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Pathways of dehydroepiandrosterone formation in rat brain glia

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Abstract

In peripheral steroidogenic tissues, dehydroepiandrosterone (D) is formed from pregnenolone (P) by the microsomal cytochrome P450c17 enzyme. Although some steroidogenic P450s have been found in brain tissue, no enzyme has been shown to possess P450c17 activity. We recently demonstrated the presence of an alternative, Fe^{2+}-dependent pathway responsible for D formation from alternative precursors in rat glioma cells. We and others could not find P450c17 mRNA and protein in rat brain, but demonstrate herein the presence of Fe^{2+}-dependent alternative pathway for D formation in rat brain cortex microsomes. Using primary cultures of differentiating rat glial cells, we observed that P450c17 mRNA and protein were present in O-2A oligodendrocyte precursors and mature oligodendrocytes. In the presence of P, O-2A and mature oligodendrocytes formed D. Addition of Fe^{2+} together with submaximal concentrations of P increased D formation by these cells. Treatment of oligodendrocytes with the P450c17 inhibitor SU 10603 in the presence or absence of P failed to inhibit D production. These data suggest that D formation in oligodendrocytes occurs independently of the P450c17 protein present in the cells. In isolated type I astrocytes we did not find neither P450c17 mRNA nor protein. These cells responded to Fe^{2+} by producing D and addition of P together with Fe^{2+} further increased D synthesis. SU 10603 failed to inhibit D formation by astrocytes. Taken together these results suggest that in differentiating rat brain oligodendrocytes and astrocytes D is formed via a P450c17-independent and oxidative stress-dependent alternative pathway. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Neurosteroids; DHEA; Glia; Oxidative stress

1. Introduction

In addition to the well-defined actions in peripheral tissues, steroids have pleiotropic actions [1] on the central nervous system (CNS), where they control a number of neuroendocrine and behavioral functions. The specific interactions of steroids with binding sites on neuronal membranes and the ability of various steroids to modulate brain function has prompted investigation of the steroidogenic potential of CNS structures. The pioneering work of Baulieu and colleagues demonstrated that levels of pregnenolone (P) and dehydroepiandrosterone (D) accumulated in the rat brain persist up to 2 weeks after the removal of peripheral steroidogenic organs [2,3]. This group also demonstrated immunocytochemical localization of the cytochrome P450 side chain cleavage enzyme (P450scc) to the white matter of the rat brain [4], suggesting that oligodendrocytes may be a source of steroid synthesis in the brain. Furthermore, they demonstrated that glial cells could convert cholesterol to P and give origin to steroid metabolites, potential modulators of neuronal function [5,6]. It was then shown that oligodendrocytes, a glioma cell line, and Schwann cells have the ability to form P from mevanolactone or cholesterol precursors

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[5,7–9]. Although discrepancies exist among the levels of the enzymatic activity, the amount of immunoreactive protein, and mRNA of the P450scc present in brain [10] it is quite clear that specific brain cells have the ability to synthesize P.

D was the first neurosteroid to be described and high levels of D have been found in brain [11]. D and its sulfated form are two of the main neuroactive steroids found in brain [11]. However, there is no evidence that cytochrome P450 17α-hydroxylase (P450c17) activity and protein are present in brain [12] or glioma cells [10]. Only a transient expression of the mRNA for this enzyme during embryonic life was reported [13] and contradictory data on the presence of its mRNA in the adult has been presented [14–17]. Thus, the pathway by which D is synthesized in the brain is unknown.

In 1994, Prasad and colleagues [18] presented evidence showing that organic extracts of rat brain contain precursors that, upon treatment with various chemicals, especially $FeSO_4$, liberate D. In 1998, Cascio and colleagues [19] demonstrated that $FeSO_4$ increased D production by the C6-2B subclone of the rat C6 glioma cell line, which do not contain P450c17 protein or mRNA. Even in the presence of the P450c17 inhibitor, SU-10603, $FeSO_4$ increased the formation of D, indicating that the Fe^{2+}-sensitive process does not involve P450c17 and it may be due to the fragmentation of in situ-formed tertiary hydroperoxides [19]. Further experiments suggested that the precursor of the D produced in C6 cells is a steroid where both C-17 and C-20 are oxygenated. We now report that, in addition to glioma cells, normal rat brain cortex has the ability to synthesize D via this alternative pathway. Using primary cultures of differentiating rat brain glial cells, we investigated developmental changes in: (1) the mRNA, protein, and enzymatic activity expression of P450c17; and (2) D synthesis by the alternative pathway.

2. Materials and methods

2.1. Materials

[7-^{3}H(N)]P (sp. act. 21.1 Ci/mmol) and [1,2,6,7-^{3}H(N)]D (sp. act. 89.2 Ci/mmol) were obtained from DuPont-New England Nuclear (Wilmington, DE). SU-10603 was a gift from CIBA-GEIGY (Suffern, NY). Cell culture supplies were purchased from GIBCO (Grand Island, NY) and cell culture plasticware was from Corning (Corning, NY). Poly-L-lysine, transferrin, sodium selenate, biotin, putrescin and insulin were from Sigma (St. Louis, MO). Platelet-derived growth factor (PDGF) was from Upstate Biotechnology (Lake Placid, NY). Electrophoresis reagents and materials were supplied by Bio-Rad (Richmond, CA). Sep-Pak Silica cartridges were purchased from Waters (Milford, MA). Organic solvents were of HPLC grade purchased from Fluka (New York) and Fisher Scientific (Pittsburgh, PA). All other chemicals were of analytical quality and were obtained from Sigma.

2.2. D synthesis in microsomes

Sprague-Dawley rats were killed at postnatal day 1 (P1) by decapitation and frontal cortices were collected. Microsomes were prepared by differential centrifugation as previously described [19]. Briefly, cortices were minced and homogenized in 0.25 M sucrose, centrifuged for 10 min at 1000**g**, the pellets were discarded and the supernatants were centrifuged for 10 min at 10 000**g**. The supernatants were collected and centrifuged for 60 min at 120 000**g**. Microsomal pellets were washed and suspended in 50 mM tris-maleate buffer [pH 7.4] to a final protein concentration of 5 mg/ml.

D formation by isolated microsomes was examined as previously described [19]. In brief, in order to test the P450c17 enzymatic activity, 100 μg of the microsomal protein fraction were incubated in tris-maleate buffer in the presence of 600 μM NADPH, 10 mM glucose-6-phosphate, 1.5 KU/l glucose-6-phosphate dehydrogenase together with the substrate P (50 μM) in the presence or absence of the inhibitor of P450c17 SU-10603 (5 μM; 20). To test the presence of a Fe^{2+}-mediated activity responsible for D formation, microsomes were also incubated with 10 mM $FeSO_4$ in the presence and absence of 10 μM P with or without 5 μM SU-10603. All the incubations were carried out at 37°C in 5% CO_2 atmosphere for 20 min and stopped by addition of cold ethanol. Steroids were extracted using diethyl-ether, separated by HPLC and quantified by RIA. The identity of D under these conditions was previously established by Gas Chromatography-Mass Spectrometry [19].

2.3. Cell culture and treatments

Purified cortical O-2A progenitor and type I astrocytes were prepared by modification of the methods described by Gallo and Armstrong [21]. In brief, P1 Sprague-Dawley rats were killed by decapitation and their cortices were removed under aseptic conditions. Upon removal of the meninges, the cortices were mechanically dissociated and filtered through 85 μm nylon mesh. Cells were resuspended in Dulbecco's Modified Eagle's Medium (DMEM) containing high glucose, 10% heat-inactivated fetal bovine serum and 3% heat-inactivated calf serum and plated in T75 plastic flasks (one cortex per flask) at 37°C and 5% CO_2 in air. Media were changed 3 days later. On day 7, a monolayer of type I astrocytes was present and oligodendrocyte progenitor cells began to appear growing on top of

the astrocyte layer. On day 10, media were changed and the O-2A progenitor cells were detached by overnight shaking. Contaminating microglial cells were further eliminated by plating this fraction on culture dishes for 90 min. The non-adherent O-2A progenitor cells were collected and plated at a density of 250 000 cells per 35 mm dish coated with poly-L-lysine. Cortical O-2A progenitor cells were cultured for 7 days in DMEM supplemented with insulin (10 mg/ml), 15% heat-inactivated calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin in order to obtain mature oligodendrocytes. On some occasions O-2A progenitor cells were cultured up to 3 d in DMEM-N1 media containing 50 mg/ml transferrin, 10 μg/ml biotin, 25 μM sodium selenate, 10 mg/ml insulin, 2 M putrescine, 280 μM progesterone, 100 units/ml penicillin and 100 μg/ml streptomycin. These cells were treated every 24 h with PDGF (10 ng/ml) in order to obtain oligodendrocytes at an early differentiation state. Cultures enriched in different cell types were characterized immunocytochemically by using cell-specific markers: A2B5 cell surface antigen for the O-2A precursors, PDGF-receptor for the bipolar immature oligodendrocytes, galactocerebrosidase C (Gal-C) for the mature oligodendrocytes, and glial fibrillary acidic protein (GFAP) for the type I astrocytes. Using these markers, cell purity for the various glia cell types isolated could be assessed at $\sim 95\%$.

2.4. Immunohistochemistry

For immunohistochemistry tissues were removed, fixed in 10% formaldehyde, dehydrated, and then embedded in paraffin. Sections of 5 μm thickness were cut, deparaffinated and hydrated. Streptavidin-biotin-peroxidase immunostaining was carried out using Histostain-SP kits (Zymed Laboratories, South San Francisco, CA). Dilutions of 1:100 of the P450c17 antiserum [22] and 1:400 of the IgG fraction of S-100 protein antiserum (Sigma) were used. Normal rabbit serum at the same dilution as P450c17 was used as control.

2.5. Immunocytochemistry

Cell cultures used for immunostaining were grown on coverslips coated with poly-L-lysine or they were grown in four chambered slides (VWR, W. Chester, PA). Cell suspensions were collected and fixed on a slide with the aid of a cytospin. Cells were fixed with 70% ethanol at 4°C for 15–30 min. Slides were incubated with primary antibody in 10% calf serum for 1 h at room temperature at the following dilutions (polyclonals) or concentrations (monoclonals): P450c17 polyclonal at 1:200 [22], GFAP mouse monoclonal IgG fraction at 1 μg/ml (Boehringer Mannheim, Indianapolis, IN), Gal-C mouse monoclonal IgG fraction at 1 μg/ml (Boehringer Mannheim); A2B5 mouse monoclonal IgM at 5 μg/ml (Boehringer Mannheim). HRP-linked anti-rabbit IgG and HRP-linked anti-mouse IgG (Transduction Laboratories, Lexington, KY) were applied at a dilution of 1:2000 and 1:500 respectively for 1 h at room temperature, and HRP substrate 3-amino-9-ethyl carbazole (Sigma) was used to detect peroxidase reaction. Phosphatase-labeled anti-mouse IgM (KPL, Gaithersburg, MD) and HistoMark Red assay kit (KPL) were used to detect phosphatase reaction (A2B5). Slides were counterstained with hematoxylin (Sigma).

2.6. Glia cell D formation, isolation and measurement

To determine the amount of D synthesized, cells were washed and pretreated with serum-free medium for 30 min to remove preexisting steroids. Media was replaced and cells were treated for 2 h with or without the indicated concentrations of $FeSO_4$, with and without SU 10603 (10 μM). At the end of the incubation, culture medium was quickly removed, the remaining cells were collected by scraping and sonicated in 1 ml ice-cold deionized water. Aliquots of these sonicates were reserved for protein assay and the remainder recombined with the culture medium to be processed for steroid extraction and isolation as previously described [19,23]. In brief, samples were extracted with diethyl-ether/ethyl acetate (1:1, vol:vol), the organic phases were collected and then evaporated to dryness. In all samples radiolabeled steroids were added to correct for the recovery of the extraction. The dried residues were resuspended in n-hexane, applied to Sep-Pak Silica cartridges, and the steroids of interest were eluted with n-hexane/isopropyl alcohol (95:5, vol:vol). The steroids were then separated by HPLC (Beckman System Gold, Fullerton, CA) using a Beckman ultrasphere XL 3 μm Spherical 80 A pore column (Phenomenex, Torrance, CA) equilibrated with methanol (50%) in water and eluted with a 1 ml/min flow rate on a 50–100% gradient of methanol. Steroids were identified by respective retention times compared with radiolabeled standards (Rt P = 28 min, Rt progesterone = 24 min, Rt D = 18 min). In previous experiments, steroids in these fractions were identified as P, progesterone and D, respectively, by Gas Chromatography-Mass Spectrometry [19]. Steroid contents were quantified using specific RIAs. Antisera to D were obtained from ICN (Costa Mesa, CA) and the assays were performed as described by the manufacturer. The sensitivity of the RIAs was 10 pg. The analysis of the RIA data was performed using the IBM-PC RIA data reduction program (version 4.1) obtained from Jaffe and Associates (Silver Spring, MD).

2.7. Reverse transcriptase-PCR

Total cellular RNA from astrocytes, O-2A progeni-

tors, oligodendrocytes and MA-10 Leydig cells was isolated by acid-guanidium thiocyanate-phenol-chloroform extraction method using the RNAzol B reagent (Tel-Test, Friendswood, TX). Reverse transcription and PCR were carried out as previously described [19] using the Gene Amp RNA PCR kit (Perkin Elmer, Foster City, CA), 1 μg of total RNA as template, and 20 μM of specific primers. The primers used for PCR amplification for the P450c17 were: sense CCCATCTATTCTCTTCGCCTGGGTA and antisense GCCCCAAAGATGTCTCCCACCGTG. PCR products were resolved on 1.5% agarose electrophoresis gels containing 1 μg/ml ethidium bromide. The amplified fragments were recovered and purified using the Quiaquick gel extraction kit (Quiagen, Chatsworth, CA). The identity of the generated PCR products was confirmed by automatic sequencing carried out using the ABI Prism Dye Terminator Cycle Sequencing ready reaction kit (Perkin Elmer). DNA sequencing was performed at the Lombardi Cancer Center Sequencing Core Facility (Georgetown University Medical Center).

2.8. Miscellaneous

Protein concentration was determined by the protein-dye binding assay of Bradford [24] using bovine serum albumin as standard. Statistical analysis of the data was performed by either ANOVA or unpaired *t*-test using the Instat (version 3.0) package from GraphPad (San Diego, CA).

3. Results

Immunohistochemical studies in postnatal day 1 (P1; Fig. 1(B)) and 70 day old adult (Fig. 1(D)) rat brains failed to demonstrate the presence of P450c17 immunoreactivity. We used various dilutions of the antiserum and the data shown is with a 1:100 antibody dilution, deliberately chosen in order to detect any immunoreactivity in rat brain. At the same dilution (1:100) the anti-P450c17 antiserum gave a strong positive reaction in the P1 (Fig. 1(F)) and 70 day old (Fig. 1(H)) rat testes, where in addition to the interstitial Leydig cells, some germ cells were immunopositive. Control sections incubated with normal rabbit serum were negative (Fig. 1(E, G)). S-100 was used as the positive control for brain and strong reactions were seen in both the P1 (Fig. 1(A)) and adult rat brains (Fig. 1(C)). These results suggest that P1 rat brain may not have the ability to form D. We have recently demonstrated the presence of an alternative pathway for D formation in rat C6 glioma cells [19]. Following a similar protocol, we examined the ability of microsomes from the cortex of postnatal day 1 (P1) rats to make D by this alternative pathway. Microsomes were isolated from whole cortex as described and incubated with or without 50 μM P, 10 mM $FeSO_4$ and 10 μM SU 10603, a specific P450c17 inhibitor. Cortical microsomes alone or with 50 μM P do not make any detectable D (Table 1). Incubating microsomes with $FeSO_4$ alone causes the production of 116 pg/mg protein D. This production of D is further increased by addition of P and $FeSO_4$. This Fe^{2+}-dependent D formation, in the presence or absence of P, is not inhibited by SU 10603, indicating that this is a P450c17-independent process similar to that previously seen in rat C6 glioma cells.

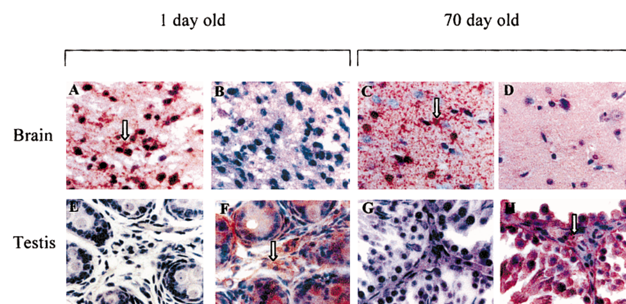

Fig. 1. Absence of P450c17 (B, D, F, H) immunoreactivity in 1 day (B) and 70 day-old (D) rat brain. Various brain areas were examined and none was found to contain P450c17 immunoreactivity. S-100 immunolocalization in 1 day (A) and 70 day-old rat brain (C) serves as a positive control for brain immunostaining. For P450c17 note the strong staining in 1 day (F) and 70 day-old (H) rat testis. Normal rabbit serum (E,G) serves as a negative control for testis immunostaining. Magnification: 400 ×.

Because of the recently published data indicating that P450c17 expression may be a developmentally regulated process in vivo [13,25], we examined P450c17 mRNA,

Table 1
D formation by isolated brain cortex microsomes from 1 day-old (P1) rats[a]

Treatment of P1 cortex microsomes	D, pg/mg protein
Control	ND
Pregnenolone (50 μM)	ND
$FeSO_4$ (10 mM)	116 ± 44
$FeSO_4$ (10 mM) + SU 10603 (10 μM)	174 ± 69[b]
Pregnenolone (50 μM) + $FeSO_4$ (10 mM)	350 ± 20[c]
Pregnenolone (50 μM) + $FeSO_4$ (10 mM) + SU 10603 (10 μM)	380 ± 35[c,d]

[a] Incubations and D measurement were performed as described in Section 2. Results shown are means ± SD from three independent experiments ($n = 9$). ND, non detectable.
[b] Significant ($P = 0.0494$) compared to $FeSO_4$ treatment.
[c] Highly significant ($P < 0.0001$) compared to $FeSO_4$ treatment.
[d] Significant ($P = 0.0402$) compared to pregnenolone + $FeSO_4$ treatment.

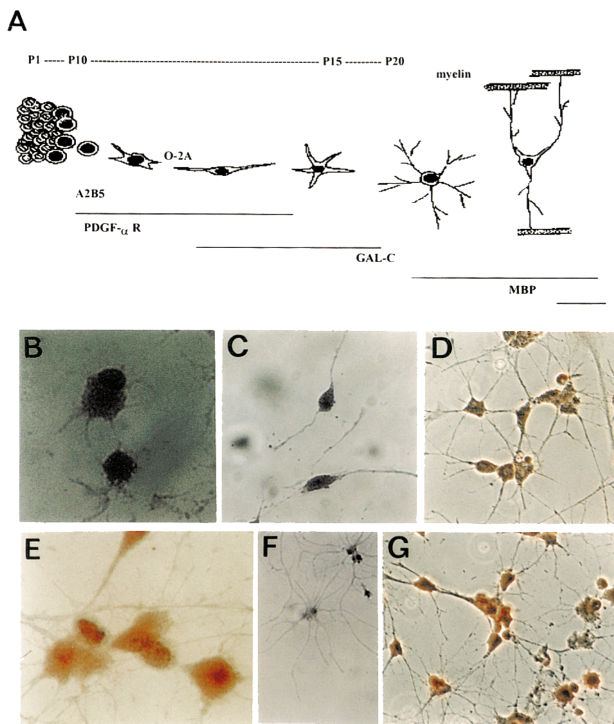

Fig. 2. Developing O-2A progenitors and oligodendrocytes were analyzed using immunocytochemistry to characterize the state of differentiation. Panel A shows the characteristic morphology of glia cells during differentiation and development from postnatal day 1 (P1) to myelinization and the specific time-frame where various markers are expressed. O-2A progenitor cells are A2B5 positive (B, 400 × magnification) and P450c17 positive (E, 400 × magnification). Immature oligodendrocytes express PDGF receptor (C, 250 × magnification). Mature oligodendrocytes express both GalC (D, 250 × magnification) and P450c17 (G, 250 × magnification). Omission of the primary antibody was used as a control (F, 250 × magnification).

protein and activity in primary cultures of differentiating oligodendrocytes and astrocytes. In Fig. 2 we characterize the different stages of oligodendrocyte differentiation (Fig. 2(A)) in our model system. O-2A progenitor cells are A2B5 immunopositive (Fig. 2(B)) and P450c17 immunopositive (Fig. 2(E)). Immature oligodendrocytes express PDGF receptor (Fig. 2(C)). Mature, fully differentiated oligodendrocytes express both galactocerebrosidase (GalC, Fig. 2(D)) and P450c17 (Fig. 2(G)). Control staining of differentiated oligodendrocytes using normal rabbit serum is shown in Fig. 2(F).

We looked for expression of mRNA for P450c17 in these differentiating cell types and in Type I astrocytes. After 35 cycles of RT-PCR, we found that both mature oligodendrocytes and the O-2A progenitor cells express P450c17 mRNA (Fig. 3). The generated 743 bp fragment was sequenced and found to be identical to the reported rat P450c17 nucleotide sequence [26] and 85% identical to the mouse P450c17 sequence. Surprisingly, we also found a strong signal for P450c17 mRNA in the MA-10 Leydig tumor cells. These cells express very

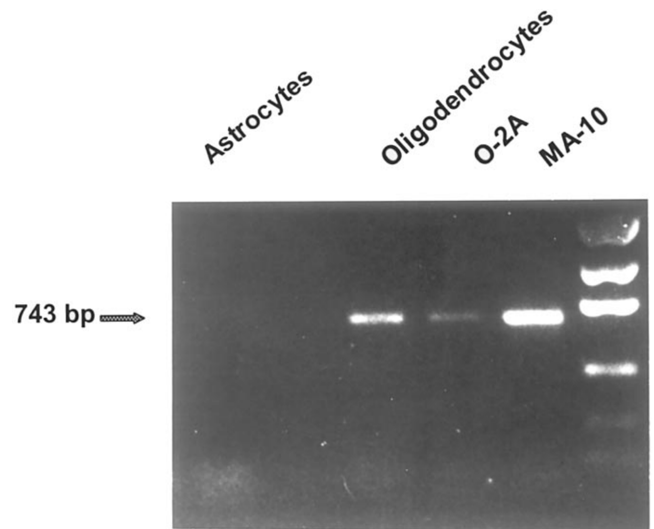

Fig. 3. P450c17 mRNA expression in differentiating rat brain glia cells. mRNA for P450c17 was isolated and amplified using specific primers. Both O-2A progenitor cells and mature oligodendrocytes express message for P450c17, as do mouse MA10 Leydig tumor cells (positive control). Type I astrocytes do not express message for P450c17, even after 35 cycles of PCR.

low amounts of P450c17 and their major product is progesterone and not testosterone [27]. In our hands, these cells make large amounts of progesterone, in response to hCG (data not shown), and do not contain an immunoreactive P450c17 protein (data not shown), suggesting that the expression of P450c17 mRNA, which may have been induced by the culture conditions used, does not result in the production of an active enzyme.

Fig. 4 shows that O-2A progenitor cells, which are A2B5 positive, and mature oligodendrocytes, which express immunoreactivity for GalC but not for A2B5, make D from exogenous P. Interestingly, the O-2A progenitor cells make more D than the mature oligo-

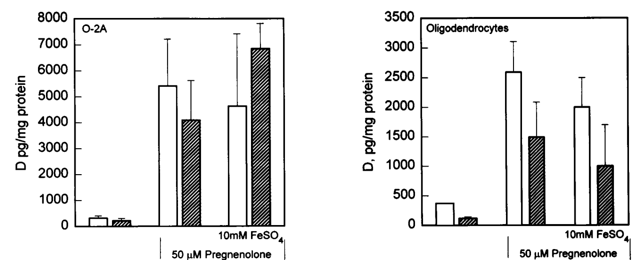

Fig. 4. D formation by O-2A progenitor and mature oligodendrocytes. Cells were incubated in the absence (open bars) or presence (hatched bars) of 10 μM SU 10603. D was extracted, isolated and measured as described under materials and methods. Cells have very low levels of D under basal conditions. However, both cell types will make D from exogenous P (50 μM) ($P < 0.001$). This activity is not affected by application of the competitive P450c17 inhibitor SU 10603 or by addition of 10 mM $FeSO_4$. Results shown are means ± SD from three independent experiments ($n = 9$).

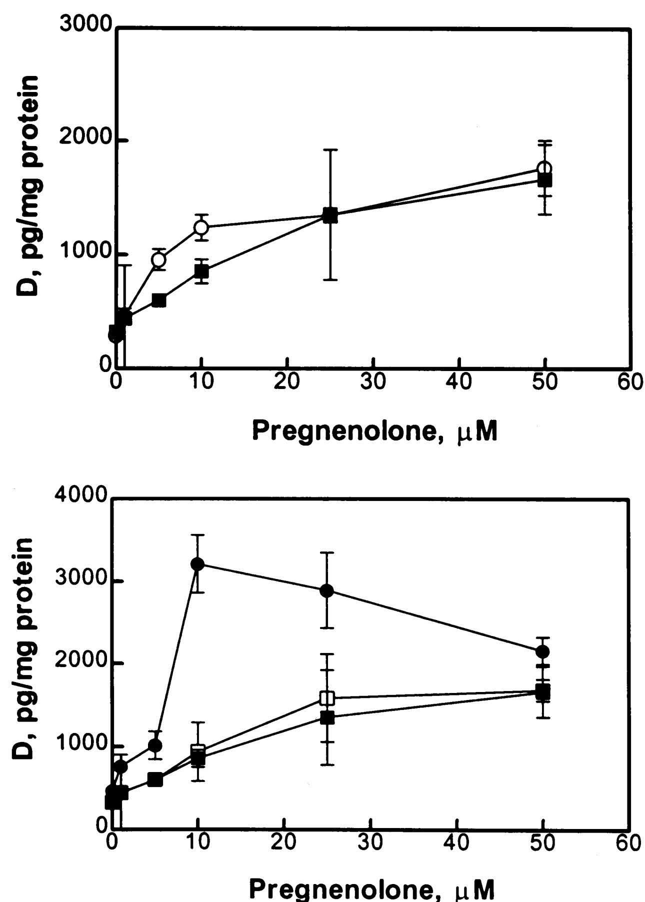

Fig. 5. Effect of increasing concentrations of exogenous P on D formation by mature oligodendrocytes. Addition of increasing concentrations of P to mature oligodendrocytes results in increased D formation (closed squares, both panels). Top panel shows the effect of the competitive P450c17 inhibitor SU 10603 (10 μM), added together with P, on D synthesis. Closed squares, P alone; open circles, P + SU-10603. Bottom panel shows the effect of 3 mM (open squares) and 10 mM (closed circles) $FeSO_4$, added together with P, on D formation. Closed squares, P alone. Results shown are means ± SD from a representative experiment ($n = 3$–4). Similar results were obtained in two independent experiments.

dendrocytes, providing support for a role for D during development. In these studies, we observed that D formation in the presence of 50 μM P was not affected by application of $FeSO_4$, indicating that this may be due to a P450c17-dependent activity. However, application of SU 10603 does not block D production. This may be due to the relatively large amount of P (50 μM) applied to the cultures. SU 10603 is a competitive inhibitor of P450c17 [20]; in this scenario, the exogenous P may overcome the ability of SU 10603 to block the enzyme activity. To test this hypothesis we performed dose-response studies of P in the presence and absence of SU 10603. Fig. 5 (top panel) shows that SU 10603 (10 μM) did not inhibit the formation of D induced by either 1, 3, 5, 10, 25, or 50 μM P. Further studies (Fig. 5 bottom panel) indicated that in the presence of submaximal concentrations of P, $FeSO_4$ increased by 3–4-fold D formation ($P = 0.028$), indicating the presence of a P450c17-independent pathway for D formation, which it may be inhibited by the large amounts (50 μM) of the precursor P used in our initial studies (Fig. 4). It should be noted that in parallel experiments we verified the biological activity of SU-10603 using isolated rat Leydig cells; SU-10603 inhibited the LH-stimulated testosterone production (data not shown).

We also examined the expression of P450c17 message and activity in type I astrocytes. Type I astrocytes are GFAP immunopositive (Fig. 6(B)) but P450c17 immunonegative (Fig. 6(C)). Moreover, they do not contain P450c17 message (Fig. 3). Type I astrocytes will produce D in a dose-dependent manner when treated with $FeSO_4$, indicating the presence of the alternative pathway (Fig. 6(D)). This response is maximal at 10 mM $FeSO_4$, which gives a 12-fold increase over basal D levels (Fig. 6(D)). Treatment of the cells with $FeSO_4$ and P further increased (19-fold) the amount of D formed (Table 2). Application of SU 10603 does not block neither the Fe^{2+}-dependent nor the Fe^{2+}- and P-dependent D production (Table 2).

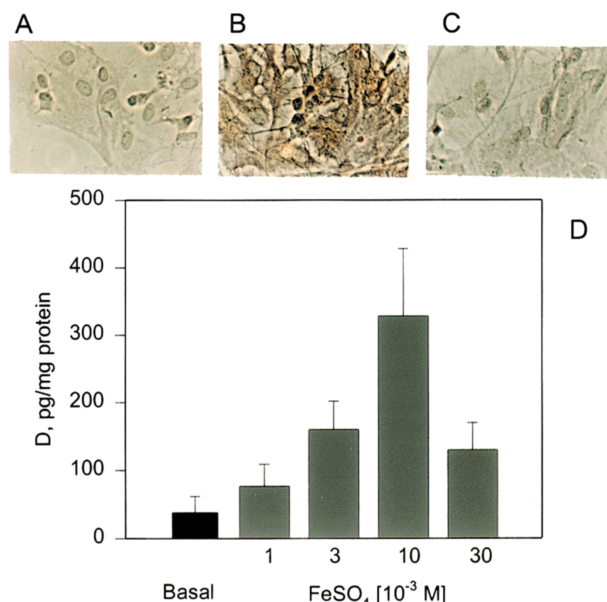

Fig. 6. P450c17 protein and alternative pathway activity in type I astrocytes. Type I astrocytes are immunopositive for GFAP (B) but do not express immunoreacitve P450c17 (C). (A) is control staining omitting the primary antibody. When type I astrocytes are incubated with $FeSO_4$ (D), they respond by producing large amounts of D. This response peaks at 10 mM $FeSO_4$. Results shown are means ± SD from three independent experiments ($n = 9$). The effect of $FeSO_4$ was significant ($P < 0.001$) at all concentrations used above 1 mM.

Table 2
D formation by isolated type I astrocytes, incubations and D measurement were performed as described in Section 2[a]

Treatment of astrocytes	D, pg/mg protein
Control	38 ± 27
$FeSO_4$ (10 mM)	329 ± 101[b]
$FeSO_4$ (10 mM)+SU 10603 (10 μM)	315 ± 130[b,c]
Pregnenolone (50 μM)+$FeSO_4$ (10 mM)	730 ± 143[b,d]
Pregnenolone (50 μM)+$FeSO_4$ (10 mM)+SU 10603 (10 μM)	782 ± 214[b,e]

[a] Results shown are means ± SD from two independent experiments ($n = 6$–8).
[b] Highly significant ($P < 0.0001$) compared to control.
[c] Non significant compared to $FeSO_4$ treatment.
[d] Highly significant ($P < 0.0001$) compared to $FeSO_4$ treatment.
[e] Non significant compared to pregnenolone+$FeSO_4$ treatment.

4. Discussion

There is controversy in the field of neurosteroid biosynthesis concerning the mechanism by which D is produced in the brain. There have been attempts to demonstrate the presence of P450c17 in the brain, but evidence for enzymatic activity has proven more elusive. We have recently demonstrated the existence of an alternative pathway for D synthesis in both rat [19] and human [23] glioma cells but not in other cell types, such as human breast cancer cells [19], isolated normal rat Leydig cells [19] and human neurons [23]. This pathway is modulated by levels of reactive oxygen species and can be stimulated by either ferrous ions or β-amyloid peptide [23]. Based on a series of biochemical studies we proposed that D formation is due to the fragmentation of an in situ formed tertiary hydroperoxide reacting to Fe^{2+} to yield a ketone, D [19]. This hydroperoxide may a 17-hydroperoxide of P or a 17,20-hydroperoxide of cholesterol [19]. It has recently been proposed that D may play a role in neurodevelopment, due to transient expression of P450c17 [13] and the potential ability of D to aid in the neuronal pathway formation [25]. We therefore examined the neurosteroid biosynthetic pathways for D in developing rodent brain.

Microsomes from P1 cortex do not contain immunoreactive P450c17 protein and do not have the ability to form D in the absence or presence of the substrate P. However, when these microsomes are incubated with ferrous ions, we found an increase in the production of D. In agreement with our data on the C6 glioma cells [19], addition of P to these microsomes further increases the amount of D formed in the presence of Fe^{2+}, suggesting that the precursor of D may be a hydroperoxide of P [19]. Addition of the specific P450c17 inhibitor SU 10603 [20] failed to block the Fe^{2+}-induced D formation in either the presence or absence of P, suggesting that the reaction responsible for D formation is not mediated by the P450c17 enzyme.

Le Goascogne et al. failed to show the presence of P450c17 immunoreactivity in rat or guinea-pig brain [12]. Compagnone et al. demonstrated expression of P450c17 immunoreactivity in embryonic day 13.5 rat and 10.5 [13]. This immunoreactivity decreases with age and is absent in the adult brain. This group has further shown a potential role for D in modulating neurite outgrowth in the neocortex [25]. These data suggest that P450c17 expression and activity may be a developmentally regulated process, present in the embryonic brain but disappearing after birth. In agreement with both studies we also failed to show the presence of P450c17 immunoreactivity in neonatal and adult rat brain even when high concentrations of the anti-P450c17 antiserum were used. We therefore examined P450c17 mRNA, protein and activity in developing oligodendrocytes at different stages of differentiation, as well as in type I astrocytes in primary cultures. We found that both O-2A progenitors (A2B5 positive) and mature (GalC positive) oligodendrocytes express message for P450c17, contain a P450c17 immunoreactive protein and are able to form D in the presence of P. Because we did not observe P450c17 protein and activity in P1 cortical tissue, from where the cells were isolated, it is possible that the process of cell isolation and culture in vitro may be responsible for the induction of P450c17 expression. Further studies using increasing concentrations of P demonstrated the dose-dependent synthesis of D by oligodendrocytes. Although these data initially indicated to us that P450c17 may be involved in D biosynthesis by oligodendrocytes in vitro, two observations suggested that D formation by these cells may not be related to the P450c17 activity. First, the O-2A progenitors make two times more D than the mature oligodendrocytes. However, the progenitors contain 5–10 times less P450c17 mRNA compared to mature oligodendrocytes and no difference could be seen in the amount of immunoreactive P450c17 protein present in these two cell types. Although we recognize that RT-PCR is a semiquantitative procedure to measure mRNA levels, the difference between O-2A and mature oligodendrocytes is striking, considering that we used the same amount of total RNA as template. Second, we noted that the specific P450c17 inhibitor SU 10603 was unable to block D formation by oligodendrocytes, as was the case with the P1 cortex microsome D synthesis, suggesting that the mechanism underlying this substrate-induced D synthesis is not mediated by the P450c17 enzyme. It is possible that oligodendrocytes may have high endogenous free radical and/or ferrous ion levels to induce D formation from P in the absence of $FeSO_4$ and thus in a SU-10603-resistant manner.

Surprisingly, in our initial studies we observed that in the presence of 50 μM of exogenous P these cells do not produce more D in response to ferrous ions, suggesting that they may lack the alternative pathway for D synthesis. However, in the presence of submaximal concentrations of P, $FeSO_4$ increased D formation, indicating the presence of a P450c17-independent pathway for D synthesis, which may be inhibited by the large amounts (50 μM) of the substrate P used in our previous experiments. Because at present we do not know the process by which D is formed from P and we also do not know whether P is the best substrate for this oxidative stress-induced D formation, we cannot provide yet an explanation for the blocking effect of high concentrations of P. Studies are in progress in our laboratory to characterize this alternative pathway and identify the endogenous substrate(s) used to form D.

In addition to looking at P450c17 in oligodendrocytes, we examined the role of this enzyme in D production by astrocytes. We found that type I astrocytes do not contain message or protein for P450c17. However, they do make D, both in the absence and presence of $FeSO_4$. The basal production of D may be due to endogenous reactive oxygen species. This ferrous-induced D formation was not inhibited by SU 10603. This indicates that type I astrocytes can make D in a P450c17-independent manner, via the alternative pathway. This pathway is very responsive to ferrous ions, with a 12-fold increase in D production over basal levels in the presence of 10 mM $FeSO_4$. This ferrous-induced D formation by the astrocytes was further increased by addition of exogenous P. However, in contrast to oligodendrocytes, astrocytes do not convert exogenous P to D in the absence of ferrous ions (data not shown). These data suggest that there is a major difference between brain microsomes and astrocytes in one hand and immature and mature oligodendrocytes on the other hand in their ability to form D in the presence of exogenous P. Considering that both pregnenolone and cholesterol could serve as substrates for D formation [18,19] one possible explanation of this difference may be that in oligodendrocytes endogenous cholesterol may be the preferred precursor. Interestingly, it has been reported that oligodendrocytes produce more cholesterol than astrocytes further supporting the above presented hypothesis [28].

Although the high concentrations of ferrous ions used to detect the alternative pathway is of concern, these concentrations allowed us to demonstrate a brain-specific [19] mechanism of D formation distinct of the classic pathway present in peripheral steroidogenic tissues. First, we should note that concentrations up to 10 mM of $FeSO_4$ were not toxic to glia cells (unpublished results). Second, it is possible that this mechanism may not reflect physiological but pathological situations. Iron is a significant component of the senile plaques and iron encrustation of blood vessels is common in Alzheimer's disease [29]. Iron levels are elevated and iron mobility decreased in Alzheimer's disease affected brains [29]. Moreover, β-amyloid was shown to increase reactive oxygen species [30] and increased oxidative stress has been shown in Alzheimer's disease brain [31,32]. In support of a role of the alternative pathway of D formation in neuropathology, we recently reported that β-amyloid-induced reactive oxygen species and D production in human glioma cells in culture and that both free radical and D formation were blocked by the antioxidant Vitamin E [23]. In addition to Alzheimer's pathology, in head trauma and stroke massive bleeding could significantly produce iron-mediated oxidative stress and neurodegeneration [33]. If our hypothesis were correct one would expect that the brain D levels would be elevated in patients with neurodegenerative disorders that involve oxidative stress. Indeed, in preliminary studies we found that D levels are significantly higher in all regions of Alzheimer's disease brain compared to control [34].

During the preparation of this manuscript, two papers appeared by Zwain and Yen on the biosynthesis of neurosteroids and D in rat brain [35,36]. These authors provided evidence that D is biosynthesized in the brain, and more specifically, in astrocytes, through the P450c17 enzymatic pathway. They explain the inability of previous groups to demonstrate P450c17 activity to be due to microglial contamination of their culture system, which may inhibit the expression of P450c17. Although cell-cell interaction may modulate neurosteroid production by rat glial cells, these authors did not prove their suggestion, they did not investigate the effect of adding isolated microglia back to the glia cell cultures. Concerning the role of microglia, we would like also to note that the isolated glial cells used in our studies were devoid of microglia contamination as determined using specific antibodies recognizing rat microglia cells. We also observed that isolated microglia do not make neurosteroids (data not shown).

These authors also detected P450c17 mRNA, but not the enzyme, in astrocytes and neurons but not in oligodendrocytes. They also reported that the levels of P450c17 mRNA were very low, but the production of D by astrocytes was high, suggesting a potent enzymatic activity for these cells. Although there are some common findings between our study and those of Zwain and Yen, there are important methodological differences. (1) The types of oligodendrocytes used in Zwain and Yen's experiments were not characterized. (2) In our hands, in contrary to what Zwain and Yen reported, the application of RIA analyses on raw, unpurified cell media or extracts from rat oligodendrocytes, rat astrocytes, rat C6 glioma cells, human glioma

cells, normal human astrocytes and human neurons never gave reliable assays of D. In our experiments, reliable detection and estimation of D required extensive purification before the use of RIA. Each sample was extracted with organic solvents, purified on two columns, silica and C18 HPLC, to isolate D. The concentration of D was then determined by specific radioimmunoassay with a sensitivity of 15 pg. Under these conditions, purified D was also identified by GC-MS. By contrast GC-MS of the total cell culture media indicated the presence of a number of unknown compounds, anyone of which might crossreact with various commercial anti-D antibodies. (3) Considering our recent data that human astrocytes produce four times the levels of reactive oxygen species than glioma cells in response to Fe^{2+} [23], we could anticipate the data presented by Zwain and Yen, they found that astrocytes have 4 times more P450c17 activity than oligodendrocytes. However, this 4-fold difference is not due to the presence of P450c17 enzyme. (4) As expected for every enzymatic reaction, the addition of increasing concentrations of substrate should saturate the response. This does not happen in the data shown by Zwain and Yen, suggesting that the anti-D antibodies used, may crossreact with other cell constituents. Thus, although our data agrees with that of Zwain and Yen that astrocytes make D, we still disagree on the mechanisms used in this production.

As noted above, addition of exogenous P is an artificial way to demonstrate the physiological role of P450c17 activity. Therefore, it is important to perform experiments on alternative pathways of steroid synthesis in the absence of the 'ideal' precursor in order to see alternative activities. We believe that the final answer to the question of how D is made in the brain will hinge on the isolation and characterization of the brain P450c17 or the Fe^{2+}-dependent activity, as well as on the generation of P450c17 knockout mice.

We have recently demonstrated that in human brain, all cell types (neurons, oligodendrocytes and astrocytes) express message and protein for P450c17 [23]. However, no activity could be found. Nevertheless, addition of Fe^{2+} or β-amyloid peptide, which increase cellular reactive oxygen species, resulted in the formation of D [23]. These data obtained in human cells support the data presented herein on the ability of astrocytes and oligodendrocytes to form D. Thus, in separate brain model systems, in three species, including human [23] and rat [19] glioma cell lines, primary cultures of human [23] and rat (this study) oligodendrocytes and astrocytes, microsomes from rat brain (this study), rat brain organic extracts [18] and bovine brain microsomes (unpublished results), the presence of a Fe^{2+}-dependent, P450c17-independent process of D formation has been demonstrated.

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