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Central nervous system myelination in vitro

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A revue concerning tissue culture methods and myelination in vitro is made on the basis of our own results and of the other authors' data. Different in vitro systems are used for revealing the complex mechanisms of myelination: organotypic, aggregated, dissociated cultures and cultures of bulk-isolated myelinating cells. Myelinogenesis in vitro corresponds to this process in vivo with respect to its morphological and biochemical parameters. The myelin marker lipids, the proteins and the enzymes responsible for their synthesis, have an ontogenetic profile and time-sequence which are similar to the described in vivo. In this connection the tissue cultures provide a convenient system to study factors regulating the cell interactions and expression of myelin components during the myelinogenesis.

Key words: CNS, myelination, tissue culture, cell markers.

The myelin membrane is a unique structure characteristic of the nervous system which has definite morphological, biochemical, physiological and immunological properties. The isolated myelin contains 70-85% of lipids (cholesterol, phospholipids and galactolipids) and 15-30% of proteins [52]. About 50% of the total proteins are proteolipid proteins (PLP), including DM-20 proteins; 30-35% are myelin basic proteins (MBP); 5% are 2′-3′-CNP and 1% are myelin-associated glycoproteins (MAG) [25]. The myelin sheath is formed during ontogenesis as a result of a complex and multilateral process in the nervous system — myelinogenesis — whose mechanisms have not been totally clarified yet. The priority role in this process is attributed to the oligodendrocytes in the central nervous system and to the Schwann cells in the peripheral nervous system, but the interactions between the different elements in the nervous system are rather complicated. The introduction of new methods and techniques filled in many gaps concerning the mechanism of myelin-formation. Particularly important in this respect was the introduction of tissue and cell cultures in neurobiology. The importance of these systems is suggested by the fact that the participation of the satellite Schwann cell in the formation of spirally wrapped structures around the axons was demonstrated for the first time in cultures of spinal ganglia [31].

In vitro systems of myelination

Several methods can be applied, depending on the objectives of the study:

Organotypic cultures. Small organs (spinal ganglia) or tissue fragments, about 1 mm^3 in size, are explanted on a collagen or polylysine substrate under suitable conditions. The organotypic cultures are maintained in a medium with serum, embryonal extract, glucose, etc., most often in Maximov's assemblies at temperature of about 34,5°C [16, 20, 21, 55]. The explants preserve their optimal cell composition in the organotypic relations between the cells of which they consist. The shortcoming of this method is that the cells are not clearly visualized in a living state and selective access to them is not possible. The method is suitable for morphological studies and for inducing demyelination [2, 13, 34].

Aggregated cultures. Using the tendency of the freely dispersed animal cells in a liquid medium to migrate and form aggregates, Moscona [48, 49, 50] introduced the method of aggregated cultures. Seeds [68] modified the method by introducing eddy movement of the suspension with the aim of facilitating the aggregation processes. The aggregates are obtained in the first 24 hours and can be maintained under suitable conditions for up to several months. The orientation of the cells in aggregates is not accidental. They tend to take a natural position and orientation, characteristic of the histotypical organization of the original tissue [79], and to establish definite contacts which are closest to the contacts in vivo [69].

This system of cultivation is suitable for applying biochemical and morphological methods in the studies of myelination (TEM and SEM) [42]. It eliminates some of the shortcomings of the dissociated cultures: the cells occupy more suitable positions, but preserve some of the disadvantages of the organotypic cultures — difficult access for direct observation and manipulation.

Cultures of bulk-isolated myelinating cells. Methods have been proposed recently for obtaining enriched fractions of oligodendrocytes which could be preserved and investigated in vitro [28, 58, 59]. This system finds an ever wider application for studying myelination by adding other cell types (e.g. neurons) to the enriched fraction of oligodendrocytes and then using them for transplantation [51].

Dissociated cultures. The dissociation can be enzymatic or mechanical, using nylon sieves or tubes with definite dimensions. The suspension obtained is cultivated over substrate in flasks, at a temperature of 36-37°C and 95% humidity. The nutrient medium is chosen depending on the aims of the study [33, 42, 65].

The dissociated cultures have several advantages. This system is suitable for performing biochemical investigation for which large quantities of material are needed: there exists a possibility for direct observation of the cells and for studying the metabolic parameters, as well as for obtaining enriched or pure cell population.

When this culture system is used for investigating myelination in vitro, it is necessary to observe the following requirements: the most suitable object for dissociation should be the neonatal brain, because its cells are not yet differentiated, more specifically definite brain areas whose composition is known; obligatory identification of the isolated cell types should be made [46, 47].

Due to the big changes which the cells undergo in the processes of isolation and cultivation, one cannot rely on the morphological characteristics only of a concrete cell population. Specific immunological markers have been proposed, which guarantee a reliable identification of the cells.

It is necessary to take into account the fact that when dissociated cultures are used, initially the neurons manifest a process of differentiation and then of regression to the primitive state of neuroblasts. Moreover, it is possible that no normal myelination would occur.

The main cell types in the composition of the nerve tissue — neurons, oligodendrocytes and astrocytes — repeat along general lines in vitro the morphological and metabolitic properties of the respective population, although certain peculiarities may also be observed depending on the culture system chosen.

Behaviour of CNS cells in culture

Neurons. An important condition for the normal development of the nerve cells in an in vitro system for studying myelination is the choice of a suitable stage in their development in the brain for isolation and explantation. The most suitable period for culturing neurons of rodents (mice and rats) has been proved to be the 14th-15t-16th embryonal day [40, 65]. By using cell markers for the neuroblasts and neurons, they can be identified even on the third day in vitro [37]. A number of specific markers have been proposed, the principal ones among them being the antisera against concrete substances or receptors contained in the nerve cells only: 1) usage of antiserum against neurofibrils (NSF) [37] monoclonal antibodies RT 97 has already been proposed; 2) identification of neuron-specific enolase (NSE) [12, 40], considered to be an intracellular neuronal marker; 3) identification of receptors against tetanus toxin [45]; 4) labelling of the body of the neurons with antisynapsin I-antiserum, especially in the early terms [40]; 5) labelling of the GABA-receptors with ^{3}H-muscimol, which is a specific label for neurons, neuroblasts and neuronal processes [67]; 6) determination of the acetylcholinesterase activity during the later terms of cultivation [37].

Neurons develop an extensive network of neurites in vitro and form synaptic contacts. The closest morphological similarity with definite neuronal types in vivo is found in nerve cells cultivated in organotypic cultures. During the first week of organotypic culture the neurons are differentiated and acquire the morphological characteristics of those in vivo [20, 21]. They contain large light nuclei, well developed endoplasmic reticulum, free ribosomes scattered in the cytoplasm and multiple Golgi appartus. Numerous neurofibrils which pass into the processes are demonstrated in the cytoplasm.

In spite of their poor mobility, neurons in monolayer cultures tend to group, so that small cell aggregates can be seen in the first 24 hours. Processes begin to grow, becoming longer with the increasing duration of the culture, the dendritic network becomes denser and dendrite groups in bundles appear on the 15th day in vitro [33]. The neurons are differentiated during the first and second week in vitro [76], after which they tend to degenerate [8, 33, 42, 40, 66].

The metabolic activities of the nerve cells in vitro are still insufficiently studied.

In organotypic culture from the central nervous system the glial cells are localized either in the dense zone close to the neurons, or they migrate to the growth zones where there are no nerve cells. Their numerous processes participate together with the axons in the formation of the growth zone [20, 21, 36].

Astrocytes. In dissociated cultures of all cell elements of the nervous tissue, the most frequent cell population obtained consists of astrocytes. Mechanical isolation through a nylon sieve with a definite mesh size results in a cell

suspension containing 95 per cent of the astrocytes. Their number increases during the culturing [5], consisting of large flattened cells with branching fibrous growths containing large light nuclei.

A characteristic feature of astrocytes is that they contain glial fibrillar acid protein (GFAP) which is detected as early as during the first week in vitro and is considered to be astroglial cell marker [46, 75]. Protein S-100 is also specific for the astrocytes, but it remains at a low level (compared with its content in vivo) throughout the entire culturing period [70]. The supporting and trophic function of the astroglia has been recognized for a long time. There is still no clarity about their participation in the process of myelinogenesis in the central nervous system. It has been demonstrated, however, that the astrocytes are localized among the neurons in cerbellum cultures on the 7th day in vitro, with numerous processes prior to the myelination [38] among which the neurons are localized. A description is also given of the process of fasciculation, which is associated with myelinogenesis [83].

Oligodendrocytes. In organotypic culture the oligodendrocytes are localized in the inner part of the explants. From the very first days of their cultivation they migrate and participate together with the astrocytes in the formation of the growth zone. The introduction of the methods of dissociated cultures resulted in the discovery of new possibilities for detailed study of the morphological and biochemical specificities of oligodendrocytes. Great difficulties are involved in the obtaining and maintaining of pure oligodendrocyte cultures, therefore, until recently the term "enriched" oligodendrocyte culture was used. On the basis of some properties of the oligodendrocytes, Araceli Espinosa de los Monteros et al. [27] recently proposed a method for obtaining and for maintaining a pure culture of metabolically active oligodendrocytes.

During the first days, in mixed cultures the oligodendrocytes lie over the layer of astrocytes [70]. In pure cultures they are scattered over the surface of the substrate, having long thin processes which exceed several times the diameter of the cell body. In an in vitro system the vital oligodendrocytes manifest the same specific features as in vivo. They are small cells, with dark nucleus and relatively dense cytoplasm, and numerous branching processes. Under phase contrast the cytoplasm is strongly granular [70]. Their ultrastructural pattern identifies them as cells with relatively electron-dense cytoplasm, with many mitichondria and free ribosomes having an electron-dense round nucleus and peripherally positioned heterochromation [59].

Cell criteria have been established for identifying oligodendrocytes in tissue and cell cultures, namely: 1) they have a positive response to the action of antigalactocerebroside serum, considered to be a surface cell marker [61, 62]; 2) the myelin basic protein (MBP) is considered to be an intracellular oligodendrocyte marker [74, 83]; 3) the enzyme 2'3'-CNP is found to be a marker for oligodendrocytes and for the myelin sheath [80, 87].

Both oligodendrocytes and neurons degenerate quickly in mixed cell culture and after the second week in vitro they are replaced by fibrous astrocytes [70].

The principal function of oligodendrocytes in the central nervous system is their participation in the formation and maintenance of the myelin sheaths.

CNS myelinogenesis in culture

Different in vitro systems have been used for revealing the complex mechanisms of myelination. In organotypic culture myelinogenesis corresponds to this process in vivo with respect to its morphological and biochemical parameters [15, 16, 20, 56].

Growth cones are identified from the very first hours of the explanting [20, 21]. The proliferation and migration of glioblasts starts on the 1st-2nd day in organotypic culture and around the 4th day in vitro in aggregated cerebellar culture [42], being induced according to Wood and Bunge [82] by the growing axons. The neurons end their differentiation before the beginning of myelination in organothypic cultures — around the 8th day in vitro, and slightly later in dissociated cultures. The amount of ceramideglu-cosyltransferase in them increases. This enzyme catalyses the formation of glucosylceramide a precursor of the ganglioside synthesis, considered to be a marker of the neuronal differentiation [42].

The onset of myelination depends, above all, on the brain area cultivated and on the culture system chosen. In organotypic cultures myelination starts around the 10th day [20, 73]. In dissociated and aggregated cultures the process of myelinogenesis is delayed, starting around the 2nd-3rd week [63, 65, 66]. Orientation of the glial cells along the nerve fibres is observed before the visualization of the myelin sheaths [20]. Cell nests are formed in monolayer culture [63], with filamenous surface and later with an uneven surface [66].

Most accurate data on the course of the synthesis processes during in vitro myelination have been obtained by applying radioactive precursors of the myelin components. Irrespective of certain variations resulting from the use of different systems and conditions of culture, the myelin-associated marker lipids, the proteins and the enzymes responsible for their synthesis, have an ontogenetic profile which is similar to the one described in vivo. They appear exactly at definite intervals and in a definite order, and perhaps the coordinating control is performed by a common central cell source, i. e. probably by the oligodendrocytes [66], because this order is preserved in a purely oligodendrocyte culture as well, although the quantities of the different components are smaller [63]. One cannot rule out the influence of some neuronal factors on the myelinating oligodendrocytes [6]. The cholesterol synthesis shows a manifold increase during myelination, with a peak of deposition in organotypic culture from newborn mouse cerebellum around the 15th day [57]. Galactolipids-cerebrosides and sulphatides are barely identifiable around the 4th day in vitro, but they increase considerably between the 20th and 30th day in vitro [17]. The peak in their deposition coincides with the period of active myelinogenesis [39, 63, 72]. As compared with cerebrosides, the sulphatides are synthesized and metabolized more actively [66]. The data on the synthesis of the myelin lipid components coincide with the data established in vivo for the respective animal species [22, 43].

During the whole period of myelinogenesis, and especially during active myelination, there is a strong activation of the enzymes included in the synthesis of myelin lipids: galactosylceramide sulfotransferase (E.C. 2.8.2.11) and ceramidegalactosyltransferase (E.C. 2.4.145) [42, 65, 66].

The main myelin proteins: myelin basic proteins (MBP), proteolipid proteins (PLP) and myelin-associated glycoprotein (MAG), are synthesized in vitro slightly later than the myelin lipids (about one week after the galactolipids) [25], but they follow the order established in vivo.

MAG is a minor transmembrane glycoprotein of the myelin in the central nervous system, which is limited to the periaxonal and noncompact areas of the myelin sheath [78]. Applying immunocytochemical techniques, it has been found that MAG appears in the oligodendrocytes later than MBP (on the 4th day in vitro in mixed cultures of dog cerebellum) and is assumed to play a certain role in the interactions between the action and the myelin-forming cells. Oligodendrocytes express MAG, establishing a contact with the axon as one of the first steps of

myelination, bofore the other components have been formed [90]. These assumptions were confirmed by the findings of Dubois-Dalcq et al. [25], who have found galactolipid-positive cells in oligodendrocytic culture from the brain of 16 to 17-day-old mouse embryos in the very first days, and MAG-positive cells around the 5th-7th day. MAG is localized perinuclearly and it is the first of the myelin proteins to be observed in the oligodendrocyte processes around the 10th day.

MBP is synthesized on the free ribosomes in the cytoplasm and in the processes of the oligodendrocytes [64], being identified immunocytochemically in myelinating oligodendrocytes, diffusely in the cytoplasm [74], and in the major dense line of the myelin sheath [53].

During the first days the MBP content in rat fetal brain aggregated culture is very low. After the 18th day their amount begins to increase — more than ten times until the 40th day [41]. In dissociated cultures in the cytoplasm of the oligodendrocytes MBP is seen during the first week [25], but only after the second week all cells have a positive reaction to anti-MBP antiserum [63] and this antigen is detected in the processses as well, i.e. one week after the first visualization in the cytoplasm [25].

PLP, the major myelin proteins, are involved in the process of increasing the density and stabilization of myelin. They are synthesized on membrane-bound ribosomes, mainly in the oligodendrocytic cell body, becoming acylated after passing through the Golgi apparatus and being incorporated in the myelin membranes [77]. They are demonstrable immunocytochemically in the myelinating cells 1-2 days after MBP [19], as well as in vivo. Maximum PLP synthesis is observed several days later, as compared with MBP.

The enzyme 2′-3′-cyclic nucleotide phosphohydrolase, (EC 3.1.2.37), which is specific for myelin, increases during the whole period of myelination, where its maximum activity coincides with the period of active myelinogenesis [42, 57].

The visualization of the myelin sheaths using different histochemical and electron microscopic methods depends on the cultivation system and coincides with the time of the deposition of myelin components in the sheath. It becomes denser with time and acquires a compact lamellar structure, with alternation of the major dense and intraperiod lines, as in the normal myelin [21].

The myelination process in organotypic cultures is completed towards the end of the third week [20]. In aggregated and dissociated monolayer cultures it is difficult to determine the exact terms, because a degeneration occurs in the neurons and oligodendrocytes after 3-4 weeks in vitro [42, 65, 66].

Many publications appeared recently on the formation of myelin membranes in pure oligodendrocyte culture, in the absence of neurons [1, 18, 25, 60]. The term "myelin-like" is most frequently used, because there is still no absolute clarity on the degree to which these membranes correspond to the mature myelin in vivo. They possess all components characteristic of the mature compact myelin, but the correlation between them is rather different. The evidence about CNP is contradictory [1, 60]. As compared to the proteins, the quantities of cerebrosides and sulphatides are twice higher in the myelin-like fraction than in the compact myelin [60]. The MBP synthesis is considerably delayed and reduced [1]. The morphological picture of the myelin-like membranes does not correspond always to that of the mature myelin. There often appear loosely wrapped lamellae, without a clearly identifiable major dense and intraperiod lines, referred to as "premature myelin" by Althaus et al. Many results indicate a similarity between the myelin-like lamellae and the mature compact myelin (density, enzyme activity

and lipids), but there are also distinct differences, mainly in the protein content, and these membranes may be precursors to mature compact myelin [60].

There are still no exact data on the participation of the cell elements in the synthesis of the myelin components and on their interaction during in vitro myelinogenesis. Similar to the in vivo system, the priority is given to the oligodendrocytes. There is no evidence about the direct participation of neurons in this in vitro process. Little is known about the nature of the processes which induce or regulate the changes in the oligodendrocytes, mainly with a view to their proliferation and the onset of the synthesis of the myelin components. Direct cell contacts are found to influence some biochemical parameters of the cell. Neuronal stimulation results in increased proliferation of the glial cells. The molecular mechanisms involved in the cell proliferation have not been studied yet, but it is assumed that the changes on the cell surface due to the "cell-cell" contact influence this process of increased synthesis of cell proteins [80], although it is not known to what extent these mechanisms are under genetic control. Transferrin, which is considered to be a neurotrophic factor [4], is synthesized by the oligodendrocytes and increases during myelination. It probably plays some special role in this process, with a stimulating effect on the neurons [9]. Evidence has been found recently about the stimulating influence of some growth factors on the individual cell elements of the nerve tissue, although they are not yet associated with the myelination process: nevertheless, they may influence its course indirectly. A stimulating effect on the astrocytes is observed for EGF [81], interleukin 1 [32], PDGF [35], $AGF_{(A)}$ and $AGF_{(B)}$ [56] and thrombin [54]. Oligodendrocytes are stimulated by the brain extract [23], FGF[8,26], IGF_1 [44]. The problem concerning the stimulating effect of some factors on neuroblasts and neurons is least studied. The action of two factors has been proved for the time being: of $FGF_{(B)}$ [29] and meningeal extract [30]. The unsuccessful attempts connected with the isolation, survival and maturing of the neurons can explain the fact that mature myelin sheaths are seldom formed in dissociated brain cultures [83]. The role of the neuron in this process is probably much more active. Zaprianova [88, 89] expressed a view about the existence of neuron-glia interactions in the product of CNS myelin phospolipids in vivo. The stimulating effect of the nerve cells in the proliferation of the glia, prior to the beginning of visible myelination, has been proved [10, 11]. Another important factor influencing the process of normal in vitro myelination is the obligatory presence of astrocytes in the cultivation system. The beginning of myelination is preceded by fasciculation of the glia, with the formation of bundles on which the mature oligodendrocytes are localized, which have a positive reaction to antiserum against galactocerebrosides [83]. The fasciculation results in the appearance of the necessary micromedium which leads to extensive myelination [83]. No fasciculation is observed in dissociated cultures in which the viable neurons are few or lacking, whereas astrocytes with their processes form a continuous network on which the oligodendrocytes are localized [70]. This fact clearly demonstrates the influence of the neurons on this process. The signal for the onset and for the discontinuation of myelination, and for the proliferation of oligodendrocytes probably comes from the neuron, either directly or through the axon, because myelination of all axons simultaneously is never observed and, moreover, the division of the oligodendrocytes is also asynchronous [5, 73].

There exist data on the influence of the thyroid hormones on myelination. The direct effect of 3′-triiodothyronin (T_3) on myelination, in its capacity of monitor of the synthesis of some lipids closely connected with myelin, is demonstrated by using an experimental model in which mixed cell cultures grow in a medium containing serum of calves after thyroidectomy. Low degree of

synthesis of sulphatides and CNP is observed in hypothyroid serum [6, 7, 71, 86] which is restored to its normal level after addition of normal serum or of T_3. The activity of the lipid sulphotransferases is disturbed by more than 30 per cent [6]. A dose-dependent effect is observed: the sulphatide synthesis increases with the increase of the doses of T_3 and T_4 (thyroxine). T_3 (the active form of the hormone) is 30 times more active than T_4. It has been proved that the maximum effect of the thyroid hormones on the expression of CNP and sulphatides is found in the earlier cultures.

In vitro myelinogenesis can be delayed or totally inhibited by changing the culture medium. Myelinogenesis is inhibited when antisera against components of the central nervous system are added to the nutrient medium, e.g. antiserum against the white matter of the brain [14], antiserum against galactolipids [84, 85], and antiserum against gangliosides [84, 85]. The mechanisms of action of the antisera on myelinogenesis are not yet known.

Although many studies have been devoted to the myelination in vivo and in vitro, many problems connected with this process still remain unsolved.

1. It has not been completely clarified where the myelin components and their precursors are synthesized, and what control of the synthesis is realized.
2. Which cell types participate in the synthesis of the myelin components and what interactions exist between them?
3. What is the exact role of the neuron in the in vitro myelinogenesis?
4. What biologically active substances participate in the regulation of the cell interactions during myelinogenesis?
5. What is the mechanism of action of the endogenous brain stimulators?
6. Which elements of the nerve tissue are influenced by the antiserum myelin-inhibiting factors?

In this connection, the tissue culture provides a convenient system to study factors regulating neuron-glia interaction and expression of myelin components during myelinogenesis.

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