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Demonstration of Progesterone Receptors in Rat Schwann Cells

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We have recently shown that progesterone promotes myelin formation in peripheral nerves of rodents. In this study, we demonstrate the presence of progesterone receptors (PR) in primary cultures of rat Schwann cells, the glial cells of the PNS, prepared from sciatic nerves of 4–5 days old rats. After 3 weeks of culture, the presence of PR was measured by whole cell assay after incubating living cells for 1 h at 37°C with [^{3}H]-Organon 2058 as a ligand, and about 5000 specific binding sites per cell were found. In contrast to the PR of rat glial cells from the central nervous system (CNS), which is induced by estrogens, treatment of Schwann cells with estradiol did not increase the PR-binding, even after exposure of cells to high doses of estrogen under various culture conditions. Progesterone receptors were also visualized in Schwann cells by indirect immunofluorescence staining with a monoclonal anti-PR antibody. Again, treatment of the cells with estradiol did not increase the immunofluorescence staining of the PR. Specific PR binding was also measured in sciatic nerves of adult female rats. Cytosol was prepared and labeled with [^{3}H]-Organon 2058 for 15 h at 2°C. After treatment with dextran-coated charcoal, specific ligand binding was about 30 fmol/mg cytosolic protein. When castrated adult female rats were treated with estradiol (20 μg EB/day for 3 days), no PR-induction was observed in the cytosol of sciatic nerves. In contrast, PR-binding sites in cytosols prepared from pituitary gland and uteri of the same animals were significantly increased by the estrogen. Copyright © 1996 Elsevier Science Ltd.

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INTRODUCTION

Progesterone is a steroid hormone that influences the growth, development and function of female reproductive tissues such as uterus and mammary gland [1]. It is also well established that progesterone plays an important role in the brain and hypophysis, where it regulates female reproductive behavior and gonadotropin secretion [for review see [2, 3]]. We have shown that progesterone can also modulate cell growth and the synthesis of myelin proteins in rat glial cells of the central nervous system (CNS) [4]. The biological effects of progesterone and of other steroid hormones are mediated by specific receptor proteins, which, after hormone binding, function as activated transcription factors that bind to specific nuclear response elements to modulate transcription of target genes [5, 6]. The brain contains receptors for all classes of steroid hormones [2], and we have identified the presence of progesterone, glucocorticoid, estrogen and androgen receptors (PR, GR, ER and AR) in primary cultures of glial cells from newborn rat forebrains. Among these receptors, only the PR was estrogen-inducible and this PR-induction was stronger in female cultures [4, 7]. Progesterone has multiple effects on central glial cells (composed of oligodendrocytes and astrocytes) in primary culture and influences cell growth and differentiation. In particular, a striking increase of myelin-specific proteins such as MBP (myelin basic protein) and CNPase (cyclic nucleotide phosphodiesterase) was observed in oligodendrocytes, the myelinating glial cells of the CNS, when cultured in presence of progesterone [4, 8]. Recently, we have studied the role of progesterone in peripheral nerve repair and it was found that progesterone also plays an important role in myelinisation of sciatic nerves after freeze lesions [9, 10]. This observation led us to investigate whether rat Schwann cells in culture, or rat sciatic nerves, possess specific progesterone binding sites and whether these receptors are regulated by estrogen.

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EXPERIMENTAL

Primary cultures of rat Schwann cells

The preparation and purification of Schwann cells has been described in detail [11]. Briefly, sciatic nerves from 4–5 days old Sprague–Dawley rats were dissected, minced into small fragments and digested in Dulbecco's modified Eagle's medium (DME) containing 0.25% trypsin and 0.1% collagenase type A (Sigma) for 40 min at 37°C in a cell incubator. The derived cells were plated onto poly-L-lysine coated tissue culture flasks (PLL, mol. wt. > 300,000, Sigma, 20 μg/ml distilled water). After 2 days of culture in DME supplemented with 10% heat-inactivated fetal calf serum (FCS), fibroblasts were killed by treatment for 2 days with 10^{-5} M cytosine arabinose. Cell growth was then stimulated by adding insulin and forskolin to the culture medium (respectively 4.8 and 2 μg/ml), since we found that insulin-like growth factor 1 (IGF-1) is a potent mitogen for Schwann cells in presence of forskolin or dbc-AMP [11]. Residual fibroblasts were killed by treatment with anti-Thy 1.1 antibody and rabbit complement (Cederlane, Canada) as described by Brockes *et al.* [12]. By this procedure, highly purified Schwann cells were obtained, as shown by indirect immunofluorescence staining of the S-100 antigen as described in details previously [13]. Indeed, the Ca^{2+} binding protein S-100 is a specific marker for Schwann cells and is found at high levels in peripheral nerves [14]. These purified Schwann cells can be kept in culture for several weeks, they were multiplied by serial trypsinations and dilutions.

For immunofluorescence studies, aliquots of purified Schwann cells were plated onto PLL-treated glass cover slips, placed in Petri-dishes, and cultured under the described conditions.

Immunofluorescence staining of PR

The glass coverslips were taken out of the Petri-dishes, rinsed in PBS and fixed in 3% formaldehyde in PBS for 30 min. After washing and permeabilizing cells with Triton X-100 (1% in PBS) for 4 min and thoroughly washing in PBS, cells were stained with a monoclonal anti-progesterone receptor antibody (IgG_1) which reacts with the PR from human, calf, rat and mouse tissue but does not cross react with ER, AR or GR (Affinity Bio Reagents). The coverslips were incubated in a humid chamber for 1 h at room temperature. The anti-PR antibody was utilized at 1 μg/ml PBS. After washing, the second fluorescent antibody was applied for 30 min. The coverslips were stained with Evans Blue and mounted in Moviol. The cells were observed and photographed with a Leitz Laborlux D photomicroscope.

Progesterone receptor binding in Schwann cells

Before the experiments, confluent Schwann cell cultures were kept for 48 h in DME medium complemented with 10% charcoal-treated calf-serum and then for 16 h in serum-free medium. The cells were recovered by gently scraping the Petri dishes, centrifuged and resuspended in 0.5 ml DME containing various concentrations of [^{3}H]-Organon 2058 (51 Ci/mmol, NEN) ranging from 0.5-5 nM with or without 1 μM unlabeled progesterone. Cells were labeled for 1 h at 37°C (1 confluent 100 mm Petri dish per experimental point, corresponding to about 2×10^6 cells). After the incubation, cells were kept on ice, recovered by centrifugation and washed three times with 2 ml of ice-cold PBS. During the washing cycles, cells were kept for 5 min on ice to allow non-specifically bound [^{3}H]-Organon 2058 to diffuse out of the intact cells. The washed pellets were eluted with 1.0 ml ethanol and the radioactivity was counted. Specific binding was expressed as the difference between total binding and non-specific binding (measured in the presence of competitor). Separate dishes were counted to determine the number of cells, and the results were expressed as dpm of [^{3}H]-Organon 2058 bound/1×10^6 cells.

Progesterone receptor binding in sciatic nerves

Adult female Sprague Dawley rats were killed by decapitation, sciatic nerves were dissected and collected in ice-cold PBS, weighed, minced into small fragments and homogenized in TED buffer using a glass-glass Potter homogenizer (TED: Tris 10 mM, EDTA 1 mM, Dithioerythritol 1 mM, glycerol 10%, pH 7.8). The homogenate was centrifuged at 105,000 ***g*** for 1 h at 4°C, the cytosol was recovered and aliquots were incubated for 15 h at 2°C with various concentrations of [^{3}H]-Organon 2058, from 1.0–6.0 nM in the absence or presence of 1 μM unlabeled progesterone. The cytosol was then treated with dextran-coated charcoal for 5 min (0.5 g charcoal, Norit A, 0.05 g dextran T60/100 ml TED, vol/vol) and after centrifugation, aliquots of the charcoal-treated cytosol were counted in a scintillation counter. Specific binding was expressed as femtomoles of [^{3}H]-Org 2058 bound per mg cytosol protein, after deduction of the non-specific binding. The protein concentration was measured by using the technique of Lowry.

For the measure of PR-induction by estradiol in sciatic nerves, pituitaries and uteri, adult female rats were ovariectomized 2 weeks before estradiol treatment. The animals were then injected subcutaneously for 3 days with 20 μg/day estradiol benzoate (EB) in 50 μl sesame oil or the oil vehicle alone. On the fourth day, the animals were killed by decapitation, their sciatic nerves, pituitaries and uteri immediately removed and placed in ice-cold PBS. All subsequent procedures were carried out as described above. Receptor levels were expressed as femtomoles of [^{3}H]-Organon 2058 bound per mg of cytosol protein.

RESULTS

Demonstration of progesterone binding sites in primary cultures of rat Schwann cells

To determine whether Schwann cells contain receptors for progesterone, whole cell assays were used. After purification of Schwann cells by Thy 1.1 treatment as described in Experimental, the cells were plated on PLL-treated 100 mm Petri dishes and allowed to grow until they became confluent, in the presence of 10% FCS, insulin and forskolin. Before the experiments, cells were kept for two to three days in presence of 10% charcoal-treated calf serum, and then for 16 h in the absence of serum to avoid occupation of progesterone binding sites by progestins, possibly present in FCS. Schwann cells were labeled with the synthetic progestin [^{3}H]-Organon 2058, known for its high affinity and specificity for the PR, for 1 h at 37°C at different ligand concentrations, ranging from 1.0–6.0 nM. Non-specific binding was determined in the presence of 1 μM excess unlabeled progesterone, and parallel Petri dishes were counted so that the results could be expressed as bound [^{3}H]-Organon 2058 per 10^6 cells. A series of seven experiments was performed, each one using a new primary culture and the experiments gave similar results. The average amount of specific PR binding was 12 $\pm$ 1.1 fmol bound Organon 2058 per 10^6 cells, which corresponds to about 5450 $\pm$ 520 sites per cell (mean $\pm$ SEM) (Fig. 1). We then determined if PR-binding sites in Schwann cells are also estrogen-inducible as they are in primary cultures of glial cells of the CNS. After purification of Schwann cells by Thy 1.1-treatment, usually at day 6 after primary culture, the cells were grown in DME-medium containing 10% charcoal-treated calf-serum, insulin and forskolin, and 100 nM estradiol dissolved in ethanol was added daily. Control cultures were treated with ethanol alone. After one, two or three weeks of estradiol treatment, PR binding was measured by whole cell assay. Surprisingly, no difference in the amount of specific PR binding sites was observed, as can be seen in Fig. 1. We also increased the concentrations of estradiol from 200–500 nM and each experiment was carried out with different primary cultures and here

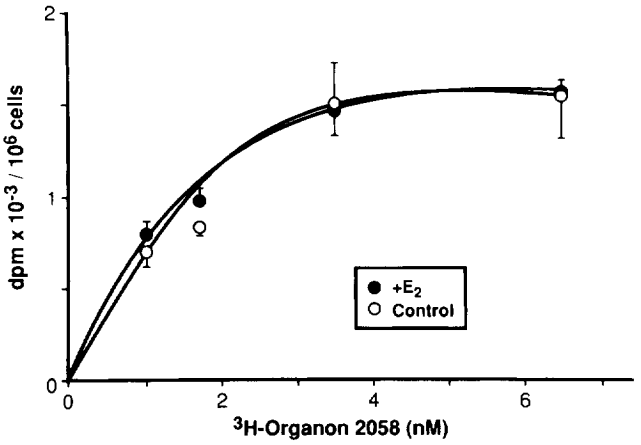

Fig. 1. Binding analysis of [^{3}H]-Org 2058 in cultured rat Schwann cells. Schwann cells were incubated for 1 h at 37°C with increasing concentrations of [^{3}H]-Org 2058, either alone or in the presence of 1 μM unlabeled progesterone. Specific PR-binding sites were measured by whole cell assay as described in Experimental. Results are expressed as dpm bound per 1×10^6 cells. Non-specific binding corresponded to about 30% of total binding. ●—● Schwann cells cultured for 2 weeks prior the PR measurement in the presence of 100 nM estradiol. ○—○ Control cultures, treated with vehicle only. Experiments were done in triplicates (3 measures for each experiment), mean values $\pm$ S.D. are indicated.

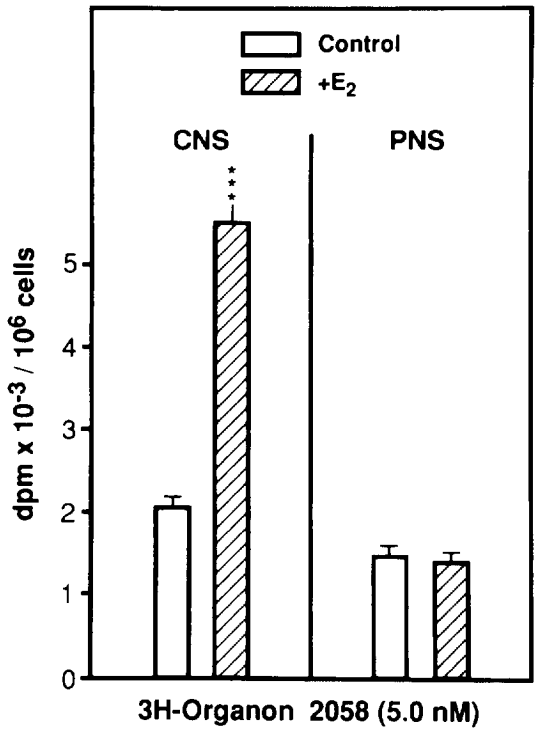

Fig. 2. Progesterone receptors in primary cultures of rat glial cells from the CNS and the PNS. Glial cells from the CNS were prepared from cerebral hemispheres of newborn Sprague–Dawley rats (1 day after birth). After removal of meninges and blood vessels, the brain tissue was mechanically dissociated and the cell suspension was plated on poly-L-lysine coated 100 mm Petri-dishes and cultured in DMEM supplemented with 10% heat-inactivated calf serum, antibiotics and insulin (4.8 μg/ml) as discribed in detail in [4]. The cultures consist of a bedlayer of astrocytes overlaid by process-bearing oligodendrocytes. Cells were treated (or not) from days 10 to 27 of primary culture with 100 nM. estradiol. Glial cells from the PNS (Schwann cells) were prepared from 4-5 days old Sprague–Dawley rats as described in Materials. Pure Schwann cells were cultured from days 10 to 27 of primary culture in the presence or absence of 100 nM estradiol. At day 28, specific PR binding was measured in both cell types by whole cell assay. Cells were incubated for 1 h at 37°C with 5.0 nM [^{3}H]-Organon 2058, in the absence or presence of 1 μM unlabeled progesterone. Parallel Petri-dishes were counted to determine the number of cells. Specific PR-binding was calculated after substraction of non-specific binding and the results expressed as bound [^{3}H]-Organon 2058 (dpm) per 1×10^6 cells. Experiments were done in triplicate (3 measures for each experiment), bars indicate means $\pm$ SD. ***$P \leq 0.001$ when compared to the control group by Student's t-test.

again, no PR-induction was observed in Schwann cells treated with estradiol.

Since the PR-induction by estradiol in rat glial cells from the CNS was observed in mixed primary cultures composed of oligodendrocytes and astrocytes [7], and since another group has reported that estrogen-induction of PR in primary cultures of normal mouse mammary epithelial cells was only observed in the presence of stromal fibroblasts [15], we wondered if the non-inducibility of PR in Schwann cells was due to the culture conditions, in particular to the absence of other cell types. Therefore, PR binding was measured in non-purified cultures of Schwann cells, which also contained fibroblasts (about 30%) and macrophages (about 5%). Cells were treated from the beginning of the primary culture with estradiol and PR-binding was measured after 5–10 days of culture, or after 2–3 weeks of culture. In none of these experiments, PR-binding sites were significantly modulated by estradiol. Finally, PR-binding was measured in parallel in primary cultures of glial cells from newborn rat forebrains and in primary cultures of rat Schwann cells, both cell types were cultured in the presence or absence of estradiol (100 nM). As shown in Fig. 2, only glial cells from the CNS contained estrogen-inducible PR-binding sites.

Immunofluorescence staining of PR

For the immunohistochemical detection of PR, Schwann cells were cultured at low density on PLL-coated glass coverslips in DME medium containing 10% calf-serum and insulin. With the use of a monoclonal anti-PR antibody, which does not interact with other steroid receptors, PR immunofluorescence staining was mainly found in the cytoplasm of the cells, but some nuclear staining was also observed. Exposure of Schwann cells to 200 nM of progesterone 1 h before immunofluorescence staining resulted in an increase of nuclear PR localization (Fig. 3). Treatment of the cells with estradiol (200 nM) did not increase the intensity of the PR-immunofluorescence when compared to control cultures, thus confirming the non-inducibility of PR by estrogen observed during binding studies. Positive PR-staining was restricted to spindle-shaped cells which also expressed the S-100 antigen.

Demonstration of PR in cytosolic extracts of rat sciatic nerves

Schwann cells may not respond to estrogen because of the culture conditions. To circumvent this problem, we measured PR binding in cytosols prepared from female rat sciatic nerves. The concentration of specific [^{3}H]-Organon 2058 binding in cytosol of freshly isolated sciatic nerves was 30 ± 5 fmol/mg protein at saturating ligand concentrations (6 nM). As it was described that cytoplasmic PR-binding sites are significantly increased in female rat pituitary after estrogen treatment [16], we examined in parallel cytosols from sciatic nerves and pituitaries in control- and EB-treated ovariectomized rats. The results of these experiments are shown in Fig. 4. Basal PR-levels of sciatic nerves and pituitaries were similar (35 and 60 fmoles/mg cytosol protein respectively, at saturating ligand concentration). Whereas, PR-binding sites in sciatic nerves remained unchanged after estrogen treatment, a 6 fold increase of PR concentration was measured in pituitaries. The absence of estrogen stimulation of progestin receptor levels in sciatic nerves was confirmed in our additional experiment by measuring PR-induction in cytosols from sciatic nerve, pituitary and

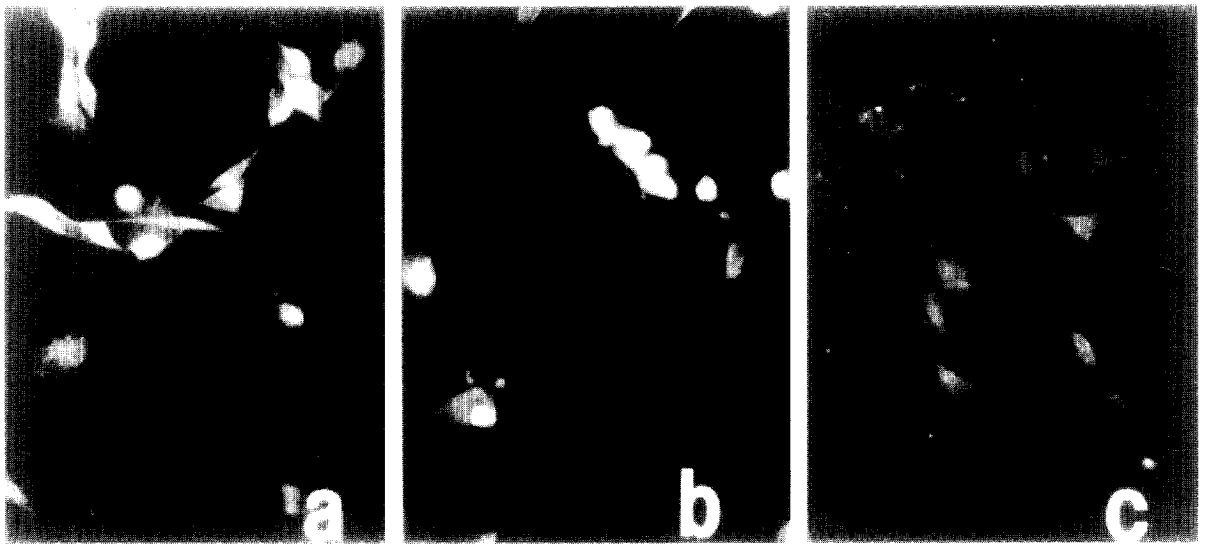

Fig. 3. Progesterone receptors in rat Schwann cells in primary culture: indirect immunofluorescence staining. Schwann cells were cultured on glass cover slips in hormone-free medium. 1 h before immunofluorescence staining, cells shown in (b) were cultured in the presence of progesterone (100 nM). Note the increased nuclear localization in (b). The immunofluorescence staining of PR was done as described in Experimental. Control experiments done with non-specific immunoglobulins (non immune) show only background staining (c).

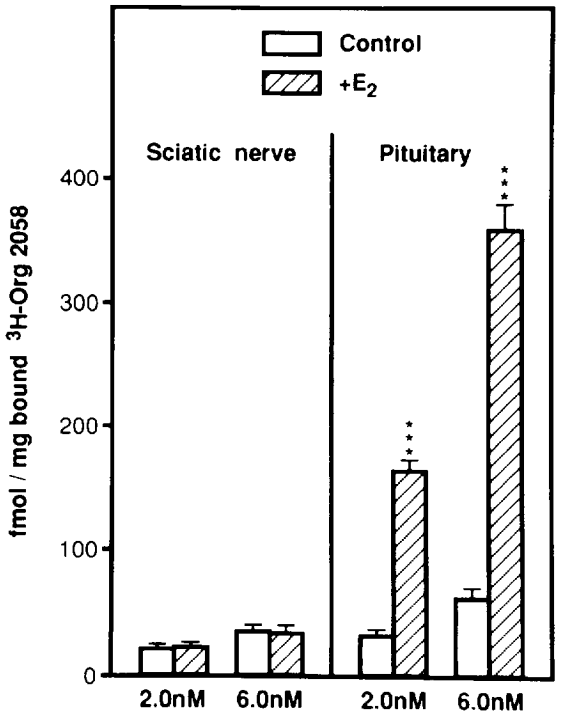

Fig. 4. Effect of estradiol treatment on specific [^{3}H]-Org 2058 binding in rat sciatic nerve and pituitary. Cytosols were prepared from ovariectomized adult rats, treated (or not) for 3 days before sacrifice with 20 μg/day of estradiol benzoate in sesame oil. Aliquots of the cytosols were incubated for 15 h at 2°C with 2.0 or 6.0 nM [^{3}H]-Org 2058, in the absence or presence of excess unlabeled progesterone. Bound [^{3}H]-Org 2058 was determined by the dextran-coated charcoal assay as described in Materials. Experiments were done in triplicates (3 measures for each experiment), bars indicate means ± SD. ***P ≤ 0.001 when compared to the control group by Student's *t*-test.

uterus. The basal PR-levels in uterus cytosol were about 10 times higher than in cytosol from nervous tissue, and after estrogen treatment, only the PR-binding in uterus and pituitary cytosol increased significantly. The results are shown in Table 1.

Table 1. Effects of estradiol treatment on the concentration of specific [^{3}H]-Org 2058 binding sites in sciatic nerve, pituitary and uterus cytosol

[^{3}H]-Org 2058 binding sites (fmol/mg cytosol protein)		
	Control	+ EB
Sciatic nerve	35 ± 4	33 ± 6^{ns}
Pituitary	61 ± 9	358 ± 20 ***
Uterus	450 ± 18	1115 ± 43 ***

Cytosols were prepared from tissues of ovariectomized rats treated for 3 days before sacrifice with either 20 μg / day of estradiol benzoate (EB) in sesame oil or with the vehicle alone (control). Cytosols were incubated with 6.0 nM [^{3}H]-Org 2058 for 15 h at 2°C, in the absence or presence of a 500-fold molar excess of progesterone. Experiments were done in triplicate (3 measures for each experiment), mean values ± SD are indicated. ***$P \leq 0.001$ when compared to the control group by Student's *t*-test.

DISCUSSION

These data demonstrate the presence of progesterone receptors in rat Schwann cells, and extend our previous studies on steroid hormone receptors and steroid hormone action in myelinating glial cells [4, 7, 8, 11]. The demonstration of specific PR-binding in cultured Schwann cells was done by whole cell assay. Using [^{3}H] Organon 2058 as a ligand, an average number of 5400 progestin-binding sites per cell was found. The presence of PR in Schwann cells was also demonstrated by indirect immunofluorescence staining using specific anti-PR antibodies. The immunofluorescence staining of PR was mainly present in the cytoplasm of spindle-shaped Schwann cells which express the S-100 antigen, but the nuclear staining was increased after treatment of the cultures with 200 nM progesterone for 1 h before the measurement. The cytoplasmic staining of PR can be explained by the presence of unliganded, loosely bound PR which is extracted from the nucleus during the immunohistochemical procedure. Similar results were obtained when ER in Schwann cells and PR in rat oligodendrocytes were demonstrated by immunofluorescence staining [7, 13]. As shown previously and confirmed here, PR-levels in primary cultures of glial cells from rat forebrain were significantly increased after treating the cells with estradiol. This PR-induction was even stronger in cells prepared from female rat pups [4]. However, we were not able to demonstrate a similar PR-induction by estradiol in primary cultures of Schwann cells. PR-binding sites were measured after short- or long-term estrogen treatment, and increasing doses of estradiol, ranging from 100–500 nM, were applied, but despite the different treatments the values of PR-binding sites in estrogen-treated cells were never significantly different from the untreated controls. To make sure that this non-inducibility of PR was not due to long-term cultures of purified Schwann cells which might alter the responsiveness of the cells to hormone stimulation, experiments were done using non-purified, 1 week-old Schwann cells. Again, under these conditions, the PR was not estrogen-inducible. The absence of estrogen responsiveness observed in Schwann cell cultures could not be due to the lack of ER since we have previously reported the presence of functional ER in the same cells [13].

In addition to these *in vitro* studies, the induction of PR by the estrogen was measured *in vivo*, after administration of estradiol to ovariectomized adult rats, in sciatic nerves, pituitaries and uteri. Whereas PR-binding sites in cytosol of pituitaries and uteri were significantly increased after estradiol treatment, no PR-induction could be measured in cytosol of sciatic nerves.

It is well known that the PR is differently regulated by estrogen in distinct regions of the rodent brain. For example, estradiol increases PR binding in the hypothalamus and pituitary gland, but not in the cerebral

cortex and cerebellum [16]. Whereas estradiol induces PR in glial cells prepared from neonatal rat brains [7], it has no effect on the PR present in cultured Schwann cells and in sciatic nerve. The mechanisms involved in the different regulation of the PR by estrogen are unknown. It is generally accepted that the PR is encoded by a single gene, having multiple estrogen responsive elements in its promoter region [17]. It is thus possible that cell-specific factors regulate the estrogen-responsiveness of the PR.

In conclusion, no estrogen-induction of the PR could be shown in Schwann cells, neither in pure primary cultures, nor in mixed primary cultures containing also fibroblasts and macrophages. The duration of culture (5 days to 3 weeks) did not influence the results. This non-inducibility of the PR by estrogen in Schwann cells was confirmed *in vivo* by measuring the receptor in sciatic nerves of rats treated with estradiol.

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