The Glio-Axonal Interaction and the Problem of Regeneration of Axons in the Central Nervous System – Concept and Perspectives

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Lesion of the central nervous system in man is generally believed to be incurable. However, in the last time evidence accumulated that axonal growth occurs after a lesion if the growing neurites encounter a permissive environment. Since astrocytes play a considerable role as environmental factor in the CNS, the astrocytes from regenerative as well as from non-regenerative species were compared. The concept proposed here postulates that interactions between astrocytes and axons are of basic significance for fiber regeneration and have changed qualitatively during phylogeny: in lower vertebrates astrocytes guide growing and regenerating axons; in higher vertebrates including man the glioaxonal interactions were possibly deteriorated by the appearance of new compounds in the astrocytic membrane.

The regeneration of injured nerve fibers in the central nervous system is of fundamental importance from a theoretical as well as from a clinical point of view. The response to central nerve fiber lesions in birds and mammals including man is an abortive axonal sprouting which does not succeed in axonal elongation and pathfinding to the target [1, 2]. In contrast, the lower vertebrates such as fish and amphibia are able to regenerate their axons and to reconstitute central fiber tracts after axotomy [3–5]. A variety of theories have been put forward to account for these species specific differences to regenerate injured fiber tracts in vertebrates [6, 7]. Within the framework of the subsequently proposed concept, we favour the hypothesis of astroglial involvement in the regeneration process [8]. In fact, between regenerating and non-regenerating vertebrates there are striking differences in the behaviour of astrocytes to regenerating axons. After CNS injury, the astrocytes in fish and amphibia proliferate and form tunnel-like structures in association with the regrowing fibers [9–12]. In mammals, the astrocytes are obviously unable to build equivalent structures [13]; many authors believe that the glial scar may be responsible for the deficiency of regeneration in mammalian CNS [8].

Transplantation experiments performed recently by several groups [14–20] have clearly shown that axons in mammalian CNS are capable to grow after axotomy. Axonal elongation only occurs if the growing neurites encounter a permissive environment. The peripheral nerve implanted into the brain or the spinal cord is obviously such a permissive environment: it enables axonal growth of intrinsic neurons. After successful growth through the grafted tissue, axonal growth extends for only short distances beyond the graft-host tissue-interface [16]. In contrast, CNS fibers in fish and frog do not need any PNS-grafts for axonal growth, and therefore it may be assumed that the environment around nerve fibers in their CNS differs from that in mammals. Since astrocytes play a considerable role as environmental factor in the CNS, a comparison between astrocytes and especially astrocytic membranes from regenerative as well as from non-regenerative species would be interesting.

The astrocytic membrane in mammals: orthogonal arrays of particles

From freeze-fracture observations it is well known that the astrocytic membrane is – among other features – characterized by the occurrence of so-called orthogonal arrays or assemblies of particles (OAP) [21–23]. These OAP which are dispersed between the normal intramembranous particles (IMP) are associated with the protoplasmic fracture-face (P-face). On surface replicas they are not demonstrable which suggests that they are real intramembranous compounds [24]. The present exclusiveness of the

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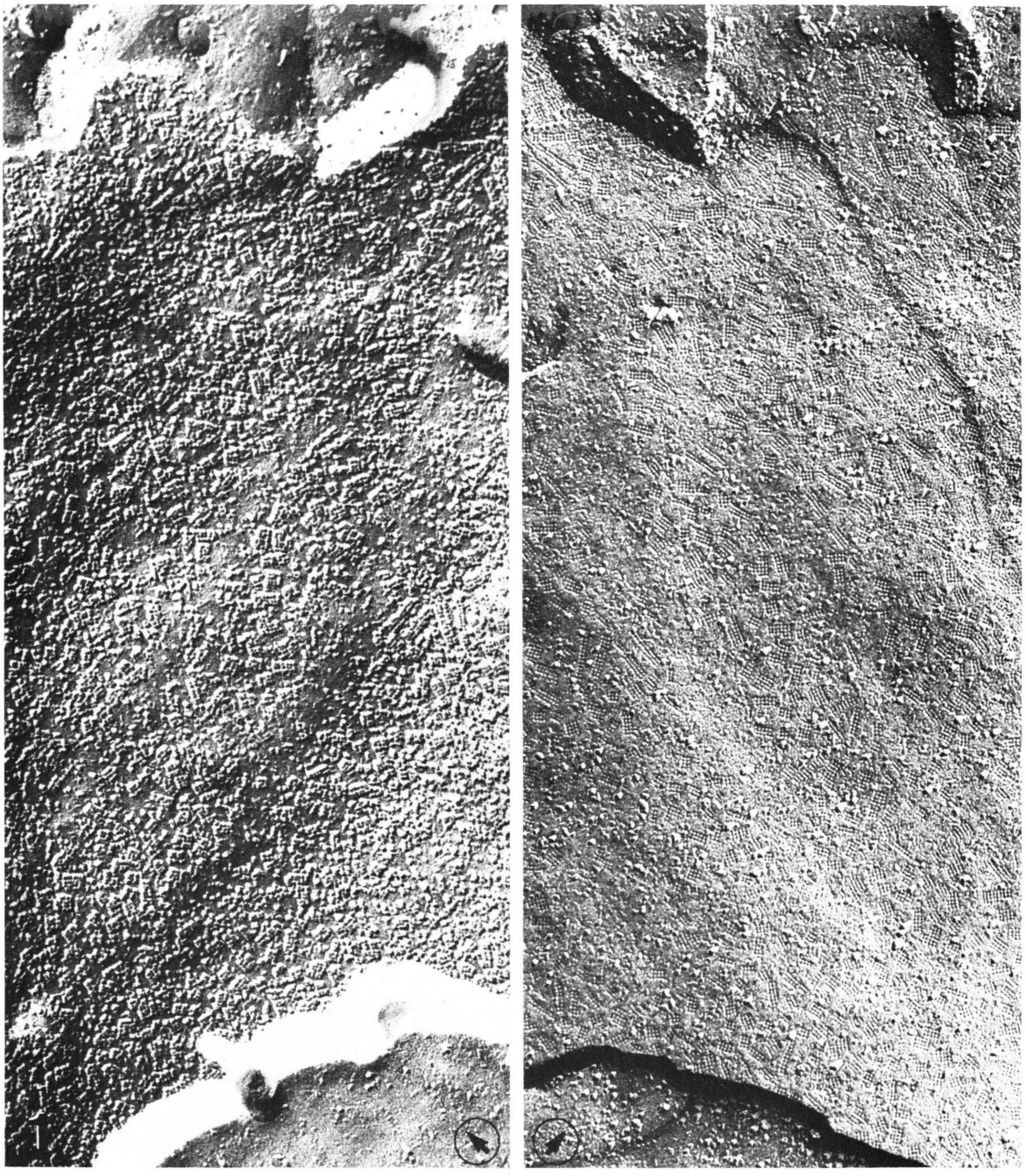

Fig. 1. Freeze-fracture replica from an astrocytic endfoot in the adult rat brain. On the left a protoplasmic fracture face (P-face) is shown. Numerous orthogonal arrays of particles are associated with the P-face. On the right a complementary replica is shown revealing the external leaflet of this membrane (E-face). The orthogonal arrays of particles leave pits in the membrane. The encircled arrows indicate the direction of shadowing. 100000:1.

freeze-fracture method for studying the OAP is possibly the reason for the negligible interest in these arrays of particles among non-morphologists engaged in the neurobiology of the glia. Presumably the OAP are of proteinaceous nature, since they disappear after using protein synthesis inhibitors [24, 25]. It was hypothesized but not proven that they could be involved in water and ion fluxes [26], because they accumulate in large concentrations in astrocytic membranes forming interfaces to fluid-containing mesenchymal spaces such as capillaries (Fig. 1), interfascicular spaces or the subpial spaces at the surface of the brain ("marginal glia"; [27–29]). In contrast, within the neuronal parenchyma, the membranes of the astrocytes reveal only few OAP ([27]; Fig. 2) which is also true for the perinodal processes of astrocytes [30]. Thus, it seems that the expression of OAP in the astrocytic membranes depends on the environment: mesenchymal environment stimulates and neuronal environment inhibits the expression of astrocytic OAP. Whether the expression of OAP is modulated by *de novo*-synthesis, assembly of their subunits, incorporation into the membrane or a combination of these mechanisms, remains completely unknown. The assumption that environmental factors determine the density of OAP in the membrane is further reinforced by *in vitro* observations. The uniform distribution of the OAP without a recognizable preference of any membranous domain of the astrocytes [23, 31] may reflect the lack of environmental compartmentalization of the *in vitro* conditions (Fig. 3).

Since the OAP arise *in vivo* near or around birth [22] and most astrocytic cultures are established from neonatal animals it is not surprising that most published data deal with a low density of OAP in membranes of cultured astrocytes ([31]; Fig. 3). It is not known whether the maturation of cytoskeleton, cellular shape and metabolic activity during differentiation of astrocytes *in vitro* is accompanied by the alteration of the astrocytic membrane architecture. Recently, in collaboration with M. Sensenbrenner and her group, we could demonstrate [32] that it is possible to manipulate the membrane architecture of astrocytes *in vitro* by exposing them to a neuron-derived so-called astroglial growth factor (AGF; [33–35]). AGF is composed of two factors which are similar to, if not identical with, acidic and basic fibroblast growth factors (FGFs; [36]). Both factors decrease the density of OAP dramatically even after

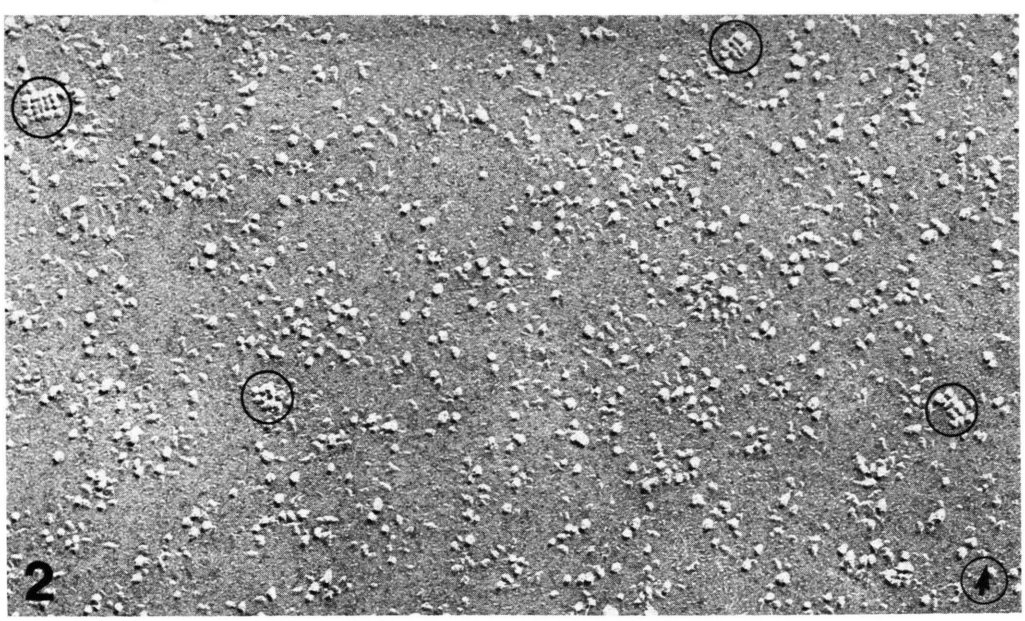

Fig. 2. Freeze-fracture replica of the P-face of an astrocytic perikaryon from the brain of a three day old rat. The density of orthogonal arrays of particles (encircled) is extremely low. Especially in the left upper corner the subunits forming an OAP are clearly visible. 160000:1.

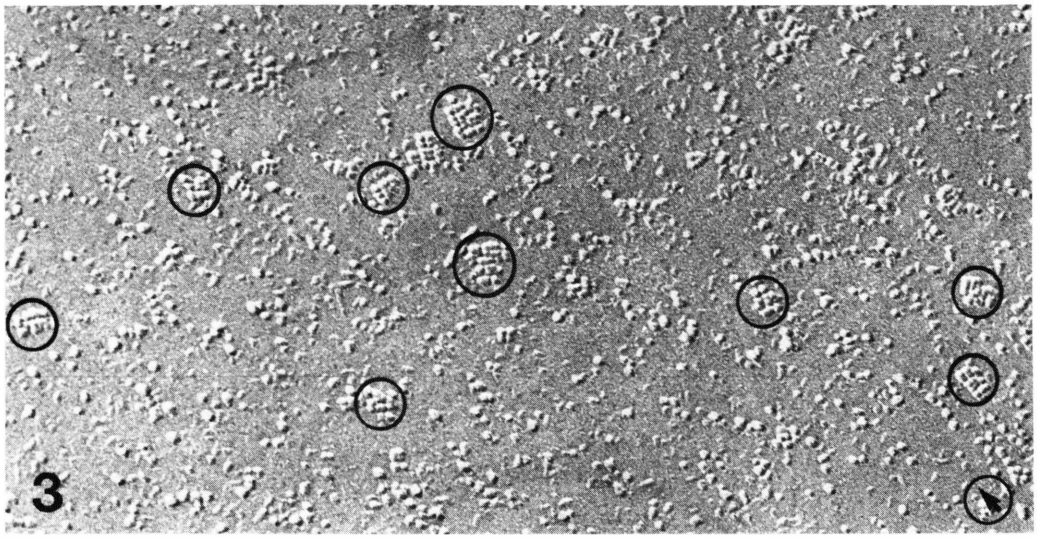

Fig. 3. Freeze-fracture replica of the P-face of an astrocyte cultured in serum-free medium for 15 days from a neonatal rat. Some OAP are encircled. In collaboration with M. Sensenbrenner. 160000:1.

24 h. Therefore, the neuron-derived FGF AGF seems to act on astrocytic membranes in a similar way as the neuronal environment *in vivo* does, namely preventing the expression of OAP [32].

The reactive astrocyte

In response to various types of brain injury, for example, after any insult including infarct, infection or tumour, the astrocytes of mammals proliferate and show signs of hypertrophy and hyperplasia. The volume of the cell increases, the number of processes is augmented, and the cytoskeleton, especially the glial filaments consisting of the glial fibrillary acidic protein (GFAP) is markedly pronounced [37]. Such astrocytes forming gliotic scars are generally called reactive astrocytes.

Freeze-fracture observations on reactive astrocytes *in vivo* suggest that the OAP density in the astrocytic membrane is increased [22, 24, 28]. However, these studies were performed on the CNS of experimentally injured neonatal animals. Kästner [38] has found in the transected adult rat optic nerve astrocytes which revealed all morphological features of reactive astrocytes and had developed a glial scar. The mean density of OAP of these astrocytes dropped with time after eye enucleation in comparison with controls. The OAP density in a glial scar 100 days after eye enucleation in an adult rat was nearly the same (42 OAP/μm^2 membrane area) as in astrocytes of an untreated rat at day 4 after birth (46 OAP/μm^2 membrane area). According to the literature [22, 24, 28], in glial scars induced in neonatal animals, the OAP density was continuously augmented. This suggests that the term reactive astrocyte circumscribes a different structural and metabolic modification of the cell. Liesi [39] has recently shown that in the developing CNS the radial glial cells which are thought to be progenitor cells for astrocytes [40], contain the intermediate filament protein vimentin and express the extracellular matrix protein laminin. In the adult rat brain, laminin is restricted to basal lamina and not expressed by astrocytes [41, 42]. However, after spinal cord and brain injury, laminin is transiently reexpressed by reactive astrocytes [42, 43]. It seems therefore, that reactive astrocytes recapitulate former stages of development. In the light of these findings Kästner's results appear plausible that reactive astrocytes would express as few OAP in their membranes as early astrocytes do.

Fedoroff *et al.* [44, 45] have shown that dibutyryl-cAMP-treated astrocytes in primary culture resemble reactive astrocytes in the injured brain. However, dbcAMP-treated astrocytes *in vitro* produce an increased number of OAP [46]. Thus, there is no direct correlation between what is called the reaction

of the astrocyte, and the astrocytic membrane architecture. This does not exclude that the membrane architecture reflects the metabolic state of the cell, but we are at present not able to analyse this relationship. Moreover, the environmental factors in a culture dish with dbcAMP-treated astrocytes differ considerably from those in an injured brain. These factors as a whole trigger the constitution and expression of certain membrane compounds which may be important for the interactions between astrocytes and nerve fibers.

The astrocytic membrane in fish and amphibia

It is well-known that fish and amphibia can regenerate interrupted fiber tracts in the CNS. In the opinion of several authors the astrocytes play a fundamental role in stimulating fiber growth and guiding growing axons [4, 9, 10, 12, 28, 47]. After injury, the astrocytes do also proliferate as in mammals; in contrast to the situation in the latter, the axons of amphibia are able to penetrate even a glial scar [48]. The axo-glial interaction in lower vertebrates seems to be basically different from that in birds and mammals. In a freeze-fracture analysis of the optic nerve regeneration in the goldfish [12, 38] we have found three different morphological aspects of the astrocytic membrane associated with regenerating axons: 1. The membrane was smooth, and the normal intramembranous particles (IMP) randomly distributed; 2. simultaneously, other membranes were undulated showing longitudinal convexities and concavities according to the course of the axons, and the IMP were distributed non-randomly. 3. After the regeneration process had terminated the situation was similar to the untreated control: the astrocytic membrane was undulated and the IMP nearly randomly distributed. It seems to us that we have observed the morphological manifestation of an axo-glial "conversation" which enables the axons to grow through the glial scar, because the astrocyte understands the "language" of the neuron.

Since, in principle, as we have mentioned above, even axons in the mammalian CNS can grow but are impeded to elongate and to be guided, the possibility arises that the axo-glial conversation became deficient in the course of phylogeny. The astrocytes of adult mammals are possibly no more able to understand the neuronal language. This assumption implies a difference in the composition of astrocytic membranes between regenerative and non-regenerative species or systems. It is striking that the astrocytic membranes in fish and amphibia do not reveal any OAP [12, 28, 38, 47, 49–51]. Even the astrocytic endfeet around capillaries and the marginal glia, in mammals crowded with OAP, are completely free of OAP in lower vertebrates.

Is there a connection between OAP absence and regenerative capability?

In order to decide whether or not there is a systematic connection between astrocytic membrane architecture and regenerative capability of the CNS, we have investigated the normal astrocytes of several species. In birds (chicken and pigeon) and mammals (rat) the OAP are regularly found [38]. These species can not regenerate their CNS after lesion [2]. In the turtle and lizard optic nerve OAP were also found [38]; no data are available on the regenerative capability of this system. However, it is known that the thoracic spinal cord of the lizard Anolis is not able to regenerate after lesion, whereas the caudal spinal cord is (Anolis-paradox; [52]). The astrocytes in the thoracic spinal cord of Anolis bear OAP, those in the caudal spinal cord do not [53].

In another approach the olfactory system of the adult rat was investigated: It is the only site in the whole nervous system of mammals where neurons are continuously replaced and sensory axons invade repeatedly the CNS and make synaptic connections with intrinsic neurons [54]. In the olfactory nerve the axons are bundled by glial cells which reveal several features of typical astrocytes [55, 56]. In the olfactory bulb the invading axons are ensheathed by a special glial type called superficial glia by Raisman [57]. This glial ensheathment protects the olfactory axons from the neuropil in the olfactory bulb. The astroglia-like glial cells of the olfactory nerve as well as the superficial glial cells in the olfactory bulb do not contain any OAP in their membranes [58].

Furthermore, neonatal mammals are able to regenerate central fiber tracts [59]. Unfortunately, there are no systematic investigations looking for conventional and freeze-fracture electron microscopy of astrocytes in the lesioned and regenerating part of the CNS of neonatal mammals. However, descriptive investigations not related to the problem of regeneration suggest that the astrocytic OAP appear not before birth [22]. Taken together, the facts

favour the assumption that where axons grow or regenerate *in vivo*, the associated glial cells do not contain any OAP (Table I).

Astrocytes in culture have long been implicated in the stimulation of neuritic growth. There is a large and growing body of evidence that cultured astroglial cells exert neuronotrophic and neurite-promoting activity [60–66]. A preformed layer of astrocytes provides a trophic support for neuronal cells [67–69] and recently astrocytes in culture were shown to produce laminin [70], one of the most potent neurite-promoting extracellular matrix compounds [71–76]. Cultured astrocytes from early rats produce laminin, the longer the earlier they were isolated from the animals. It is not known what may be responsible for the cessation of laminin production in astrocytes *in vitro*. The glio-neuronal interaction as a prerequisite of continuous laminin production has to be taken into consideration.

The membrane of cultured astrocytes is impoverished in OAP; however, experiments pursuing the interrelationship between astrocytic membrane structure and neurite-promoting activity *in vitro* are completely lacking. From the *in vivo* observations mentioned above one should expect that astrocytes *in vitro* when cocultured with neurons, are inhibited to express OAP; this remains to be demonstrated. The interesting question whether OAP-rich astrocytes would still be able to exert their neurite-promoting activity, can probably not be answered by *in vitro* experiments, because strong OAP expression and presence of neuronal elements exclude each other mutually.

Recently, *in vivo* observations have shown that in regenerative systems such as the visual system of goldfish and frog and the olfactory system of the rat the astrocytes express laminin, and that laminin is absent in astrocytes of the normal adult mammalian brain [41, 77]. However, it is reexpressed transiently in reactive astrocytes after adult brain injury [43]. It seems therefore that where astrocytes are able to promote neuritic regeneration or sprouting they tend to produce laminin and to suppress OAP. Currently, it is a matter of speculation whether or not both phenomena are related to each other.

Although the marginal processes of astrocytes in the mature rat spinal cord are covered by a basal lamina, laminin is recognizable by antilaminin immunocytochemistry only on basal lamina of endothelial and pial cells [42]. However, some days after hemisection of the spinal cord astrocytes transiently secrete laminin. After 10 days laminin could not be found immunocytochemically in astrocytes of the injured region [42]. Bernstein [78] assumes that the basal lamina of the mammalian CNS is a "no-growth barrier". Considering the promoting effect of laminin on neuritic growth one has to render into account that basal lamina of astrocytes differ from endothelial and other basal lamina. Astroglia-derived laminin in the goldfish optic nerve was suggested to play

Table I. Inverse correlation between regenerative capability of axons in different species or systems and the occurrence of orthogonal arrays of particles (OAP) in the membranes of corresponding astrocytes.

	Axonal regeneration after injury	References	Occurrence of astroglial OAP	References
teleosts	+	[1–7, 12, 77]	–	[12, 38, 47, 50]
amphibia	+	[9, 10, 11]	–	[28, 38, 47]
reptiles				
turtle optic nerve	unknown		+	[38, 47]
lizard optic nerve	unknown		+	[38, 53]
lizard thoracic spinal cord	–	[52]	+	[53]
lizard caudal spinal cord	+	[52]	–	[53]
birds				
pigeon optic nerve	unknown		+	[38, 47]
chick optic nerve	unknown		+	[38]
mammals adult	–	[1, 8, 13]	+	[21–27, 29, 30, 47]
mammals neonatal	+	[59]	just arising	[23, 25]
rat olfactory system	+	[54]	–	[58]

an important role in nerve regeneration [77]. It seems probable that production by astrocytes of laminin correlates with OAP-suppression, but that intercalation of other than astrocytic basal lamina induces stimulation of OAP in astrocytes. Therefore, the production of endothelial and pial basal lamina or other extracellular matrix compounds would exert opposite effects on OAP expression in comparison with the production of astrocytic basal lamina compounds. This would provide new insights in the understanding of the interference of axonal growth and regeneration with environmental factors.

Conclusions and perspectives

We have seen that intrinsic axons in the CNS of lower vertebrates are able to regenerate, and that this capability is not lost in higher vertebrates. It can be reactivated by an appropriate environment. An important constituent of the neuronal environment is the astroglia. Astrocytes of the fish optic nerve are intimately associated with growing and regenerating fibers. It is likely that this morphological association reflects metabolic interaction between both cell types enabling permanent growth. These astrocytes probably produce laminin continuously [41, 77] which is believed to be an important neurite-promoting matrix substance in the CNS as well as in the peripheral nervous system [18, 79]. Another point is the absence of the orthogonal arrays of particles in astrocytic membranes of regenerative species, and their presence in astrocytes of birds and mammals. In these species, the frequency of intramembranous OAP-expression is dependent on the kind of environment: at the interface to mesenchymal spaces the OAP-expression may be stimulated, whereas within the neuropil it may be largely prevented. Astrocytes in the adult mammalian brain do not produce laminin. Thus, there is some evidence – from *in vitro* as well as from *in vivo* observations – that production of laminin by astrocytes on the one hand and the absence of OAP on the other are connected with a capability to axonal regeneration. However, in the injured brain the astrocytes can be induced to express laminin. It is known from many investigations that axons sprout in response to injury ([80, 81], for example). It may now be postulated that this sprouting is improved by astrocytic laminin production. Whereas in lower vertebrates the laminin production is a continuous process and increased after injury, in mammals it is stopped several days after lesion. The reason for this is completely unclear. It may be speculated that the sustained laminin production implies a successful and long-term interaction between astrocytes and axons such as described in the goldfish optic nerve [12]. This long-term interaction may be impaired during both phylogeny and ontogeny by the appearance of the astrocytic OAP. In a metaphorical sense this would mean that the astrocytes would have "forgotten" to understand the "language" of the neuron, and the result would be the limited production or availability of astroglial laminin or other glia-derived neurite-promoting substances [82, 83], respectively, and thus the end of neuritic sprouting.

This concept modifies the idea of the glial scar as the element impeding neuritic growth. The astrocytes of all vertebrates are obviously neurite-promoting elements. Only in higher vertebrates which have introduced the OAP in their astrocytic membranes the mesenchymal environment would "transform" them into cells which are no more able to promote neuritic growth. This concept would explain why after mild injury of the spinal cord without formation of a mesenchymal scar the regeneration of spinal axons is improved [84]: the less mesenchymal cells proliferate the smaller is their interface with astrocytes, the less OAP are induced and the more laminin can be produced.

Finally, it should be stressed that a causal relationship in a strict sense between the occurrence of the orthogonal arrays of particles in astrocytes and the incapability for axonal growth is unproven. Imaginable as well is the formation of the OAP as an epiphenomenon of non-regenerating nervous systems. Possibly the OAP only indicate the incapability for central nerve fiber regeneration without being causally involved in any regeneration mechanism. However, if there is a strict correlation of OAP presence and non-regeneration, it should be possible to improve the regeneration process by depressing the number of orthogonal arrays of particles. On principle, it is possible to depress the number of OAP experimentally, as we have shown above [32].

The mechanisms effective in the proposed relations are unknown. Since the OAP are intramembranous complexes they are not directly associated with the membrane surface. Any effect of the OAP can therefore be imagined only on the basis of OAP-associated surface compounds. Several lines of experiments should be traced in order to test the pro-

posed concept: 1. to study whether decrease of the density of OAP improves the glio-neuronal interaction *in vitro* and *in vivo* and therefore the laminin-improved elongation of neurites; 2. to study the biochemical and immunological properties of OAP and the putative OAP-associated compounds in order to establish antibodies against them.

Future research will show whether or not this approach will give new insights into the process of fiber regeneration in the central nervous system.

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