Smad proteins are targets of Transforming Growth Factor β 1 in immortalised GnRH releasing neurones.

Mariarita Galbiati*, Simona Saredi, Nicola Romanò, Luciano Martini, Marcella Motta, Roberto

Cosimo Melcangi

Department of Endocrinology, University of Milan, Milan, Italy.

*Correspondence should be addressed to:

Mariarita Galbiati

Department of Endocrinology, University of Milan

Via Balzaretti, 9

20133 Milan – Italy

ph. +39 02-5031.8237

fax +39 02-5031.8204

e-mail: rita.galbiati@unimi.it

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ABSTRACT

Transforming growth factor β (TGFβ) is one of the growth factors involved in the neuroendocrine control of the GnRH neurones. It is produced and released by the astrocytes surrounding GnRH neurones and directly controls their secretory activity. TGFβ signaling is based on a complex of two receptors that transduces the signal through peculiar intracellular substrates, the Smad proteins, which, upon activation, move into the nucleus, and modify the transcription of TGFβ responsive genes. The aim of the present study was to verify whether TGFβ1 is able to regulate the Smad pathway in GT1-1 cells (i.e., an immortalised neuronal cell line releasing GnRH). We show that: a) GT1-1 cells express Smad 2, 3, 4, and 7; b) TGFβ1 enhances the phosphorylation of Smad 2 and 3 at short times of exposure (15-30 min); c) TGFβ1 induces the synthesis of the inhibitory Smad 7 at longer times (60-120-240 min); d) the conditioned medium of type 1 astrocytes enhances the phosphorylation of Smad 2 and 3 in GT1-1 cells and a TGFβ1 neutralising antibody counteracts this effect. The results hereby reported, indicate that Smads are targets of TGFβ1 and that astrocytes are able to modulate Smads proteins in GT1-1 cells through the release of TGFβ1. Taken together, the data provide new evidence that glial cells are important regulators of the GnRH neuronal activity.

INTRODUCTION

The hypothalamic neurones synthesising and secreting the gonadotropin hormone releasing hormone (GnRH) form, with other neuronal systems and the surrounding glia, the so-called GnRH network. Recent *in vitro* and *in vivo* observations have shown that glial cells may control the activity of the GnRH secreting neurones, via the release of different growth factors, like transforming growth factor α, transforming growth factors β1 and β2 (TGFβ1, TGFβ2), basic fibroblast growth factor, etc (see for review 1). In previous works, we demonstrated that the conditioned medium of type 1 astrocytes (A1-CM) and TGFβ1 are able to modulate the release and the gene expression of GnRH in GT1-1 cells, a line of immortalised hypothalamic GnRH-secreting cells, widely used as a model to study GnRH neurones (2,3,4).

Signals of TGFβ are transduced through heteromeric complexes of transmembrane serine/threonine kinases, formed by type I and type II receptors (TGFβ RI and TGFβ RII) (5). In particular, data available in literature indicate that TGFβ RII binds the ligand, and phosphorylates TGFβ RI. The subsequent signaling events are mediated by the activation of a peculiar family of proteins, the Smad proteins. Currently, 9 Smads are known; those involved in the signaling pathway of TGFβ are Smad 2, 3, 4 and 7. Smad 2 and 3 are receptor regulated (R-Smads) and following activation, form a complex with Smad 4 (6). This complex moves into the nucleus to modulate the transcription of TGFβ responsive genes, either acting directly, or co-operating with other distinct transcription factors (5). Smads can function as transcriptional regulators binding to a specific DNA sequence. An optimal Smad binding sequence has been identified, and repeated copies of this sequence (Smad Binding Element; SBE) are sufficient to confer the responsiveness to TGFβ (7). In addition, the inhibitory-Smad 7 has also been

described.

Our and others' previous observations have shown the presence of the mRNA coding for both TGFβ RI and RII and of the related proteins in the GT1-1 cells (4,8). Receptors binding TGFβ are also expressed by GnRH neurones *in vivo*. Prevot and co-workers have demonstrated that GnRH secreting neurones express the mRNA coding for a type I serine-threonine kinase able to bind TGFβ and activin (9), and that GnRH neuronal bodies localised in the preoptic area express both TGFβ RII and Smad 2/3 (10). Finally, by an "in silico" analysis, we have determined the presence of different copies of SBE on the promoter region of human, mouse and rat GnRH gene, supporting the possibility of a direct effect of TGFβ on the GnRH gene (1).

Despite the data implicating TGFβ in the control of GnRH dynamics, little is known regarding the mechanism of action of this growth factor on GnRH neurones. Thus, we have studied whether GT1-1 neurones express Smad proteins and whether TGFβ1 is able to activate this intracellular signaling pathway. In addition, we have verified whether A1-CM is able to mimic the effect of TGFβ1 on Smad proteins in GT1-1 cells.

MATERIALS AND METHODS

Cell culture

GT1-1 cells, kindly 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; Seromed, Biochrom KG, Berlin, Germany) supplemented with 10% heat-inactivated foetal bovine serum (FBS, Invitrogen, San Giuliano Milanese, Italy), 1 mM glutamine, 100 UI/mL penicillin G K-salt, and 100 µg/mL streptomycin sulphate, and grown in 10 cm dishes.

Primary cultures of type 1 astrocyte (A1) were obtained from the hypothalamus of neonatal rats as previously described (11). Briefly, after mechanical dissociation of hypothalamic tissue, mixed glial cells were plated at high density (20×10^6 cells) in 75-cm^2 flasks, and cultured for 2 weeks. The bedlayer, which consists of type 1 astrocytes, was separated by shaking and plated as 3×10^6 cells/dish (10 cm diameter) in DMEM supplemented with 10% FBS, 50 UI/mL penicillin G K-salt, and 50 µg/mL streptomycin sulphate. A1 cultures were routinely more than 95% pure astrocytes, as assessed by glial fibrillary acidic protein immunostaining.

Conditioned medium

The conditioned medium of type 1 astrocyte (A1-CM) was obtained from purified cultures of A1 at 2-4 day *in vitro*. To obtain the A1-CM, cells were cultured without FBS for 24 hours, then the medium was centrifuged at 1000 g (to avoid the possibility of cells eventually being present), and frozen at -20°C.

Treatments

The GT1-1 cells were plated 3×10^6 cells/Petri dish (10 cm diameter) and used when at 80% confluence, when they were still dividing. In all the experiment series, GT1-1 cells have been

utilised before the 30th passage. GT1-1 cells were maintained overnight in serum free conditions and then exposed to 100% A1-CM or to TGFβ1 (0.5, 1, or 5 ng/mL). TGFβ1 was supplied by Sigma Chemical, Milan, Italy. In the experiments utilising A1-CM, the GT1-1 cells were grown in the A1 medium. To neutralise the TGFβ1 activity, 1 hour before the treatment of GT1-1 cells, different doses (0.1, 1, 5 μg/mL) of a TGFβ1 antibody (R&D Systems, Minneapolis, USA) was added to A1-CM. At the end of the selected period of exposure, the cells were scraped and processed for RNA or protein extraction.

Total RNA preparation

The cells were harvested in 4M guanidium isothiocyanate (containing 25 mM sodium citrate pH 7.5, 0.5 % sarcosyl and 0.1 % 2-mercaptoethanol) and total RNA isolated by phenol-chloroform extraction according to the method of Chomczynski and Sacchi (12). Quantification was carried out by absorption at 260 nm.

Reverse Transcription and PCR

For the Reverse Transcription (RT), an aliquot of GT1-1 total RNA (1 μg) was treated for 15 min at room temperature with 1 U of DNaseI (Sigma Chemical, Milan, Italy). The samples (after having heat-inactivated DNaseI) were reverse transcribed at 37° C for 60 min using 200 U of M-MLV reverse transcriptase (Invitrogen, San Giuliano Milanese, Italy), in the presence of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, dNTP (0.5 mM each), 40 U RNase inhibitor, and random hexamer primers (250 ng, Invitrogen, San Giuliano Milanese, Italy) in a 35 μl reaction. Reverse transcription efficiency was tested using primers designed on HPRT mouse cDNA sequence with experimental conditions previously reported (4). The PCR reactions were carried out in a final volume of 25 μl containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl,

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1.5 mM MgSO$_4$, dNTP (0.2 mM each), 1 U Taq DNA polymerase (Invitrogen, San Giuliano Milanese, Italy), 1 μM of each primer and 2 μl of cDNA as template (that correspond to 60 ng of total RNA reverse-transcribed). Primer pairs used were as follows: Smad 2: forward primer, 5'-GCCCCAACTGTAACCAGAGA-3'; reverse primer, 5'-AAGGGGATCCCATCTGAGTT-3'A; Smad 3: forward primer, 5'-GAAGAAGGGCGAGCAGAAC-3'; reverse primer, 5'-CGTAATTCATGGTGGCTGTG-3'; Smad 4: forward primer, 5'-AACATTGGCCATTGGTTTTC-3'; reverse primer, 5'-TGCTAGGATGAGCTCCATTTG-3'; Smad 7: forward primer, 5'-CCTCCTGCTGTGCAAAGTGTT-3'; reverse primer, 5'-TGGTTGCTGCATGAACTCGT-3'. The following cycling parameters were used: denaturation at 94°C for 1min, annealing at 60°C for 1min, and extension at 72°C for 1min, for a total of 30 cycles, followed by a 7-min final extension at 72°C. The annealing temperature for Smad 7 was 63°C. Additional samples of GT1-1 cells were subjected to RT-PCR in the absence of reverse transcriptase, as negative controls. The PCR products were gel purified by QIAquick gel extraction kit (Qiagen, Milan, Italy) and sequences were determined by MWG (Florence, Italy). Database searches for homologies were carried out using Blast algorithm at NCBI (National Center for Biotechnology Information).

<u>Semiquantitative RT-PCR</u>

The quantitative approach used in this investigation was according to the relative quantitative RT-PCR protocol from Ambion (Austin, TX, USA). The protocol consisted of several steps, including the determination of the linear range and the optimum ratio of 18S primers to competimers, before undertaking relative quantitative PCR. The PCR reactions were labelled by adding [α-^{32}P] dATP (Amersham, Cologno Monzese, Italy) to the reaction. Products were

resolved by electrophoresis on a 6% polyacrilamide gel; this was transferred to blotting paper, dried and imaged by autoradiography. Next, the gel was lined up with the autoradiography and the bands excised with a scalpel and counted in a scintillation counter. Each sample was loaded twice. The results have been normalised with 18S (co-amplified in the same sample). The mean value of the control within a single experiment was set to 100 and all the other values were expressed as percent *versus* the levels detected in controls. Values of controls from different experiments were within 10% of each other.

Western blot analysis

Proteins were extracted as previously described (11). Briefly, GT1-1 cells were washed with cold phosphate buffered saline (PBS), harvested and collected by centrifugation. Pellets were sonicated on ice in lysis buffer, that consists of: Tris HCl 50 mM pH 7.4, NaCl 150 mM, EDTA 1 mM, containing a cocktail of protease inhibitors (10μg/mL pepstatin A, 0.5mM PMSF, 1μg/mL benzamidine, 2μg/mL aprotinin, 10μg/mL leupeptin, 2μg/mL antipain, 2μg/mL chymostatin, 1 mM sodium vanadate, 1 mM sodium fluoride), and 1% NonidetP40. All these chemicals were purchased by Sigma Chemical (Milan, Italy). The samples were clarified by centrifugation at 10,000 g for 15 min, and the protein content of the supernatants was measured according to the Bradford method (13). Cell lysates (20-50 μg) were denatured in loading buffer, boiled for 3 min, and resolved by a 7.5% sodium dodecyl sulfate polyacrilamide gel electrophoresis (SDS-PAGE) under reducing conditions. After electrophoresis, proteins were electrophoretically transferred (100 V for 2 h) to a nitrocellulose membrane (Biorad, Segrate, Italy). Membranes were blocked in TBS-T (Tris-HCl 20 mM pH 7.4; NaCl 150 mM; Tween 20 0,1%) and 5% non fat milk or 5% bovine serum albumin; then membranes were incubated

overnight at 4°C with the primary antibodies diluted in TBS-T: anti-Smad 2/3 (Upstate, Lake Placid, NY, USA), 1:500; anti-phosphoSmad 2 (detecting phosphorylation on Ser465 and on Ser467, Cell Signaling, Beverly, MA, USA), 1:1,000; anti-phosphoSmad 3 (kindly provided by Prof. EB Leof, Mayo Clinic College of Medicine, Rochester, MN, USA), 1:3,000; anti-Smad 7 (abcam, Cambridge, UK), 1:1,000; anti α-tubulin (Sigma Chemical, Milan, Italy), 1:2,000. After washing with TBS-T, membranes were incubated with the right secondary antibody for 1 hour at room temperature (1:2,000), washed again, and developed with ECL Plus chemioluminescence system (Amersham, Cologno Monzese, Italy) and autoradiography. All samples were normalised to α-tubulin to control for equal starting protein. Samples of all experimental groups were loaded on the same gel to minimise the inter-assay variations.

Statistical analysis

The data have been analysed by ANOVA, using Tukey's test for multiple comparison. Statistical tests were calculated using the statistical package "Systat" (Systat Inc, Evanston, IL) on an iMac computer (Apple). When appropriate, data were analysed by the Student's t test for independent samples.

RESULTS

Expression of Smads in GT1-1 cells.

Fig. 1 shows that GT1-1 cells express the mRNAs for Smad 2, 3, 4, and 7 (lanes G). The identities of the RT-PCR products were confirmed by sequencing and by database searches for homologies carried out using Blast algorithm (a.n. U 60530 for Smad 2, a.n AB 008192 for Smad 3, a.n. U 79748 for Smad 4, a.n. AF 015260 for Smad 7). Since Smad 4 is constitutively present in different cellular systems and is not activated in response to TGFβ (14-16), in the following experiments we have focused our attention on Smad 2, 3, and 7.

Activation of R-Smads by TGFβ1.

In GT1-1 cells, TGFβ1 is able to increase the levels of the phosphorylated form of Smad 2 (P-Smad 2) and Smad 3 (P-Smad 3) (Fig. 2), measured by Western blot analysis. TGFβ1 has been utilised at the concentration of 5ng/mL (i.e., the dose we had previously demonstrated to be effective in modulating the synthesis and the secretion of GnRH in GT1-1 cells) (2,3). The increase of P-Smad 2 is maximal within 15 and 30 min and is sustained through 60 min of treatment. A similar pattern of phosphorylation is shown in the case of P-Smad 3 (Fig. 2), the highest levels of phosphorylation being detected after 15 and 30 min of treatment, while, at longer times (60 and 120 min), P-Smad 3 returns to basal levels. There were no differences among the controls at all times considered.

Based on these observations, a dose-response curve has been performed at 30 min. The addition of TGFβ1 at the concentrations of 1 and 5 ng/mL for 30 min induces a significant increase of the phosphorylation (Fig. 3). The lowest dose of TGFβ1 (0.5 ng/mL) does not increase the level of P-Smad 2, while the same dose induces a significant enhancement of P-Smad 3 levels, although

not at the maximal level.

The possibility that TGFβ1 might also modulate the synthesis of Smad 2 and 3 has been investigated evaluating Smad 2 mRNA levels, through a quantitative RT-PCR approach (Fig. 4 A), and measuring Smad 2/3 protein levels by Western blot analysis (Fig. 4B). Smad 2 mRNA levels are unaffected by TGFβ1 treatment (5 ng/mL) at any time considered (30, 60, 120, and 240 min). The same treatment is unable to significantly modify the protein levels of Smad 2 and 3. The antibody utilised for this experiment detects both Smad 2 and 3 (respectively at 58 and 52 kDa).

Regulation of Smad 7 expression by TGFβ1.

In addition to regulating Smad 2 and 3 phosphorylation, TGFβ1 is able to affect also the inhibitory Smad 7 in GT1-1 cells. Treatment of these cells with TGFβ1 (5 ng/mL) for 60, 120, and 240 min results in a marked induction of Smad 7 gene expression, as evaluated by quantitative RT-PCR (Fig. 5). Smad 7 mRNA levels remain unaffected after 30 min of treatment (Fig. 5). Moreover, TGFβ1 is also able to induce an increase of Smad 7 protein levels after 120 and 240 min, while the levels remain unchanged after 30 and 60 min of treatment (Fig. 6).

A1-CM affects Smad proteins through TGFβ1.

Based on our previous *in vitro* observations that hypothalamic A1 act on GnRH dynamics by producing and releasing TGFβ1 (2,3), the ability of A1-CM to modulate the phosphorylation and/or the protein levels of Smads so far considered, has been investigated. Fig. 7 indicates that the A1-CM (frozen and thawed to convert the latent form of TGFβ1 into the active form), like TGFβ1 itself (see Fig. 2), is able to induce the phosphorylation of Smad 2 and 3 after 30 min of treatment. Unexpectedly, A1-CM does not affect Smad 7 protein levels after 120 minutes of

treatment (i.e., the time at which TGFβ1 has the maximal effect in stimulating the synthesis of Smad 7 in GT1-1 cells).

To verify whether TGFβ1 is the astrocyte-derived factor mediating the effects of the A1-CM on Smad proteins activation in GT1-1 cells, we neutralised the TGFβ1 activity in the A1-CM utilising a TGFβ1 antibody. According to the manufacturer's protocol, we utilised the antibody at the concentrations of 1 and 5 μg/mL, which allow for obtaining respectively 50 and 90% of the maximal inhibition of the cytokine activity. As reported in Fig. 8, the antibody is able to diminish or abolish the activity of A1-CM on Smad 2 and 3 phosphorylation. Additionally, in this case, differences between P-Smad 2 and P-Smad 3 pattern of phosphorylation are evident. In fact, for P-Smad 3 only the highest dose of the antibody counteracts the effect of TGFβ1.

In all the series of experiments conducted, the effectiveness of TGFβ1 and A1-CM treatments on the release of GnRH by GT1-1 cells has been verified by measuring the amount of GnRH released in the media by RIA. The results indicate that, in agreement with our previous observations (2,3), both TGFβ1 and A1-CM are able to increase the release of the decapeptide from GT1-1 cells. In particular, TGFβ1 significantly increases the release of GnRH after 60, 120, and 240 min of treatment (percent increases: $213.4\pm11.2\%$ at 60 min, $158.6\pm10,2\%$ at 120 min, 149.1 ± 5.2 at 240 min). Treatments shorter than 60 minutes (e.g. 15, and 30 min) are ineffective.

DISCUSSION

The present study has been addressed to elucidate the intracellular mechanisms through which TGFβ1 might exert its effects on GT1-1 cells. We have shown that these cells express Smad 2, 3, 4, and 7, and that TGFβ1 regulates the phosphorylation and the expression of these mediators. Moreover, we have reported that the conditioned medium of A1 (i.e., the *in vivo* source of TGFβ1 for GnRH neurones) additionally affects the R-Smads phosphorylation and that a TGFβ1 neutralising antibody counteracts this effect.

In detail, we have shown that the induction of Smad 2 and 3 phosphorylation by TGFβ1 is time and dose dependent. A quick activation of these two Smads by phosphorylation seems to be in agreement with the stimulatory effect of TGFβ1 on the GnRH release by GT1-1 cells that is exerted after 1 hour of exposure, as reported in our previous work (2) and confirmed in the present study. None effect is evident at shorter times of exposure. Nevertheless, differences in the activation of these two forms of R-Smad are present. In fact, TGFβ1 exerts a more prolonged action on Smad 2, while lower doses of TGFβ1 are able to activate Smad 3. Although highly homologous, Smad 2 and Smad 3 have many distinguishing features that contribute to the unique pattern, through which they induce gene activation. They both bind to TGFβ RI through an identical loop and are phosphorylated with similar kinetics in most cells. Smad 2 has an insertion of 30 amino acids in the MH1 domain that precludes its direct binding to DNA, as does Smad 3 (17,18). This suggests that only Smad 3 might directly modulate GnRH gene transcription through its direct binding to SBEs. On the other hand, since TGFβ1 also activates Smad 2, it is possible to hypothesise that the modulation of the dynamics of GnRH in GT1-1 cells might also be affected through the interaction of Smad 2 with other transcription factors that, at present,

remain to be identified. In this context, it is important to highlight that although Smads are the main signaling pathway in most TGFβ actions, other signaling systems might also be activated directly by TGFβ. For example, TGFβ is able to activate Rho GTPases (19), protein phosphatase 2A (20) and MAP kinases (21,22) in a Smad-independent manner. Interestingly, it has been demonstrated that the MAP kinase pathway may also converge on Smads (23), and that the phosphorylation of Smads may be carried out also by JNK and PKC although on sites different from that utilised by TGFβ1 (24,25). The role of these pathways in the control of the TGFβ1-induced GnRH release remains to be elucidated in GT1-1 cells.

Null mice for both Smad 2 and 3 are described; while null mice for Smad 2 are not viable, the ones for Smad 3 survive untill the adulthood. Moreover, in the case of Smad 3 two different null mice have been developed and these express different characteristics: adult homozygous mutants with a targeted disruption in exon 2 (26) maintain the reproductive capacity, while mice with a targeted disruption in exon 8 (27) have a reduced fertility. The reason for this discrepancy is not yet known; it is possible that different domains of the Smad 3 protein are involved in activation of different downstream pathways, as already suggested by different *in vitro* studies (28,29). However, the reduced fertility of null mice with a targeted disruption in exon 8 support our hypothesis of an involvement of Smad 3 in the control of GnRH dynamics and the reproductive phenomena (27).

TGFβ1 proved able to stimulate the mRNA (after 60, 120, and 240 min of exposure) and protein (after 120 and 240 min) levels of Smad 7, which is well known to turn off the effects of the signal provided by TGFβ. In fact, lacking the C-terminal phosphorylation sites and competing with R-Smads for the binding to TGFβ RI, the Smad 7 protein acts as negative feedback

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inhibitor of the TGFβ signal (30-32). Interestingly, since the Smad 7 promoter contains SBEs, it is possible to hypothesise that the activation of this Smad may be mediated through the activation of Smad 3. This is supported also by the fact that, in other cellular systems, it has been demonstrated that TGFβ1 can stabilise the messenger levels of Smad 7 and can also activate Smad 7 transcription (33).

Based on our previous observations which has shown that A1-CM acts on the secretion of GnRH through the release of TGFβ1 (2), we have also evaluated the ability of the A1-CM to activate the Smad pathway (Fig. 7). The present data indicate that A1-CM, like TGFβ1 itself, is able to induce an increase of the phosphorylated form of Smad 2 and 3. On the contrary, the treatment with A1-CM did not modify the levels of the Smad 7 protein (Fig. 8). A possible explanation may be the weak activity of A1-CM on Smad 3, which, as mentioned above, might be liable for the activation of Smad 7 transcription (33). However, the effects induced by A1-CM on the phosphorylation of R-Smads are less intense than those induced by TGFβ1 itself; this difference might depend on the levels of TGFβ1 present in the A1-CM which are lower than those utilised in the present experiments. Actually, the lowest dose of TGFβ1 induces only a slight increase of P-Smad 2 and 3 (see Fig. 3), and Buchanan and colleagues reported that the content of TGFβ1 in the 24 hours CM of hypothalamic astrocyte cultures is around 250 pg/mL (8). The possibility that in the A1-CM other factors, able to modulate GT1-1 cells signaling pathways, might be present should also be taken in consideration. These signals impinging on the cells might enhance, suppress, or modify the effects of TGFβ and/or its signaling pathway.

The effects of the conditioned medium are due to the astrocyte-derived TGFβ1, since a TGFβ1-neutralising antibody is able to abolish its effects (Fig. 8). This result agrees with the study of

Zwain and colleagues (34) demonstrating that the same antibody is able to counteract the effect of TGFβ1 on GnRH release by GT1-7 cells.

As previously mentioned, we have demonstrated that the treatment with TGFβ1 or with A1-CM increases GnRH release in immortalized GnRH neurones rapidly (i.e., after 60 min) (2,3). Consequently, the observations here reported suggest that Smad proteins might mediate the effects of TGFβ1 on GnRH release. A possible hypothesis is that phosphorylation of R-Smads occurring after 15-30 min may be responsible for the increase of GnRH observed after 60 min, while the increase of Smad 7, which occurs later (i.e., after 120 min), may prevents further phosphorylation of R-Smads due to the continuous presence of TGFβ1. Future studies with dominant negative Smad proteins transfected in GT1-1 cells will be useful to establish this point. In conclusion, the results here reported demonstrate that GT1-1 neurones express the Smad proteins and that these are targets of TGFβ1. Moreover, the present study provides further evidence that astrocytes, through the release of TGFβ1, are important modulators of the GnRH neuronal activity and consequently of the reproductive function.

REFERENCES

1. Melcangi RC, Martini L, Galbiati M. Growth factors and steroid hormones: a complex interplay in the hypothalamic control of reproductive functions. Prog Neurobiol 2002; 67: 421-449.

2. Melcangi RC, Galbiati M, Messi E, Piva F, Martini L, Motta M. Type 1 astrocytes influence luteinizing hormone-releasing hormone release from the hypothalamic cell line GT1-1: is transforming growth factor-β the priciple involved? Endocrinology 1995; 136: 679-686.

3. Galbiati M, Zanisi M, Messi E, Cavarretta I, Martini L, Melcangi RC. Transforming growth factor-β and astrocytic conditioned medium influence luteinizing hormone-releasing hormone gene expression in the hypothalamic cell line GT1. Endocrinology 1996; 137: 5605-5609.

4. Messi E, Galbiati M, Magnaghi V, Zucchi I, Martini L, Melcangi RC Transforming growth factor beta 2 is able to modify mRNA levels and release of luteinizing hormone releasing hormone in an immortalised hypothalamic cell line (GT1-1). Neurosci Lett 1999; 270: 165-168.

5. ten Dijke P and Hill CS. New insights into TGF-β-Smad signaling. Trends Biochem Sci 2004; 29: 265-273.

6. Lagna G, Hata A, Hemmati-Brivanlou A, Massaguè J. Partnership between DPC4 and SMAD proteins in TGF-beta signaling pathways. Nature 1996; 383: 832-836.

7. Zawel L, Dai JL, Buckhaults P, Zhou S, Kinzler KW, Vogelstein B, Kern SE. Human Smad3 and Smad4 are sequence-specific transcription activators. Mol Cell 1998; 1: 611-617.

8. Buchanan CD, Mahesh VB, Brann DW. Estrogen-astrocyte-luteinizing hormone-releasing hormone signaling: a role for transforming growth factor β1. Biol Reprod 2000; 62: 1710-1721.

9. Prevot V, Bouret S, Croix D, Takumi T, Jennes L, Mitchell V, Beauvillain JC. Evidence that members of the TGF superfamily play a role in regulation of the GnRH neuroendocrine axis: expression of a type I serine-threonine kinase receptor for TGFβ and activin in GnRH neurones and hypothalamic areas of the female rat. J Neuroendocrinol 2000; 12: 665-670.

10. Bouret S, De Seranno S, Beauvillain J-C, Prevot V. Transforming growth factor β1 may directly influence Gonadotropin-Releasing Hormone gene expression in the rat hypothalamus. Endocrinology 2004; 145: 1794-1801.

11. Galbiati M, Martini L, Melcangi RC. Oestrogens, via Transforming Growth Factor α, modulate basic Fibroblast Growth Factor synthesis in hypothalamic astrocytes: in vitro observations. J Neuroendocrinol 2002; 13: 483-489.

12. Chomczynski P and Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; 162: 156-159.

13. Bradford MM. A rapid and sensitive method for the quantitation of microgram of protein utilizing the principle of protein-dye binding. Ann Biochem 1976; 72: 248-254.

14. Massague J. TGF-beta signal transduction. Annu Rev Biochem 1998; 67: 753-791.

15. Poncelet AC, de Caestecker MP, Schnaper HW. The transforming growth factor-beta/SMAD signaling pathway is present and functional in human mesangial cells. Kidney Int. 1999; 56: 1354-1365.

16. Liu C, Gaca MD, Swenson ES, Vellucci VF, Reiss M, Wells RG. Smads 2 and 3 are

differentially activated by transforming growth factor-beta (TGF-beta) in quiescent and activated hepatic stellate cells. Constitutive nuclear localization of Smads in activated cells is TGF-beta-independent. J Biol Chem 2003; 278: 11721-11728.

17. Yang YC, Piek E, Zavadil J, Liang D, Xie D, Heyer J, Pavlidis P, Kucherlapati R, Roberts AB, Bottinger EP. Hierarchical model of gene regulation by transforming growth factor beta. Proc Natl Acad Sci USA 2003; 100: 10269-10274.

18. Datto MB, Frederick JP, Pan L, Borton AJ, Zhuang Y, Wang XF. Targeted disruption of Smad3 reveals an essential role in transforming growth factor beta-mediated signal transduction. Mol Cell Biol 1999; 19: 2495-2504.

19. Bhowmick NA, Ghiassi M, Bakin A, Aakre M, Lundquist CA, Engel ME, Arteaga CL, Moses HL. Transforming growth factor-beta1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. Mol Biol Cell 2001; 12: 27-36.

20. Petritsch C, Beug H, Balmain A, Oft M. TGF-beta inhibits p70 S6 kinase via protein phosphatase 2A to induce G(1) arrest. Genes Dev 2000; 14: 3093-3101.

21. Yu L, Hebert MC, Zhang YE. TGF-beta receptor-activated p38 MAP kinase mediates Smad-independent TGF-beta responses. EMBO J 2002; 21: 3749-3759.

22. Itoh S, Thorikay M, Kowanetz M, Moustakas A, Itoh F, Heldin CH, ten Dijke P. Elucidation of Smad requirement in transforming growth factor-beta type I receptor-induced responses. J Biol Chem 2003; 278: 3751-3761.

23. Massaguè J. How cells read TGF-β signals. Nat Rev Mol Cell Biol 2000; 1: 169-178.

24. Wong C, Rougier-Chapman EM, Frederick JP, Datto MB, Liberati NT, Li JM, Wang XF. Smad3-Smad4 and AP-1 complexes synergize in transcriptional activation of the c-Jun promoter by transforming growth factor beta. Mol Cell Biol 1999; 19: 1821-1830.

25. Yakymovych I, ten Dijke P, Heldin CH, Souchelnytskyi S. Regulation of Smad signaling by protein kinase C. FASEB J 2001; 15: 553-555.

26. Zhu Y, Richardson JA, Parada LF, Graff JM. Smad3 mutant mice develop metastatic colorectal cancer. Cell 1998; 94: 703-714.

27. Tomic D, Brodie SG, Deng C, Hickey RJ, Babus JK, Malkas LH, Flaws JA. Smad 3 may regulate follicular growth in the mouse ovary. Biol Reprod 2002; 66: 917-923.

28. Nagarajan RP, Liu J, Chen Y. Smad3 inhibits transforming growth factor beta and activin signaling by competing with Smad 4 for FAST-2 binding. J Biol Chem 1999; 274: 31229-31235.

29. Hayes SA, Zarnegar M, Sharma M, Yang F, Peehl D, ten Dijke P, Sun Z. Smad3 represses androgen receptor mediated transcription. Cancer Res 2001; 61: 2112-2118.

30. Itoh S, Itoh F, Goumans M-J, ten Dijke P. Signaling of transforming growth factor-β family members through Smad proteins. Eur J Biochem 2000; 267: 6954-6967.

31. Kavsak P, Rasmussen RK, Causing CG, Bonni S, Zhu H, Thomsen GH, nd Wrana JL. Smad 7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF-β receptor for degradation. Mol Cell 2000; 6:1365-1375.

32. Suzuki C, Murakami G, Fukuchi M, Shimanuki T, Shikauchi Y, Imamura T, Miyazono K. Smurf1 regulates the inhibitory activity of Smad7 by targeting Smad7 to the plasma membrane. J Biol Chem 2002; 277: 39919-39925.

33. Denissova NG, Pouponnot C, Long J, He D, Liu F. Transforming growth factor beta-inducible independent binding of SMAD to the Smad7 promoter. Proc Natl Acad Sci USA 2000; 97: 6397-6402.

34. Zwain IH, Arroyo A, Amato P, Yen SSC. A role for hypothalamic astrocytes in dehydroepiandrosterone and estradiol regulation of gonadotropin releasing hormone (GnRH) release by GnRH neurons. Neuroendocrinology 2002; 75:375-383.

FIGURE LEGENDS

Fig. 1

GT1-1 cells express the mRNAs coding for different Smad proteins. Mk: molecular weight marker. Lanes + represent positive control (mouse brain); lanes G represent GT1-1 cells and lanes − show negative controls (samples performed without reverse transcriptase).

Fig. 2

Temporal pattern of Smad 2 and Smad 3 phosphorylation induced by TGFβ1 (5ng/mL) in GT1-1 cells. Representative Western blot analyses are reported in panel A; panel B shows quantitative data expressed as percent *vs.* the levels found in controls, after normalisation with α-tubulin. Each bar represents the mean ± SEM of four determinations. The experiment has been repeated in two independent cultures for verification of results. Different superscripts denote significant differences between groups ($P < 0.05$, by one-way ANOVA, Tukey *post hoc* test).

Fig. 3

Dose-dependent action of TGFβ1 (0.1, 1, 5 ng/ mL) on phospho-Smad 2 and 3 induction. GT1-1 cells were treated with TGFβ1 for 30 min. A) Representative Western blot analyses are reported; B) quantitative data (after normalisation with α-tubulin) are expressed as percent *vs.* the levels found in controls. Each bar represents the mean ± SEM of four determinations and groups with different superscripts are significantly different from each other ($P < 0.05$, by one-way ANOVA, Tukey *post hoc* test). The experiment has been repeated in two independent cultures for verification of results.

Fig. 4

Effect of TGFβ1 (5 ng/mL) on Smad 2 gene expression (A) and on Smad 2/3 protein levels (B) in GT1-1 cells. A) Smad 2 mRNA levels measured by semiquantitative RT-PCR; data are expressed as percent *vs.* the levels found in controls after normalisation with 18S rRNA. Each bar represents the mean ± SEM of six determinations performed; B) a representative Western blot analysis for Smad 2/3 protein expression. Two immunoreactive bands corresponding to Smad 2 (58 kDa) and to Smad 3 (52 kDa) are evident.

Fig. 5

Temporal pattern of induction of Smad 7 mRNA by TGFβ1 (5ng/mL) in GT1-1 cells. Data obtained by semiquantitative RT-PCR are normalised with 18S rRNA and expressed as percent *vs.* the levels found in controls. Each bar represents the mean ± SEM of six determinations performed. Different superscripts denote significant differences between groups (P < 0.05, by one-way ANOVA, Tukey *post hoc* test). The experiment has been repeated in two independent cultures for verification of results.

Fig. 6
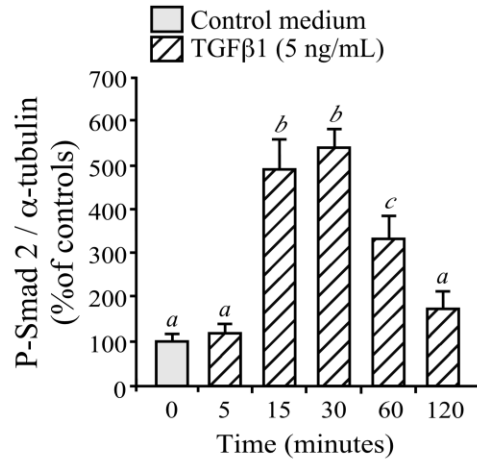
Effect of different times of treatment with TGFβ1 (5 ng/mL) on Smad 7 protein levels in GT1-1 cells. A) A representative Western blot analysis is reported; B) quantitative data (after normalisation with α-tubulin) are expressed as percent *vs.* the levels found in controls. Each bar represents the mean ± SEM of four determinations and groups with different superscripts are

significantly different from each other (P < 0,05, by one-way ANOVA, Tukey *post hoc* test). The experiment has been repeated in two independent cultures for verification of results.

Fig. 7

Effect of type 1 astrocyte conditioned medium (A1-CM) on different Smad levels in GT1-1 cells. Panel A: effect on the P-Smad 2 induction; Panel B: effect on the P-Smad 3 induction; Panel C: effect on the protein levels of Smad 7. In all panels, a representative Western blot analysis and quantitative data (expressed as percent *vs.* the levels found in controls, after normalisation with α-tubulin) are reported. Each bar represents the mean ± SEM of four determinations. The experiment has been repeated in two independent cultures for verification of results. *a* P<0.01, *b* P<0.05 *vs.* control medium (Student's *t* test).

Fig. 8

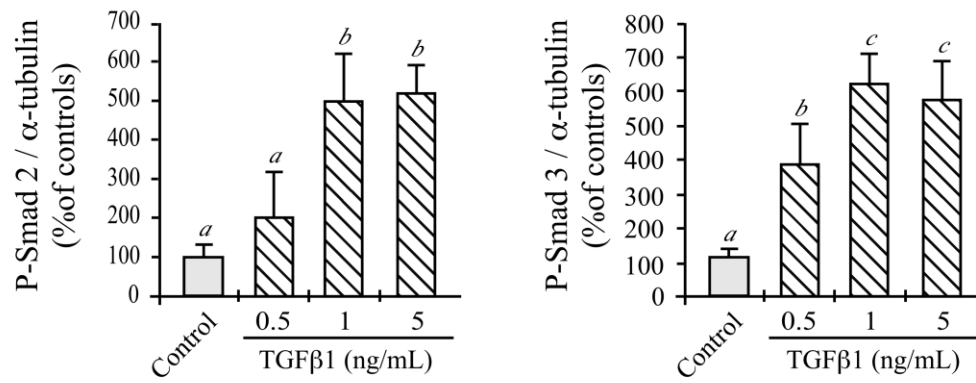TGFβ1 neutralising antibody counteracts the effect of A1-CM on the phosphorylation of Smads in GT1-1 cells. The antibody was added to the thawed A1-CM 1 hour before the treatment of the cells for 30 min. Panel A: representative Western blot analyses are reported; Panel B) quantitative data (after normalisation with α-tubulin) are expressed as percent *vs.* the levels found in controls. Each bar represents the mean ± SEM of four determinations performed and groups with different superscripts are significantly different from each other. The experiment has been repeated in two independent cultures for verification of results. (P < 0.05, by one-way ANOVA, Tukey *post hoc* test).
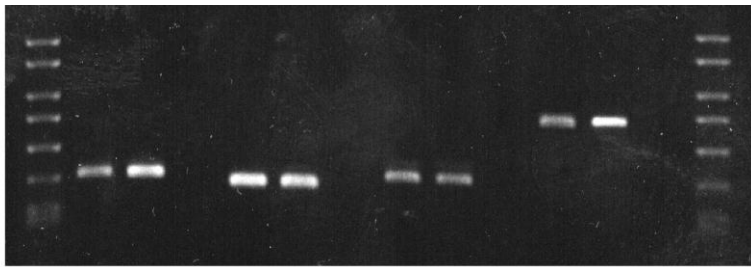
Mk + G −   + G −   + G −   + G −   Mk
Smad 2 Smad 3 Smad 4 Smad 7
303 bp 258 bp 297 bp 700 bp

FIGURE 1

A

B

FIGURE 2

A

P-Smad 2 ➔ [blot image] 58-60 kDa

α-tubulin ➔ [blot image] 55

Control   0.5   1   5
        TGFβ1 (ng/mL)

P-Smad 3 ➔ [blot image] 52-54 kDa

α-tubulin ➔ [blot image] 55

Control   0.5   1   5
        TGFβ1 (ng/mL)

B

Figure 3

A

B

FIGURE 4

A

FIGURE 5

A

Smad 7 → [band] — 45 kDa

α-tubulin → [band] — 55

Control | TGFβ1
30 60 120 240
Time (minutes)

B

FIGURE 6

A P-Smad 2

B P-Smad 3

C Smad 7

FIGURE 7

A

B

Figure 8