocytes regulate the numbers of Purkinje cell axosomatic and axospinous synapses, induce Purkinje cell dendritic spine proliferation, and promote the structural and functional maturation of Purkinje cells.

Key words: Organotypic cultures, Development, Synapse, Dendritic spines, Astrocytic sheath

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Introduction

Many functions have been attributed to astrocytes, including structural support for neurons (Varon and Somjen, 1979), isolation of neuron receptive surfaces (Palay, 1966), ion uptake during neuronal activity (Hertz, 1978), synthesis, metabolism, release and uptake of neurotransmitters (Iverson and Kelly, 1975; Schousboe, 1981), phagocytosis (Westrum, 1969), glial scar formation (Eng et al., 1987), antigen presentation (Fontana et al., 1984), serving as guides for neuronal migration (Rakic, 1971) and axonal pathfinding (Singer et al., 1979; Silver and Sidman, 1980), secretion of a variety of factors that promote neuronal survival and neurite outgrowth (Rudge et al., 1985; Müller et al., 1990), neuronal rescue (Bernstein and Goldberg, 1991), a facilitating role in myelination and remyelination (Franklin et al., 1993; Waxman et al., 1993) and, most recently, controlling the number of synapses that develop in central nervous system (CNS) cultures (Ullian et al., 2001). Still additional functions of these versatile cells have come to light in studies with mouse organotypic cerebellar cultures, including astrocytic regulation of Purkinje cell axosomatic and axospinous synapses, induction of dendritic spine proliferation, and promotion of morphological and electrophysiological maturation of Purkinje cells. These additional properties will be the focus of this review.

Purkinje cells are among those neurons with ultrastructurally recognizable glial sheaths, a property not shared by all neurons (Peters et al., 1991). The Purkinje cell somata, dendrites and even the synapses with dendritic spines are completely covered by astrocytic sheaths, except where axon terminals penetrate to make synaptic contacts (Figs. 1, 2). It is evident from Figs. 1, 2 that the astrocytic ensheathment

of Purkinje cells is preserved in organotypic cerebellar cultures. As defined by Palay and Chan-Palay (1974), the sheaths are formed predominantly by processes of Golgi epithelial cells, the astrocytes that give rise to the radial Bergmann fibers that extend through the molecular layer of the cerebellar cortex and provide a lattice for granule cell migration (Rakic, 1971). These astrocytes are often, though inappropriately, referred to as "Bergmann glia." Purkinje cells are the only neurons in the cerebellar cortex that are fully ensheathed by astrocytes, whereas basket and stellate cells have only partial sheaths and Golgi and granule cells (Fig. 3) are generally unensheathed (Palay and Chan-Palay, 1974). It is this close relationship between Purkinje cells and astrocytes that is reflected in some neuron-glia interactions that are not necessarily typical, but may be characteristic of more tightly coupled cells. With this caveat in mind, we will examine the evidence for these particular astrocytic properties.

The cerebellar culture models

Parasagittally oriented cerebellar explants derived from newborn (within 24 hours after birth) Swiss-Webster mice and maintained in Maximow chambers for 2 or more weeks exhibit many of the structural and functional characteristics of the cerebellum *in vivo* (Seil, 1972; Leiman and Seil, 1973; reviewed in Seil, 1979,

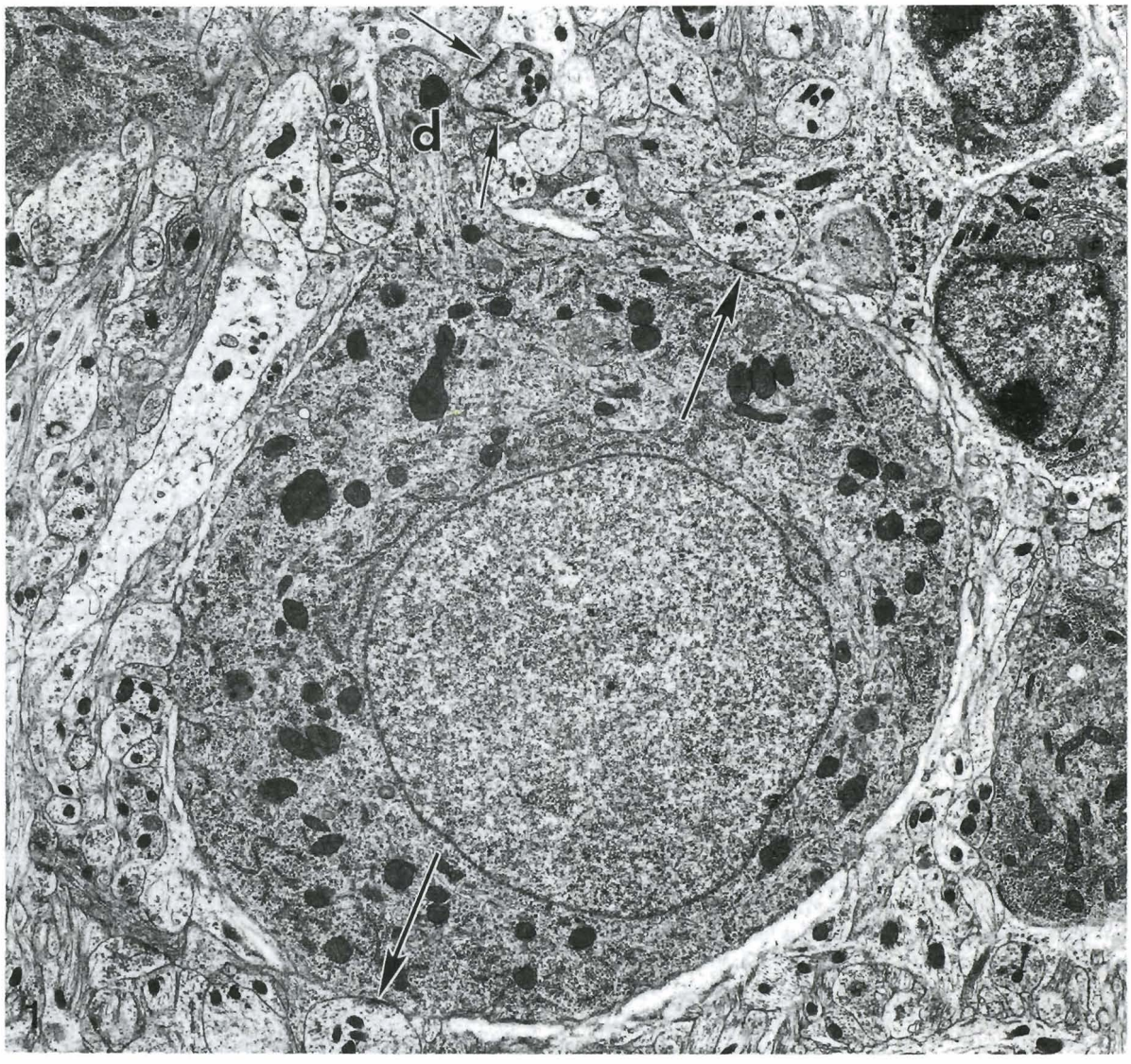

Fig. 1. Purkinje cell from an untreated control cerebellar culture, 15 DIV. Shown is the soma and a portion of a dendrite (d) extending upward. The soma is surrounded by an astrocytic sheath, which appears as a relatively clear area immediately adjacent to the smoothly contoured soma membrane. Five axon terminals interrupt the sheath to contact the soma, two of which are synaptic contacts (large arrows). The astrocytic sheath extends to the dendrite, where there is an axodendritic synapse on a smooth portion and synapses on two dendritic spines (small arrows). x 6,000

1996). The five major cortical neuronal types are represented, namely Purkinje cells, inhibitory neurons whose axons constitute the only projection from the cerebellar cortex and whose axon collaterals project to all other cortical neurons, including other Purkinje cells; granule cells, the only excitatory cortical neurons, whose function in the intact cerebellum is to relay excitatory impulses from extracerebellar afferent mossy fibers to all other cortical neurons via their axons, the parallel fibers; and the inhibitory interneurons, including the basket, stellate and Golgi cells. Basket cells innervate the somata and proximal dendrites of Purkinje cells and stellate cell axon terminals synapse with the smooth portions of distal Purkinje cell dendrites. Golgi cells project complex multibranched axons that synapse with granule cell dendrites. Absent or greatly reduced in cerebellar explants are the extracerebellar afferents, including mossy, climbing, catecholaminergic and serotoninergic fibers. Very few synapses are present in the mouse cerebellar cortex at birth, but the expected synaptic development takes place in culture (Seil and Leiman, 1979; Herndon et al., 1981) and the characteristic cerebellar cortical circuitry is fairly well established by 15 days *in vitro* (DIV).

Typical neuron-glia interactions also develop in culture (Seil, 1979). Oligodendrocytes begin to wrap myelin-receptive axons, such as Purkinje cell axons and collaterals, at 6-7 DIV, a process that is largely completed by 12 DIV. Axons that do not myelinate *in vivo*, such as parallel fibers in the mouse, also do not myelinate *in vitro* (Fig. 2). Astrocytic ensheathment of Purkinje cells is underway by 6 DIV and progresses to complete ensheathment of somata and dendrites 3-4 days later (Herndon et al., 1981; Seil et al., 1991). As already noted, synapses between parallel fiber terminals and Purkinje cell dendritic spines are included within the

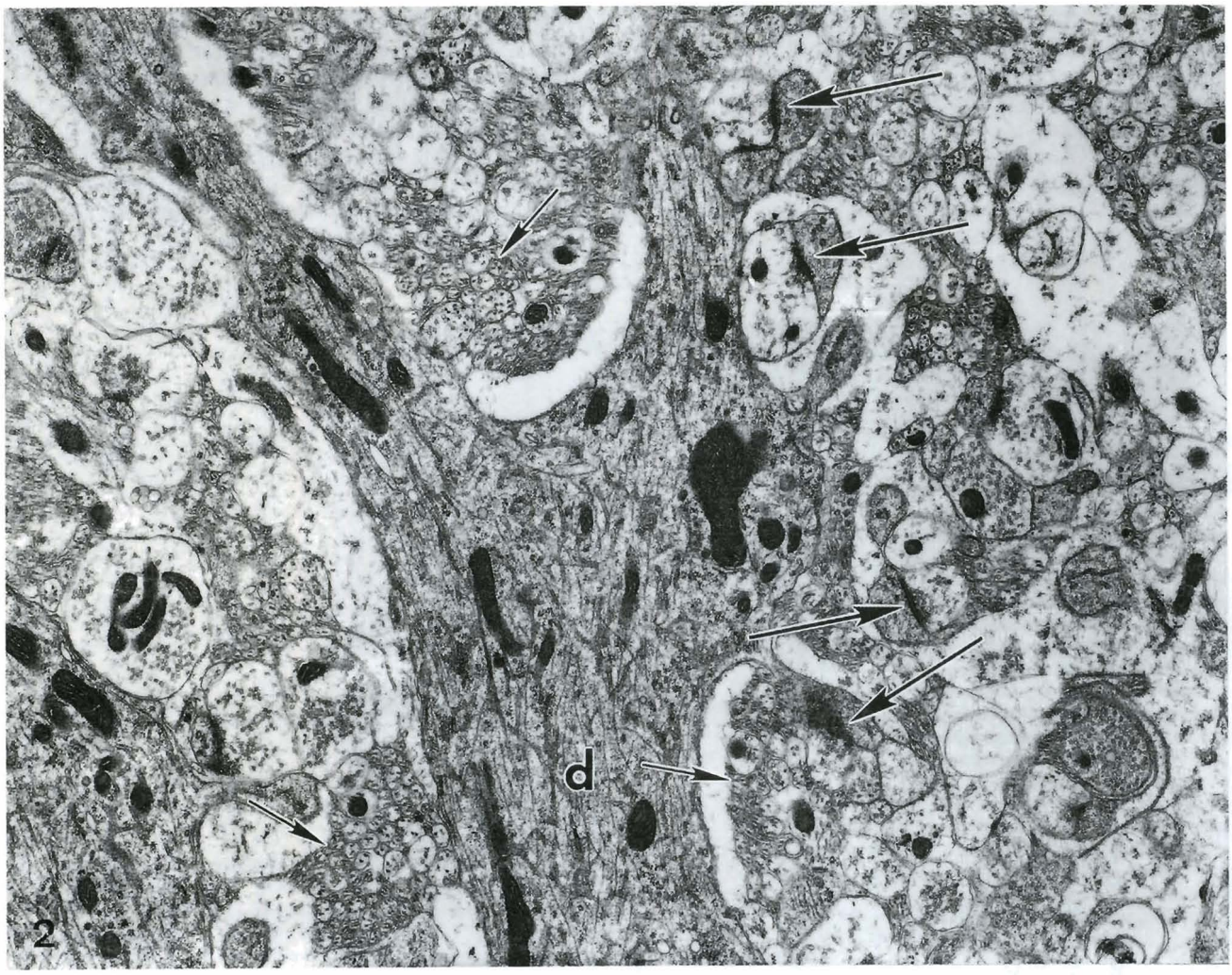

Fig. 2. Purkinje cell dendrite (d) and branches from an untreated control cerebellar culture, 15 DIV. Relatively clear astrocytic processes appose the dendrite and its branches and completely or partially cover dendritic spine synapses with terminals of parallel fibers (granule cell axons). Several axospinous synapses are indicated by large arrows. The small arrows point to bundles of unmyelinated parallel fibers. x 13,000

sheaths (Fig. 2). Consistent with ultrastructural findings *in situ* (Palay and Chan-Palay, 1974), it would appear from light micrographs of cerebellar cultures reacted with antibody to glial fibrillary acidic protein (GFAP) that processes from several astrocytes contribute to each Purkinje cell glial sheath (Seil et al., 1992a).

This organotypic tissue culture model was profoundly altered by exposure for the first 5 DIV to cytosine arabinoside (Ara C), a DNA synthesis inhibitor that destroys oligodendrocytes and granule cells (Seil et al., 1980, 1991; Blank et al., 1982). As a result of such exposure, the cultures failed to myelinate and the cortical neurons became reorganized, the central feature of which was a sprouting of Purkinje cell recurrent axon collaterals that projected inhibitory terminals to the dendritic spines of other Purkinje cells, sites usually occupied by excitatory parallel fiber terminals. Such heterotypical synapses were functional, as indicated by electrophysiological studies (Seil et al., 1980). A preparation of Ara C manufactured for the Sigma Chemical Co. (St. Louis, MO) and purchased in 1978 exhibited an additional property, namely reducing the population of astrocytes in the cerebellar explants and functionally compromising those that survived, so that they were unable to appose neuronal membranes (Blank et al., 1982). Purkinje cells in such cultures were unensheathed by astrocytes (Fig. 4). By contrast, a more recently purchased Ara C preparation from Pfanstiehl Laboratories (Waukegan, IL) also destroyed oligodendrocytes and granule cells, but did not compromise astrocytes, and Purkinje cells in cultures exposed to this preparation did have astrocytic sheaths (Seil et al., 1992a). The influence of astrocytes on Purkinje cells could thus be compared in cultures that were otherwise similarly altered from control explants.

A useful model for investigating Purkinje cell-astrocyte interactions was the "transplant" model. In this case, cerebellar cultures were exposed for the first 5 DIV to kainic acid, a glutamic acid analog that destroyed all cortical neurons except granule cells, but without compromising the glia (Seil et al., 1979), and then superimposed at 9 DIV on 9 DIV cerebellar cultures that had been treated with Sigma Ara C for the first 5 DIV (Seil and Blank, 1981; Seil et al., 1983; Blank and Seil, 1983; Seil, 1997). The kainic acid treated explants were complementary to the Ara C exposed cultures, containing the granule cells, oligodendrocytes and functional astrocytes that were missing in the latter. The expected cellular migrations from the superimposed explants into the host explants occurred, allowing evaluation of the circuit reorganization and neuron-glia interactions that ensued when the missing elements were restored. Variants of this model were superimposition of glia without granule cells, in the form of optic nerve fragments (Meshul et al., 1987), and granule cells without glia, in the form of cerebellar cultures exposed to kainic acid for the first 5 DIV to eliminate all neurons but granule cells and then to Sigma Ara C for the next 4

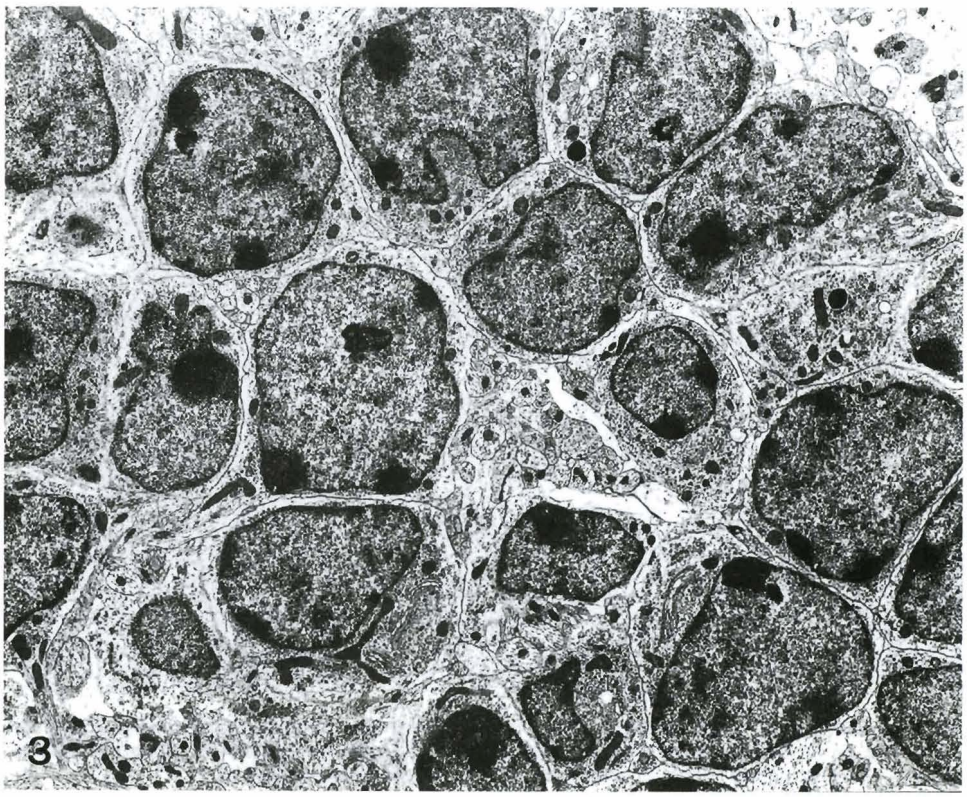

Fig. 3. Closely packed granule cells in a control cerebellar culture, 15 DIV. Granule cells are the most numerous and the only excitatory cortical neurons. Their cytoplasm is sparse and they are not ensheathed by astrocytic processes. Granule cell axons, the parallel fibers, project to all other cortical neurons. x 5,000

DIV to compromise the glia (Seil, 1994).

Regulation of synapses

In control cultures, the glially ensheathed somata of Purkinje cells have smooth contours and an average ratio of 2.2 axosomatic synapse profiles per somatic profile after 15 DIV (Fig. 1), the presynaptic terminals being about equally divided between those originating from basket cell axons and those derived from Purkinje cell recurrent axon collaterals (Blank and Seil, 1982; Seil and Drake-Baumann, 1994; Seil, 1996). Purkinje cell somata in Sigma Ara C treated cultures were devoid of astrocytic sheaths and were partially scalloped by multiple abutting axon terminals, some of which formed synapses (Fig. 4), so that the average ratio of axosomatic synapse to soma profiles in these hyperinnervated cells was 4.9, more than twice that of control cultures (Blank et al., 1982; Seil et al., 1991; Seil and Drake-Baumann, 1995). The excess axon terminals were mostly derived from Purkinje cell recurrent axon collaterals, which had sprouted profusely in the absence of granule cells, and which also projected to Purkinje cell dendritic spines, forming functional inhibitory synapses (Seil et al., 1980; Blank et al., 1982). These heterotypical synapses developed in place of the homotypical parallel fiber-Purkinje cell dendritic spine synapses, appearing at about the same developmental time (Seil et al., 1991). As was the case with the Purkinje cell somata in Sigma Ara C treated cultures, the heterotypical synapses were not covered by astrocytic sheaths (Blank et al., 1982).

In Pfanstiehl Ara C treated cerebellar cultures, in which astrocytes were not compromised, Purkinje cells were enveloped by astroglial sheaths and their somata

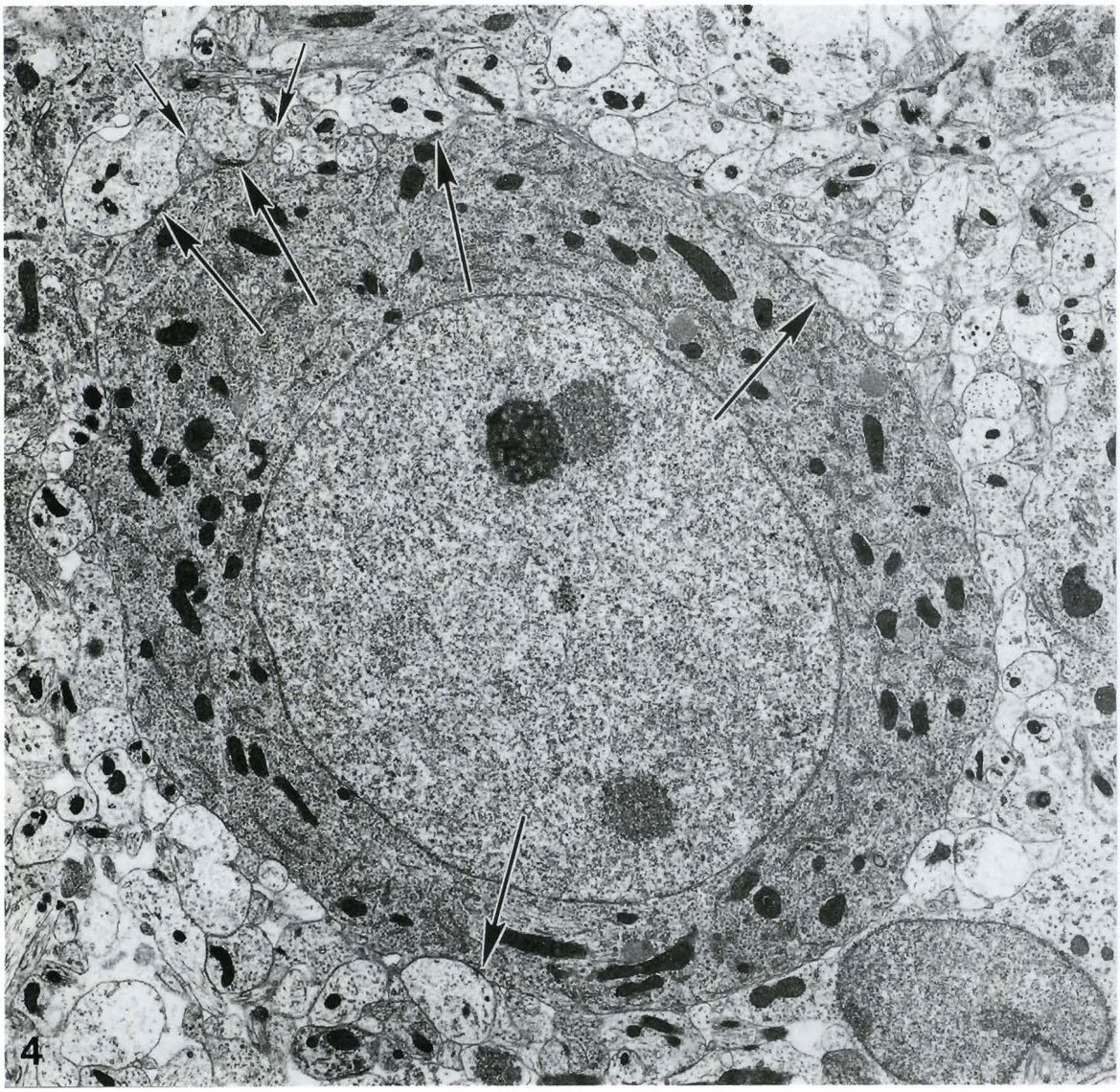

Fig. 4. Purkinje cell from a Sigma Ara C treated cerebellar culture, 15 DIV. No astrocytic ensheathment is present and multiple axon terminals abut the soma and indent it at several points (more severe scalloping is evident in Fig. 5). Five of the abutting axon terminals synapse with the soma (large arrows). One of these axosomatic synapses is between two persistent somatic spines, indicated by small arrows. x 6,000

were not hyperinnervated by recurrent axon collateral terminals (Seil et al., 1992a). Purkinje cell recurrent axon collaterals did sprout in such cultures and were present in great excess, with numerous projections to Purkinje cell dendritic spines, but hyperinnervation of Purkinje cell somata did not occur in the presence of astrocytic sheaths. Heterotypical synapses were functionally inhibitory and were also glially ensheathed, suggesting that they were formed at a time when astrocytes were immature and supportive of synapse development.

Axosomatic synapse reduction

By 10 days after the superimposition of kainate treated cerebellar cultures onto explants that had been exposed to Sigma Ara C, Purkinje cells had acquired astrocytic sheaths and the complement of axosomatic synapses was similar to that of control cultures (Blank and Seil, 1983). The sprouted recurrent axon collaterals were also reduced, as heterotypical synapses were replaced by homotypical parallel fiber-Purkinje cell dendritic spine synapses. It was therefore not clear from this study whether the reduction of axosomatic synapses was related to astrocytic ensheathment of Purkinje cells or to the loss of the excess recurrent axon collaterals. In subsequent experiments in which 7-day-old mouse optic nerve fragments were superimposed on Sigma Ara C treated cerebellar cultures, the density of sprouted recurrent axon collaterals was not appreciably changed in the absence of granule cells, but Purkinje cells acquired astrocytic sheaths and the ratio of axosomatic synapse profiles to soma profiles was markedly reduced (Meshul et al., 1987; Meshul and Seil, 1988; Seil et al., 1988). As the loss of axosomatic synapses occurred in the presence of persistent excess recurrent axon collateral terminals, it appeared that astrocytes played a role in the regulation of synapse density, at least with regard to glially ensheathed neurons. It was also notable that Purkinje cells in this case were ensheathed by optic nerve astrocytes, indicating that ensheathment of Purkinje cells was not a property exclusively of Golgi epithelial cells and suggesting that the signal for ensheathment emanates from the Purkinje cell. The signaling molecule remains to be identified, but it is not the neural cell adhesion molecule, N-CAM, as antibodies to N-CAM failed to inhibit Purkinje cell glial ensheathment (Seil and Herndon, 1991).

The means by which Purkinje cell axosomatic synapses are reduced after addition of functional astrocytes to Sigma Ara C exposed cerebellar explants were further elucidated in a timed ultrastructural study (Seil, 1997). Within 1 day after superimposition of kainic acid treated cultures on Sigma Ara C exposed explants, astrocytes had invaded the host cultures and extended processes to appose Purkinje cells (Fig. 5) and to interpose between axon terminals and somatic membranes, beginning the process of axosomatic synapse reduction. By 2 days after superimposition, the ratio of axosomatic synapse profiles to soma profiles had been reduced from 4.9 to 3.9 by the process of astrocytic stripping of the synapses and separation of axon

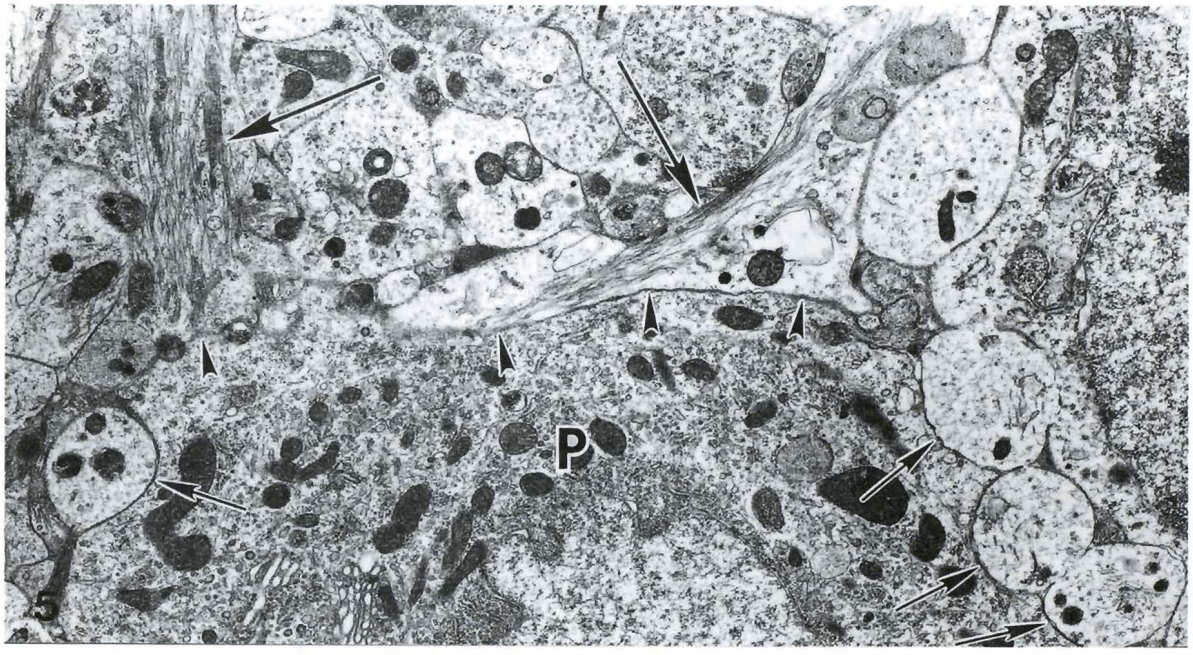

Fig. 5. Purkinje cell (P) in a Sigma Ara C treated cerebellar culture, 10 DIV, 1 day after transplantation with granule cells and glia from another source. Marked scalloping of unensheathed portions of the soma by axon terminals is evident (small arrows). Transplanted astrocytes have invaded the host culture, and intermediate filament filled astrocytic processes (large arrows) have contacted the soma (arrowheads) as the process of ensheathment begins. x 10,000

terminals from the somatic membranes (Fig. 6) as ensheathment continued. By 4 days after superimposition, astrocytic ensheathment had extended to Purkinje cell dendrites and axospinous synapses, and the ratio of axosomatic synapse profiles to somatic profiles had been reduced to 2.5, a point from which little further change occurred (the final value was 2.4). Ensheathment was complete by 6 days after superimposition (15 DIV), at which time Purkinje cell somatic contours were fully rounded and the cells appeared like Purkinje cells in control cultures at the same *in vitro* age (Fig. 1).

These studies indicated that in the absence of astrocytic sheaths, Purkinje cell somata were hyperinnervated by sprouted recurrent axon collaterals, and if reensheathment occurred, excess axosomatic synapses were reduced by an astrocytic stripping mechanism consisting of an interposition of astrocytic processes between the pre- and postsynaptic elements. This form of astrocytic regulation of synapse numbers is not unique to cerebellar cultures, as Tweedle and Hatton (1984) described a similar sequence of somatic hyperinnervation and synapse reduction in magnocellular supraoptic nucleus neurons in rats in different hydration states. Such neurons also have astrocytic sheaths. During dehydration, the astrocytic processes retracted from the cell somata, and single afferent terminals formed so-called "double synapses" by synapsing with two adjacent magnocellular neuron somata instead of one. Reensheathment of the neurons occurred with rehydration, and the double synapses were reduced. Goshgarian et al. (1989) also described formation of double and even quadruple synapses on dendrites of rat phrenic nucleus motoneurons in association with withdrawal of astrocytic processes after spinal cord hemisection, suggesting an element of glial regulation of synapse numbers in this situation as well.

Axospinous synapse reduction

After addition of granule cells and glia to Sigma Ara C exposed cerebellar cultures, homotypical parallel fiber-Purkinje cell synapses replaced most of the heterotypical axospinous synapses formed in Ara C treated explants, although some heterotypical synapses persisted 10 days after introduction of the missing cellular elements (Blank and Seil, 1983). From this study it was not at all clear that astrocytes had any role in the reduction of heterotypical synapses, as it was assumed that the observed changes were entirely due to replacement by homotypical synapses. However, when optic nerve fragments as a source of glia without granule cells were superimposed upon Sigma Ara C treated cultures, synapses in the cortical neuropil were reduced to approximately half, while sprouted recurrent axon collaterals, the source of most of the presynaptic components of these synapses, were not appreciably lessened (Meshul and Seil, 1988). Synapses in the cortical neuropil were not sorted into axospinous vs. axodendritic (on the smooth portions of the dendrites) in this study, but the former considerably outnumbered the latter. Subsequently, when only granule cells were

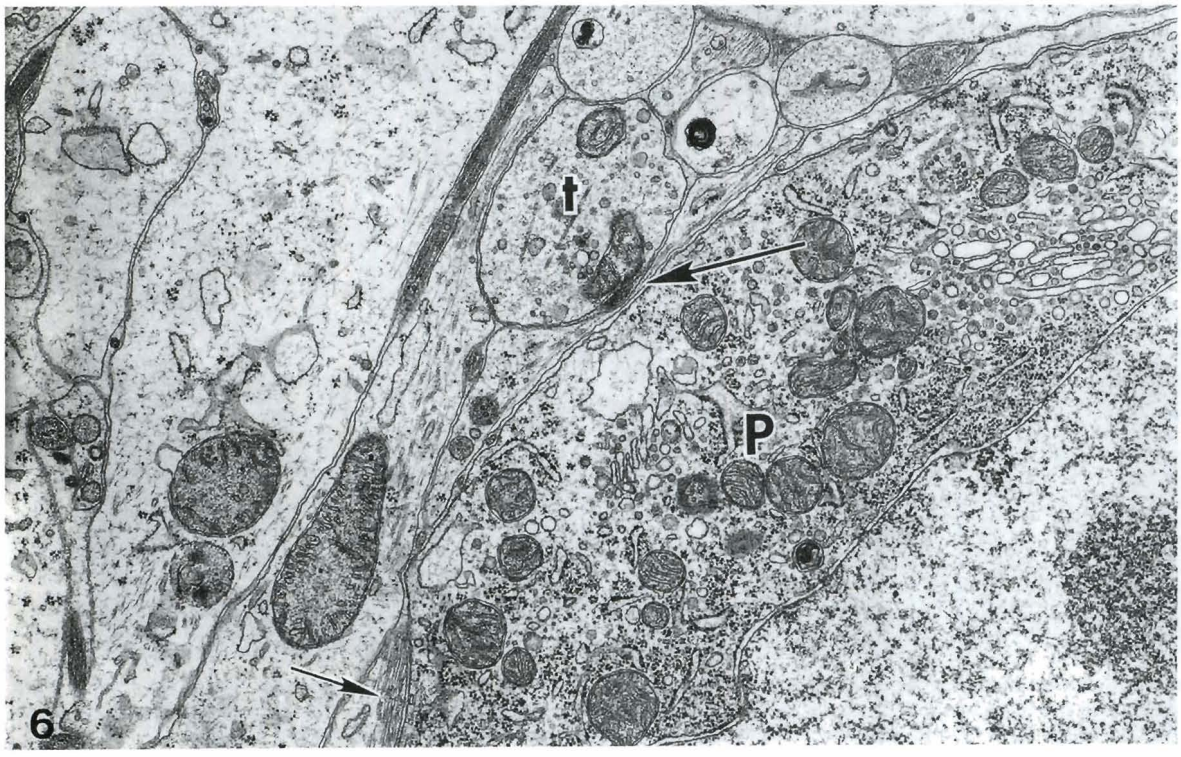

Fig. 6. Purkinje cell (P) in a Sigma Ara C treated cerebellar culture, 11 DIV, 2 days after transplantation with granule cells and glia. An astrocytic process (large arrow) has inserted itself between an axon terminal (t) and the somatic membrane, lifting the terminal from the soma as astrocytic ensheathment of Purkinje cells continues. A bundle of intermediate filaments in the astrocytic process is indicated by the small arrow. x 16,000

superimposed upon Sigma Ara C treated cultures (Seil, 1994), Purkinje cells did not acquire astrocytic sheaths, sprouted recurrent axon collaterals were reduced but not to control levels, homotypical axospinous synapses were present in numbers comparable to those found with addition of both granule cells and glia, but twice the number of heterotypical synapses persisted. The combination of heterotypical synapse reduction when only glia were superimposed on Sigma Ara C treated explants and increased persistence of heterotypical synapses when no functional astrocytes were present pointed to a role for astroglia in the elimination of heterotypical axospinous synapses.

How heterotypical synapses were removed was not evident from either of the above studies nor from the timed ultrastructural study after addition of granule cells and glia to Sigma Ara C exposed cultures (Seil, 1997). In the latter study, astrocytic processes were seen to envelop both homotypical and heterotypical axospinous synapses, but in no case were glial processes observed to be interposed between pre- and postsynaptic elements, as with axosomatic synapses. This does not, of course, rule out the possibility of such direct astrocytic intervention. There is another difference, however, between axospinous and axosomatic synapse reduction that suggests a possibility of more than one mechanism. Neither the acquisiton of Purkinje cell astrocytic sheaths nor the reduction of excess axosomatic synapses are dependent on the presence of neuronal activity, as both occurred after addition of granule cells and glia to Sigma Ara C treated explants in activity blocked cultures (Seil and Drake-Baumann, 1996). But there was increased persistence of heterotypical synapses in activity blocked transplanted cultures, to a level comparable to that with absence of functional astrocytes, suggesting a component of neuronal activity-dependence in heterotypical synapse reduction. Thus the mechanism of heterotypical axospinous synapse elimination may be more complex than glial stripping, perhaps involving an astrocyte secreted factor, but this mechanism remains to be elucidated.

Induction of dendritic spine proliferation

Initially observed in Sigma Ara C treated cultures with superimposed fragments of optic nerve were

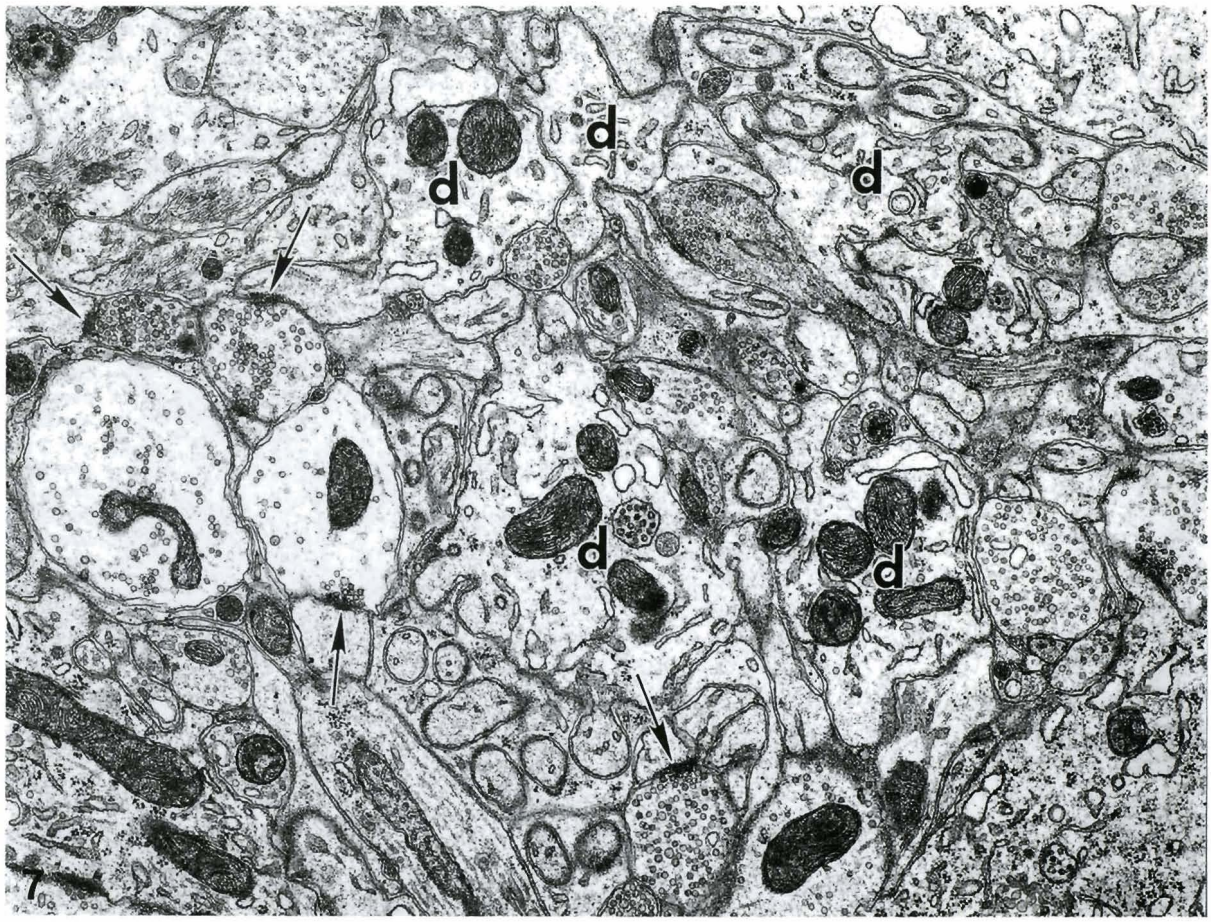

Fig. 7. Purkinje cell dendritic branches (d) in a Sigma Ara C treated cerebellar culture, 13 DIV, 4 days after transplantation with granule cells and glia. The dendrites are surrounded by proliferated spines, most of which are unattached by presynaptic elements and are encased in astrocytic processes. Some of the spines are forming synapses with parallel fiber terminals, several examples of which are indicated by arrows. x 16,000

clusters of Purkinje cell dendritic spines without attached presynaptic elements (Meshul and Seil, 1988). The clusters of unattached dendritic spines persisted throughout the period of observation, up to 35 DIV. The reason why such unattached dendritic spines had not been observed 10 days after addition of granule cells and glia to Sigma Ara C treated cultures (Blank and Seil, 1983) became evident in a later timed ultrastructural study (Seil, 1997). Proliferation of Purkinje cell dendritic spines began 2 days after Sigma Ara C exposed explants were superimposed with granule cells and glia, and usually appeared in the vicinity of astrocytic processes. Spine proliferation had accelerated by 4 days after superimposition, at which time occasional synapses with presumptive parallel fiber terminals were present (Fig. 7). Synapse formation subsequently increased among the clusters of newly proliferated spines, and by 7 days after introduction of granule cells and glia, the number of spine clusters appeared to be reduced. By 2 days later, clusters of unattached dendritic spines were sparse, as most of the available spines had formed synapses with parallel fiber terminals. Parallel fiber-Purkinje dendritic spine synapses also developed in Sigma Ara C treated cultures superimposed with granule

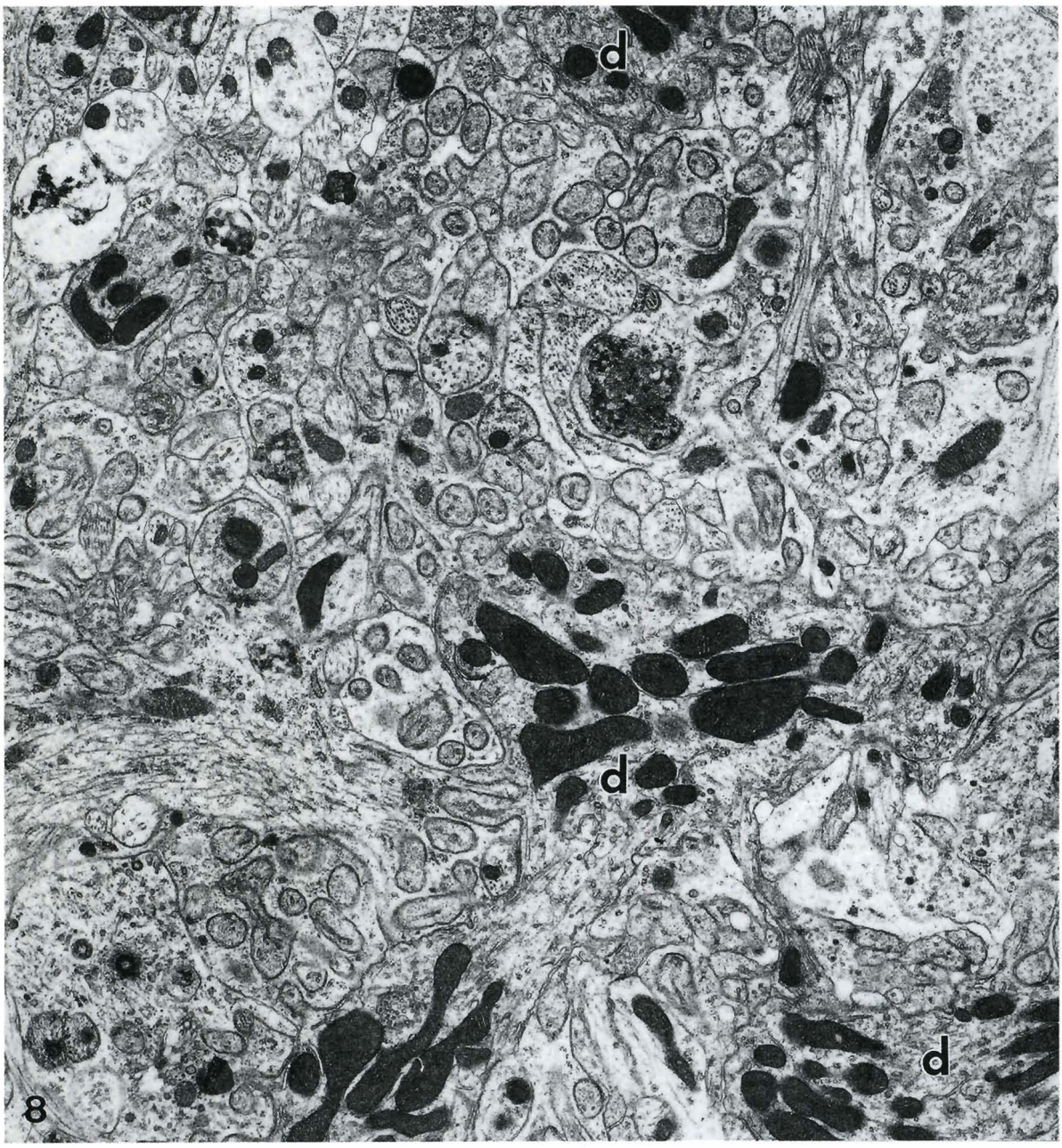

Fig. 8. Purkinje cell dendrites (d) from a Sigma Ara C treated cerebellar culture, 15 DIV, after 6 days of exposure to 20 μg laminin/ml nutrient medium. Numerous dendritic spines have proliferated in response to laminin exposure, most of which are unattached and surrounded by astrocytic processes. x 12,000

cells only, but clusters of unattached dendritic spines were not observed in the absence of functional astrocytes (Seil, 1994). No dendritic spine proliferation was observed in Sigma Ara C treated cultures transplanted with purified dissociated oligodendrocytes (Seil et al., 1989).

The occurrence of unattached dendritic spines in clusters suggested sprouting of spines in local regions of dendritic branches, as if in response to diffusible factors secreted by near-by astrocytes. The possibility of induction of dendritic spine proliferation by an astrocyte secreted factor was tested by exposure of Sigma Ara C treated cultures to astrocyte conditioned medium. The ensuing proliferation of Purkinje cell dendritic spines was diffusely distributed throughout cortical regions of the explants (Seil et al., 1992b). This result established the link between dendritic spine proliferation and astrocytes. Subsequently a series of known neurite promoting factors likely to be secreted by astrocytes were screened for dendritic spine proliferation-inducing capability by application to Sigma Ara C exposed cultures (Seil, 1998). The extracellular matrix molecule, laminin, was found to promote a vigorous proliferation of dendritic spines (Fig. 8). Purkinje cell dendritic branching was not affected, thus dissociating spine proliferation from dendritic growth. Sprouting of dendritic spines was not induced by exposure of cultures to two laminin-derived peptides with known neurite promoting effects, indicating that dendritic spine proliferation was induced by a different site on the laminin molecule than the neurite outgrowth promoting domains.

Laminin is a secretory product of astrocytes (Matthiesen et al., 1989), but its production changes with astrocyte differentiation, so that it is present during development and supportive of neurite growth, and downregulated with maturation (Liesi, 1985; Ard and Bunge, 1988). As a developmental molecule, laminin in its various forms may also have a role in synapse formation, both at the neuromuscular junction (Martin et al., 1995) and in the CNS (Tian et al., 1997). If the mature CNS is injured, glia may proliferate so that an astrocytic population consisting of a mixture of astroblasts, immature and differentiated astrocytes is present (Hatten et al., 1991). Immature astrocytes may reassume characteristics that were downregulated in differentiated astrocytes, including production of laminin (Liesi, 1985). Thus laminin may be available during repair of the mature nervous system as well as during development.

Maturation of Purkinje cells

Morphological maturation

One of the features of the morphological maturation of Purkinje cells in organotypic cerebellar cultures is the loss of somatic spines (Herndon et al., 1981; Blank and Seil, 1982; Seil et al., 1991). Although multiple somatic spines were present at earlier stages of development, few, if any, spines remained on the somata of Purkinje cells in control cultures by 15 DIV (Fig. 1). This situation was altered, however, when cultures were exposed to Sigma Ara C for the first 5 DIV. Unensheathed Purkinje cells in such cultures had multiple persistent somatic spines, some of which formed synapses with terminals of recurrent axon collaterals (Fig. 4). By contrast, glially ensheathed Purkinje cells in Pfanstiehl Ara C treated cerebellar explants, which also had excess sprouted recurrent axon collaterals, did not have persistent somatic spines (Seil et al., 1992a). The developmentally programmed loss of somatic spines appeared to be related to glial ensheathment.

Support for this concept was provided by the results of superimposition of Sigma Ara C treated cultures with granule cells and glia. Somatic spines had disappeared in ensheathed Purkinje cells when initially examined at 10 days after transplantation (Blank and Seil, 1983). Somatic spines and synapses thereon were already markedly reduced by 5 and 6 days after superimposition of granule cells and glia, their reduction correlating with the progression of astrocytic ensheathment, and were rare by 9 days after transplantation (Seil, 1997). In

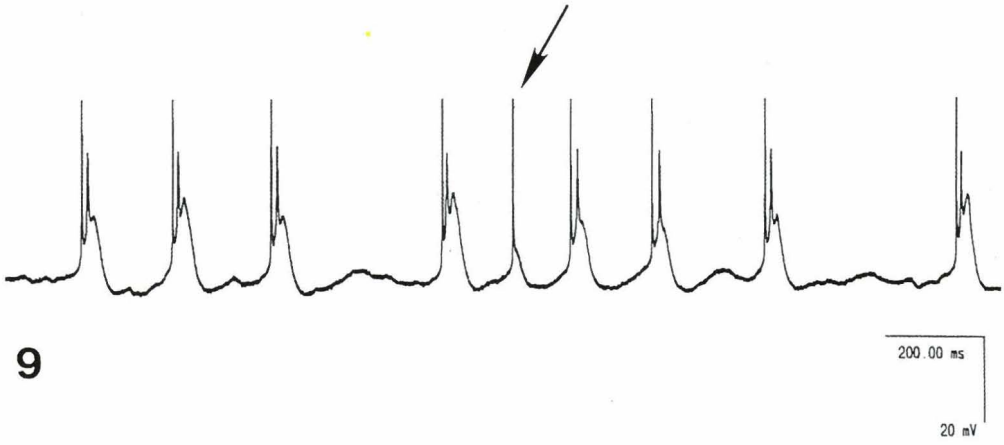

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Fig. 9. Spontaneous spike discharges recorded intracellularly from a Purkinje cell in a control cerebellar culture at 14 DIV. Complex spikes predominate in control cultures, with occasional simple spikes (arrow) interposed. The spikes are all followed by afterhyper-polarizations. (Trace courtesy of Rosemarie Drake-Baumann. Reproduced from Seil, 1996 with permission).

addition to linking somatic spine reduction to glial ensheathment, these studies showed that an interrupted developmental schedule for somatic spine disappearance could be resumed with the introduction of functional astrocytes.

Electrophysiological maturation

Purkinje cells in organotypic cerebellar cultures are spontaneously active, with characteristically phasic discharges that reflect synaptic interactions with other neurons (Leiman and Seil, 1973; Seil and Leiman, 1979). Intracellular recordings from Purkinje cells in control cultures after two weeks *in vitro* revealed a pattern of predominantly complex spike discharges with occasionally interposed simple spikes (Fig. 9) (Drake-Baumann and Seil, 1995). The complex spikes consisted of fast action potentials followed by prolonged afterdepolarizations on which one or more spike-like components were superimposed. Complex spikes were characteristic of mature Purkinje cells in culture (Gruol and Franklin, 1987). In the absence of glial sheaths, as in Sigma Ara C treated cultures, only spikes consisting of simple fast action potentials were evident at resting membrane potentials at 14 DIV (Drake-Baumann and Seil, 1995). Such spikes were characteristic of immature Purkinje cells in microexplant (Gruol and Franklin, 1987) and dissociated (Hockberger et al., 1989) cerebellar cultures. Simple spikes could be changed to more complex waveforms by hyperpolarization with injection of negative current, suggesting that there may be a difference in the voltage dependence for activation of a low-threshold Ca^{2+} current in glially ensheathed vs. unensheathed Purkinje cells.

As other differences between control and Sigma Ara C treated cultures, such as the presence or absence of granule cells and parallel fiber-Purkinje cell dendritic spine synapses, could also have contributed to Purkinje cell spike configuration, Sigma Ara C exposed cultures were compared with Pfanstiehl Ara C treated explants, the only difference between the two being the presence of functional astrocytes in the latter (Drake-Baumann and Seil, 1999). Purkinje cells in Pfanstiehl Ara C treated cultures had astrocytic sheaths and a pattern of predominantly complex spike discharges, similar to that of Purkinje cells in control cultures. Thus complex spike activity at resting membrane potentials developed when Purkinje cells were ensheathed by astrocytes.

Differences were also evident in passive membrane properties when comparing ensheathed and unensheathed Purkinje cells (Drake-Baumann and Seil, 1995, 1999). Although resting membrane potentials were similar in Purkinje cells from control explants and the two Ara C treated preparations, the input resistance of unensheathed Purkinje cells in Sigma Ara C exposed cultures was significantly lower. The implication of lower input resistance is a reduced sensitivity to inhibitory innervation, thus explaining why Purkinje cells in Sigma Ara C treated cultures discharged spontaneously at the same rate as Purkinje cells in control cultures, in spite of the markedly increased inhibitory input (Seil et al., 1980). Consistent with this notion was a slower discharge rate in glially ensheathed Purkinje cells in Pfanstiehl Ara C exposed cultures, whose input resistance was comparable

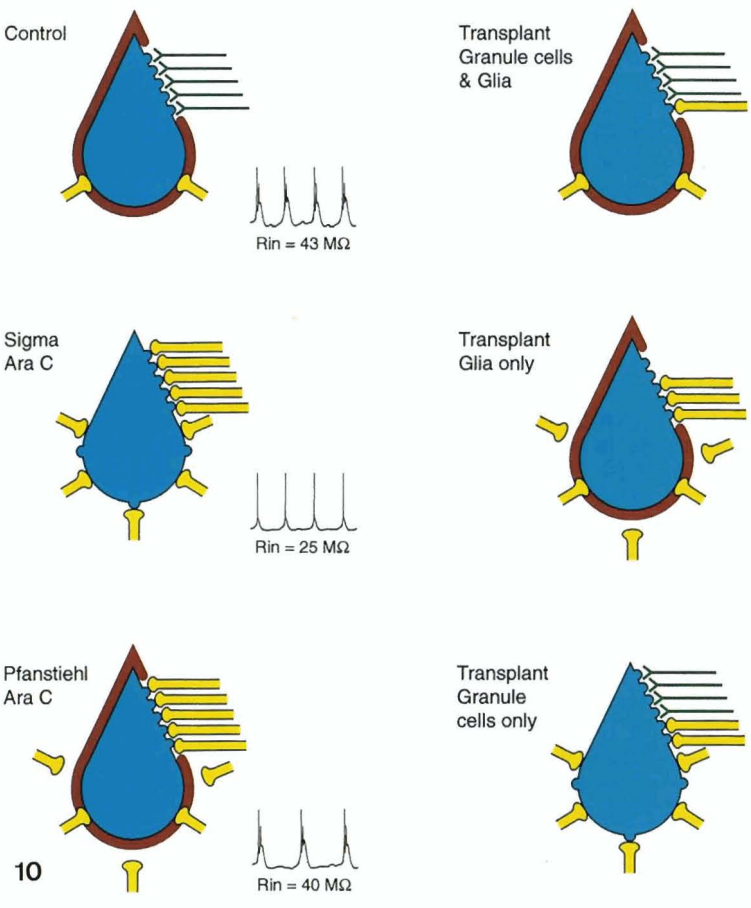

Fig. 10. Diagrammatic representation of Purkinje cells (blue) and their astrocytic sheaths (red) and axosomatic and axospinous synapses in control cerebellar cultures and in various experimental conditions. Although not illustrated, axospinous synapses are also covered by astrocytic processes whenever functional astrocytes are present. Persistent somatic spines are indicated when astrocytic sheaths are absent. Excitatory parallel fibers and terminals are shown in green and inhibitory axons and terminals (predominantly of recurrent axon collateral origin in cultures exposed to Ara C) are colored yellow. Intracellularly recorded Purkinje cell spontaneous spike discharge patterns are shown for control and Sigma and Pfanstiehl Ara C treated cultures, and values are indicated for Purkinje cell membrane input resistances (Rin) in these conditions. In all cases of transplanted cultures, the cerebellar explants were exposed to Sigma Ara C for the first 5 DIV, and then transplanted at 9 DIV or later with cellular components as indicated. Details of the experimental conditions are presented in the text (summarized in the first paragraph of the Discussion).

to that of control Purkinje cells (Drake-Baumann and Seil, 1999). Although Purkinje cells in Pfanstiehl Ara C treated explants had a normal complement of axosomatic synapses, they received excess inhibitory input in the form of recurrent axon collateral projections to their dendritic spines, accounting for the slower rate of spontaneous discharges. The development of higher membrane input resistance was also evidently related to glial ensheathment.

Discussion and conclusions

The various neuron-glia interactions that have been described in cerebellar cultures are summarized diagrammatically in Fig. 10. In the absence of granule cells and functional glia, as in the Sigma Ara C treated preparation, Purkinje cells lacked astrocytic sheaths and their somata had persistent spines and were hyperinnervated by sprouted recurrent axon collaterals, terminals of which also formed heterotypical synapses with Purkinje cell dendritic spines. The membrane input resistance of such cells was reduced, the spikes were all simple, and the discharge rate was similar to that of control Purkinje cells. If functional astrocytes were present in otherwise identical conditions, as in Pfanstiehl Ara C exposed explants, Purkinje cells were ensheathed by astrocytes, somatic spines had disappeared and the complement of axosomatic synapses was like that of control cells, in spite of the presence of numerous sprouted recurrent axon collaterals, which formed heterotypical synapses with Purkinje cell dendritic spines that were glially ensheathed. The membrane input resistance was similar to control Purkinje cells, complex spikes predominated, but the discharge rate was reduced. If Sigma Ara C treated cultures were transplanted with granule cells and glia, Purkinje cells acquired astrocytic sheaths, somatic spines disappeared, axosomatic synapses and sprouted recurrent axon collaterals were reduced to control levels, and most heterotypical dendritic spine synapses were replaced with homotypical synapses with parallel fibers. If only glia were transplanted, sprouted recurrent axon collaterals were not appreciably reduced, but Purkinje cells were ensheathed, somatic spines were lost and excess axosomatic synapses were eliminated. Heterotypical synapses were partially reduced, even in the absence of granule cells, and there were persistent clusters of proliferated unattached Purkinje cell dendritic spines (not shown). If only granule cells were transplanted, sprouted recurrent axon collaterals were not appreciably reduced and continued to hyperinnervate unensheathed Purkinje cell somata with persistent somatic spines. Heterotypical dendritic spine synapses were replaced by homotypical synapses, but twice as many heterotypical synapses persisted as when glia were included in the transplant.

It is evident that astrocytes are very much involved with the control of synapse numbers, primarily by containment in the models described, as in preventing hyperinnervation of Purkinje cell somata or elimination of excess axosomatic or axospinous synapses, the former by a stripping mechanism and the latter by an as yet undetermined process. Can such astrocytic regulation of synapse numbers be overridden? The answer appears to be yes in the case of Purkinje cell axosomatic synapses, which are all inhibitory. When cerebellar cultures were continuously exposed to anti-GABA agents such as picrotoxin or bicuculline to increase neuronal activity during development, Purkinje cells had more that twice the control number of axosomatic synapses after 2 weeks *in vitro*, in the presence of intact astrocytic sheaths (Seil et al., 1994). The spontaneous discharge rate of such cells was less than that of control Purkinje cells, indicating the effectiveness of the inhibitory hyperinnervation in protecting the cells from excess neuronal activity. The signal for the override mechanism would appear to originate from the Purkinje cell, which is also the probable source of the signal for ensheathment, as suggested by the fact that optic nerve astrocytes were able to ensheath Purkinje cells as well as the more specialized cerebellar Golgi epithelial cells.

How is control of synapse numbers by reduction compatible with the promotion of synaptogenesis, as in the case of cultured retinal ganglion cells, where the presence of astrocytes was required for the development and maintenance of synapses (Ullian et al., 2001)? Astrocytes are known to have dual functions in different maturational stages, such as inhibition of axonal growth in the mature nervous system (Eng et al., 1987) and promotion of axonal growth (Rudge et al., 1985; Müller et al., 1990) and guidance (Singer et al., 1979; Silver and Sidman, 1980) during development. Laminin secreting astrocytes may also promote dendritic spine proliferation during development, and contribute to synapse formation by elaborating postsynaptic membranes, ensuring the availability of target sites for arriving axons. Thus immature astrocytes may have synapse promoting or, as in the case of heterotypical synapse formation in Pfanstiehl Ara C treated cultures, permissive properties, while having an opposite function at a later maturational stage. As both axosomatic synapse reduction and Purkinje cell dendritic spine proliferation appeared to take place simultaneously (Seil, 1997), it is conceivable that signals emanate from Purkinje cells that attract astrocytes in different maturational stages to different sites on the same cell. It is known that astrocytes in various stages of differentiation may co-exist (Hatten et al., 1991), and the functions of any given astrocyte may be dictated by its state of differentiation.

Astrocytes may support Purkinje cell maturation by secreting factors that promote neuronal survival and differentiation (Rudge et al., 1985; Müller et al., 1990). Glial ensheathment, with apposition of glial and neuronal membranes, appears to be a more directly interactive process, although contact effects could also be mediated by secreted molecules. Spines persisted on the somata of Purkinje cells in the absence of glial

ensheathment, and over 20% of the excess number of axosomatic synapses were with somatic spines (Seil, 1997). This percentage was markedly reduced with glial ensheathment by transplanted astrocytes, and most of the somatic spines disappeared. Not all of the persistent somatic spines had synapses, however, so that astrocyte-induced loss of afferent innervation, as by synapse stripping, does not explain all cases of somatic spine reduction. Moreover, Purkinje cell somatic spines were devoid of synapses during maturation of control cerebellar cultures (Privat and Drian, 1976; Seil et al., 1991). Some other maturational trigger must also be involved, one possibly released by a contact effect of glial processes on neuronal cell membranes.

Astrocytic ensheathment affected the development of at least one passive Purkinje cell membrane property, namely input resistance. Membrane input resistance was lower in the absence of ensheathment, resulting in reduced sensitivity to inhibitory innervation (Drake-Baumann and Seil, 1995, 1999). Another neuronal membrane effect of astrocytic ensheathment was to allow expression of a low-threshold Ca^{2+} conductance, which is involved in the generation of complex spikes. Thus the development of complex spikes by Purkinje cells may have been the outcome of a membrane change induced by glial ensheathment. The specific membrane events set in motion by contact cellular interactions and their molecular mechanisms remain to be determined.

Astrocytes continue to be fascinating subjects for study. Many properties of these cells have been described, but many more will undoubtedly be discovered. Cells that were once thought to be far less interesting than neurons are rapidly increasing in importance in our concept of how the nervous system functions, and still further revisions in our thinking are highly probable.

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