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Even in culture, oligodendrocytes myelinate solely axons

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ABSTRACT **Cerebral hemispheres from mouse embryos at 15 days of gestation were dissociated and maintained in culture for several weeks in a medium which permitted homochronic and homotypic oligodendrocytes and neurons to interact in the presence of other central nervous system cells. After 13–14 days in culture a few oligodendrocytes changed from highly branched, "sun-like," nonmyelinating cells to sparcely branched myelinating cells. The number of fibers myelinated per oligodendrocyte ranged from 1 to 10, similar to that described previously *in vivo* in the corpus callosum. When an oligodendrocyte began to myelinate, it immediately myelinated a maximum number of fibers, suggesting that the number of axons to be myelinated by the oligodendrocyte was predetermined. When only one fiber was in the vicinity of a myelinating oligodendrocyte, whorls of myelin-like figures were seen at the tip of oligodendrocyte processes that had not reached an axon. Myelinated fibers were unambigously identified as axons both by immunostaining and by electron microscopy. Myelin was not observed around astrocyte processes or around dendrites. The exclusive myelination of axons suggests the existence of a specific axonal recognition signal which attracts oligodendrocyte processes.**

In the central nervous system (CNS), oligodendrocytes have the unique ability to elaborate large amounts of membrane. These membranous sheaths wrap around axons and compact to form mature myelin. Adjacent myelin sheaths are separated on the axon by the nodes of Ranvier, permitting saltatory conductance.

Much has been learned about the sequences of events related to the differentiation of glial progenitor cells into oligodendrocytes (1–3) and the maturation of newly differentiated oligodendrocytes into mature oligodendrocytes (4, 5). These studies have been performed on purified or enriched cultures of oligodendrocyte progenitors or mature oligodendrocytes. Although these preparations are virtually devoid of neurons, oligodendrocytes in culture can produce myelin-specific lipids and proteins and synthesize myelin-like figures (6–9). These results suggest that the last stages of oligodendrocyte maturation preceding myelination are regulated intrinsically within the oligodendrocyte itself. However, while in the peripheral nervous system the signal for nerve engulfment and ensheathment is known to reside in axons with which Schwann cells interact (10), very little is known about the factors which induce or regulate myelination in the CNS.

We report here a reproducible system of *in vitro* myelination using dissociated cultures from embryonic mouse brain. In these cultures, all types of CNS cells are present, as *in vivo*, and some aspects of *in vivo* myelination, such as the number of segments myelinated by a single oligodendrocyte and exclusive myelination of axons, are mimicked.

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MATERIALS AND METHODS

Antibodies. Mouse monoclonal anti-galactosylceramide (GalC) antibody-containing hybridoma culture supernatant was kindly provided by B. Rantsch (11) and diluted 1:30 in Dulbecco's modified Eagle's medium (DMEM, Seromed, Munich) containing 10% fetal bovine serum (FBS; Flow Laboratories). Mouse monoclonal antibodies (ascites fluid) against microtubule-associated protein 2 (MAP-2 antibody, IgG1) (12) and neuron-specific β-tubulin (TuJ1 antibody, IgG2a) (13) were produced by one of us (A.F.) and diluted 1:120 (MAP-2) or 1:150 (TuJ1). A mouse monoclonal antibody directed against the six consecutive phosphorylated repeats of the high molecular weight subunit of neurofilament (RMO-24, IgG1, culture supernatant) (14) was produced (V.M.-Y.L.) and diluted 1:10. Rabbit polyclonal anti-myelin basic protein (MBP) antiserum was produced (H.V.) and diluted 1:100. Anti-MBP, anti-MAP-2, TuJ1, and RMO-24 antibodies were diluted in DMEM/10% FBS/0.2% Triton X-100. Rhodamine-conjugated goat antibody against mouse IgG3, fluorescein-conjugated goat antibody against mouse IgG2a or IgG1, and fluorescein-conjugated goat anti-rabbit IgG affinity-purified antibodies were from Southern Biotechnology (Birmingham, AL) and diluted 1:100. Rhodamine-conjugated swine anti-rabbit IgG antibody (Dakopatts, Glostrup, Denmark) was diluted 1:100. All conjugated antibodies were diluted in DMEM/10% FBS.

Cell Cultures. Forebrains were removed from 15-day mouse fetuses, dissociated first mechanically and then by digestion (15 min at 37°C) with 0.05% trypsin (Seromed) in Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution (GIBCO) for 20 min at 37°C, washed, and passed gently through nylon mesh (63-μm pores). After washing, the pellet was suspended in DMEM/10% FBS/0.028% bovine serum albumin (Miles). Cells were plated on poly(L-lysine) (Sigma) -coated 14-mm diameter glass coverslips in 24-well plates (Costar), at 5×10^4 cells per coverslip. The cells were layered in 10 μl of medium for 30 min in an air/CO_2 incubator to facilitate attachment, and then 500 μl of the final culture medium was added to each well. The culture medium consisted of B-S medium (15), supplemented with 1% FBS, 1% penicillin/streptomycin solution (Seromed), and recombinant platelet-derived growth factor AA (UBI, New York) at 10 ng/ml. All cultures were kept in a humidified incubator with a 5% CO_2 atmosphere at 37°C. Culture medium was changed twice a week.

Immunostaining. For indirect immunofluorescence, cells were fixed in 4% paraformaldehyde, rinsed, incubated for 30 min in normal sheep serum diluted 1:1 in DMEM/10% FBS, and then incubated for 25 min with anti-GalC antibody and for 25 min with an anti-IgG3 rhodamine-conjugated antibody. Coverslips were briefly rinsed, ethanol-fixed for 4 min at

Abbreviations: CNS, central nervous system; FBS, fetal bovine serum; GalC, galactosylceramide; MBP, myelin basic protein; MAP-2, microtubule-associated protein 2.

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room temperature, and rinsed before incubation for 25 min with a mixture of anti-MBP and either TuJ1, MAP-2, or RMO-24 antibodies. Excess antibodies were washed off and the cells were incubated with a mixture of rhodamine-conjugated anti-rabbit IgG and fluorescein-conjugated anti-mouse IgG2a or IgG1 antibodies. Anti-GalC and -MBP staining were revealed by using second antibodies conjugated to the same fluorochrome (rhodamine) in order to improve the visualization of the connection between the myelinating oligodendrocytes and the myelin sheaths. Coverslips were then mounted in Gelvatol (Monsanto) containing *n*-propyl gallate (Sigma) to prevent fading of fluorescence (16). Controls performed either by omitting the first antibodies or by incubating one of the three first antibodies with a mixture of the two nonrelated labeled antibodies were always negative.

Electron Microscopy. Culture medium was removed and the cells were washed with phosphate-buffered saline (PBS: 0.15 M NaCl/0.01 M/sodium phosphate, pH 7.4) before

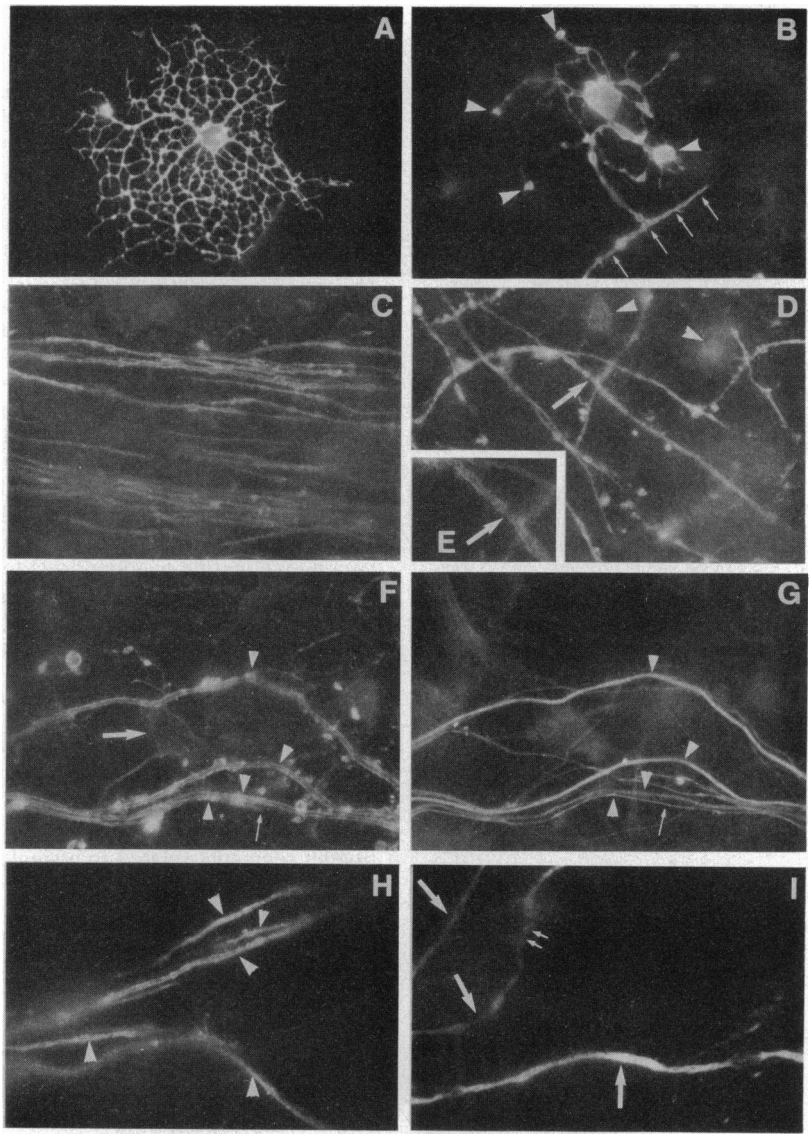

FIG. 1. Myelination of neurites in culture. Dissociated cultures from embryonic day 15 mouse brain were cultivated for 2 weeks and immunostained with either anti-GalC antibody revealed by a rhodamine-labeled anti-mouse IgG3 (*A*) or a combination of anti-GalC and anti-MBP antibodies revealed by a mixture of rhodamine-labeled anti-mouse IgG3 and anti-rabbit IgG antibodies (*B–F* and *H*). Mature, nonmyelinating oligodendrocyte (*A*) has an extremely dense network of processes composed of primary (emerging from the cell body), secondary (branched on the primary process), and tertiary (interconnecting primary and secondary processes) processes. A myelinating oligodendrocyte (*B*) has been dramatically altered in morphology by complete loss of tertiary processes and by a decrease in the number of secondary and even primary processes. This cell (*B*) is myelinating one fiber (arrows) and the other processes are forming whorls of myelin-like figures at their tips (arrowheads). Both parallel arrays (*C*) and unordered fibers (*D*) were myelinated. Myelinating oligodendrocytes were usually not easy to see in these fields of myelinated fibers, as they were located above the fibers. Arrowheads (*D*) point to the cell bodies of myelinating oligodendrocytes that are out of focus. Arrow (*D*) points to a node of Ranvier, better seen in *E*, which is an enlargement of this part of the photograph. Neurites were labeled with TuJ1 antibody revealed by a fluorescein-conjugated anti-mouse IgG2a antibody (*G*). Arrowheads point to four myelinated fibers which were $GalC^+/MBP^+$ (*F*) and $TuJ1^+$ (*G*). The cell body of the myelinating oligodendrocyte (large arrow in *F*) is barely visible because it was not in the same plane of focus. Note the perfect colocalization of the $TuJ1^+$ fibers in the middle of the double line of the $MBP^+/GalC^+$ myelin sheaths. These fibers are not myelinated on their whole length and the small arrow indicates, for the fiber in the lower part of the picture, where the myelin sheath ends (*F*). In *G*, other nonmyelinated $TuJ1^+$ fibers are also seen. Absence of myelination around dendrites was shown by using MAP-2 antibody to specifically label dendrites (*I*). Arrowheads point to five myelinated fibers ($GalC^+/MBP^+$) (*H*), which do not colocalize (*I*) with any of the MAP-2 dendrites (arrows). Paired small arrows (*I*) point to a $MAP\text{-}2^+$ neuronal cell body. (*A–C*, ×275; *D*, ×300; *F* and *G*, ×425; *H* and *I*, ×400; *E*, ×780.)

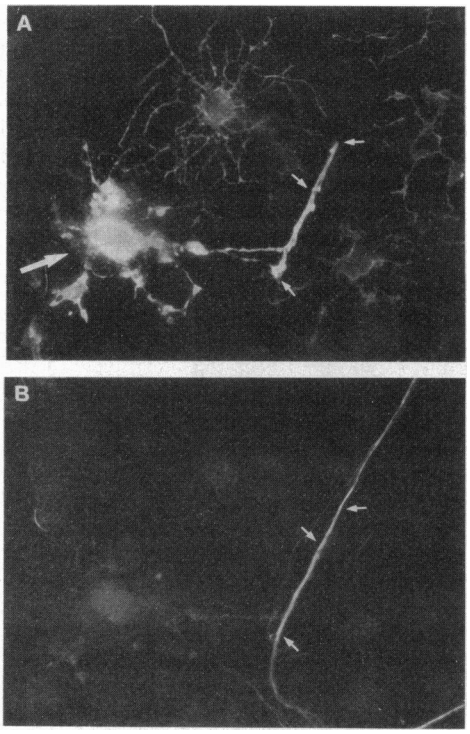

FIG. 2. Oligodendrocyte myelinating an axon. Oligodendrocyte cell body and processes were labeled with anti-GalC and anti-MBP antibodies (*A*). Axons were labeled with RMO-24 antibody (*B*). In this field several oligodendrocytes can be seen, but only one (large arrow in *A*) is myelinating; small arrows (*A* and *B*) point to the part of the axon which is wrapped in a myelin sheath. Only one axon is in the vicinity of the myelinating oligodendrocyte and, as illustrated in Fig. 1, the other processes of the myelinating oligodendrocyte end with myelin-like whorls. (×330.)

fixation with 2.5% glutaraldehyde in PBS for 2 hr at 25°C. After three washes in PBS, cells were postfixed in 2% OsO_4 for 30 min. After a brief passage in distilled water and dehydratation in a graded series of ethanol solutions, the cultures still on the coverslips were embedded in araldite. The blocks of araldite were separated from the coverslips by thermal shock with liquid nitrogen. Ultrathin sections were cut parallel to the surface of the cultures. After counterstaining with lead citrate, sections were examined with a JEOL 1200 EX electron microscope operated at 70 kV.

RESULTS

Identification of Myelinating Oligodendrocytes. During week 2 in culture, the oligodendrocyte morphology changed dramatically. This change was apparent with anti-GalC. The number of processes increased and most of the oligodendrocytes rapidly acquired a typical stellate appearance with numerous primary processes emerging from the cell body and then dividing to form secondary processes and even tertiary ones which established connections between the primary and secondary branches (Fig. 1*A*). The highly branched oligodendrocytes also expressed MBP. Then, at 13–14 days *in vitro*, some of the oligodendrocytes resorbed most of their processes. In some instances, it was possible to see one of the remaining processes divide and run in opposite directions along the length of fibers that were identified as neurites (see below). These processes wrapped around the neurites to form a myelin sheath easily recognizable by the typical double outline, which could best be observed with a combination of anti-GalC and anti-MBP (Fig. 1*B*). Immunostaining with anti-GalC allowed clear visualization of oligodendrocyte cell bodies and processes (Fig. 1*A*) but failed to consistently demonstrate the presence of a myelin sheath (data not shown). In contrast, anti-MBP clearly immunolabeled myelin sheaths but poorly stained the oligodendrocyte cell bodies, because MBP migrates out of the cell bodies of the myelinating oligodendrocytes (17, 18). Preparations were therefore double-labeled with both anti-GalC and anti-MBP. In addition, when an oligodendrocyte myelinated only one or two fibers, the processes that had not contacted a neurite ended in a whorl intensely stained with anti-GalC and anti-MBP (Fig. 1*B*). These round formations were unambiguously identified as myelin-like figures by electron microscopy (see below and Fig. 3*D*).

Myelination in Culture. Myelination almost always occurred in regions of high cell density where there were large bundles of fibers. Myelinated fibers were rarely isolated. Even when myelination had just started, several fibers in proximity, either intermingled or disposed in parallel in a bundle (Fig. 1 *C* and *D*), were myelinated, suggesting that a single oligodendrocyte started to myelinate segments of several fibers at the same time. Most often, only one segment was myelinated on each fiber, although in a few cases, on a single fiber, several adjacent myelinated segments separated by a short nonmyelinated space were observed (Fig. 1*E*). These images were suggestive of nodes of Ranvier. Despite double GalC/MBP immunolabeling, when a field of myelin fibers was observed it was often difficult to unambiguously follow all the connections between a myelinating oligodendrocyte and all the fibers it myelinated. There were two reasons for this. Numerous oligodendrocytes were located close to the myelinated segments, and discrimination between myelinating oligodendrocytes and nonmyelinating oligodendrocytes was based primarily on their distinct morphology. Oligodendrocytes were located above the myelinated fibers, and the oligodendroglial cell body was rarely in the same plane of focus, making it difficult to follow the oligodendroglial processes all the way from the cell body to the myelin segment. When an unambiguous relationship between one oligodendrocyte and myelinated segments was observed, the myelinated segments were counted. The mean number of segments myelinated by a single oligodendrocyte was 4–5, with ratios ranging from 1:1 to 1:10. The number of myelin segments related to a single oligodendrocyte did not vary with time in culture. Indeed, when myelination was first observed after 13–14 days *in vitro*, patches of 10 myelinated segments per oligodendrocyte were already apparent and the maximal ratio of myelinated fibers per oligodendrocyte did not increase with time in culture. However, the number of myelinated fibers per unit of surface increased gradually between 13 and 22 days. The number of myelinated segments per 50 mm^2 was 113 ± 48, 315 ± 152, and 405 ± 153 at 15, 18, and 22 days, respectively (mean ± SEM, n ≥ 10 cultures). At 30 days, the number of myelinated fibers per 50 mm^2 dropped to 165 ± 45 as a result of demyelination, which was confirmed by the appearance of small $GalC^+/MBP^+$ droplets.

Myelin Is Deposited Along Axons. In preliminary experiments (data not shown) we never observed myelination of astrocyte (glial fibrillary acid protein-positive) processes. We therefore assumed that myelinated fibers were most probably neurites. To verify this observation, we attempted to identify myelinated fibers with the TuJ1 antibody, which is specific for neuronal β-tubulin and stains all neurites (axons and dendrites) as well as the neuronal cell body. $GalC^+/MBP^+$ myelinated fibers were always $TuJ1^+$ (Fig. 1 *F* and *G*). Moreover, $TuJ1^+$ myelinated neurites were long, straight, and often organized in bundles, strongly suggesting that these myelinated fibers were axons. To characterize more specifically the myelinated neurites, we labeled the $GalC^+/MBP^+$ myelinated fibers with either the MAP-2 antibody, which recognizes the somatodendritic domain of neurons, or the

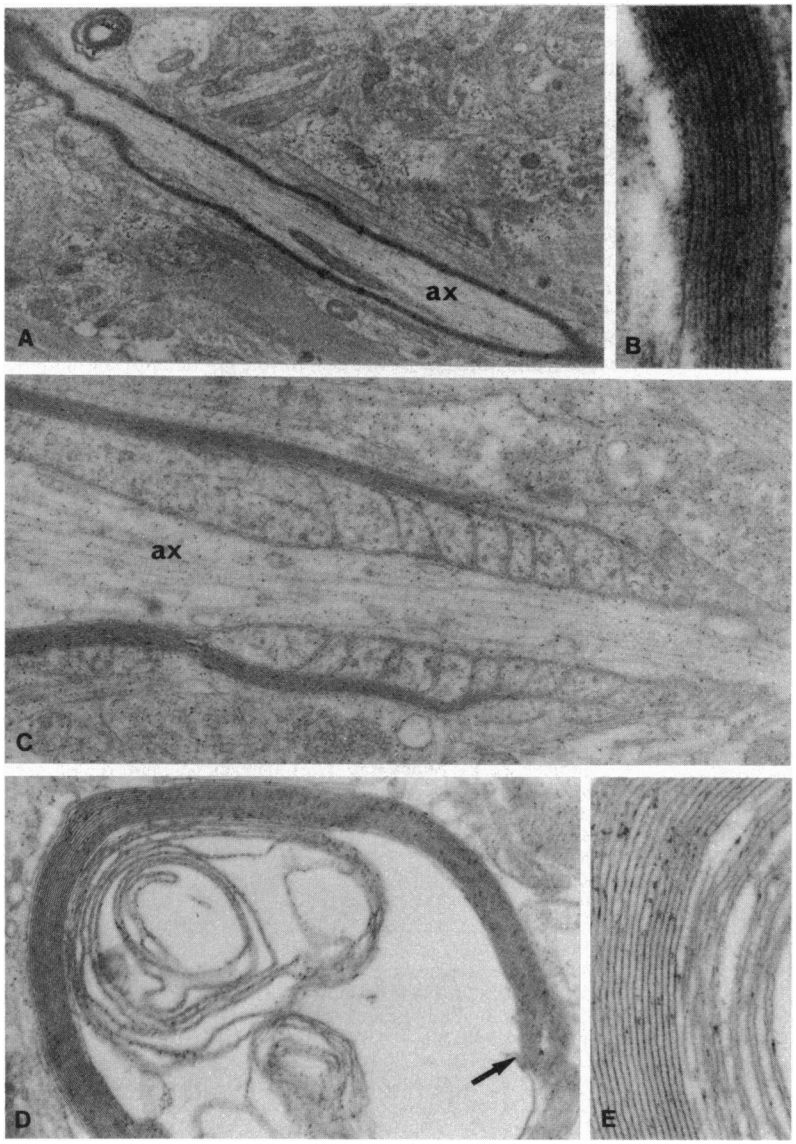

FIG. 3. Transmission electron micrographs of myelinated axons and myelin-like structures in culture. Sublongitudinal section (*A*) shows microtubules and a mitochondrion in the axon (ax). Myelin deposited around the axons is well compacted as illustrated by the juxtaposition of major dense lines and intraperiodic lines (*B*). The paranodal region of a longitudinally sectioned myelinated axon (*C*) illustrates the region where the myelin sheath ends and becomes progressively thinner as the lamellae terminate. Each lamella extends beyond the one beneath it and opens up to enclose paranodal cytoplasm. When an oligodendrocyte process has not encountered an axon to myelinate, it wraps around itself and piles up to form a myelin-like figure. Arrow (*D*) points to the region where the oligodendrocyte process folds up in several layers to form this myelin-like figure, which is not as compact (*E*) as when deposited around an axon (*B*). (*A*, ×8250; *B*, ×180,000; *C*, ×30,000; *D*, ×26,250; *E*, ×75,000.)

axon-specific RMO-24 antibody. Myelin sheaths were never seen around MAP-2$^+$ dendrites (Fig. 1 *H* and *I*). In contrast, GalC$^+$/MBP$^+$ myelin sheaths colocalized with RMO-24$^+$ axons (Fig. 2), indicating that only axons were myelinated in culture. Moreover, when preparations were analyzed with a dual-band optical filter set (19), which allowed simultaneous observation of fluorescein and rhodamine fluorochromes, the fluorescein-labeled RMO-24 axons were clearly fringed with the double outline of a GalC/MBP rhodamine-labeled myelin sheath.

Ultrastructural Analysis. Myelinated fibers were unambiguously identified as axons by the presence of microtubules streaming in parallel lines and by the absence of ribosomal clusters (Fig. 3 *A–C*). Myelin deposited around axons appeared to have a typical trilamellar aspect, with a succession of major dense lines and intraperiodic lines separated by a clear space (Fig. 3*B*). In some instances, nodes of Ranvier were observed, with the myelin sheath of the paranodal region becoming progressively thinner as successive lamellae terminated to enclose pockets of paranodal cytoplasm (Fig. 3*C*). Aside from typical myelin circularly arranged around axons, configurations of other types, such as redundant loops and double concentric sheaths, were also observed (data not shown). In addition to myelin deposited around axons, a second type of myelin figure was seen, corresponding to oligodendrocyte processes winding around themselves (Fig. 3 *D* and *E*). These myelinated structures, which correspond most probably to the GalC$^+$/MBP$^+$ whorls seen at the optical level (Fig. 1*B*), had two characteristics. (*i*) The oligodendrocyte process did not really wind around itself but, rather, folded up on itself, resulting in the piling up of segments of the process to form a myelin-like structure (Fig. 3*D*). (*ii*) These myelin figures were not densely compacted, as illustrated by the absence of intraperiodic lines (Fig. 3*E*). Myelin was not observed around dendritic or astrocytic processes.

DISCUSSION

To study neuron–oligodendrocyte interactions, we have developed dissociated primary cell cultures derived from day 15 embryonic mouse brains in which myelination occurs after 12–13 days *in vitro*.

Most of the earlier work describing *in vitro* myelination utilized long-term CNS organotypic cultures and was essentially aimed at studying demyelinating agents present in the serum of animals with experimental allergic encephalomyelitis or of patients with multiple sclerosis (20). Because cell density is high in organotypic cultures, they are not well suited to study cell–cell interactions. Myelination in dissociated CNS cultures has already been reported in cultures derived from embryonic day 15 rat brain (21); myelin was detected by phase-contrast and electron microscopy after 10

days *in vitro*. Later, Bunge and coworkers (22) developed a coculture system in which mature oligodendrocytes were added to dorsal root ganglion neurons and successfully enveloped neuritic processes, forming myelin sheaths. This elegant system demonstrated that mature oligodendrocytes are able to remyelinate *in vitro* (23), that astrocytes affect myelination (24), and that dorsal root ganglion axons have a mitogenic effect on mature oligodendrocytes (25, 26). One limitation of the dorsal root ganglion myelination system is that only part of dorsal root ganglion axons are in the CNS, so that some specific properties of CNS axons may be absent or difficult to detect.

In the peripheral nervous system, Schwann cells myelinate only one segment of an axon, whereas in the CNS, the number of axons myelinated by a single oligodendrocyte is quite variable. This was already noted in the initial description by Del Rio-Hortega (27) of oligodendrocytes related either to several small axons or to single large axons. The number of myelin segments elaborated by a single oligodendrocyte remains controversial, however. Peters and Proskauer (28) calculated that, in the optic nerve, an individual oligodendrocyte may elaborate and maintain up to 30–50 separate myelin sheaths. Matthews and Duncan (29) reported an oligodendrocyte/myelin segments ratio of 1:40–60. To explain discrepancies among these earlier studies, heterogeneity within the oligodendrocyte population or in axon diameter has been invoked. More recently, Remahl and Hildebrand (30) described different regional patterns for the glial unit. They reported that, in the cat spinal cord, the relationship between compacted myelin segments and oligodendrocytes is 1:1, while in the corpus callosum an average of 9 segments can be related to one oligodendrocyte. These results are in agreement with the 1:10 ratio reported in the present study, since our cultures were derived from cerebral hemispheres, where the majority of oligodendrocytes myelinate the corpus callosum. The frequently observed smaller patches (1–4 segments) might correspond to oligodendrocyte processes that had not managed to contact axons. The observation of whorls of myelin at the tip of the processes of oligodendrocytes myelinating only one fiber is consistent with this hypothesis (Figs. 1*B* and 3 *D* and *E*). The number of myelinated segments per oligodendrocyte did not increase with time in culture, as if, when an oligodendrocyte starts to myelinate, it myelinates from the start a maximum of fibers. In this respect, it would be of interest to study the ratio of oligodendrocytes to myelinated segments in cultures from other CNS regions known to have a different ratio of oligodendrocytes to myelinated fibers, such as the spinal cord or the optic nerve.

Although it is well accepted that only axons are myelinated *in vivo*, several authors have reported that oligodendrocytes are able to elaborate myelin-like figures in culture in the absence of neurons (6–9). Formation by oligodendrocytes of myelinated structures around carbon fibers has also been described (31). These results suggest that the myelination process in oligodendrocytes could be at least partially independent of axonal influence. We also observed formation of myelin whorls at the tips of oligodendrocyte processes (Fig. 1*B*). Nevertheless, and as already reported by others, these structures are myelin-like figures, not well-compacted myelin (Fig. 3 *D* and *E*).

In contrast, under our culture conditions, except for these myelin-like whorls, myelination occurred only around neurites, as demonstrated by immunostaining of myelinated fibers with the TuJ1 antibody. Further, only axons and not dendrites were myelinated. This was shown both by electron microscopy and by the specific labeling of myelinated neurites with the axon-specific RMO-24 antibody and the absence of colocalization of myelinated fibers with the MAP-2 antibody. RMO-24 recognizes highly phosphorylated neurofilament epitopes and preferentially stains axons (14). MAP-2 staining is restricted to the neuronal somatodendritic domain (32, 33). This spatial distribution of MAP-2 is not observed during the first week *in vitro* (i.e., before myelination starts) but is evident in older cultures (33). The selective myelination of axons suggests the existence of a recognition signal which attracts oligodendrocyte processes.

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