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Metabolism of Steroids in Pure Cultures of Neurons and Glial Cells: Role of Intracellular Signalling

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In the brain, the 5α-reductase converting testosterone (T) is present both in neurons and in glial cells, even if it prevails in neurons; the 3α-hydroxysteroid-dehydrogenase (3α-HSD), the enzyme converting dihydrotestosterone (DHT) into 3α-diol, is particularly concentrated in type 1 astrocytes. In glial cells, since the 5α-reductase is activated by a cAMP analogue, PKA seems to be involved in the control of this enzyme, postulating that nervous inputs utilizing cAMP as the second messenger might modify the activity of this enzyme in glial cells. Moreover, the results indicate that, in type 1 astrocytes, both the 5α-reductase and the 3α-HSD are stimulated by the co-culture with neurons and by the addition of neuron-conditioned medium, suggesting that secretory products released by neurons might intervene in the control of glial cell function.

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INTRODUCTION

In the central nervous system (CNS) of all animal species investigated so far [1, 2] testosterone (T) is converted into its "active" metabolite 5α-androstan-17β-ol-3-one (dihydrotestosterone, DHT), by the action of the enzyme 5α-reductase. In the brain, DHT may be further metabolized into 5α-androstan-3α,17β-diol (3α-diol) by the enzyme 3α-hydroxysteroid-dehydrogenase (3α-HSD). A large group of observations indicate that, the 5α-reductase is widely distributed in several CNS structures (e.g. the hypothalamus, the midbrain, etc.), even if its concentrations may be variable [1, 2]. For instance, it has been observed that this enzymatic activity is particularly elevated in nervous structures mainly composed of white matter (like the corpus callosum, the corticospinal tract, etc.) [3–6], as well as in purified myelin membranes [6, 7].

Previous studies of this laboratory have evaluated the distribution of the 5α-reductase and of the 3α-HSD among the different cellular components of the brain (neurons, astrocytes, oligodendrocytes). Two types of experimental preparations have been used: cells isolated by differential gradient centrifugations [8] and neurons and glial cells in culture [8, 9]. These studies have shown the preferential localization of the enzyme 5α-reductase in the neuronal component; however, this enzyme is also present in glial cells (particularly in type 2 astrocytes and in oligodendrocytes). On the contrary, the enzyme 3α-HSD appears to be mainly, if not exclusively, present in type 1 astrocytes [9]. A similar cellular distribution of the 5α-reductase and of the 3α-HSD has been found when progesterone (P) rather than T was used as the substrate [10].

In parallel studies, it has been found that the aromatase, the enzyme which transforms T into estrogens, is present only in neurons [11].

SIGNALLING SYSTEMS CONTROLLING THE 5α-REDUCTASE IN GLIAL CELLS

It is now well established that cells respond to extracellular signals reaching their membrane receptors by translating the information into different kinds of intracellular second messengers. A classical example is provided by the adenylatecyclase system; in this system, cyclic AMP (cAMP), which is formed from ATP by the membrane-bound enzyme adenylatecyclase, acts as a second messenger by activating the intracellular protein kinase A (PKA); a chain of phosphorilations follows. Another intracellular signal transduction mechanism is represented by the phosphatidylinositol (PI) system, in which the enzyme phospholipase C induces the breakdown of membrane linked precursors, leading to the formation of two different second

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messengers, inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DG). DG transports towards the cell membrane and activates the phosphorilating enzyme protein kinase C (PKC). The final events modulated by these two second messengers are the mobilization of calcium from intracellular stores (e.g. mitochondria) and/or the facilitation of the entry of extracellular calcium into the cell. Glial cells exhibit a large variety of receptors on their surface, the activation of which brings about either an increase (e.g. β_1-adrenergic, adenosine, PGE_1 receptors, etc.), or a decrease (e.g. α_2-adrenergic, somatostatin and melatonin receptors) [12] of the formation of cAMP. It has also been demonstrated that other types of glial receptors (e.g. muscarinic, α_1-adrenergic, glutamate, histamine, serotonin and bradykinin receptors) [12], utilize the phosphatidylinositol signalling pathways.

On the basis of these considerations, it was deemed of interest to analyze if manipulations of the two second messenger systems might be able to modify the 5α-reductase-3α-HSD activity present in cultured glial cells. The approach selected was to use a molecule able to activate PKC (the phorbol ester 12-*O*-tetradecanoyl-phorbol-13-acetate, TPA), in order to mimic the effects of the activation of the phosphatidylinositol pathway, and a cAMP analogue (8-Br-cAMP) in order to duplicate the effects of the activation of the adenylatecyclase. The 5α-reductase and 3α-HSD activities present in cultures of glial cells have been evaluated at different time intervals after treatment, utilizing labelled T and DHT as specific substrates for the two enzymes. In these experiments mixed glial cell cultures were used. They were obtained from the brain of neonatal rats as previously described [13].

Figure 1 shows the formation of DHT from labelled T in glial cultures treated for different times with TPA or with an inactive phorbol ester (4α-Ph). It is evident that neither TPA nor 4α-Ph are able to induce significant alterations of the 5α-reductase activity of the cells at any time interval considered. The minor decreases observed at 3 h and at 24 h are not statistically significant. Figure 2 shows the formation of DHT from labelled T in glial cell cultures at different time intervals after exposure to 10^{-3} M of 8-Br-cAMP. A statistically significant increase in the formation of DHT is evident after 6 h; the formation of DHT appears to be further stimulated after 12 h; at 24 h a stimulation is still present, but this is lower than that found at 12 h and similar to that observed at 6 h. Figure 3 shows the results of the study in which the possible effect of 8-Br-cAMP on the formation of 3α-diol was analyzed utilizing [^{14}C]DHT as the labelled substrate. It is apparent that, in cultured glial cells, there are no changes in the activity of 3α-HSD at any time considered.

The present results indicate that, in cultures of mixed glial cells, the formation of DHT is not modified

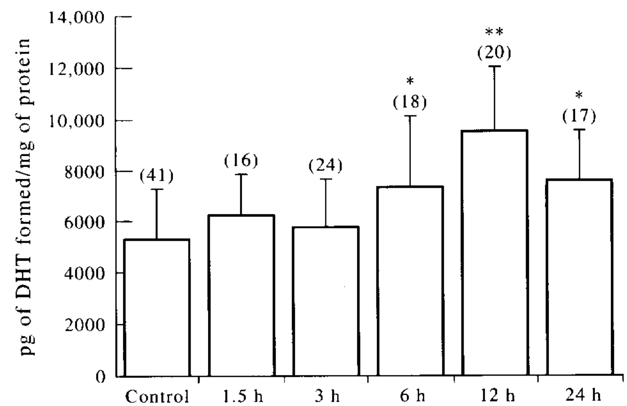

Fig. 2. DHT formation in cultured glial cells incubated for different time in presence of 8-Br-cAMP. Data were analyzed by ANOVA; Tukey HSD test for multiple comparisons. $^{**}P < 0.01$ vs control; $^{*}P < 0.05$ vs control. The numbers in parentheses represent the number of Petri dishes analyzed. Data are expressed as mean ± SD.

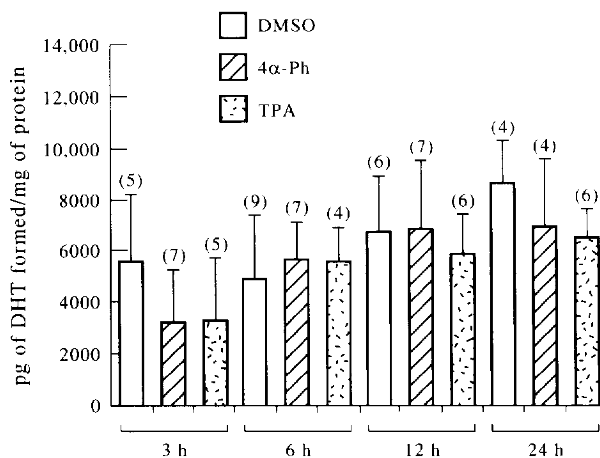

Fig. 1. DHT formation in cultured glial cells incubated for different times in the presence of active (TPA) and inactive (4α-Ph) phorbol esters. Data were analyzed by ANOVA. The numbers in parentheses represent the number of Petri dishes analyzed. Data are expressed as mean ± SD.

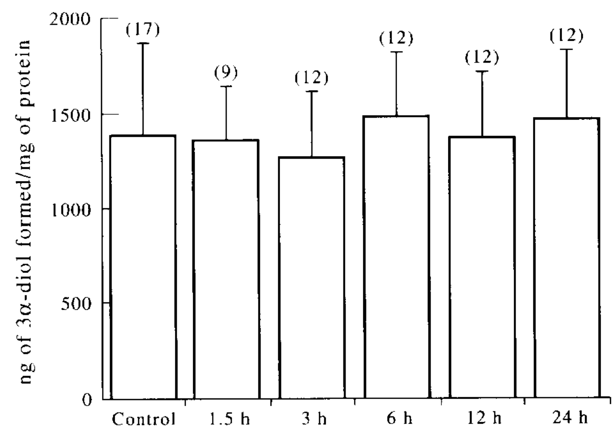

Fig. 3. 3α-diol formation in cultured glial cells incubated for different times in presence of 8-Br-cAMP. Data were analyzed by ANOVA. The numbers in parentheses represent the number of Petri dishes analyzed. Data are expressed as mean ± SD.

by the addition of phorbol esters, indicating that PKC probably is not involved in the intracellular signalling system controlling the enzyme 5α-reductase in these cells. On the contrary, a statistically significant increase in 5α-reductase activity over control levels has been observed after incubation with 8-Br-cAMP. The effect of the cAMP analogue appears to be specific for the 5α-reductase, since the activity of 3α-HSD did not show any variation. The results reported here are germane to a few previous data indicating that cAMP may participate in the control of other enzymatic systems in the brain. Callard [14] has found that dibutyril cAMP and a phosphodiesterase inhibitor, when added to primary cultures of the brain of adult turtles (*Chysemys picta*), increase the conversion of T to estradiol, stimulating the aromatase activity present in these cultures. Baulieu and Robel [15] have suggested that the addition of cAMP to rat mixed glial cell cultures may increase the synthesis of pregnenolone and 20α-OH-pregnenolone from mevanolactone. As mentioned in the Introduction, there is abundant evidence indicating that the glia receives a lot of nervous inputs, which use cAMP as the second messenger [12, 16]. Among these β_1-adrenergic and α_2-adrenergic are particularly relevant. On the basis of the data presented here, one might hypothesize that all these inputs play a role in modulating the 5α-reductase activity present in glial cells.

NEURONAL SIGNALS CONTROLLING THE 5α-REDUCTASE IN GLIAL CELLS

For many years glial cells have been considered to represent only a mechanical support for neurons. The existence of important functional relationships between specific glia components (oligodendrocytes, astrocytes, etc.) on one side, and neurons on the other have become apparent only recently. The interactions of glial cells with neurons have been shown to play an important role in neuronal migration, in neurite outgrowth, and in axonal guidance during ontogenetic development [17–21]. Moreover, it has been demonstrated recently that glial cells are able to synthesize, and possibly to release, an array of bioactive principles, like neurotransmitters, growth factors, interleukins, prostaglandins, excitatory amino acids, polyamines, neurosteroids, etc. [for review see 22]. It is probable that several of these principles may exert definite and specific influences on neuronal activity.

The glia–neuronal interactions are not a one-way phenomenon, since several data indicate that neurons may interfere with the proliferation and the maturation of glial elements. For example, neuronal activity has been shown to up-regulate the expression of the glial fibrillary acidic protein (GFAP), an astroglia intermediate filament protein which is considered a specific marker of astrocytes [23]. Moreover, other parameters of astrocytic function, like the enzyme glutamine synthetase [24], the formation of gangliosides [25] and the assembly of voltage-sensitive calcium channels [26] are activated only in the presence of neurons.

This peculiar compartmentalization of the two enzymes (5α-reductase and 3α-HSD) which characterize the 5α-reductive metabolism of androgens and progestagens in the brain (see Introduction) has prompted us to investigate whether humoral mechanisms of communication might exist between neurons and type 1 astrocytes (the site of the highest concentration of the enzyme 3α-HSD).

To examine the reciprocal interaction between astrocytes and neurons the following two approaches have been utilized: (1) a convenient co-culture system has been established, this allows the free transfer of secretory products from one type of co-cultured cells to the other, even if these remain physically separated; and (2) the addition of neuron-conditioned medium (CM) to cultures of astrocytes, and vice versa of astrocyte-conditioned medium to cultures of neurons. In both types of experiments, the activity of the two enzymes was studied using as precursors either labeled T (mainly to quantitate the formation of DHT, 5α-reductase activity), or labeled DHT to quantitate the formation of 3α-diol (3α-HSD activity).

The pure neuronal and glial cultures used in the present experiments were characterized by immunocytochemical procedures, using monoclonal antibodies against specific cellular markers (NFP, MAP_2 for neurons; GFAP for type-1 astrocytes). For more information the reader is referred to the text and the figures of a previous paper [9], which show cultures identical to the ones utilized in the present experiments. The formation of DHT and 3α-diol (expressed as picograms of steroids per mg of protein after 2 h of incubation) was first evaluated using T as the substrate in neurons and type 1 astrocytes, co-cultured for 4 days; neurons and type 1 astrocytes grown in similar conditions, but not co-cultured, represented the controls. As indicated in Fig. 4, control neurons showed an important formation of DHT, and a smaller formation of 3α-diol; the co-culture with type 1 astrocytes did not significantly affect the formation of either metabolite. Type 1 astrocytes, in the control culture, were able to form DHT, even if in quantities lower than those found in neurons, and 3α-diol in quantities similar to, or slightly higher than, those found in the neuronal cultures (Fig. 4). In the co-culture experiments, the formation of both 5α-reduced derivatives (DHT and 3α-diol) was increased in the astrocytes; however, only the formation of DHT was significantly higher than that recorded in the controls. However, if one considers, as an indication of the 5α-reductase activity, the sum of the two 5α-reduced metabolites (DHT plus 3α-diol) formed from T, it appears that their formation ($51{,}880 \pm 8946$) is significantly higher ($P < 0.01$) in the astrocytes co-cultured with neurons than in the controls ($34{,}417 \pm 11{,}308$).

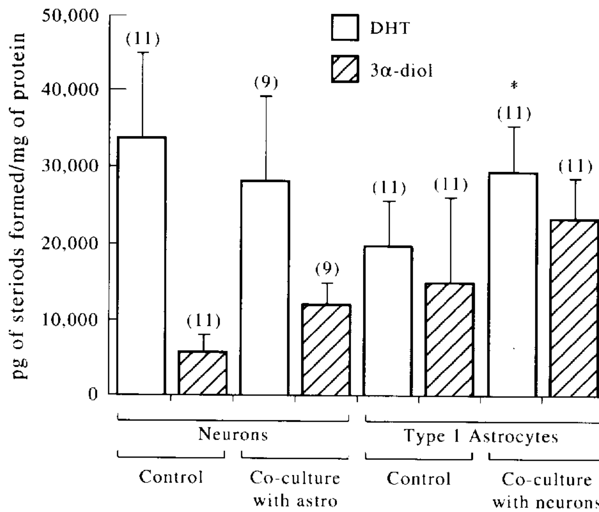

Fig. 4. Effect of 4 days of co-culture of neurons and type 1 astrocytes on DHT and 3α-diol formation (incubation with testosterone). Numbers in parentheses represent the numbers of determinations performed. $*P < 0.05$ vs the corresponding controls (Tukey HSD test). The data are expressed as means ± SD.

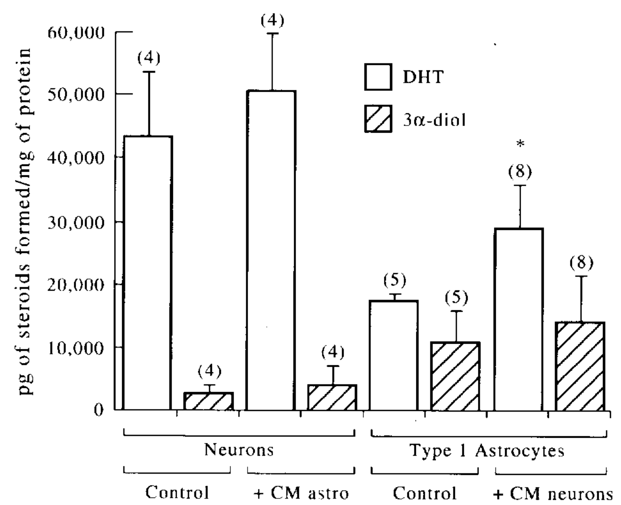

Fig. 6. Effect of a 2-day exposure to conditioned media (CM) on DHT and 3α-diol formation in neurons and type 1 astrocytes (incubation with testosterone). Conditioned media have been obtained after 2 days of culture. Numbers in parentheses represent the numbers of determinations performed. $*P < 0.01$ vs the corresponding controls (Tukey HSD test). The data are expressed as means ± SD.

In a subsequent experiment, the formation of 3α-diol was separately evaluated using DHT as the substrate (Fig. 5). As in previous experiments [9], the transformation of DHT into 3α-diol was very low in neurons, and the simultaneous presence of type 1 astrocytes in co-culture did not significantly influence this process. Again as in the previous experiments [9], 3α-diol appeared to be a major metabolite of DHT in type 1 astrocytes; the formation of 3α-diol was slightly, even if significantly, increased when the cells were co-incubated with neurons. The effects observed in the co-culture studies have been in general reproduced in the experiments in which neurons and astrocytes have been cultured separately, but have been exposed to the CM of the other type of cells. Figure 6 shows that the presence of the CM of type 1 astrocytes for 2 days does not influence the formation of either DHT or 3α-diol in neurons incubated with T as the precursor. On the contrary, the presence, for 2 days, of the CM of neurons significantly increased the formation of DHT in the astrocytes. Figure 7 reports the effect of the CM on the direct formation of 3α-diol from DHT in neurons and in astrocytes. As in the co-culture experiments, there was no effect of the glial cell medium on

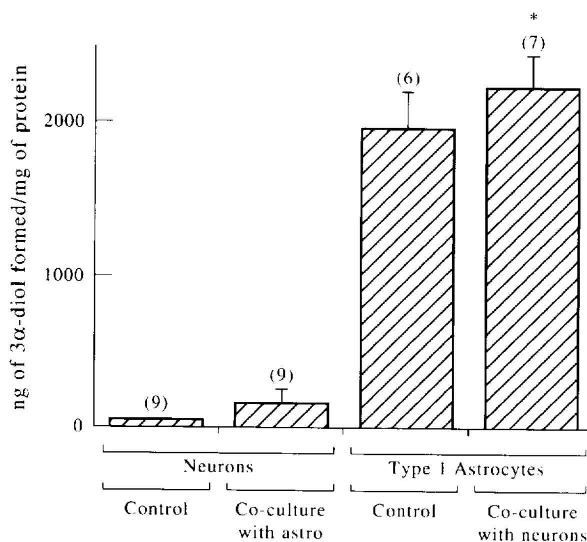

Fig. 5. Effect of 4 days of co-culture of neurons and type 1 astrocytes on 3α-diol formation (incubation with dihydrotestosterone). Numbers in parentheses represent the numbers of determinations performed. $*P < 0.05$ vs the corresponding controls (Tukey HSD test). The data are expressed as means ± SD.

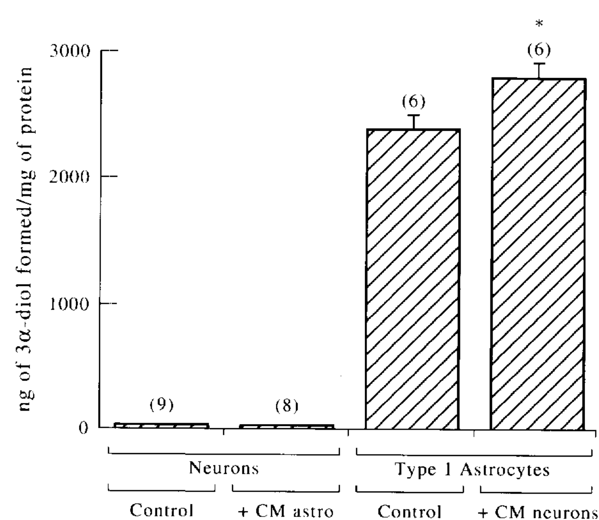

Fig. 7. Effect of a 2-day exposure to conditioned media (CM) on 3α-diol formation in neurons and type 1 astrocytes (incubation with dihydrotestosterone). Conditioned media have been obtained after 2 days of culture. Numbers in parentheses represent the number of determinations performed. $*P < 0.01$ vs the corresponding controls (Tukey HSD test). The data are expressed as means ± SD.

the neuronal 3α-HSD. On the contrary, a significant, even if small, increase in the formation of 3α-diol was observed in type 1 astrocytes exposed for 2 days to the CM of neurons.

The data presented here confirm, with minor quantitative differences, the previous observations mentioned in the Introduction [9], which have indicated that cultured neurons possess a higher 5α-reductase activity than type 1 astrocytes, and that, on the contrary, 3α-HSD is more elevated in type 1 astrocytes than in neurons. The present data indicate in addition that some principle(s) produced by the neurons may influence the activities of both enzymes in astrocytic cultures. First of all, it has been shown that, when T is used as the substrate, the presence of neurons in co-culture produces a significant stimulation of the 5α-reductase activity of type 1 astrocytes (as evaluated on the basis of the formation of DHT, or of the sum of DHT plus 3α-diol). In these experiments, the apparent increase in the formation of 3α-diol was probably due to two parallel effects; the stimulation of the 3α-HSD (as shown in the experiments using DHT as the substrate, Fig. 5), and the enhanced availability of the immediate precursor of 3α-diol (DHT formed in higher amounts because of the increased 5α-reductase activity). These results were substantially confirmed by the experiments in which astrocytes were exposed to the CM of neurons. In these experiments, no variation in the metabolic pattern of neurons was observed when these were co-cultured for 4 days with type 1 astrocytes, or were exposed for 2 days to the conditioned medium of type 1 astrocytes.

It appears from these results that one or more soluble substance(s), which is (are) able to stimulate the two enzymes present in the astrocytes, and which transform T into its 5α-reduced metabolites (DHT and 3α-diol), is (are) released by neurons in culture. It is difficult, at the moment, to speculate on the chemical nature of the substance(s) released. The 0.45 μm pore size of the septum which separates the two types of cells in co-culture, allows an unselective diffusion of molecules, and the conditioned media utilized were not fractionated in any way. Experiments are presently in progress designed to try to elucidate the nature of the compound(s) involved.

Since the physiological role of the 5α-reductase–3α-HSD system in neurons and in glial cells is largely unknown, it is difficult to speculate on the biological significance of the existence of a neuronal mechanism able to stimulate the metabolism of steroid hormones in glial cells. However, it appears pertinent to underline that while the formation of DHT is usually considered a mechanism for potentiating androgenic actions, the subsequent conversion to 3α-diol is a mechanism of steroid catabolism, since 3α-diol is much less effective than T as an androgen, and does not bind to the androgen receptor [2]. Consequently, neurons appear to be simultaneously able to increase and to decrease the androgenic potential of astrocytes.

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