Pergamon

J Steroid Biochem Molec Biol. Vol. 48, No. 1, pp. 145–154, 1994

0960-0760/94 $6.00 + 0 00

0960-0760(93)E0012-V

Actions of Steroid Hormones and Growth Factors on Glial Cells of the Central and Peripheral Nervous System

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Primary cultures of oligodendrocytes and astrocytes and purified cultures of Schwann cells were prepared respectively from forebrain and sciatic nerves of newborn rats. The effects of steroid hormones and growth factors on glial cell growth and on the production of myelin-specific proteins and lipids were investigated. Progesterone (P, 100 nM) decreased the proliferation of glial cells of the central nervous system. This inhibitory effect of P was abolished by the simultaneous administration of the antagonist RU486, thus suggesting a receptor-mediated action of the hormone. The expression of myelin-specific proteins, including the myelin basic protein (MBP) and the 2′,3′-cyclic nucleotide-3′-phosphodiesterase (CNPase), and of a myelin-specific lipid, galactocerebroside (Gal C), was also measured during cell differentiation under different hormonal conditions. The expression of MBP in oligodendrocytes was increased by P, and this effect was not blocked by RU486. The combined application of P and insulin promoted a synergistic stimulation of MBP expression. Insulin, by itself, also increased the number of MBP-positive oligodendrocytes in culture. The effects of P and insulin appeared to be selective as dexamethasone, dehydroepiandrosterone, pregnanolone and epidermal growth factor (EGF) had no effect. Only estradiol (E_2, 500 nM) increased the number of MBP-immunoreactive cells, but in contrast to P, only a small synergism between E_2 and insulin on MBP expression was observed. The expression of CNPase, another myelin-specific protein, was also increased by P and, here again, a synergy between P and insulin could be observed. In contrast, the expression of Gal C, a myelin-specific lipid, was not modified by P or other steroid hormones. Moreover, the increase in Gal C-positive cells observed in response to insulin alone was not further potentiated by P. Glial cells of the peripheral nervous system, namely Schwann cells, are also sensitive to steroid hormones. Schwann cells contain estrogen receptors, and E_2 stimulates their proliferation in the presence of forskolin or dibutyryl cyclic AMP (dbcAMP). The mitogenic effect of E_2 was abolished by the pure antiestrogen ICI-164,384. Insulin, at micromolar concentration, also stimulated Schwann cell growth when forskolin or dbcAMP were present in the culture medium. The mitogenic effect of insulin was mediated by insulin-like growth factor I (IGF-I) receptors. Indeed, at a physiological nanomolar concentration, IGF-I but not insulin or IGF-II, increased the proliferation of Schwann cells in synergy with forskolin. In addition, Schwann cells express receptors for IGF-I.

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INTRODUCTION

Steroid hormones have profound influences on the growth, maturation, differentiation and functioning of brain cells. The action of steroid hormones requires activation of gene transcription, which is mediated by specific high-affinity intracellular receptors [1]. The presence of receptors for steroid hormones within different regions of the brain has been described by several groups (for review see [2, 3]), but in glial cells, only the presence of glucocorticoid receptors (GR) has been reported [4]. Using primary cultures of rat glial cells, we have shown that glial cells contain, in addition to GR, receptors for progesterone (PR), estrogen (ER) and androgen (AR). We also demonstrated the estrogen-inducibility of PR during primary culture as well as sex differences of this PR induction. Indeed, estradiol (E_2) substantially increased the amount of PR in glial cell cultures established from newborn female

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Received 8 June 1993; accepted 11 Oct. 1993

pups, while very little PR induction was observed after E_2 treatment in male cultures [5, 6].

In agreement with the presence of intracellular receptors for steroids, glial cells of the CNS are sensitive to the actions of steroid hormones. For instance, P inhibits and E_2 stimulates cell proliferation. Both steroid hormones also influence glial cell morphology and differentiation, as was seen by an increased synthesis of myelin proteins in oligodendrocytes [6]. In the central nervous system (CNS), myelin is formed by oligodendrocytes. These cells are responsible for the maintenance of myelin, a multilayered assembly of membranes, which permits rapid and safe conduction of nerve impulses along axons [8]. In rodents, central myelin is composed of about 70% lipids [9], among which galactocerebroside (Gal C) is a specific marker for oligodendrocytes [10], and of about 30% proteins. The myelin proteins are mainly composed of proteolipid protein, myelin basic protein (MBP), 2′,3′-cyclic nucleotide-3′-phosphodiesterase (CNPase) and myelin-associated glycoproteins [9, 11]. Very little is known about the influence of steroid hormones on the expression of myelin-specific proteins during glial cell differentiation. In the present work, with the use of specific antibodies to Gal C, MBP and CNPase, we report studies of the expression of these specific oligodendrocyte markers during the first 10 days of primary culture in the presence or absence of steroid hormones and/or antihormones.

In the peripheral nervous system (PNS), myelin is formed by Schwann cells. As oligodendrocytes, Schwann cells from neonatal rat sciatic nerves can be grown in culture and secondary cultures preserve basic Schwann cell functions [12]. However, in contrast to oligodendrocytes, which differentiate and produce myelin proteins in cell culture in the absence of neurons, Schwann cells remain undifferentiated. However, under these conditions, the effects of mitogens can be tested [12]. The proliferation of Schwann cells plays an important role during the development and regeneration of myelin. Schwann cells were the first cells for which cyclic AMP (cAMP) was recognized as a mitogen in culture [13]. It was found that cAMP is not mitogenic by itself, but it promotes the division of Schwann cells by increasing their sensitivity to peptide growth factors [14, 15].

During studies of the action of steroid hormones on Schwann cell proliferation, we found that E_2 increased cell growth in synergism with cAMP [16]. This mitogenic effect was abolished by the antagonist ICI-164,384, a pure antiestrogen compound [17]. As we have demonstrated the presence of specific ER in Schwann cells [16], it is possible that the mitogenic action of E_2 is mediated by intracellular receptors.

We have also shown that insulin-like growth factor I (IGF-1) stimulates the proliferation of Schwann cells but again only in the presence of elevated levels of cAMP [18]. Using binding studies and immunofluorescence staining, we showed that Schwann cells express receptors for IGF-I and that the amount of binding sites for IGF-I was increased by cAMP [18]. We now describe additional studies of the action of steroid hormones and growth factors on Schwann cell proliferation.

EXPERIMENTAL

Glial cells of the CNS

Primary cultures of glial cells were established from newborn Sprague–Dawley rats as described in detail previously [5, 6]. At the day of birth (day 1 of culture), cerebral hemispheres were mechanically dissociated in culture medium containing serum [Dulbecco's Modified Eagle medium, containing 10% fetal calf serum (FCS), glucose (1.8 mg/ml), penicillin (100 UI/ml) and streptomycin (100 μg/ml)]. This medium will be named DME-F. For the measure of steroid hormone effects, FCS was replaced by 10% charcoal-treated calf serum (DME-CX). After cell dissociation, the cell suspension was filtered and plated on poly-L-lysine (PLL)-coated plastic dishes, or onto PLL-treated glass coverslips. When hormone effects were measured, cells were allowed to attach in DME-F overnight, and the following day, DME-CX was given and the corresponding hormones, antihormones or growth factors were added as will be described for each experiment. Media were changed every 2 to 3 days, hormones were added daily.

Immunofluorescence staining of myelin-specific proteins. The immunofluorescence procedure has been described in detail previously [7, 19]. Briefly, cells were cultured on glass coverslips in Petri dishes. For the measure of MBP and CNPase, coverslips were washed, fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 30 min, and after washing, cells were permeabilized with a 1% solution of Triton X-100 in PBS for 4 min. After washing, the monoclonal antiMBP (Boehringer, Mannheim, Germany), or the monoclonal antiCNPase (Sigma Immunochemicals) was applied for 30 min, followed, after washing, by the fluorescein coupled goat antimouse IgG, diluted 1:20, for another 30 min.

Immunofluorescence staining of Gal C was done using a monoclonal antibody to Gal C (clone m Gal C from Boehringer, Mannheim). AntiGal C was added to living cells for 30 min and, after fixing in 4% paraformaldehyde, the second fluorescein coupled goat antimouse IgG was applied. The coverslips were stained with Evans blue and mounted in Moviol. The cells were observed with a Leitz Laborlux D photomicroscope.

The number of MBP-, Gal C- and CNPase-positive cells was estimated by counting the number of fluorescent cells among unlabeled oligodendrocyte-like cells. About 100 random grids on different coverslips were counted. Unlabeled oligodendrocytes and astrocytes can be distinguished easily after staining with Evans blue at the end of the immunofluorescence

labeling, and the number of antigen-positive cells among 2000 oligodendrocyte-like cells are indicated. Counts were done in triplicate for each experiment and analyzed by non-parametric statistics (Kruskal-Wallis one-way analysis of variance and Mann-Whitney U tests for between-group comparisons). Experiments were repeated several times, each one with a new primary culture.

Cell growth experiments. At day 6 of culture, cells were subcultured and replated in PLL treated 60 mm Petri dishes in DME-CX medium in the absence of hormone. 24 h later, when cells were attached, hormones or antihormones were added to the medium. Media were changed every 2 days, hormones or antihormones were added every day. At the indicated days of culture, triplicate dishes were counted with a hemocytometer. The numbers of cells determined were analyzed by one-way ANOVAS followed by Tukey's tests. For each cell growth experiment, a new primary culture was used and experiments were repeated several times.

Glial cells of the PNS

Schwann cells from peripheral nerves were established as described in detail previously [16]. Briefly, sciatic nerves from 4- to 5-day-old Sprague–Dawley rats were minced into small fragments and the tissue was digested in tissue culture medium containing 0.25% trypsin and 0.1% collagenase type A (Sigma C-9891) for 40 min at 37°C by gently stirring in a cell incubator. After dissociation, FCS was added to the suspension and cells were recovered by centrifugation. After washing the pellet in DME-F, cells were plated onto 25 cm^2 tissue culture flasks precoated with PLL. After 2 days of culture, cytosine arabinoside (10^{-5} M) was added for 48 h to eliminate contaminating fibroblasts. The cultures were then washed and grown in DME-F supplemented with forskolin (Sigma, 2 μg/ml) to stimulate Schwann cell growth. When cell confluency was reached, residual fibroblasts were eliminated by antiThy 1.1 treatment and complement, as was described previously [20]. Cells were recovered by trypsin, centrifuged, and the pellet resuspended in 0.2 ml antiThy 1.1 monoclonal antibody (Cederlane, Canada), diluted 1:4 in DME-F. After 30 min incubation at 37°C, cells were centrifuged, the pellet recovered in 0.1 ml DME-F and 0.1 ml Low-Tox-M Rabbit complement (Cederlane) and incubated for 30 min at 37°C. Cells were then centrifuged, washed in DME-F and plated in PLL-treated plastic flasks. By this procedure, highly purified Schwann cells were obtained which were multiplied by serial trypsination and dilution and were kept in culture for several weeks.

Cell growth experiments were done with pure Schwann cells, plated on PLL-treated 60 mm Petri dishes as described for glial cells of the CNS. For each cell growth experiment, a new primary culture was used and experiments were repeated several times.

RESULTS

Glial cells of the CNS

Effect of progesterone on glial cell proliferation. Primary cultures of rat glial cells contain two main cell types, oligodendrocytes and astrocytes. Generally the cultures consisted of 50–60% oligodendrocytes and 40–50% astrocytes. Some ependymal cells were also present. These mixed glial cultures were trypsinized on day 6 and replated at low density (220,000 cells per 60 mm Petri dish) in DME-CX medium. After attachment, P (100 nM), or the antagonist RU486 (200 nM), or both together, were added to the culture medium and the number of cells was determined after different days of treatment. As shown in Fig. 1, P decreased cell multiplication and this decrease was inhibited when the anti-progestin RU486 was present together with the hormone. RU486 had a slight stimulatory effect by itself, but only on days 17 and 20, when high cell density was reached.

As can be seen in table 1, the amount of MBP-expressing cells was consistently higher in the presence of P when compared to control cultures. As we had

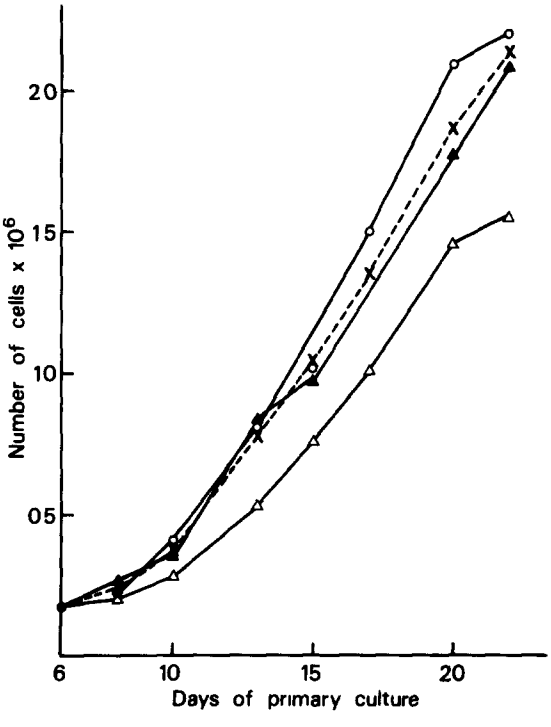

Fig. 1. Growth of rat brain glial cells in primary culture. Primary cultures were established at day 1 (day of birth) as described in Experimental. At day 6, cells were subcultured and replated in 60 mm Petri dishes in the absence of hormone. 24 h later, hormone-containing medium was given. Media were changed every 2 days, hormones were added daily. Triplicate dishes were counted with a hemocytometer and mean values are indicated, not differing one from another by >10%. One-way ANOVAS showed a significant effect of treatment on days 13 to 22, when progesterone significantly inhibited cell growth (control vs progesterone: $P \leq 0.01$ on each day by Tukey's tests). RU486 increased cell proliferation on day 20 ($P \leq 0.05$). (X---X) control; (△—△) +P (100 nM); (○—○) +RU486 200 nM; (▲—▲) +P (100 nM) and +RU486 (200 nM).

Table 1 MBP-positive oligodendrocytes at different days after primary culture

Days of culture	MBP-positive cells/2000 cells median (range)			
	Control	+P	+RU486	+P + RU486
3	0 (0–0)	1 (1–4)	0 (0–0)	0 (0–0)
4	0 (0–0)	5 (3–6)	1 (0–2)	3 (2–4)
6	2 (1–3)	7 (5–7)	6 (4–8)	5 (5–8)
7	10 5 (9–13)	25.5* (15–28)	18* (15–21)	20* (17–25)
9	38 (33–40)	88* (85–92)	60* (58–62)	90* (80–93)
10	59 (49–62)	111* (98–120)	88 5* (85–92)	126* (125–128)

Glial cells were plated on coverslips the day of birth (day 1), and cultured in the absence (control) or presence of P, 500 nM, or RU486, 1 μM, or in the presence of P (500 nM) + RU486 (1 μM) At the indicated days, MBP was measured by indirect immunofluorescence staining using specific antibodies to MBP. The number of MBP-positive cells per 2000 counted oligodendrocyte-like cells was determined The counterstaining of coverslips with Evans blue permits to distinguish easily astrocytes which were not taken into account. The median and range of 3 independent measures (corresponding to different coverslips) are shown. *$P \leqslant 0.05$ when compared to the control group by Mann–Whitney U tests after Kruskal-Wallis one-way analysis of variance

Table 2. Immunofluorescence intensity of MBP in oligodendrocytes at different days of culture in the presence or absence of progesterone

Days of culture	Spot metering fluorescence (time of automatic exposure) median (range)	
	Control	+Progesterone
3	—	—
6	17 (10–26)	10** (7–14)
8	10 (7–13)	4** (2–7)
10	7 (5–10)	3** (2–6)

Glial cells were plated on coverslips at day 1 and cultured in the absence (control) or presence of P (500 nM). At the indicated days of culture, MBP was measured by indirect immunofluoresence staining and the fluorescence intensity of MBP within a single cell was determined by spot metering with a Leitz sensor. The sensor signal, converted to an electrical signal, was expressed as seconds necessary for automatic exposure. Reduced exposure time corresponds to increased fluorescence intensity The median intensities of 20 tested oligodendrocytes are indicated **$P \leqslant 0$ 01 when compared to control groups by Mann-Whitney U tests.

observed an antagonistic effect of RU486 during cell proliferation experiments, we examined whether the induction of MBP-expressing cells by P was inhibited in the presence of the antagonist. Surprisingly, the antiprogestin RU486 did not inhibit the induction of MBP by P, but instead showed some stimulatory effects by itself (Table 1).

When the immunofluorescence intensity of MBP-expressing cells within single oligodendrocytes was measured by spot metering with a Leitz sensor, the degree of fluorescence, reflecting weak, moderate or strongly labeled cells, was consistently higher in oligodendrocytes cultured in the presence of P (Table 2).

Estradiol also increased the number of MBP-expressing cells, although to a lesser extent than P. When the antiestrogen ICI-164,384, a pure antagonist devoid of estrogenic activity in rats and mice [17] was tested together with E_2, the MBP-expression induced by E_2 was not modified by the antagonist. ICI-164,384 alone had no significant effect on the amount of MBP-positive cells (Table 3).

Other steroids, such as pregnanolone, dehydroepiandrosterone or dexamethasone had no significant effect on MBP-expression (not shown).

As "*in vitro*" studies have shown that polypeptide growth factors influence glial cell growth [21], we tested the effect of EGF which has been reported to inhibit MBP expression [22]. In our experimental conditions, EGF, at 20 or 100 ng/ml, had no significant influence on MBP-expression during the first 10 days of primary culture (not shown).

It was reported that insulin increases MBP in oligodendrocytes [23] and that insulin and IGF-I support oligodendrocyte development [24–26]. In our previous studies of steroid hormone receptors in these glial cells [5, 6], we routinely added insulin to the culture medium which resulted in oligodendrocyte-enriched cultures. In the present studies, we measured the effects of insulin and steroid hormones on MBP expression. The glial cultures were treated from day 1 to 10 with P or E_2 in the presence or absence of insulin, or with insulin alone. On days 4, 7, 9 and 10, MBP-positive oligodendrocytes were counted and the results are shown in Fig. 2. The increase in MBP-positive cells in the presence of P or E_2 was similar to the experiments described above. In the presence of insulin, a

Table 3. MBP-positive oligodendrocytes at different days of primary culture

Days	Median (range)			
	Control	+E_2	+ICI	+E_2 + ICI
7	10* (10–11)	22* (20–24)	9 (8–10)	18* (16–21)
9	29 (28–35)	45* (39–48)	41 (35–45)	45* (35–48)
10	60 (57–62)	85* (82–91)	60 (52–68)	82* (77–90)

Glial cells were plated on coverslips at day 1 (birth of pups) and treated or not with E_2 (500 nM), or ICI-164,384 (ICI, 1 μM), or both together MBP was measured by indirect immunofluorescence staining at the indicated days of culture. The number of MBP-positive cells per 2000 counted oligodendrocyte-like cells was determined The median and range of 3 independent measures are shown *$P \leqslant 0$ 05 when compared to the control group by Mann-Whitney U tests after Kruskal-Wallis one-way analysis of variance

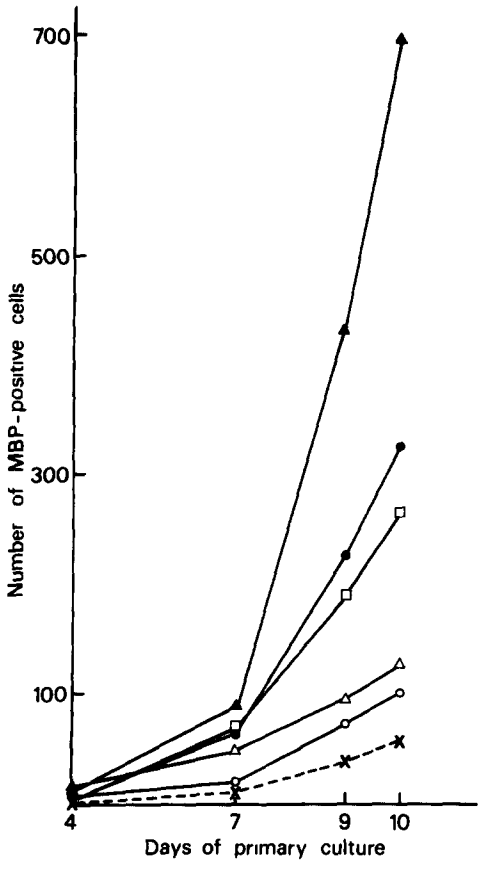

Fig. 2. MBP-expression in rat oligodendrocytes in primary culture. Glial cells were plated at day 1 on PLL-treated glass coverslips, as described in Experimental, and grown in DME-CX medium in the absence (X- - -X), or in the presence of P, 500 nM (△—△); E_2, 500 nM (○—○); insulin, 4.5 μg/ml (□—□); P, 500 nM +insulin, 4.5 μg/ml (▲—▲); or E_2, 500 nM + insulin, 4.5 μg/ml (●—●). Hormones were added daily, media were changed every 2 days. At the indicated days, immunofluorescence staining of MBP was done with the monoclonal antiMBP antibody as described in Experimental. Labeled and unlabeled cells were then counted and the number of MBP-positive cells per 2000 oligodendrocyte-like cells are indicated. The median and range of 3 independent measures are shown. On days 9 and 10, there were significantly more MBP-positive oligodendrocytes on coverslips treated with P, E_2, or insulin when compared to controls and on coverslips treated with insulin + progesterone when compared to insulin alone. For each comparison, $P \leq 0.05$ by Mann-Whitney tests after Kruskal-Wallis one-way analysis of variance. E_2 did not significantly increase the number of MBP-positive cells in the presence of insulin.

5- to 6-fold increase in MBP-positive oligodendrocytes was observed, but when P and insulin were present together in the culture medium, a 14-fold increase in MBP-expressing oligodendrocytes was observed, especially on days 9 and 10. The experiments were repeated several times, each one with a new primary culture. Representative immunofluorescence-stained cultures are shown in Fig. 3. In contrast to P, E_2 did not significantly increase the effect of insulin.

Expression of CNPase and Gal C. In separate experiments, the modulation of different myelin components, namely, MBP, CNPase and Gal C, was measured in oligodendrocytes cultured under different hormonal conditions. One of these experiments is shown in Fig. 4. Cells from the same primary culture were treated from days 1 to 10 with P or E_2 in the presence or absence of insulin, or with insulin alone. The number of antigen-expressing oligodendrocytes was then determined. As was observed for MBP expression, P alone slightly increased CNPase-expressing cells and again, the effect of P was potentiated by insulin. A 5- to 6-fold increase in CNPase expression was observed by insulin alone, whereas the addition of P to insulin resulted in a 10- to 11-fold increase in CNPase-positive cells. Estradiol had little effect by itself on CNPase expression and it did not potentiate the effect of insulin. In contrast to MBP and CNPase, Gal C expression was only increased by insulin and not by the steroid hormones.

Glial cells of the PNS

Effect of estradiol on Schwann cell proliferation. Purified Schwann cells were obtained by exposing primary cultures to cytosine arabinose and by treating them with an antiserum to Thy 1.1 and rabbit complement as described in Experimental. These purified Schwann cells in secondary culture expressed the neuroectodermal S-100 antigen, a specific marker for Schwann cells [16, 27]. In our recent studies we showed that Schwann cells contain specific and saturable binding sites for E_2. Treatment of the cells with forskolin, a potent activator of the cAMP-generating system [28], resulted in a 2-fold increase in ER [16]. We have also shown that E_2, at a 100 nM concentration, stimulated Schwann cell growth, but only in the presence of forskolin (5 μM) or dbcAMP (1 μM). One of these experiments is shown in Fig. 5. The addition of E_2 alone had no effect on cell growth, however, in the presence of forskolin, which had some stimulatory effect by itself, E_2 became a potent mitogen. The effects of lower doses of E_2 (1, 10 and 100 nM) on cell growth were then tested in the presence of dbcAMP (1 μM). The mitogenic effect of E_2 was already maximal at 1 nM (Fig. 6). The specificity of E_2 action on Schwann cell growth was tested by using the antagonist ICI-164,384, which completely blocked the mitogenic action of E_2 [16].

Effect of IGF-I on Schwann cell proliferation. We have recently shown that Schwann cells express receptors for IGF-I [18]. These receptors were visualized on the Schwann cell surface by indirect immunofluorescence staining using anti IGF-I receptor antibodies, and they were measured by binding assays. Scatchard analysis showed a single class of high-affinity receptors, and the amount of binding sites was increased by forskolin, from about 5000 to 12,000 binding sites/cell. IGF-I stimulated cell growth, but only in the presence of forskolin or dbcAMP [18]. In other experiments, we tested the effect of nerve growth factor (NGF) at the same concentration as used for IGF-I (20 ng/ml). As was seen in previous experiments, IGF-I strongly increased Schwann cell growth, whereas NGF, even in the presence of forskolin, had no effect (Fig. 7).

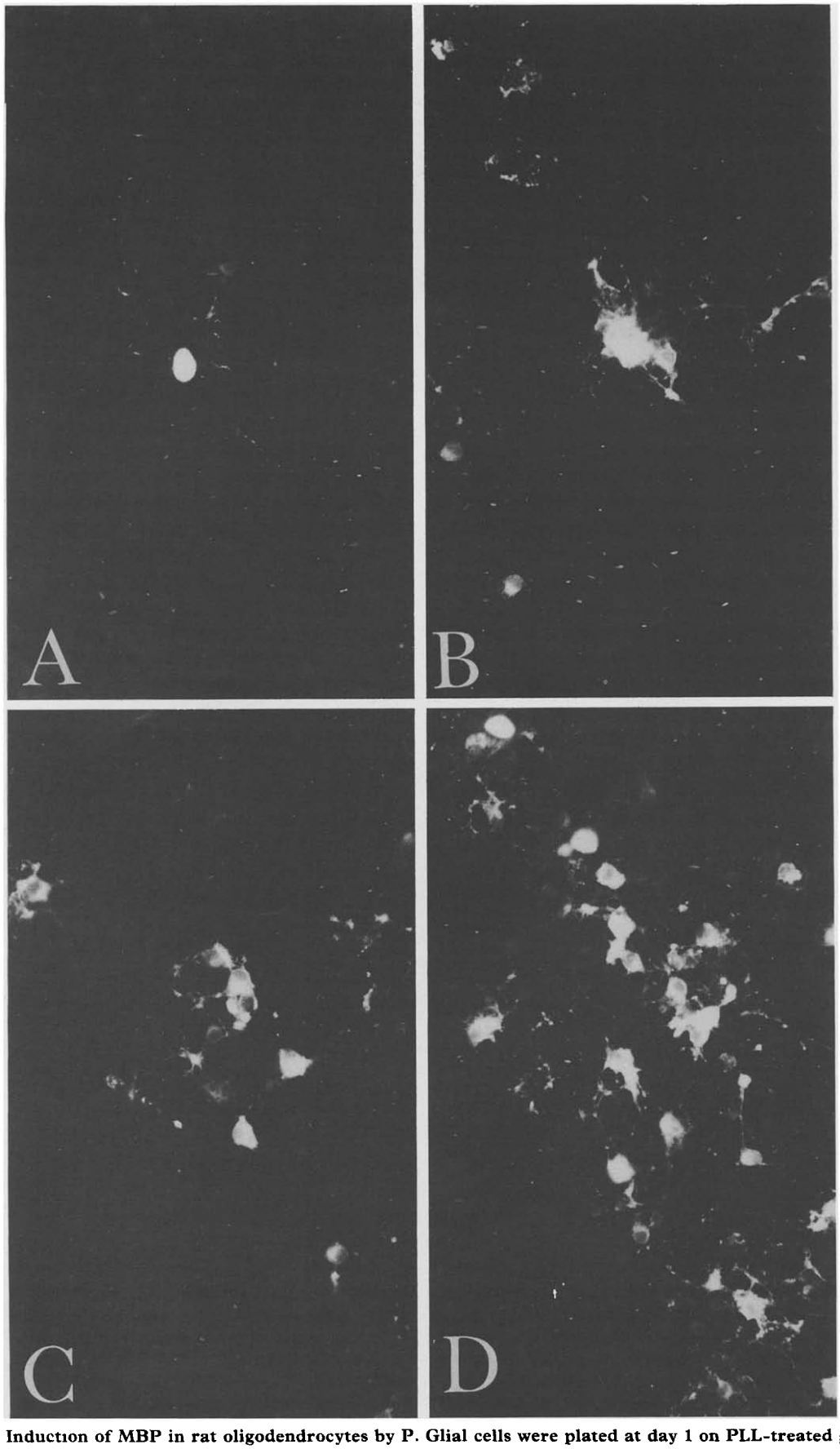

Fig. 3. Induction of MBP in rat oligodendrocytes by P. Glial cells were plated at day 1 on PLL-treated glass coverslips and cultured in DME-CX medium without (A), or in the presence of P, 500 nM (B); insulin, 4.5 μg/ml, (C); or both P (500 nM) and insulin (4.5 μg/ml) (D). Hormones were added daily, media were changed every 2 or 3 days. At day 9, cells were fixed and immunostained for MBP as described in Experimental. Representative microscope fields are represented. Most fields of control cultures (A) contained no MBP-positive cells; (×250).

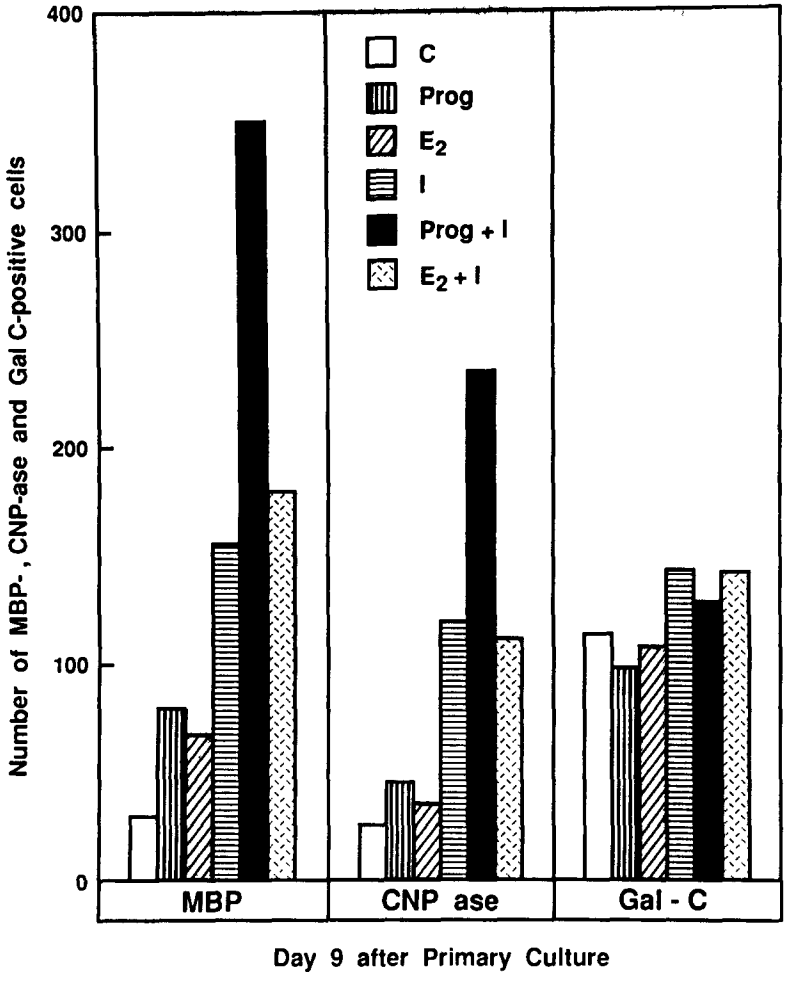

Fig. 4. Expression of MBP, CNPase and Gal C in rat oligodendrocytes in primary culture. Glial cells were plated at day 1 on PLL-treated glass coverslips and cultured in DME-CX medium in the absence (□ C); or in the presence of P, 500 nM, (▥ Prog); E_2, 500 nM (▨ E_2), insulin, 4.5 μg/ml (▤ I); P, 500 nM + insulin, 4.5 μg/ml (■ Prog + I); or E_2, 500 nM + insulin, 4.5 μg/ml (▩ E_2 + I). Hormones were added daily, media were changed every 2 or 3 days. At day 9, immunofluorescence staining of MBP, CNPase and Gal C was done as described in Experimental. Labeled and unlabeled cells were then counted and the number of antigen-expressing cells per 2000 oligodendrocyte-like cells are indicated. The median of 3 independent measures are shown. Insulin significantly increased the number of oligodendrocytes expressing MBP, CNPase and Gal C ($P \leqslant 0.05$). By contrast, P increased the number of MBP- and CNPase-positive cells ($P \leqslant 0.05$) but not of Gal C-positive cells. Cells stained for MBP and CNPase were more numerous on coverslips treated with insulin + P when compared to insulin alone ($P \leqslant 0.05$). Although E_2 slightly increased the number of MBP- and CNPase-positive cells when compared to the control group ($P \leqslant 0.05$) the hormone did not significantly potentiate the effect of insulin (Mann-Witney tests after Kruskal-Wallis one-way analysis of variance).

DISCUSSION

In the present study, we focused part of our experiments on the effect of P on central glial cells, since preliminary studies indicated that P increases the expression of MBP in oligodendrocytes [6]. After treatment of glial cells from days 1 to 15 of primary culture with P (100 or 500 nM), we have demonstrated by immunofluorescence staining an about 2-fold increase in MBP-positive cells between days 6 and 10. After that time, the percentage of MBP-positive oligodendrocytes was too high for a precise evaluation of their number. It is unlikely that the maturation effect of P corresponds to an increased number of oligodendrocytes in culture, since glial cell proliferation was slightly inhibited by P, as was seen in cell growth experiments. Also, the number of oligodendrocytes expressing the specific marker Gal C was not influenced by P. By measuring the immunofluorescence intensity within single oligodendrocytes, we showed that P not only increased the percentage of MBP-expressing cells within a culture, but also the amount of MBP per cell.

The inhibition of cell multiplication by P was completely abolished by the antagonist RU486, thus suggesting a receptor-mediated action of the hormone. Interestingly, the induction of MBP expression by P was not inhibited by RU486, suggesting that this effect of P may not be mediated by the classical PR. The antagonist had even a slight agonistic effect in comparison with control cultures. The molecular basis of the agonistic activities of RU486 is not currently

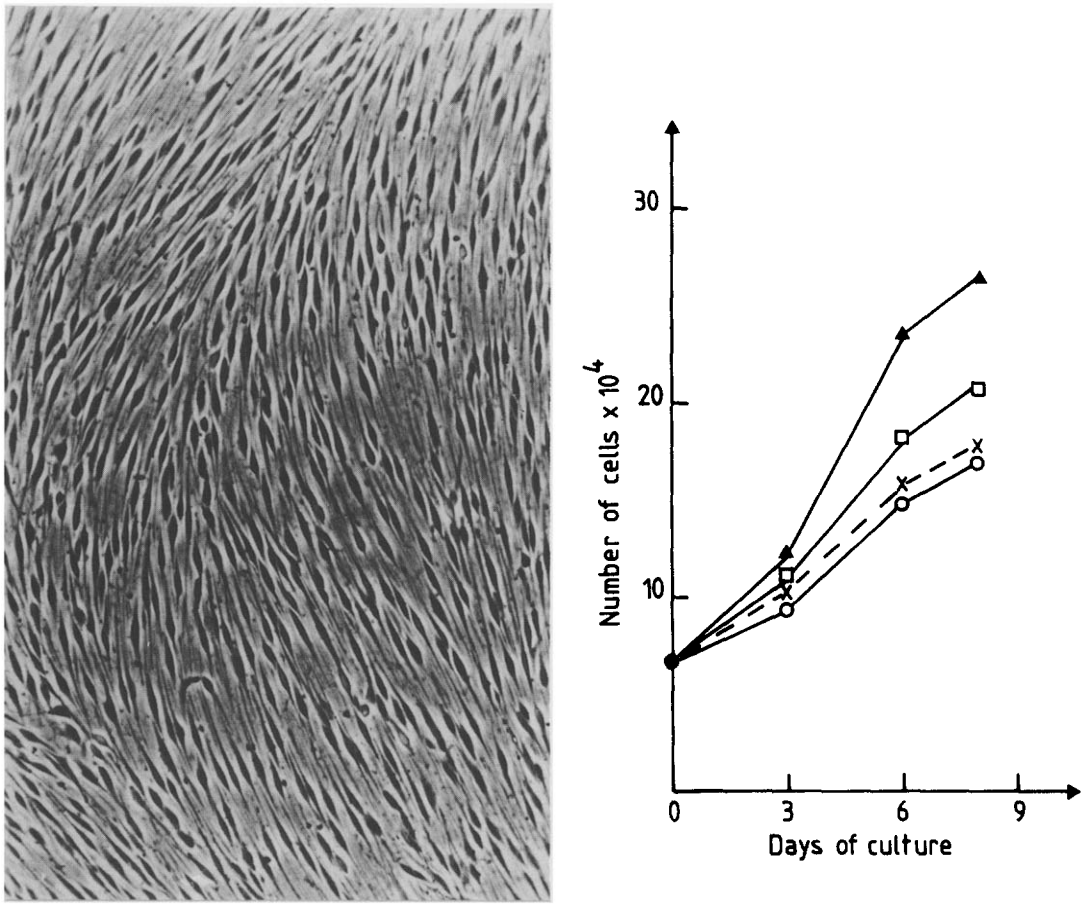

Fig. 5. Primary culture of rat Schwann cells, effect of E_2 and forskolin on cell growth. *Left*: phase contrast microscopy of purified Schwann cells, 2 weeks after primary culture. Fibroblasts were eliminated by treatment with antiThy-1.1 and complement, as described in Experimental (×200). *Right*: purified Schawnn cells were plated in 60 mm Petri dishes and cultured in DME-CX medium in the absence (X- - -X), or in the presence of E_2, 100 nM (○—○), forskolin, 5 μM (□—□), or E_2, 100 nM + forskolin, 5 μM (▲—▲). Media were changed and hormones and growth factors were added every 2 days. At the indicated days of culture, cells were counted. The mean values of triplicate dishes are indicated, not differing one from another by >10%. One-way ANOVA showed a significant effect of treatment on day 6 and 8 ($F = 76$, $df = 12$, $P \leqslant 0.001$) (control vs E_2: $P \geqslant 0.05$; control vs forskolin: $P \leqslant 0.05$; E_2 + forskolin vs forskolin: $P \leqslant 0.01$ by Tukey's tests).

understood. Its antagonistic activity *in vivo* is well established [29], but when transcriptional activity of the RU486–PR complex was measured in a receptor mediated transcription system *in vitro* and compared with that of the agonist R5020–PR complex, some transcriptional activation by RU486 was observed, corresponding to about 25% of that elicited by the progestin agonist [30].

Estradiol (100 and 500 nM) also had some agonistic effects on MBP expression, and here again, the antagonist ICI-164,384, a pure antiestrogen [17], did not influence the estrogen activity. Other steroids such as dexamethasone, pregnanolone or dehydroepiandrosterone, or epidermal growth factor (EGF) failed to induce MBP expression.

It is known that insulin increases MBP in oligodendrocytes in primary culture in a dose-dependent manner. The concentration of insulin which induced a maximal increase in MBP protein was 5 μg/ml [23]. When P was added together with insulin to the primary cultures, a dramatic increase in the number of MBP expressing oligodendrocytes was observed. This effect was not additive, but synergistic. At the high concentration of insulin used (4.5 μg/ml), it is likely that insulin acts via IGF-I-receptors, which have been demonstrated in rat oligodendrocytes [26]. Dose–response experiments with insulin, IGF-I and P are in progress for a better understanding of the underlying mechanism.

Among myelin-specific markers, CNPase and Gal C are also expressed early in oligodendrocytes in primary culture. In order to evaluate whether steroid hormones also alter the expression of other myelin proteins and lipids in oligodendrocytes, CNPase or Gal C levels were measured in parallel with MBP in oligodendrocytes cultured under different hormonal conditions. P alone had little effect on CNPase immunoreactivity, but in the presence of insulin, the hormone strongly potentiated its expression. In contrast, no effect of P was observed on Gal C expression, even in the presence of insulin. Thus, steroid hormones selectively increase the expression of distinct components of central myelin.

Our observations that oligodendrocytes synthesize steroids [7], that E_2 stimulates and P inhibits oligodendrocyte proliferation, and that these steroids increase

the expression of myelin-specific proteins, suggest that oligodendrocytes may use an autocrine mechanism to regulate their own proliferation and differentiation.

Schwann cells, the glial cells of the PNS, do not express MBP in primary culture in the absence of neurons [12]. But cell proliferation can be measured and we found that E_2 increases cell growth, but only in the presence of forskolin or dbcAMP. This synergism corresponds probably to a mechanism by which cAMP could increase the sensitivity of Schwann cells to E_2, since treatment with forskolin for 2 to 3 days caused a 2-fold increase in E_2 binding sites [16]. As Schwann cell proliferation is an important step in myelin repair, E_2 might play an important role in promoting the myelination of peripheral nerves during development or during pathological states.

Schwann cells are also targets for several growth factors, some of which are produced by Schwann cells themselves [31]. We found that IGF-I is a potent mitogen for rat Schwann cells at a low concentration (2.6 nM), but again, only in the presence of forskolin or dbcAMP. The up-regulation of IGF-I receptors by cAMP may explain the synergism between IGF-I and cAMP in stimulating Schwann cell growth. In contrast, NGF had no mitogenic effect, even in the presence of forskolin.

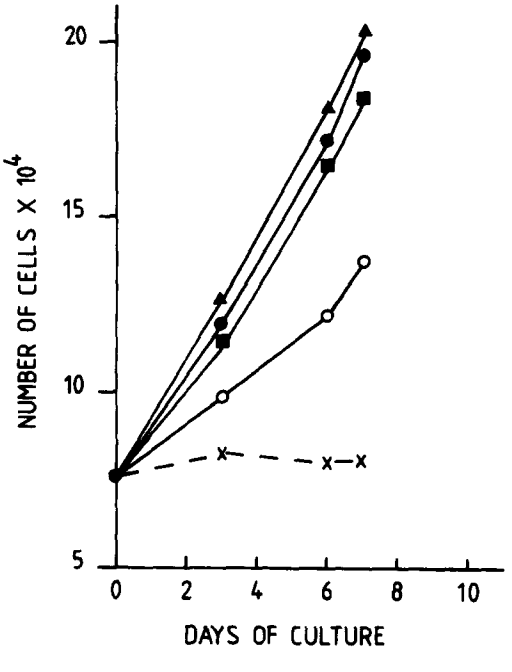

Fig. 6. Growth curves of Schwann cells: effect of E_2 and dbcAMP. Purified Schwann cells were prepared as described in the legend of Fig. 5; plated in 60 mm Petri dishes and cultured in DME-CX medium. Media were changed every 2 days. dbcAMP (1 μM) was added daily and E_2 (1, 10 or 100 nM) was added twice per day. At the indicated days of culture, cells were counted and mean values of triplicate dishes are indicated, not differing one from another by >10%. One-way ANOVA showed a significant effect of treatment on day 3, 6 and 7 ($P \leqslant 0.001$) [control vs dbcAMP: $P \leqslant 0.01$; E_2 (all concentrations) + dbcAMP vs dbcAMP alone: $P \leqslant 0.001$ on day 6 and 7 by Tukey's tests]. (X---X) control; (○—○) dbcAMP 1 μM; (▲—▲) E_2 1 nM + dbcAMP 1 μM; (●—●) E_2 10 nM + dbcAMP 1 μM; (■—■) E_2 100 nM + dbcAMP 1 μM.

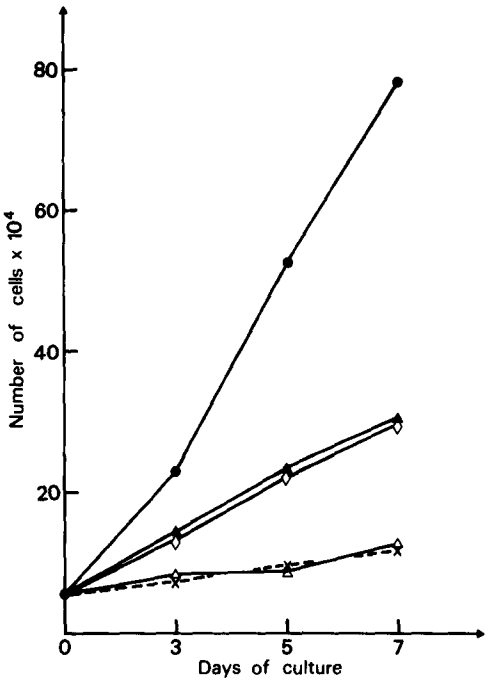

Fig. 7. Growth curves of Schwann cells: effect of IGF-I, NGF and forskolin. Purified Schwann cells were cultured in DME-CX medium as described in the legend of Fig. 5. During the experiment, media were changed and growth factors were added every 2 days. At the indicated days of culture, cells were counted and mean values of triplicate dishes are indicated, not differing one from another by >10%. One-way ANOVA showed a significant effect of treatment on day 3, 5 and 7 ($P \leqslant 0.001$) (control vs forskolin alone: $P \leqslant 0.01$; IGF-I + forskolin vs forskolin alone: $P \leqslant 0.01$ on all days by Tukey's tests). (X---X), control; (△—△) IGF-I 20 ng/ml; (◇—◇) forskolin 5 μM; (▲—▲) NGF (20 ng/ml) + forskolin 5 μM; (●—●) IGF-I 20 ng/ml + forskolin 5 μM.

From the data presented in this paper we conclude that glial cells from the CNS, oligodendrocytes, and glial cells from the PNS, Schwann cells, are targets for steroid hormones. E_2 stimulates, whereas P inhibits brain glial cell growth. However, P stimulates the expression of the myelin-specific proteins MBP and CNPase in oligodendrocytes. An important finding was the strong synergism between P and insulin in stimulating the expression of myelin-specific proteins.

E_2 is also a mitogen for Schwann cells. A possible interaction between E_2 and growth factors as IGF-I in stimulating the proliferation of Schwann cells is under current investigation.

Acknowledgements—We thank C Legris and F. Boussac for excellent assistance in preparing the manuscript, J. C. Lambert and L Outin for preparing the figures and B. Delespierre for skilful technical assistance. This work was supported by INSERM and partially by the Association Française Contre les Myopathies (A.F.M.).

REFERENCES

1. Gorski J., Toft D., Shyamala G, Smith D. and Notides A.: Hormone receptors. studies on the interaction of estrogen with the uterus. *Recent Prog. Horm. Res.* 24 (1968) 45–80
2. McEwen B. C., Biegon A., David P. G., Krey L. C., Luine V. N., McGinnis Y., Paden C. M., Parsons B and Rainbow T C · Steroid hormones: humoral signals which alter brain cell

properties and functions. *Recent Prog. Horm. Res.* **38** (1982) 41–83.
3. Kato J.: Progesterone receptors in brain and hypophysis. In *Current Topics in Neuroendocrinology*. Springer-Verlag Berlin, Heidelberg, Vol. 5 (1985) pp. 31–38.
4. Kumar S. and De Vellis J.: *Glial Cell Receptors* Raven Press, New York (1988) pp. 243–264.
5 Jung-Testas I, Renoir J. M., Gasc J. M and Baulieu E. E. Estrogen-inducible progesterone receptor in primary cultures of rat glial cells. *Exp. Cell Res.* **193** (1991) 12–19.
6. Jung-Testas I, Renoir M., Bugnard H., Greene G L. and Baulieu E. E.. Demonstration of steroid hormone receptors and steroid action in primary cultures of rat glial cells. *Steroid Biochem. Molec. Biol* **41** (1992) 815–821.
7. Jung-Testas I., Hu Z. Y., Baulieu E. E. and Robel P . Neurosteroids biosynthesis of pregnenolone and progesterone in primary cultures of rat glial cells. *Endocrinology* **125** (1989) 2083–2091
8. Ritchie J. M : Physiological basis of conduction in myelinated nerve fibers In *Myelin* (Edited by P. Morell). Plenum Press, New York (1984) pp. 117–145.
9. Norton W T. Biochemistry of myelin. In *Advances in Neurobiology* (Edited by S. G. Waxman and J. M Ritchie). Raven Press, New York (1981) pp. 93–121.
10. Raff M C, Mirsky R., Fields K. L., Lisak R. P., Dorfman S H., Silberberg D. H., Gregson N A, Leibowitz S and Kennedy M C.. Galactocerebroside is a specific cell-surface antigenic marker for oligodendrocytes in culture *Nature* **274** (1987) 813–816.
11. Lees M. B. and Brostoff S. W.: Proteins of myelin In *Myelin* (Edited by P. Morrell). Plenum Press, New York, 2nd Edn (1984) pp. 197–217.
12. Porter S, Clark M. B., Glaser L. and Bunge R. P · Schwann cells stimulated to proliferate in the absence of neurons retain full functional capability. *J. Neurosci.* **6** (1986) 3070–3078
13. Raff M. C., Abney E., Brockes J. P. and Hornby-Smith A.. Schwann cell growth factors. *Cell* **15** (1978) 813–822.
14. Morgan L, Jessen K. R. and Mirsky R.: The effects of cAMP on differentiation of cultured Schwann cells: progression from an early phenotype (04+) to a myelin phenotype (PO+, GFAP-, N-CAM-, NGF receptor-) depends on growth inhibition. *J Cell Biol.* **112** (1991) 457–467.
15. Stewart H J. S., Eccleston P. A, Jessen K. R and Mirsky R . Interaction between cAMP elevation, identified growth factors and serum components in regulating Schwann cell growth. *J. Neurosci Res* **30** (1991) 346–352.
16. Jung-Testas I, Schumacher M, Bugnard H. and Baulieu E E Stimulation of rat Schwann cell proliferation by estradiol: synergism between the estrogen and cAMP. *Dev. Brain Res.* **72** (1993) 282–290
17. Wakeling A. E. and Bowler J.: Novel antiestrogens without partial agonist activity. *J. Steroid Biochem.* **31** (1988) 645–653.
18. Schumacher M., Jung-Testas I., Robel P. and Baulieu E. E. Insulin-like growth factor I: a mitogen for rat Schwann cells in the presence of elevated levels of cyclic AMP *Glia* **8** (1993) 232–240.
19. Jung-Testas I., Alliot F., Pessac B., Robel P. and Baulieu E. E.: Localisation immunocytochimique du cytochrome P-450$_{scc}$ dans les oligodendrocytes de rat en culture. *C.R. Acad. Sci. Paris* **308** (1989) 165–170.
20 Brockes J P., Fields K. L and Raff M. C.: Studies on cultured rat Schwann cells. I. Establishment of purified populations from cultures of peripheral nerve. *Brain Res* **165** (1979) 105–118.
21 Raff M. C.: Glial cell diversification in the rat optic nerve. *Science* **243** (1989) 1450–1455.
22. Dubois-Dalcq M. and Armstrong R.: The cellular and molecular events of central nervous system remyelination. *BioEssays* **12** (1990) 569–576
23 Saneto R P, Low K G, Melner M. H. and de Vellis J. Insulin/insulin-like growth factor I and other epigenetic modulators of myelin basic protein expression in isolated oligodendrocyte progenitor cells. *J. Neurosci. Res.* **21** (1988) 210–219.
24. McMorris F A. Cyclic AMP induction of the myelin enzyme 2',3'-cyclic nucleotide 3'-phosphohydrolase in rat oligodendrocytes. *J. Neurochem.* **41** (1983) 506–515
25. Van der Pal R. H. M., Koper J. W., Van Golde L. M. G. and Lopes-Cardozo M Effects of insulin and insulin-like growth factor I (IGF-I) on oligodendrocyte-enriched glial cultures. *J. Neurosci. Res.* **19** (1988) 483–490.
26 McMorris F. A., Smith T M., DeSalvo S. and Furlanetto W. Insulin-like growth factor I/Somatomedin C: a potent inducer of oligodendrocyte development. *Proc Natn. Acad. Sci. U.S A.* **83** (1986) 822–826.
27 Moore B. W, Perez V. J. and Gehring M. Assay and regional distribution of a soluble protein characteristic of the nervous system. *J. Neurochem* **15** (1968) 265–272
28. Seamon K B., Padgett W and Daly J. W.: Forskolin: unique diterpene activator of adenylate cyclase in membranes and in intact cells. *Proc Natn Acad. Sci. U.S A* **78** (1981) 3363–3367.
29. Baulieu E E.: Editorial: RU486 and the early nineties. *Endocrinology* **127** (1990) 2043–2046
30 O'Malley B. W., Tsai S Y., Bagchi M, Weigel N. L., Schrader W. T. and Tsai M. J.: Molecular mechanism of action of a steroid hormone receptor. *Recent Prog. Horm. Res* **47** (1991) 1–26.
31 Eccleston P. A Regulation of Schwann cell proliferation: mechanisms involved in peripheral nerve development *Exp. Cell Res* **199** (1992) 1–9