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Interactions between type 1 astrocytes and LHRH-secreting neurons (GT1-1 cells): modification of steroid metabolism and possible role of TGFβ1

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Abstract

The hypothesis that type 1 astrocytes (A1) might modify the activities of the enzymes 5α-reductase (5α-R) and 3α-hydroxysteroid dehydrogenase (3α-HSD) present in the GT1-1 cells has been tested. The data obtained indicate that, utilizing a co-culture technique, A1 are able to: (1) decrease the formation of dihydrotestosterone (DHT) from testosterone (T); (2) increase the formation of dihydroprogesterone (DHP) from progesterone (P); (3) decrease the conversion of DHP into tetrahydroprogesterone (THP) in GT1-1 cells. Moreover, GT1-1 cells are able to increase the formation of DHP in A1; that of DHT was unchanged. The present data might suggest the possible existence of a third isoform of the enzyme 5α-R; details on this hypothesis are provided in the text. Interestingly, the inhibitory effect exerted by A1 on the formation of DHT in GT1-1 cells can be mimicked by transforming growth factor β1 (TGFβ1). Since TGFβ1 had been previously shown to be directly involved in the stimulatory control of LHRH secretion by GT1-1 cells, acting both on LHRH release [R.C. Melcangi, M. Galbiati, E. Messi, F. Piva, L. Martini, M. Motta, Type 1 astrocytes influence luteinizing hormone-releasing hormone release from the hypothalamic cell line GT1-1: is transforming growth factor-β the principle involved? Endocrinology 136 (1995) 679–686.] and gene expression [M. Galbiati, M. Zanisi, E. Messi, I. Cavarretta, L. Martini, R.C. Melcangi, Transforming growth factor-β and astrocytic conditioned medium influence LHRH gene expression in the hypothalamic cell line GT1, Endocrinology 137 (1996) 5605–5609], the present data also show that TGFβ1 might intervene in modulating feedback signals reaching hypothalamic LHRH producing neurons. The present findings underline once more the importance of the physiological cross-talk between A1 and neurons.

Keywords: Type 1 astrocytes; GT1-1 cells; LHRH; TGFβ1; Steroids

1. Introduction

Recent data obtained in this and other laboratories have indicated that, in the central nervous system (CNS), neurons and glia (astrocytes type 1 and 2, oligodendrocytes) must be viewed as forming a functional unit, in which each single element may exert deep effects on the others. This concept is based on several observations. For instance, neuronal influences are able to up-regulate the expression of the most specific astrocytic marker, the glial fibrillary acidic protein (GFAP) [3], and to activate other parameters of astrocytic function, like the enzyme glutamine synthetase [4], the formation of gangliosides [5], and the assembly of voltage-sensitive calcium channels [6]. Moreover, neurons, when placed in co-culture with type 1 astrocytes (A1), are able to stimulate the activity of the enzymes 5α-reductase (5α-R) and 3α-hydroxysteroid dehydrogenase (3α-HSD) [7], which are both present in the glia, even if in different concentrations [8–10]. Interestingly, molecules secreted by neurons are able to

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stimulate the conversion of testosterone (T) into dihydrotestosterone (DHT), a reaction catalyzed by the 5α-R, also in a tumoral cell line of glial origin (C6 glioma) [11]. On the other hand, glial cells release a variety of molecules, like growth factors (e.g. TNF, NGF, TGFα and β), interleukins, excitatory aminoacids, GABA, neuropeptides, neurosteroids, etc. (see for a review Ref. [12]), which are able to influence neuronal function. For instance, our laboratory has recently demonstrated that A1 in culture secrete transforming growth factor β1 (TGFβ1), a factor which is able to modulate the gene expression [2] and the release [1] of LHRH from GT1-1 cells, a neuronal cell line derived from a hypothalamic LHRH-producing tumor.

Previous work of this and other laboratories has indicated that the GT1-1 cells possess receptors for estrogens, progesterone, androgens and corticoids [13–15] and exhibit 5α-R and 3α-HSD activities [13]. GT1-1 cells do not seem to possess, on the contrary, the enzyme aromatase [13]. It appears now important to analyze whether the presence in a co-culture system of A1 might modify in the GT1-1 cells the enzymatic conversion of two substrates of the 5α-R, respectively T and P. This analysis appears of importance for clarifying whether A1 influence LHRH expression and secretion only by the direct effect of TGFβ1 [1,2] (and possibly of other activating factors), or whether they might also intervene in the control of LHRH secretion by modifying the local formation of steroids (e.g. DHT, DHP, etc.) able to intervene as negative or positive signals in the feedback control of this hypothalamic hormone. In this context, it is interesting to recall that TGFβ1 has been shown to be able to stimulate the formation of DHT at least in genital skin fibroblasts [16] and that, in various cell systems, TGFβ1 expression is controlled by sex steroids (see for a review Ref. [17]).

In order to obtain a complete picture, also the opposite possibility, that GT1-1 cells might modify steroid metabolism in A1 should be considered. As previously mentioned, sex steroid receptors are present in glial cells [18], and the 5α-reduced metabolites of T and of P have been shown to be able to influence the gene expression of a typical marker of A1, the GFAP [19].

On the basis of these considerations, the major aim of the present paper has been to evaluate, the 5α-R activity converting T and P into DHT and DHP respectively, and the 3α-HSD activity converting DHT and DHP into 5α-androstan-3α,17β-diol (3α-diol) and tetrahydroprogesterone (THP) respectively in GT1-1 cells and in A1 and to verify whether the co-culture of the two elements might bring in some modifications of these enzymatic activity respectively in GT1-1 cells and in A1. Moreover, the direct effect of TGFβ1 has been evaluated on the 5α-R and of 3α-HSD present in GT1-1 cells.

2. Materials and methods

2.1. Animals

Young (1–2-day-old) rats, born in the laboratory, were obtained from mothers (Sprague-Dawley rats, Crl:CD BR, Charles River, Calco, Italy) maintained in the Department's animal quarters with controlled temperature and humidity. The light schedule was 14 h light and 10 h dark (lights on at 6.30 h).

2.2. Type 1 astrocyte cultures

Mixed glial primary cultures were obtained from the cerebral cortex of 1–2 day-old rats as previously described [8]. The mixed glial cells were then cultured for 2 weeks, and the bedlayer (which consists of type 1 astrocytes) was separated by shaking and plated as previously described [7]. At 5 day in vitro (d.i.v.) the cultures were used for the experiments.

2.3. GT1-1 cultures

GT1-1 cells (generously provided by Dr. R.I. Weiner, San Francisco, CA, USA) were maintained in Dulbecco's Modified Eagle's Medium–4.5 g/L glucose (DMEM; Biochrom K.G., Berlin, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco, Grand Island, NY), 100 U/ml penicillin and 100 μg/ml streptomycin. The GT1-1 cells were used at 80% confluence, when they were still dividing.

2.4. Co-culture

Cell culture inserts containing purified A1 were transferred to wells in which GT1-1 cells had been plated. After 2 days of co-culture each kind of cells were scraped and incubated with the appropriate labeled precursors for the enzymatic assays. GT1-1 cell cultures in 6-well plate and type 1 astrocytes in cell culture inserts were utilized as controls.

2.5. Treatments of GT1-1 cells with TGFβ1

GT1-1 cells plated as previously described [1] were treated in serum-free condition for 24 and 48 h with TGFβ1 5 ng/ml (2×10^{-10} M). At the end of exposure, the cells were processed for the steroid enzymatic assays.

2.6. Incubation procedures and detection of metabolites

The incubations with [^{14}C] T have been performed as previously described [8]. Briefly, cells have been scraped, sonicated by Microson™ ultrasound sonicator (10% of output power for 10 s) and incubated in 250 μl of Krebs-Ringer buffer solution in the presence of a NADPH generating system (NADP, disodium salt, Boehringer Mannheim, 9.32×10^{-3} M; glucose 6-phosphate, disodium salt, Boehringer Mannheim, 11.76×10^{-2} M and glucose 6-phosphate dehydrogenase from yeast grade 1, Boehringer Mannheim, 3.5×10^{-2} U.I.) and of [^{14}C] T (3.16×10^{-6} M) (specific activity ∽56.9 mCi/mmol, Amersham, UK) as the labeled substrate. The amounts of the generating system and of [^{14}C] T selected were used for ∽100 μg of protein. Protein content was measured according to the method of Bradford [20]. Vials without cells provided the blanks. The incubation was carried out at 37°C for 2 h in a Dubnoff metabolic shaker, under a stream of O_2/CO_2 98:2. At the end of the incubation, the reaction was stopped by deep freezing of the samples. Tritium labeled DHT (about 5000 DPM each) was added to each sample in order to evaluate the recovery. The metabolites formed were extracted twice with diethyl-ether, and separated on a thin layer silica gel chromatography plate (Merck 60 F254, DC), eluted three times with dicloromethane/diethylether (11:1). The DHT spots were identified with iodine vapors, scraped and the radioactivity counted in a Packard 300 C liquid scintillation spectrometer. Quench corrected DPM of the two isotopes were obtained by a calibration standard curve. In the experiments in which the formation of 3α-diol has been evaluated, the cells were incubated in the same conditions previously described but with [^{14}C] DHT as the labeled precursor (3.4×10^{-6} M) (spec. act. ∽57 mCi/mmol, Amersham, UK). The metabolites formed were extracted, separated and the radioactivity counted as previously described. Tritium labeled 3α-diol was added to each sample in order to evaluate the recovery.

The formation of DHP and THP were analyzed in two different sets of incubations using respectively [^{14}C] P and [^{14}C] DHP, as labeled precursors. [^{14}C] P (specific activity: 57.2 mCi/mmol) was obtained from Du Pont de Nemours, NEN division (Germany), [^{14}C] DHP was prepared from [^{14}C] P by a chemical method (catalytic hydrogenation). The incubation procedure was performed as previously described in detail [11]. After 2 h of incubation at 37°C in a Dubnoff metabolic shaker under a stream of O_2/CO_2 98:2, tritium labeled DHP or THP were added to evaluate the recoveries. Then the samples were extracted twice with diethylether and separated on a thin layer silica gel chromatography plate (Merck 60 F_{254}, DC). In the incubations in which labeled P was used as substrate the plates were eluted one time with a mixture of benzene/methanol (95:5) and one time with a mixture of cyclohexane/*n*-butyl acetate (30/60); when labeled DHP was used, the elution was performed only one time with a mixture of benzene/methanol (95/5). The radioactivity was counted in a Packard 300 C liquid scintillation spectrometer. Crystallization to constant specific activity was performed at the beginning of the experiments, and repeated whenever necessary.

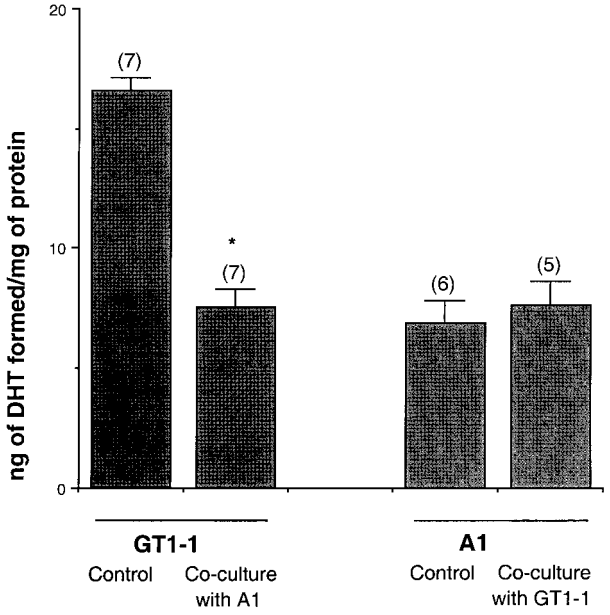

Fig. 1. Effect of 2 day of co-culture of GT1-1 cells and type 1 astrocytes on DHT formation (incubation with testosterone). Numbers in parentheses represent the number of experiments performed (see Materials and methods for details). $^*P < 0.01$ vs. control. The data are expressed as means ± S.E.M.

The amounts of androgen (DHT and 3α-diol) and progestagen metabolites (DHP and THP) were expressed as ng formed after 2 h of incubation per mg of protein. The data presented in the figures and in the tables represent the mean of the results obtained in independent experiments involving different cell preparation. The numbers given in parenthesis represent the number of the experiments performed. In each experiment the values obtained represent the mean of 2–3 determinations.

2.7. Statistics

Statistical evaluation was performed by Student's *t*-test.

3. Results

Figure 1 confirms, first of all previous data of this

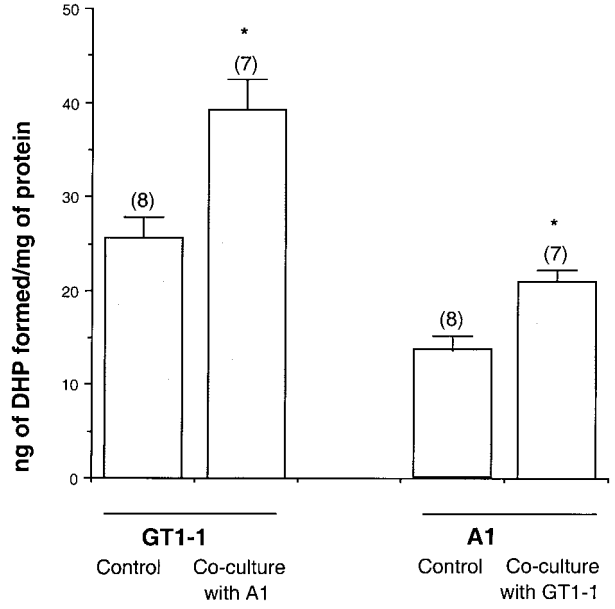

Fig. 2. Effect of 2 day of co-culture of GT1-1 cells and type 1 astrocytes on DHP formation (incubation with progesterone). Numbers in parentheses represent the number of experiments performed (see Materials and methods for details). $^{*}P < 0.01$ vs. control. The data are expressed as means ± S.E.M.

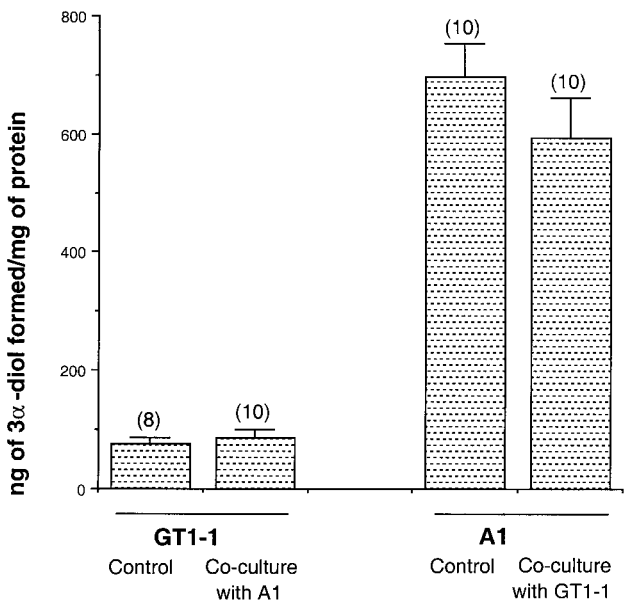

Fig. 3. Effect of 2 day of co-culture of GT1-1 cells and type 1 astrocytes on 3α-diol formation (incubation with DHT). Numbers in parentheses represent the number of experiments performed (see Materials and methods for details). The data are expressed as means ± S.E.M.

laboratory showing that T may be 5α-reduced to DHT in GT1-1 cells [13], and shows, in addition, that the formation of DHT (expressed as ng of DHT formed/mg of protein after 2 h of incubation with labeled T) in GT1-1 cells is significantly decreased when these cells are co-cultured for two days with A1. The figure also shows that A1 are able to 5α-reduce T to DHT. The 5α-R activity of A1 appears to be lower than that of GT1-1 cells, a result which is in line with our previous observations showing that glial elements usually possess a lower 5α-R activity than neurons (here represented by GT1-1 cells) [8,9]. No significant changes in the 5α-R activity were seen in A1 co-cultured with GT1-1 cells.

Figure 2 shows the results of a similar experiment, in which P rather than T was used as the substrate. It appears that also P may be 5α-reduced to DHP in GT1-1 cells; actually, like in any other system, P appears to be the preferential substrate for this enzyme [21] (note the difference of the scale between Figs. 1 and 2). The effect of the co-culture of GT1-1 cells with A1 is quite different from that observed in the experiments in which T was utilized as substrate. In fact, as shown in Fig. 2, the formation of DHP is significantly increased in GT1-1 cells when they have been co-cultured with A1 for two days. Moreover, Fig. 2 shows that also A1 are able to 5α-reduce P; also in A1, P appears to be the preferential substrate. At variance with the data obtained when T was used as the substrate, the co-culture with GT1-1 cells increased the formation of DHP in A1.

Figure 3 shows that GT1-1 cells have a very low 3α-HSD activity (expressed in this figure as formation of

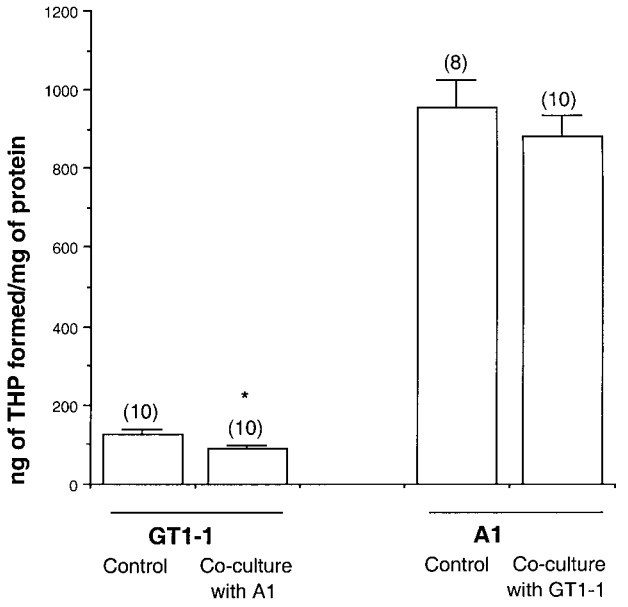

Fig. 4. Effect of 2 day of co-culture of GT1-1 cells and type 1 astrocytes on THP formation (incubation with DHP). Numbers in parentheses represent the number of experiments performed (see Materials and methods for details). $^{*}P < 0.01$ vs. control. The data are expressed as means ± S.E.M.

Table 1
Effect of 24 and 48 h treatment with TGFβl on the formation of DHT, 3α-DIOL, DHP and THP in GT1-1 cells. *$P < 0.01$ vs. control. The data (ng of steroids formed/mg of protein) are expressed as means ± S.E.M.[a]

	24 h		48 h	
	Control	TGFβ1	Control	TGFβ1
DHT	11.54 ± 1.59 (9)	5.03 ± 0.96* (6)	7.32 ± 1.85 (5)	6.85 ± 1.27 (5)
3α	28.61 ± 1.89 (5)	22.96 ± 2.69 (5)	37.49 ± 2.88 (5)	31.47 ± 2.28 (5)
DHP	25.67 ± 2.71 (10)	24.30 ± 3.00 (10)	24.39 ± 2.73 (6)	18.75 ± 1.12 (6)
THP	25.01 ± 4.72 (7)	28.34 ± 6.35 (6)	57.31 ± 4.02 (6)	39.75 ± 4.29* (5)

[a] Numbers in parentheses represent the number of experiments performed (see Materials and methods for details).

3α-diol from DHT), a result comparable to that previously found in fetal rat neurons [9]. The figure also shows that this activity does not change significantly in GT1-1 cells when they are co-cultured with A1. In confirmation of previous data of this laboratory, the figure shows that A1 have a high 3α-HSD activity [9], which is not modified by the co-culture with GT1-1 cells (Fig. 3).

The 3α-HSD activity was also measured in GT1-1 cells and in A1 alone or in co-culture using DHP as the substrate. The formation of THP was significantly decreased in GT1-1 cells co-cultured with A1. It remained unchanged in A1 co-cultured with GT1-1 cells (Fig. 4).

As shown in Table 1, the direct exposure of GT1-1 cells to TGFβ1 induced after 24 h, a significant decrease of the formation of DHT from T; the effect of TGFβ1 appeared to be transient and disappeared at 48 h. On the contrary, the formation of DHP from P remained unchanged after TGFβ1 at any time of exposure. The 3α-HSD, expressed as formation of 3α-diol from DHT, was not affected by the treatment with TGFβ1, at any time of exposure. On the contrary, the formation of THP from DHP, which was unchanged 24 h after treatment, was significantly inhibited after 48 h of exposure to TGF β1.

4. Discussion

The present results show that type 1 astrocytes are able to release in culture some factor(s) which is (are) able to modify the 5α-R and the 3α-HSD activities present in GT1-1 cells. Surprisingly, the effects observed in the experiments in which A1 were co-cultured with GT1-1 cells are different depending on the substrates utilized. In fact, utilizing T as the substrate, a significant decrease of the formation of DHT was apparent when GT1-1 cells were co-cultured with A1; on the contrary, when P was utilized as the substrate, a significant increase in the formation of DHP was observed. These observations are intriguing, since they underline the possibility that, in identical experimental conditions, there might be an unusual differential control of the enzyme(s) 5α-R depending on the substrate. It is known that two isoforms of the 5α-reductase have been cloned so far and that both are able to convert T and P as well as other Δ4-3keto steroids [21]. For both 5α-R isoforms P appears to be the preferential substrate. The differential substrate-linked effect obtained in the present experiments in the presence of A1 does not appear easy to explain on the basis of an effect on either one of the two isoforms of the 5α-R so far cloned. In the authors' opinion, the data are better explained by postulating the existence of a third 5α-R isoform, that has not been cloned so far. It is important to underline that several other observations made recently, in this laboratory, both in vitro and in vivo, seem to support such an hypothesis. For instance, it has been shown that pluripotential CNS stem cells derived from the mice striatum, when induced to differentiate into glial cells, start forming DHT from T on the 14th day of differentiation, whereas they start forming DHP from P on the 10th day of differentiation [22]. The conditioned media of the C6 glioma and of the 1321N1 human astrocytoma cell lines are unable to modify the formation of DHT from T in type 1 astrocytes, while inducing a statistically significant decrease of the formation of DHP from P [11]. The exposure of C6 cells to the conditioned media of rat fetal neurons results in a stimulation of the formation DHT but not of DHP from the respective substrates [11]. Furthermore, we have recently observed that castration decreases the formation of DHT in the sciatic nerve of adult male rats, but leaves unmodified that of DHP (V. Magnaghi et al., unpublished data). Obviously other interpretations are also possible. It is important to underline, however, that in the present experiments probably only the activity of the type 1 enzyme has been evaluated, since the experiments have all been performed at pH 7.2, which is the optimum for this isoform. It is also important to note that, with regard to the 5α-R enzymes, no substrate-linked modifications of their structures have been reported so far.

The experiments here reported have indicated not only that A1 are able to modify the enzymatic conver-

sions of steroids in GT1-1 cells, but also that factors produced by GT1-1 cells, which are neuronal in origin, may influence steroid metabolism in A1. In fact, it has been observed that, after two days of co-culture with GT1-1 cells, the formation of DHP, but not that of DHT, is increased in type 1 astrocytes. Also this observation seems to support the hypothesis of the existence of a further 5α-R isozyme, in addition to the "classical" two isoforms already cloned (see for a review Ref. [21]).

Interestingly, when the further conversion of DHT and DHP, respectively into 3α-diol and THP, was analyzed it has been observed that A1 are able to modify the 3α-HSD activity in GT1-1 cells when this is measured on the basis of the formation of THP, but not when this is evaluated on the basis of the formation of 3α-diol. From this point of view, it is important to remember that the brain possesses two forms of 3α-HSD, one cytosolic and one microsomal. Since the substrate preferences of these two enzymes have not been fully evaluated (see for a review Ref. [21]), the result here described may be explained on the basis of the effect of astrocytic molecules on only one of these two enzymes. The fact that the activity of the 3α-HSD is not modified in the co-culture system when DHT is used as substrate rules out the possibility that the decrease in the formation of DHT from T previously reported, in these same conditions, might be ascribed to an increased conversion into 3α-diol of the DHT formed.

The present data have also shown for the first time that TGFβ1 is able to influence the activity of steroid metabolizing enzymes in CNS cells. The data have indeed shown that the addition of TGFβ1, like the co-culture with A1, is able to decrease the 5α-R activity converting T into DHT in GT1-1 cells. This parallelism may obviously bring to suggest that TGFβ1 might be the factor (or one of the factors) transferred from A1 to GT1-1 cells and blocking the activity of the 5α-R.

As mentioned before, the formation of DHP in GT1-1 cells was highly stimulated by the presence of A1; however, DHP formation remained unchanged after exposure of GT1-1 cells to TGFβ1. This growth factor does not appear then to be the possible factor involved in transferring the stimulatory effect exerted by the glial elements. It is noteworthy that also this set of experiments further confirms the existence of a differential control of the enzymes 5α-reducing T and P, and consequently supports the hypothesis previously advanced of the possible presence of a third type of 5α-R activity in the CNS. TGFβ1 has also been shown to be able to decrease the formation of THP but not that of 3α-diol in GT1-1 cells; this effect may be linked to the presence of the two different 3α-HSDs (see above), and to the fact that only one of these enzymes is sensitive to TGFβ1.

The present experiments add several important elements to our understanding of the relationships between glia and the neuronal component in the control of LHRH secretion. First of all, one should remember that A1, possibly via the secretion of TGFβ1, directly increase the release [1] and the gene expression [2] of LHRH in GT1-1 cells. Second, one may recall that hypothalamic neurons may be the site of the feedback action of hormonal steroids, which may act directly on LHRH secreting neurons (receptors for androgens and progesterone have been found in GT1-1 cells), or indirectly via actions on other neuronal or glial elements which eventually transfer their feedback information to the final effector system via classical neurotransmitters. With regard to the possible direct effects of hormonal steroids on LHRH release, one must note that the present data show that A1 and TGFβ1 may decrease the formation of DHT in GT1-1 cells, and may consequently diminish the androgenic feedback signal, since T is a less potent ligand than DHT for the androgen receptor. On the other hand, if one considers that the presence of A1 in culture, or the addition of TGFβ1, decreases the formation of THP, i.e. of a progesterone metabolite which binds to the $GABA_A$ receptor (see for a review Ref. [21]), one may be brought to postulate that astrocytic molecules (via TGFβ1?) may interfere with the release of LHRH also indirectly, by modifying the GABAergic input to LHRH-neurons 3α-diol. Finally, in order to underline all the intricacies of the system, one should hypothesize that, like in other systems (see for a review Ref. [17]), sex steroids may modulate the formation of TGFβ1 in A1. The present data obviously suffer from the fact that they have been obtained in vitro on immortalized hypothalamic LHRH-secreting neurons. Unfortunately, due to the paucity of neurons in the physiological LHRH system and to their location, it would have been extremely difficult to design a similar type of experiment under in vivo conditions. It must be pointed out, however, that glial-LHRH neurons interactions have been described to occur also in vivo [23], especially in relation to the effects of growth factors on the release of LHRH [24,25].

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