HHS Public Access

Author manuscript

Mol Cell Endocrinol. Author manuscript; available in PMC 2015 April 30.

Published in final edited form as:

Mol Cell Endocrinol. 2015 January 15; 400: 129–139. doi:10.1016/j.mce.2014.10.021.

TGFβ2 regulates hypothalamic *Trh* expression through the TGFβ inducible early gene-1 (*TIEG1*) during fetal development

Miriam Martínez-Armenta[a], **Sol Díaz de León-Guerrero**[a,1], **Ana Catalán**[a], **Lourdes Alvarez-Arellano**[a], **Rosa Maria Uribe**[b], **Malayannan Subramaniam**[c], **Jean-Louis Charli**[b], and **Leonor Pérez-Martínez**[a,*]

[a]Laboratorio de Neuroinmunobiología, Departamento de Medicina Molecular y Bioprocesos, Instituto de Biotecnología, Universidad Nacional Autónoma de México (UNAM), Cuernavaca, Morelos, Mexico

[b]Departamento de Genética y Fisiología Molecular, Instituto de Biotecnología, UNAM, Cuernavaca, Morelos, Mexico

[c]Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN, USA

Abstract

The hypothalamus regulates the homeostasis of the organism by controlling hormone secretion from the pituitary. The molecular mechanisms that regulate the differentiation of the hypothalamic thyrotropin-releasing hormone (TRH) phenotype are poorly understood. We have previously shown that *Klf10* or TGFβ inducible early gene-1 (*TIEG1*) is enriched in fetal hypothalamic TRH neurons. Here, we show that expression of *TGFβ* isoforms (1–3) and both *TGFβ* receptors (*TβRI* and *II*) occurs in the hypothalamus concomitantly with the establishment of TRH neurons during late embryonic development. TGFβ2 induces *Trh* expression via a TIEG1 dependent mechanism. TIEG1 regulates *Trh* expression through an evolutionary conserved GC rich sequence on the *Trh* promoter. Finally, in mice deficient in *TIEG1, Trh* expression is lower than in wild type animals at embryonic day 17. These results indicate that TGFβ signaling, through the upregulation of *TIEG1*, plays an important role in the establishment of *Trh* expression in the embryonic hypothalamus.

Keywords

Cell differentiation; Hypothalamus; TIEG1; Klf10; TGFβ; Trh

1. Introduction

The hypothalamus is a gateway between the endocrine and nervous systems. The thyrotropin-releasing hormone (TRH) is a neuropeptide expressed in various hypothalamic nuclei. The TRH neurons within the hypophysiotropic paraventricular nucleus control

*Corresponding author. Departamento de Medicina Molecular y Bioprocesos, Instituto de Biotecnología, UNAM, A.P. 510-3, Cuernavaca, Morelos 62210, México. Tel.: +52 5255 5 6227658, fax: +52 52777 3172388., leonor@ibt.unam.mx (L. Pérez-Martínez).

[1]Present address: CONACyT, Laboratorio de Investigación en Inmunología y Proteómica. Hospital Infantil de México, Federico Gómez, México D. F.

thyroid function, but additional effects of TRH on feeding behavior, thermogenesis, and autonomic regulation may be related to other TRH neuron subpopulations (Lechan and Fekete, 2006). Despite the vast knowledge about hypothalamic TRH function, very little is known about the molecular pathways regulating hypothalamic *Trh* expression during development, and particularly during terminal differentiation, which is characterized by the expression of the phenotype specific neurotransmitter (Hobert et al., 2010). We have previously shown that fetal hypothalamic TRH neurons express transcription factors belonging to the Krüppel-like factor (KLF) family (Guerra-Crespo et al., 2011), and that Klf4 plays an important role in the establishment of the TRH phenotype (Pérez-Monter et al., 2011). Another member of the KLF family enriched in fetal TRH neurons is the transforming growth factor-β (TGFβ) inducible early gene-1 (*TIEG1*), also called Krüppel-like transcription factor 10 (Klf10) (Guerra-Crespo et al., 2011).

TIEG1 is a member of the three zinc finger family of KLF transcription factors, originally isolated as an early response gene rapidly upregulated after the treatment of human fetal osteoblast cells with TGFβ1 (Subramaniam et al., 1995). TIEG1 acts both as a potent activator or repressor of gene transcription through binding to CACCC-or GC-rich elements with in the regulatory region of its target genes (Noti et al., 2004; Ou et al., 2004). The effect of TIEG1 on gene regulation is mediated by its interaction with co-repressor molecules, such as the histone deacetylase mSin3A (Zhang et al., 2001) and the histone demethylase JARID1B (Kim et al., 2010), or with the co-activator histone acetyltransferase p300/CBP-associated factor (PCAF) (Xiong et al., 2012). *TIEG1* has a widespread expression pattern (Subramaniam et al., 2005); its expression is detected in various regions of the developing and adult murine brain (Yajima et al., 1997), with *TIEG1* mRNA levels peaking a few days after birth in the mouse brain (Jiang et al., 2010). Multiple signaling molecules increase *TIEG1* levels in the central nervous system (Consales et al., 2007; Wibrand et al., 2006). *TIEG1* is a NGF-responsive immediate early gene during PC12 cell differentiation that induces cell cycle exit without affecting terminal differentiation (Dijkmans et al., 2009; Spittau et al., 2010). In cerebellar granular neuron precursors the BMP2 pathway triggers cell cycle exit, with TIEG1 as a mediator (Alvarez-Rodríguez et al., 2007). Additionally, TIEG1 facilitates apoptosis induced by TGFβ in an oligodendroglial cell line (Bender et al., 2004). However, the role of TIEG1 in neuronal differentiation *in vivo*, and specifically in the development of hypothalamic phenotypes, is essentially unknown.

TGFβ proteins are multifunctional cytokines that control a wide variety of cellular processes including cell differentiation, proliferation, apoptosis and the specification of developmental fate (Shi and Massague, 2003). Canonical TGFβ signaling is initiated by the binding of a ligand dimer to its serine/threonine kinase receptor at the cell surface. TGFβ ligands (TGFβ1–3) bind the type II TGFβ receptor (TβRII), which causes its recruitment to the type I TGFβ receptor (TβRI). The formation of this complex allows the phosphorylation of the kinase domain of TβRI by TβRII, which in turn triggers cellular changes through transcriptional responses (Shi and Massague, 2003).

During CNS development, TGFβ immunolabeling is most prominent in zones where neuronal differentiation occurs and less intense in zones of active proliferation (Flanders et al., 1991). TGFβ has an anti-mitotic effect on embryonic neural progenitors and increases

the expression of neuronal markers in hippocampal and cortical primary cell cultures of developing mouse (Vogel et al., 2010). Similarly, TGFβ2 induces neuronal differentiation during chicken development (Garcia-Campmany and Marti, 2007). TGFβ2-3 induce motoneuron and midbrain dopaminergic neuron survival (Gouin et al., 1996; Krieglstein et al., 1995; Poulsen et al., 1994), and tyrosine hydroxylase expression in the developing chicken and mouse brain (Farkas et al., 2003; Roussa et al., 2008). Interestingly, TGFβ regulates the expression of hypothalamic neuropeptides (POMC, GnRH, AVP) in adult animals (Bouret et al., 2004; Fevre-Montange et al., 2004; Matsumura et al., 2007). The expression pattern of the TGFβ isoforms in different brain areas suggests that they might have phenotype-specific functions. However, the role of TGFβ in the development of the hypothalamus has remained largely unexplored.

Since TGFβ signaling regulates the expression of genes involved in neural development (Estaras et al., 2012), and *TIEG1* transcript is a TGFβ induced gene enriched in fetal hypothalamic TRH neurons (Guerra-Crespo et al., 2011), we tested the hypothesis that TGFβ mediated signaling promotes hypothalamic TRH neuronal differentiation. In this report we show that TGFβ2, through TIEG1 action, is part of the differentiation program leading to *Trh* expression within the hypothalamus.

2. Material and methods

2.1. Animals

Wistar rats were maintained at the institute animal facility in standard environmental conditions (lights on between 07:00 and 19:00 h, temperature 21 ± 2 °C) with rat chow and tap water *ad libitum*. Animal care and protocols followed the guidelines for the use of animals in neuroscience research of the Society for Neuroscience, USA, and were approved by the Animal Care and Ethics Committee of the Instituto de Biotecnología, UNAM.

Hypothalamic tissue was dissected from Wistar rats at various developmental stages [from embryonic day (E) 14 to E21, at postnatal (P) days 0, 7, 14 and 21 and adult (5 months)]. Tissue was stored at −80 °C until use. E17 hypothalamic tissue was dissected from TIEG1 knockout mice as previously described (Subramaniam et al., 2005). TIEG1 knockout mice were maintained on a C57BL/6 background and the genotype was corroborated by quantitative PCR.

2.2. Cell culture and transfection

Hypothalamic primary cultures were prepared and maintained in Neurobasal medium (Invitrogen) supplemented with B27 (Invitrogen) as previously described (Joseph-Bravo et al., 2002) (Guerra-Crespo et al., 2003). Hypothalamic cells were plated at $1.5 \times 10^5/cm^2$ and TGFβ isoforms (R&D Systems) were added at the indicated concentration and time periods. Cells ($1.18 \times 10^4/mm^2$) were transfected as reported (Guerra-Crespo et al., 2003) with a vector containing the *Trh* promoter sequence located between −776 and +84 bp driving firefly luciferase (Trh-Luc; 800 ng) (Balkan et al., 1998) or a mutated version, together with the pcDNA4/TO-TIEG1 expression vector or the equivalent concentration of empty pcDNA vector (basal). An SV40 promoter-driven β-galactosidase expression vector (kindly donated by Dr. Susana Castro, Instituto de Biotecnología-UNAM) was used for normalization.

Firefly luciferase and β-galactosidase activities were determined 72 h later using the Dual-Light System (Applied Biosystems). Data correspond to the ratio of firefly luciferase activity/β-galactosidase activity and are represented as the relative fold change compared to empty vector transfected cells.

CA77 (rat medullary thyroid carcinoma) cells were grown in advanced DMEM/F12 medium (Gibco-BRL, Gaithersburg, MD, USA) containing 5% FBS (Biowest International, Coinsins, Switzerland), 0.5 mM glutamine (Sigma) and 1% antibiotic–antimycotic (Gibco-BRL, Gaithersburg, MD, USA). Cells ($0.5 \times 10^4/cm^2$) were subcultured weekly and the medium was changed after 4 days *in vitro* (DIV). Cultures were maintained in a REVCO incubator at 37 °C in humidified air with 5% CO_2. For the treatments, CA77 cells were plated at $27.5 \times 10^4/cm^2$ (RT-PCR) or $4.76 \times 10^4/cm^2$ (for Western-blot) in Advanced DMEM-F12 medium supplemented with 0.2% FBS (Biowest International, Coinsins, Switzerland) in the absence or presence of TGFβ2 (R&D Systems) at the indicated concentrations and cultured for 1, 2 or 3 DIV. Where indicated, the TβRI inhibitor SB431542 (Tocris Bioscience 10 μM) was added to the culture 30 min before TGFβ2 treatment (an additional 48 h period). For promoter analyses, CA77 cells were transfected with Trh-Luc (800 ng) alone or together with the pcDNA4/TO-TIEG1 expression vector or a mutated version of TIEG1 (R440W). A cytomegalovirus promoter-driven *Renilla* luciferase expression *vector* was used for normalization. Reporter activity was measured 48 h after transfection using the Dual-Luciferase Reporter Assay System (Promega). The firefly luciferase data were normalized to the *Renilla* luciferase values, and the results reported as described earlier.

2.3. Site-directed mutagenesis

The KLF motifs in the rat *Trh* promoter were mutated using the Trh-Luc vector as template and the GeneTailor Site-Directed mutagenesis kit (Invitrogen). The mutated KEM1 (CACCC at −92/−88 bp) and the GCA (GGGCGGG at −119/−113 bp) motifs were previously described (Pérez-Monter et al., 2011). The KEM2 motif (CACCC at −332/−328 bp) was mutated using the following forward (5′-CTCCTTTATTTTGCTGCCCAGTCCCTGCATCTG-3′) and reverse (5′-TGGGCAGCAAAATAAAGGAGATAGGGACTT-3′) primers. The GCB element (GGGCGGG at −652/−646 bp) was mutated using the following forward (5′-ATTAAACATTTGCTTCTTTATGCCTAGAGGCTTGGG-3′) and reverse (5′-TAAAGAAGCAAATGTTTAATAAAAATTAAT-3′) primers. The base substitutions are underlined for each primer. The resulting constructs were sequenced to verify the mutagenesis.

2.4. Semi-quantitative RT-PCR

RNA extraction and cDNA synthesis were carried out as previously reported (Pérez-Martínez et al., 1998). The positive and negative strand primers as well as the amplicon sizes are shown in Appendix: Supplementary Table S1. PCR amplification products were analyzed by densitometry using the Fluor-S MultiImager acquisition system (Bio-Rad) and the Quantity One software (Bio-Rad, v 4.2.0). Data were normalized to *Cyclophilin* or to the non-developmentally regulated gene *Gapdh* (Pérez-Martínez et al., 2001).

2.5. Quantitative PCR

Total RNA was isolated from hypothalami as previously described (Pérez-Martínez et al., 1998). Reverse transcription was performed using M-MLV reverse transcriptase (Invitrogene). *TIEG1* and *Trh* mRNA levels were determined by real-time PCR using an ABI PRISM 7500 sequence detector (Applied Biosystems, Foster City, CA) and TaqMan reagents according to manufacturer instructions. The primers and probe sequences used for *TIEG1* were 5′-AGGCAGCCAACCATGCT-3′ (forward), 5′-ATGGACATGAGCGCTTCCA-3′ (reverse) and 5′-TTCCCCTCCGAAGCTT-3′ (probe) and for *Trh* were 5′-GATGGAGTCTGATGTCACCAAGAG-3′ (forward), 5′-GCCCTTTTGCCAGGATGCT-3′ (reverse) and 5′-CAGGCCGGAGGTTCAT-3′ (probe) designed by Applied Biosystems from the consensus sequence generated from sequences NM_031135.2 (*Rattus norvegicus*)/NM_013692.2 (*Mus musculus*) for *TIEG1* and NM_013046.3 (*Rattus norvegicus*)/NM_009426.3 (*Mus musculus*) for *Trh*. Relative expression was calculated by normalizing to *β-actin* using the ΔΔCT method (Livak and Schmittgen, 2001).

2.6. Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed as previously described (De La Rosa-Velazquez et al., 2007). The chromatin (fragmented to 100–500 bp long) was subjected to immunoprecipitation using 3 μg of rabbit polyclonal anti-TIEG1 antibody (Subramaniam et al., 1998), rabbit polyclonal anti-histone H3 (tri-methyl K4) antibody (Abcam), rabbit polyclonal anti-acetyl-histone H4 (K16) antibody (Upstate), or IgG antibody as a negative control. Immunoprecipitated DNA was analyzed by semiquantitative PCR using specific primers for regions (P1–3) encompassing the CACCC and/or GC sequences of the *Trh* gene promoter (Appendix: Supplementary Table S1 and Appendix: Supplementary Fig. S4A). Amplified products were visualized on an ethidium bromide stained 2% agarose gel.

2.7. Immunoblot analysis

Western blot analysis was performed as previously described (Pérez-Martínez and Jaworski, 2005). Briefly, CA77 cell lysates were prepared using lysis buffer (25 mM Hepes, pH 7.7, 150 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5% Triton X-100, 0.5 mM DTT, 20 mM β-glycerophosphate, 1 mM Na_3VO_4, 5 mM NaF, 4 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin). After 15 min at 4 °C incubation, cell lysates were then centrifuged at 13,000 *g* for 15 min at 4 °C. Nuclear extracts from frozen hypothalamic tissue were prepared as described (Pérez-Monter et al., 2011). Protein content was determined by the Bradford assay (Bradford, 1976).

Nuclear extracts (40 μg) or cell lysates were fractionated by SDS–PAGE and transferred to nitrocellulose membranes (Hybond-ECL, GE Healthcare Life Sciences). Membranes were blocked with 5% nonfat milk in Tris-buffer saline (TBS; 10 mM Tris–HCl, pH 7.5, 150 mM NaCl), followed by overnight incubation at 4 °C with the indicated antibody diluted in TBS with 0.05% Tween-20 (TBS-T). After three washes with TBS-T, membranes were incubated with the appropriate secondary antibody coupled to horseradish peroxidase, and immunocomplexes were visualized by ECL Western Lighting detection reagents according

to manufacturer's instructions (Perkin Elmer). Primary antibodies for Western blot included rabbit polyclonal anti-TIEG1 (1:3000), rabbit polyclonal anti-Smad2/3 (1:1000; Santa Cruz Biotechnology Inc), rabbit polyclonal anti-pSmad2 (1:200 (Ser465/467); Chemicon), mouse monoclonal anti-flag (1:1500; Stratagene), and goat polyclonal anti-actin (1:1000; Santa Cruz Biotechnology Inc).

2.8. *In situ* hybridization

Double *in situ* hybridization experiments were performed as previously described (Lazcano et al., 2012) using serial 20 μm frozen adult brain coronal sections through the antero-posterior extent of the hypothalamus. A single-stranded [^{35}S]UTP-labeled RNA probe complementary to rat *Trh* cDNA (nucleotides; 1-1241 (Sánchez et al., 1997)) and a single-stranded digoxygenin-UTP-labeled RNA probe complementary to the rat *TIEG1* cDNA (nucleotides 467–1445) were used. For digoxygenin-UTP-labeled RNA probe, cells were taken as positive if the blue purpura color was easily distinguished from the background (compared to the sense probe); a subjective evaluation of *TIEG1* mRNA levels of hypothalamic nucleus was made. Signal density was classified as either high (+++) or medium (++). For [^{35}S]-UTP-labeled probe, cells were considered positive if at least fifteen grains were detected on the cell. For each nucleus, the percentage of *Trh* mRNA cells positive for *TIEG1* mRNA was determined in two or three slices.

2.9. Radioimmunoassay

E17 mouse hypothalamic tissue derived from wild type or TIEG1 knockout mice was sonicated in 20% acetic acid. The soluble extracts were dried and TRH peptide levels were determined by radioimmunoassay as previously described using 1:10,000 anti-TRH antibody (Joseph-Bravo et al., 1979).

2.10. Statistical analysis

Results were calculated as percentage or fold induction of control. Data are shown as the mean ± SEM. Data were analyzed by ANOVA followed by Fisher's PLSD test, considered significant at $P < 0.05$. Data correspond to at least three independent experiments.

3. Results

3.1. TGFβ isoforms and receptors are expressed during hypothalamic development

Since TGFβ signaling plays critical roles in morphogenesis and cell lineage specification during CNS development (Farkas et al., 2003; Krieglstein et al., 2002; Maira et al., 2010; Roussa et al., 2008), and *TIEG1*, a target of TGFβ signaling, is expressed in the fetal hypothalamus (Guerra-Crespo et al., 2011), we determined the expression profile of the *TGFβ* isoforms (1, 2 and 3) and TGFβ receptors (*TβRI* and RII) during rat hypothalamus development. Semi-quantitative RT-PCR analyses revealed that the *TGFβ1* transcript expression was stable, from E14 until adult stage (Fig. 1A). In contrast, *TGFβ2* transcript expression was tightly regulated during development. We found that both E14 and E17 had increased levels of *TGFβ2* compared to E15. We also observed an increase at P0, P14 and P21, compared to P7 (Fig. 1B). During embryonic development, *TGFβ3* expression was transiently increased at E15 (>17-fold higher than at E16), and remained constant between

E16 and E21. At birth, *TGFβ3* expression increased and constant levels were detected until adulthood (Fig. 1C). *TβRI* expression was constant at all developmental stages examined (Fig. 1D). Similar to *TFGβ3*, a transient increase in *TβRII* mRNA was detected at E15 (~1.5-fold induction) and levels remained constant until birth; thereafter, *TβRII* expression increased gradually, peaking at P21, which then decreased at adult stage, although the differences were not significant (Fig. 1E). Thus, although the three TGFβ isoforms and both receptors were expressed *in vivo*, only *TGFβ2, TGFβ3* and *TβRII* were regulated during hypothalamic development. An interesting observation is the fact that the upregulation of the *TGFβ2* and *TGFβ3* isoforms coincides chronologically with the period of differentiation of the TRH phenotype (E11–E17 in rat) (Pérez-Martínez et al., 2001).

3.2. TGFβ2 enhances Trh and TIEG1 expression in primary cultures of hypothalamic cells

To explore the involvement of TGFβ mediated signaling during TRH phenotype differentiation, we used primary cell cultures derived from E17 rat hypothalamus. In accordance with the *in vivo* data, at 1 and 4 days *in vitro* (DIV) hypothalamic cultures expressed *TGFβ1-3* isoforms and both *TβRI* and *TβRII* (Appendix: Supplementary Fig. S1). Even though the three TGFβ isoforms where expressed in the hypothalamic cultures, *Trh* mRNA levels were increased in response to a saturating dose of TGFβ2 or TGFβ3 (2.5 ng/ml), but not by TGFβ1. TGFβ2 produced a steady and significant increase of *Trh* expression, with a peak at 3 DIV (approximately threefold) (Fig. 2A, left panel), followed by a decrease to basal levels at 4 DIV (data not shown). TGFβ3 increased *Trh* expression at 1DIV, and decreased to basal levels thereafter (data not shown), therefore we focused on the effect of TGFβ2 in the regulation of *Trh* expression. Despite the fact that the corticotropin-releasing hormone (*Crh*) neurons are originated in a similar period of time to *Trh* neurons, *Crh* mRNA levels were not affected in response to 2.5 ng/ml TGFβ2 (Fig. 2A, right panel). These data are consistent with the idea that the expression of these hypothalamic neuropeptides is regulated by different signals during development (Markakis, 2002; Markakis and Swanson, 1997).

Since TGFβ2 induced *Trh* mRNA levels, we evaluated the ability of TGFβ2 to regulate *Trh* promoter activity using primary fetal hypothalamic cells transfected with the minimal *Trh* promoter region (−776/+84 bp) driving a luciferase reporter gene (Trh-Luc). This minimal promoter drives *in vitro* reporter expression preferentially in TRH neurons (Guerra-Crespo et al., 2003). TGFβ2 treatment resulted in a 2.3-fold induction of *Trh* promoter activity compared to control cells (Fig. 2C). Interestingly, this effect was isoform specific since TGFβ3 had no effect on *Trh* promoter activity (Fig. 2C), a result that correlates with the low potential of TGFβ3 to regulate *Trh* transcript levels.

As *TIEG1* is upregulated in response to TGFβ (Subramaniam et al., 1995), and is enriched in fetal hypothalamic TRH neurons (Guerra-Crespo et al., 2011), we determined whether TGFβ2 regulates *TIEG1* expression in E17 hypothalamic cell cultures. TGFβ2 treatment induced a 5- and 10-fold increase in *TIEG1* mRNA levels at 1 and 3 DIV, respectively (Fig. 2B). These data demonstrate that *TIEG1* is a target of TGFβ2 activity in hypothalamic cells.

The overexpression of *TIEG1* mimics TGFβ action in several cell lines (Chalaux et al., 1999; Hefferan et al., 2000; Ribeiro et al., 1999; Tachibana et al., 1997). Similarly, *TIEG1*

overexpression increased *Trh* transcription (threefold) compared to control transfected cells (Fig. 2C). These results suggest that TIEG1 mediates the effect of TGFβ2 signaling on *Trh* expression.

3.3. The TGFβ signaling pathway regulates Trh expression through TIEG1 action in CA77 cells

To demonstrate that the upregulation of *Trh* levels by TGFβ2 was a direct effect on TRH cells, and not mediated by adjacent cells, the action of TGFβ2 was further studied in the rat medullary thyroid carcinoma CA77 cell line which expresses *Trh* (Sevarino et al., 1988). This cell line expressed *TGFβ1-3* isoforms, *TβRI*, *TβRII*, and *TIEG1* mRNAs (Appendix: Supplementary Fig. S2), as well as the TIEG1 protein (Fig. 5B). As in the primary hypothalamic cultures, TGFβ2 caused dose- and time-dependent increases in *Trh* mRNA levels in CA77 cells. Maximal response (2.3-fold) was observed at 2 DIV with 5 ng/ml of TGFβ2 (Fig. 3A, left panel); no effect was observed at 1 or 3 DIV (data not shown). These results indicate that TGFβ2 can upregulate *Trh* expression by a direct effect on TRH expressing cells.

TβRI is activated by TβRII receptor upon ligand binding and transduces signals into the cytoplasm through the phosphorylation of Smad (Smad2/3) proteins. To determine whether TGFβ2 signals through TβRI to regulate *Trh* expression, we used the TβRI inhibitor SB431542 (Matsuyama et al., 2003). SB431542 (10 μM) inhibited the phosphorylation of Smad2 without affecting total Smad protein (Fig. 3A, lower panel), and the increase in *Trh* mRNA levels induced by TGFβ2 treatment (Fig. 3A, left panel). These results indicate that TGFβ2 regulates *Trh* mRNA levels via TβRI signaling.

As in primary hypothalamic cultures, *TIEG1* mRNA levels were up-regulated by TGFβ2 in the CA77 cell line. TGFβ2 (5 ng/ml) treatment increased *TIEG1* levels at 2 DIV (approximately eightfold). This effect was eliminated by pre-treatment with the SB431542 inhibitor (Fig. 3A, right panel). Thus, TGFβ2 signals through TβRI to regulate *Trh* and *TIEG1* expression. Furthermore, and as in hypothalamic cells (Fig. 2C), TIEG1 overexpression in the CA77 cells was sufficient to increase *Trh* promoter activity (approximately sixfold) (Fig. 3B, lower panel), which was also reflected in an increase in *Trh* mRNA levels 4 days after transfection (Fig. 3B, upper panel). This effect was dependent on TIEG1 DNA binding activity since over-expression of a mutant version of TIEG1 (R440W) that affects the zinc finger domain and abolishes its ability to bind DNA (Alvarez-Rodríguez et al., 2007; Little et al., 1992), was unable to regulate *Trh* promoter activity (Fig. 3B, lower panel). These data indicate that the mechanism used by TIEG1 to activate the *Trh* promoter depends on its interaction with DNA. Overexpression of *Sp3*, another member of the KLF family, had no effect on the *Trh* promoter activity (Fig. 3B, lower panel), which suggests that not all members of the KLF family are able to regulate *Trh* transcription.

In accordance with the idea that TGFβ2 regulates *Trh* mRNA levels via a TIEG1 mediated mechanism, combining TGFβ2 treatment with *TIEG1* over-expression did not induce a higher activation of the *Trh* promoter than that observed with either of these signals alone (Fig. 3C). TGFβ2 mediated *Trh* promoter activity was drastically diminished (~50%) when the mutant R440W was expressed, compared to cells treated with TGFβ2 only (Fig. 3C).

Together these data indicate that TGFβ2 and TIEG1 act through the same pathway to promote *Trh* expression in CA77 cells.

3.4. A GC motif in the Trh promoter is required to regulate its activation by TIEG1 in hypothalamic cells

Based on the data presented earlier showing that TIEG1 regulates *Trh* transcription, we determined the ability of TIEG1 to bind to the rat *Trh* promoter region. Previous studies in our group have identified four potential binding sites for Krüppel-like transcription factors within the minimal promoter region of the rat *Trh* gene: two CACCC (−93/−88 and −332/−328 bp) and two GC motifs (−119/−113 and −652/−646 bp) (Pérez-Monter et al., 2011), which will be called henceforth as KEM1, KEM2, GCA and GCB, respectively.

To investigate the role of the putative KLF binding motifs on TIEG1 mediated *Trh* promoter activity, we used TRH-Luc constructs mutated in the KEM1 (*gt*CCC; ΔKEM1) and GCA (*t*G*c*C*ta*G; ΔGCA) (previously described in Pérez-Monter et al., 2011); as well as in KEM2 (*gt*CCC; ΔKEM2), and GCB (*t*G*c*C*ta*G; ΔGCB) elements to abolish binding of Krüppel-like (Higaki et al., 2002) and Sp1 (Li and Kellems, 2003) transcription factors. Primary hypothalamic cultures were co-transfected with the wild-type promoter construct (Trh-Luc) or with the mutated constructs (ΔKEM1-Luc, ΔKEM2-Luc, ΔGCA-Luc or ΔGCB-Luc), together with a *TIEG1* expression vector. Mutation of the KEM2 site neither altered *Trh* promoter basal activity nor the transcriptional properties of TIEG1 on the *Trh* gene. KEM1 site mutation (ΔKEM1) resulted in an enhanced basal *Trh* promoter activity, which was not affected by the overexpression of TIEG1 (Fig. 4B). In contrast, mutation of the GCA element abolished the TIEG1 mediated *Trh* promoter activity without affecting promoter basal activity (Fig. 4B compare bars 2 and 8). Finally, mutation of the GCB site drastically diminished basal as well as TIEG1 mediated *Trh* promoter activity (Fig. 4B). Thus the GCA site present on the minimal *Trh* promoter is critical for TIEG1 transcriptional properties in hypothalamic cells.

3.5. TIEG1 is expressed in the embryonic rat hypothalamus, binds to the TRH promoter, and its expression is necessary to maintain embryonic Trh mRNA levels in the mouse hypothalamus

The *in vitro* data indicate that TIEG1 regulates *Trh* expression in response to TGFβ2. To further characterize TIEG1 action on *Trh* expression, the *in vivo* developmental expression profile of rat hypothalamic *TIEG1* was determined by RT-qPCR and Western-blot assays. *TIEG1* mRNA levels were detected since the earliest developmental stage examined (E15), and were maintained during all stages analyzed (Fig. 5A, left panel). At the protein level, TIEG1 was detected at the earliest time point examined (E15) and increased gradually until the adult stage (Fig. 5B). In agreement with these results, the GENSAT database has recently reported the expression of *TIEG1* in the hypothalamus at E15.5, P7 and adult stages (http://www.gensat.org/).

Interestingly, TIEG1 expression (mRNA and protein) coincided chronologically with that of *Trh* in the rat hypothalamus. As previously reported (Pérez-Martínez et al., 2001), we observed that *Trh* expression was regulated during the development of the hypothalamus.

Trh mRNA expression was detected at E15, thereafter a steady increase was observed during the following days peaking at adult stage (Fig. 5A, right panel). To confirm that *TIEG1* mRNA co-localizes with *Trh* mRNA in the rat hypothalamus, *in situ* hybridization experiments were performed at the adult stage. The data show that *TIEG1* mRNA positive cells are distributed in many of the hypothalamic nuclei (paraventricular nucleus, anterior- and lateral-hypothalamic areas as well as dorsomedial- and ventromedial-nucleus) where *Trh* mRNA cells are localized. The density of *TIEG1* mRNA positive cells was relatively high, in particular in the parvocellular area of the paraventricular nucleus (Appendix: Supplementary Fig. S3 lower panel). To analyze whether *TIEG1* and *Trh* mRNAs are coexpressed in the hypothalamus, a [^{35}S]UTP-labeled RNA probe complementary to the *Trh* mRNA together with a digoxigenin-UTP-labeled RNA probe complementary to the *TIEG1* mRNA were used. *TIEG1* mRNA colocalized with *Trh* mRNA at the cellular level in various nuclei including the paraventricular nucleus and the lateral hypothalamic area. The percentage of *Trh* mRNA positive cells labeled with the *TIEG1* mRNA probe varied according to the hypothalamic nucleus; it was 22% (total number of cells counted 1052) in the paraventricular nucleus and 39% (total number of cells counted 1297) in the lateral hypothalamic area (Appendix: Supplementary Fig. S3). Altogether, these data suggest a role for TIEG1 in the hypothalamic *Trh* expression *in vivo*.

Based on the data discussed earlier, we determined the ability of TIEG1 to bind to the *Trh* promoter *in vivo*. Chromatin immunoprecipitation (ChIP) assays using chromatin derived from E17 rat hypothalamus and specific primers spanning the initial CACCC (KEM1) and GC (GCA) boxes showed an enrichment of the PCR amplification product using the TIEG1 immunoprecipitated fraction, compared to the IgG precipitate (Fig. 6A). Since both histone H4K16 acetylation (H4K16ac) and H3K4 trimethylation (H3K4me3) are marks associated with gene activation (Martin and Zhang, 2005), we determined the presence of these modifications at the proximal *Trh* promoter region. H4K16ac as well as H3K4me3 levels were enriched at the GCA and KEM1 elements (Fig. 6A). No amplification was detected using either an IgG control antibody or primers from the TβRII coding region (Fig. 6A and Appendix: Supplementary Fig. S4). Similar results were observed using specific primers spanning the second GC (GCB) or CACCC (KEM2) motifs (Appendix: Supplementary Fig. S4). Altogether, these data demonstrate that TIEG1 is able to transactivate the *Trh* gene by binding to the proximal promoter region.

To investigate whether TIEG1 is required for *Trh* expression *in vivo*, we compared *Trh* expression in the developing hypothalamus of *TIEG1*$^{-/-}$ and wild type mice. The null mutation was confirmed by RT-qPCR (data not shown). A 50% reduction in *Trh* mRNA levels was detected in the *TIEG1*$^{-/-}$ mice hypothalamus at E17 compared to *TIEG1*$^{+/+}$ (Fig. 6B). The reduction in *Trh* mRNA levels at E17 was reflected at the peptide level as well (~40%), although the decrease was not significantly different with respect to the wild-type mouse (Fig. 6C). Overall, these results point to a key role for TIEG1 in proper *Trh* gene expression during rodent fetal hypothalamic development.

4. Discussion

Neurogenesis involves progenitor proliferation and appropriately timed generation of postmitotic neurons. Cell cycle arrest correlates with the appearance of neurite outgrowth, and the synthesis of the phenotype-specific neurotransmitter is determined during the terminal phase of the differentiation. This process is exquisitely regulated by multiple signals including growth factors (FGF, EGF, VEGF), neurotrophins (BDNF, NT3) and transcription factors (reviewed in Pérez-Martínez and Charli, 2006).

In the hypothalamus different studies using thymidine [^{3}H] and bromodeoxyuridine (BrdU) have established the period in which specific neuroendocrine populations are generated (Markakis, 2002). In the rat, five neuronal phenotypes, including TRH neurons, are generated from the third ventricle with partially overlapping periods between days E11 and E17 (Markakis and Swanson, 1997). However, the identity of the signals controlling hypothalamic neuronal differentiation is poorly understood.

This study represents the first characterization of the impact of TGFβ signaling on hypothalamic *Trh* expression during the neuronal terminal differentiation period. We report that the three *TGFβ* isoforms as well as the *TβRI* and *TβRII* receptors are expressed in the rat hypothalamus, showing different temporal expression patterns during development. TGFβ exerts multiple functions in the CNS (Estaras et al., 2012; Garcia-Campmany and Marti, 2007). TGFβ2 has an antiproliferative effect in adult progenitor cells in hippocampal and cerebellar cultures (Lu et al., 2005). Additionally, TGFβ2-3 induce the expression of neuronal markers, such as HuC/D and NeuN in hippocampus and cortex (Vogel et al., 2010). Here, we demonstrate that TGFβ2, but not TGFβ3, induces *Trh* promoter activity. Additionally, TGFβ2 upregulates *Trh* expression through TβRI mediated signaling. Previous studies have reported a differential binding affinity between TGFβ isoforms and their receptors, where TβRI and TβRII bind to TGFβ1 and TGFβ3 with higher affinity (~1000-fold higher) than to TGFβ2. However, a direct relationship between binding affinity and the biological effect of the TGFβ isoforms has not been characterized (Baardsnes et al., 2009; Cheifetz et al., 1990; De Crescenzo et al., 2003, 2006). TGFβ2 binding to the co-receptor TβRIII (also called betaglycan) increases ligand affinity for TβRII (Lopez-Casillas et al., 1993, 1994). Interestingly, data from our group demonstrated that the TβRIII is consistently expressed throughout hypothalamic development (data not shown), which may facilitate the action of TGFβ2 on *Trh* expression.

Although TGFβ2 mediated *Trh* expression requires TβRI activity, a bioinformatic analysis of transcription factor binding sites on the *Trh* gene promoter does not show the presence of Smad binding elements, which suggests that *Trh* induction is not mediated directly by Smad proteins. However, since the *Trh* promoter region contains four potential binding sites for Krüppel-like transcription factors, *TIEG1* is enriched in TRH fetal hypothalamic neurons (Guerra-Crespo et al., 2011), and *TIEG1* overexpression mimics TGFβ effects in different cell types (Chalaux et al., 1999; Hefferan et al., 2000; Ribeiro et al., 1999; Tachibana et al., 1997), we propose that TGFβ2 promotes *Trh* expression by inducing TIEG1 expression and binding to a GC motif on the *Trh* promoter. In accordance with this idea, preliminary data

using chromatin derived from primary hypothalamic cultures show that TGFβ2 treatment increases the binding of TIEG1 to the *Trh* promoter (data not shown).

Our data also show that *TIEG1* expression is upregulated by TGFβ2 in hypothalamic cells where it is able to regulate *Trh* gene expression through the specific binding to the GCA element (−119/−113) in the *Trh* promoter. TIEG1 belongs to a subfamily within the KLF family characterized by the presence of three unique repression domains (R1–3), through which it is able to interact with the co-repressor mSin3A (Zhang et al., 2001) or with the demethylase JARID1B (Kim et al., 2010), thereby inhibiting the expression of multiple genes. Although several studies have reported that TIEG1 can transactivate genes, such as *p21, osteocalcin* or *Smad2* (Johnsen et al., 2002a, 2002b), its interaction with PCAF (p300/CBP Associated Factor), a transcriptional coactivator, was not described until recently (Xiong et al., 2012). This scenario becomes even more complex if we consider that Klf4, another transcription factor enriched in the transcriptome of fetal hypothalamic TRH neurons, also regulates *Trh* expression during hypothalamic development (Pérez-Monter et al., 2011). Klf4 upregulates *Trh* promoter activity in an additive manner with Sp1, which seems to contribute to transmit transient regulatory signals to the *Trh* promoter (Díaz-Gallardo et al., 2010). Unlike TIEG1, Klf4 effect on *Trh* promoter activity seems independent on its ability to bind DNA directly (Pérez-Monter et al., 2011). Considering previous reports showing that both Klf4 and Sp1 are required to form complexes to regulate the expression of its target genes (Pérez-Monter et al., 2011; Zhang et al., 1998), we propose a model in which, after TGFβ/TβRI activation, TIEG1 might form a heteromeric complex with Klf4 and Sp1 to interact with the *Trh* promoter. Additionally, this complex could recruit co-activators such as p300/CBP or PCAF (Evans et al., 2007; Xiong et al., 2012).

Interestingly, the activating transcription factor 3 (Atf3), a transcription factor regulated by TGFβ (Yin et al., 2010), is also enriched in the transcriptome of fetal hypothalamic TRH cells (Guerra-Crespo et al., 2011). Additionally, TIEG1, Klf4 and Atf3 expression is increased during PC12 cell differentiation after NGF treatment, suggesting that these transcription factors might work together during neuronal differentiation (Dijkmans et al., 2009). Furthermore, other KLF/Sp1 binding sites (the KEM1 and GC-B motifs) appear to act as negative or positive cis-elements for the basal *Trh* promoter activity. However, the transcription factors that bind to these sites, and the precise role of these elements in the initiation of *Trh* expression, remain to be determined.

While the relevance of other members of the KLF family such as Klf7, Klf9 and Klf11 for neuronal development has already been underlined (reviewed in McConnell and Yang, 2010), this is the first report that identifies a transcriptional role of TIEG1 in a specific neuronal phenotype. We found that TIEG1 expression *in vivo* is necessary for the proper expression of *Trh* mRNA in the developing hypothalamus. However, *Trh* mRNA levels were only decreased by 50% in *TIEG1*$^{-/-}$ mice and no significant difference was observed for TRH peptide levels. One possible explanation for this phenotype may result from the fact that only a fraction of the TRH positive cells may express *TIEG1* mRNA during embryonic development, as suggested by our *in situ* hybridization assays in adult animals. Alternatively, previous studies have shown that Klf3 and Klf5 play a redundant role with Klf4 during cellular reprogramming to reverse the highly differentiated state of a somatic

cell back to a pluripotent state (Jiang et al., 2008). This suggests that other KLF family members expressed in the CNS (such as Klf4, Klf7 and Klf9) (Blackmore et al., 2012; Laub et al., 2001a, 2001b; Lebrun et al., 2013; Seo et al., 2012) might play a redundant role with TIEG1 during the development of the hypothalamus.

In conclusion, this study has shown novel insights into the function of the TGFβ signaling pathway during hypothalamic development. Our observations using *in vitro* and *in vivo* approaches support a role of TGFβ2 and TIEG1 on *Trh* gene expression. We conclude that (1) *TGFβ* isoforms and receptors are expressed during development of the hypothalamus; (2) TGFβ2 regulates both *TIEG1* and *Trh* expression in a TβRI dependent manner; (3) TGFβ2 requires TIEG1 binding to specific DNA motifs to regulate *Trh* expression; and finally (4) TIEG1 expression is necessary but not sufficient for the correct expression of *Trh in vivo* in the fetal hypothalamus.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Dr. G. Pedraza-Alva (Instituto de Biotecnología, UNAM) for insightful scientific discussions and critical review of the manuscript, to M. Villa, M. Cisneros, V. Barajas, O. López-Gutiérrez and to Karla F. Meza-Sosa for technical support. We also thank to S. González for maintaining our rat colony. This work was supported by the author's institutions and by grants from DGAPA/UNAM (IN209212), CONACYT/Mexico (155290) and NIH DE 14036.

Abbreviations

TRH	thyrotropin releasing hormone
TIEG1	TGFβ inducible early gene-1
Klf10	Krüppel-like factor 10
ChIP	chromatin immunoprecipitation
TβRII	type II TGFβ receptor
TβRI	type I TGFβ receptor

References

Alvarez-Rodríguez R, Barzi M, Berenguer J, Pons S. Bone morphogenetic protein 2 opposes Shh-mediated proliferation in cerebellar granule cells through a TIEG-1-based regulation of Nmyc. J Biol Chem. 2007; 282:37170–37180. [PubMed: 17951258]

Baardsnes J, Hinck CS, Hinck AP, O'Connor-McCourt MD. TbetaR-II discriminates the high- and low-affinity TGF-beta isoforms via two hydrogen-bonded ion pairs. Biochemistry. 2009; 48:2146–2155. [PubMed: 19161338]

Balkan W, Tavianini MA, Gkonos PJ, Roos BA. Expression of rat thyrotropin-releasing hormone (TRH) gene in TRH-producing tissues of transgenic mice requires sequences located in exon 1. Endocrinology. 1998; 139:252–259. [PubMed: 9421422]

Bender H, Wang Z, Schuster N, Krieglstein K. TIEG1 facilitates transforming growth factor-beta-mediated apoptosis in the oligodendroglial cell line OLI-neu. J Neurosci Res. 2004; 75:344–352. [PubMed: 14743447]

Blackmore MG, Wang Z, Lerch JK, Motti D, Zhang YP, Shields CB, et al. Kruppel-like Factor 7 engineered for transcriptional activation promotes axon regeneration in the adult corticospinal tract. Proc Natl Acad Sci USA. 2012; 109:7517–7522. [PubMed: 22529377]

Bouret S, De Seranno S, Beauvillain JC, Prevot V. Transforming growth factor beta1 may directly influence gonadotropin-releasing hormone gene expression in the rat hypothalamus. Endocrinology. 2004; 145:1794–1801. [PubMed: 14670985]

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976; 72:248–254. [PubMed: 942051]

Chalaux E, Lopez-Rovira T, Rosa JL, Pons G, Boxer LM, Bartrons R, et al. A zinc-finger transcription factor induced by TGF-beta promotes apoptotic cell death in epithelial Mv1Lu cells. FEBS Lett. 1999; 457:478–482. [PubMed: 10471833]

Cheifetz S, Hernandez H, Laiho M, ten Dijke P, Iwata KK, Massague J. Distinct transforming growth factor-beta (TGF-beta) receptor subsets as determinants of cellular responsiveness to three TGF-beta isoforms. J Biol Chem. 1990; 265:20533–20538. [PubMed: 1700790]

Consales C, Volpicelli F, Greco D, Leone L, Colucci-D'Amato L, Perrone-Capano C, et al. GDNF signaling in embryonic midbrain neurons in vitro. Brain Res. 2007; 1159:28–39. [PubMed: 17574220]

De Crescenzo G, Pham PL, Durocher Y, O'Connor-McCourt MD. Transforming growth factor-beta (TGF-beta) binding to the extracellular domain of the type II TGF-beta receptor: receptor capture on a biosensor surface using a new coiled-coil capture system demonstrates that avidity contributes significantly to high affinity binding. J Mol Biol. 2003; 328:1173–1183. [PubMed: 12729750]

De Crescenzo G, Hinck CS, Shu Z, Zuniga J, Yang J, Tang Y, et al. Three key residues underlie the differential affinity of the TGFbeta isoforms for the TGFbeta type II receptor. J Mol Biol. 2006; 355:47–62. [PubMed: 16300789]

De La Rosa-Velazquez IA, Rincon-Arano H, Benitez-Bribiesca L, Recillas-Targa F. Epigenetic regulation of the human retinoblastoma tumor suppressor gene promoter by CTCF. Cancer Res. 2007; 67:2577–2585. [PubMed: 17363576]

Díaz-Gallardo MY, Cote-Velez A, Charli JL, Joseph-Bravo P. A rapid interference between glucocorticoids and cAMP-activated signalling in hypothalamic neurones prevents binding of phosphorylated cAMP response element binding protein and glucocorticoid receptor at the CRE-like and composite GRE sites of thyrotrophin-releasing hormone gene promoter. J Neuroendocrinol. 2010; 22:282–293. [PubMed: 20136691]

Dijkmans TF, van Hooijdonk LW, Schouten TG, Kamphorst JT, Fitzsimons CP, Vreugdenhil E. Identification of new Nerve Growth Factor-responsive immediate-early genes. Brain Res. 2009; 1249:19–33. [PubMed: 19013137]

Estaras C, Akizu N, Garcia A, Beltran S, de la Cruz X, Martinez-Balbas MA. Genome-wide analysis reveals that Smad3 and JMJD3 HDM co-activate the neural developmental program. Development. 2012; 139:2681–2691. [PubMed: 22782721]

Evans PM, Zhang W, Chen X, Yang J, Bhakat KK, Liu C. Kruppel-like factor 4 is acetylated by p300 and regulates gene transcription via modulation of histone acetylation. J Biol Chem. 2007; 282:33994–34002. [PubMed: 17908689]

Farkas LM, Dunker N, Roussa E, Unsicker K, Krieglstein K. Transforming growth factor-beta(s) are essential for the development of midbrain dopaminergic neurons in vitro and in vivo. J Neurosci. 2003; 23:5178–5186. [PubMed: 12832542]

Fevre-Montange M, Dumontel C, Chevallier P, Isnard AK, Guigard MP, Trouillas J. Localization of transforming growth factors, TGFbeta1 and TGFbeta3, in hypothalamic magnocellular neurones and the neurohypophysis. J Neuroendocrinol. 2004; 16:571–576. [PubMed: 15214860]

Flanders KC, Ludecke G, Engels S, Cissel DS, Roberts AB, Kondaiah P, et al. Localization and actions of transforming growth factor-beta s in the embryonic nervous system. Development. 1991; 113:183–191. [PubMed: 1764993]

Garcia-Campmany L, Marti E. The TGFbeta intracellular effector Smad3 regulates neuronal differentiation and cell fate specification in the developing spinal cord. Development. 2007; 134:65–75. [PubMed: 17138664]

Gouin A, Bloch-Gallego E, Tanaka H, Rosenthal A, Henderson CE. Transforming growth factor-beta 3, glial cell line-derived neurotrophic factor, and fibroblast growth factor-2, act in different manners to promote motoneuron survival in vitro. J Neurosci Res. 1996; 43:454–464. [PubMed: 8699531]

Guerra-Crespo M, Charli JL, Rosales-Garcia VH, Pedraza-Alva G, Perez-Martinez L. Polyethylenimine improves the transfection efficiency of primary cultures of post-mitotic rat fetal hypothalamic neurons. J Neurosci Methods. 2003; 127:179–192. [PubMed: 12906947]

Guerra-Crespo M, Perez-Monter C, Janga SC, Castillo-Ramirez S, Gutierrez-Rios RM, Joseph-Bravo P, et al. Transcriptional profiling of fetal hypothalamic TRH neurons. BMC Genomics. 2011; 12:222. [PubMed: 21569245]

Hefferan TE, Reinholz GG, Rickard DJ, Johnsen SA, Waters KM, Subramaniam M, et al. Overexpression of a nuclear protein, TIEG, mimics transforming growth factor-beta action in human osteoblast cells. J Biol Chem. 2000; 275:20255– 20259. [PubMed: 10816551]

Higaki Y, Schullery D, Kawata Y, Shnyreva M, Abrass C, Bomsztyk K. Synergistic activation of the rat laminin gamma1 chain promoter by the gut-enriched Kruppel-like factor (GKLF/KLF4) and Sp1. Nucleic Acids Res. 2002; 30:2270–2279. [PubMed: 12034813]

Hobert O, Carrera I, Stefanakis N. The molecular and gene regulatory signature of a neuron. Trends Neurosci. 2010; 33:435–445. [PubMed: 20663572]

Jiang J, Chan YS, Loh YH, Cai J, Tong GQ, Lim CA, et al. A core Klf circuitry regulates self-renewal of embryonic stem cells. Nat Cell Biol. 2008; 10:353–360. [PubMed: 18264089]

Jiang L, Chen Y, Chan CY, Lu G, Wang H, Li JC, et al. Dynamic transcriptional changes of TIEG1 and TIEG2 during mouse tissue development. Anat Rec. 2010; 293:858–864.

Johnsen SA, Subramaniam M, Janknecht R, Spelsberg TC. TGFbeta inducible early gene enhances TGFbeta/Smad-dependent transcriptional responses. Oncogene. 2002a; 21:5783–5790. [PubMed: 12173049]

Johnsen SA, Subramaniam M, Katagiri T, Janknecht R, Spelsberg TC. Transcriptional regulation of Smad2 is required for enhancement of TGFbeta/Smad signaling by TGFbeta inducible early gene. J Cell Biochem. 2002b; 87:233–241. [PubMed: 12244575]

Joseph-Bravo P, Charli JL, Palacios JM, Kordon C. Effect of neurotransmitters on the in vitro release of immunoreactive thyrotropin-releasing hormone from rat mediobasal hypothalamus. Endocrinology. 1979; 104:801–806. [PubMed: 35337]

Joseph-Bravo P, Perez-Martinez L, Lezama L, Morales-Chapa C, Charli JL. An improved method for the expression of TRH in serum-supplemented primary cultures of fetal hypothalamic cells. Brain Res Brain Res Protoc. 2002; 9:93–104. [PubMed: 12034328]

Kim J, Shin S, Subramaniam M, Bruinsma E, Kim TD, Hawse JR, et al. Histone demethylase JARID1B/KDM5B is a corepressor of TIEG1/KLF10. Biochem Biophys Res Commun. 2010; 401:412–416. [PubMed: 20863814]

Krieglstein K, Suter-Crazzolara C, Fischer WH, Unsicker K. TGF-beta superfamily members promote survival of midbrain dopaminergic neurons and protect them against MPP+ toxicity. EMBO J. 1995; 14:736–742. [PubMed: 7882977]

Krieglstein K, Strelau J, Schober A, Sullivan A, Unsicker K. TGF-beta and the regulation of neuron survival and death. J Physiol (Paris). 2002; 96:25–30. [PubMed: 11755780]

Laub F, Aldabe R, Friedrich V Jr, Ohnishi S, Yoshida T, Ramirez F. Developmental expression of mouse Kruppel-like transcription factor KLF7 suggests a potential role in neurogenesis. Dev Biol. 2001a; 233:305–318. [PubMed: 11336497]

Laub F, Aldabe R, Ramirez F, Friedman S. Embryonic expression of Kruppel-like factor 6 in neural and non-neural tissues. Mech Dev. 2001b; 106:167–170. [PubMed: 11472850]

Lazcano I, Uribe RM, Martínez-Chávez E, Vargas MA, Matziari M, Joseph-Bravo P, et al. Pyroglutamyl peptidase II inhibition enhances the analeptic effect of thyrotropin-releasing hormone in the rat medial septum. J Pharmacol Exp Ther. 2012; 342:222–231. [PubMed: 22532627]

Lebrun C, Avci HX, Wehrle R, Doulazmi M, Jaudon F, Morel MP, et al. Klf9 is necessary and sufficient for Purkinje cell survival in organotypic culture. Mol Cell Neurosci. 2013; 54:9–21. [PubMed: 23201237]

Lechan RM, Fekete C. The TRH neuron: a hypothalamic integrator of energy metabolism. Prog Brain Res. 2006; 153:209–235. [PubMed: 16876577]

Li M, Kellems RE. Sp1 and Sp3 are important regulators of AP-2gamma gene transcription. Biol Reprod. 2003; 69:1220–1230. [PubMed: 12801994]

Little MH, Prosser J, Condie A, Smith PJ, Van Heyningen V, Hastie ND. Zinc finger point mutations within the WT1 gene in Wilms tumor patients. Proc Natl Acad Sci USA. 1992; 89:4791–4795. [PubMed: 1317572]

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods. 2001; 25:402–408. [PubMed: 11846609]

Lopez-Casillas F, Wrana JL, Massague J. Betaglycan presents ligand to the TGF beta signaling receptor. Cell. 1993; 73:1435–1444. [PubMed: 8391934]

Lopez-Casillas F, Payne HM, Andres JL, Massague J. Betaglycan can act as a dual modulator of TGF-beta access to signaling receptors: mapping of ligand binding and GAG attachment sites. J Cell Biol. 1994; 124:557–568. [PubMed: 8106553]

Lu J, Wu Y, Sousa N, Almeida OF. SMAD pathway mediation of BDNF and TGF beta 2 regulation of proliferation and differentiation of hippocampal granule neurons. Development. 2005; 132:3231–3242. [PubMed: 15958511]

Maira M, Long JE, Lee AY, Rubenstein JL, Stifani S. Role for TGF-beta superfamily signaling in telencephalic GABAergic neuron development. J Neurodev Disord. 2010; 2:48–60. [PubMed: 20339443]

Markakis EA. Development of the neuroendocrine hypothalamus. Front Neuroendocrinol. 2002; 23:257–291. [PubMed: 12127306]

Markakis EA, Swanson LW. Spatiotemporal patterns of secretomotor neuron generation in the parvicellular neuroendocrine system. Brain Res Brain Res Rev. 1997; 24:255–291. [PubMed: 9385456]

Martin C, Zhang Y. The diverse functions of histone lysine methylation. Nat Rev Mol Cell Biol. 2005; 6:838–849. [PubMed: 16261189]

Matsumura S, Shibakusa T, Fujikawa T, Yamada H, Inoue K, Fushiki T. Increase in transforming growth factor-beta in the brain during infection is related to fever, not depression of spontaneous motor activity. Neuroscience. 2007; 144:1133–1140. [PubMed: 17156928]

Matsuyama S, Iwadate M, Kondo M, Saitoh M, Hanyu A, Shimizu K, et al. SB-431542 and Gleevec inhibit transforming growth factor-beta-induced proliferation of human osteosarcoma cells. Cancer Res. 2003; 63:7791–7798. [PubMed: 14633705]

McConnell BB, Yang VW. Mammalian Kruppel-like factors in health and diseases. Physiol Rev. 2010; 90:1337–1381. [PubMed: 20959618]

Noti JD, Johnson AK, Dillon JD. The zinc finger transcription factor transforming growth factor beta-inducible early gene-1 confers myeloid-specific activation of the leukocyte integrin CD11d promoter. J Biol Chem. 2004; 279:26948– 26958. [PubMed: 15087465]

Ou XM, Chen K, Shih JC. Dual functions of transcription factors, transforming growth factor-beta-inducible early gene (TIEG)2 and Sp3, are mediated by CACCC element and Sp1 sites of human monoamine oxidase (MAO) B gene. J Biol Chem. 2004; 279:21021–21028. [PubMed: 15024015]

Pérez-Martínez L, Jaworski DM. Tissue inhibitor of metalloproteinase-2 promotes neuronal differentiation by acting as an anti-mitogenic signal. J Neurosci. 2005; 25:4917–4929. [PubMed: 15901773]

Pérez-Martínez, L.; Charli, JL. Factors involved in the establishment of hypothalamic neuroendocrine phenotypes. In: Joseph-Bravo, P., editor. Molecular Endocrinology. Research Sign Post; Kerala, India: 2006. p. 127-147.

Pérez-Martínez L, Carreon-Rodríguez A, González-Alzati ME, Morales C, Charli JL, Joseph-Bravo P. Dexamethasone rapidly regulates TRH mRNA levels in hypothalamic cell cultures: interaction with the cAMP pathway. Neuroendocrinology. 1998; 68:345–354. [PubMed: 9822802]

Pérez-Martínez L, Charli JL, Joseph-Bravo P. Development of pro-TRH gene expression in primary cultures of fetal hypothalamic cells. Brain Res Dev Brain Res. 2001; 130:73–81.

Pérez-Monter C, Martínez-Armenta M, Miquelajauregui A, Furlan-Magaril M, Varela-Echavarria A, Recillas-Targa F, et al. The Kruppel-like factor 4 controls biosynthesis of thyrotropin-releasing hormone during hypothalamus development. Mol Cell Endocrinol. 2011; 333:127–133. [PubMed: 21182892]

Poulsen KT, Armanini MP, Klein RD, Hynes MA, Phillips HS, Rosenthal A. TGF beta 2 and TGF beta 3 are potent survival factors for midbrain dopaminergic neurons. Neuron. 1994; 13:1245–1252. [PubMed: 7946360]

Ribeiro A, Bronk SF, Roberts PJ, Urrutia R, Gores GJ. The transforming growth factor beta(1)-inducible transcription factor TIEG1, mediates apoptosis through oxidative stress. Hepatology. 1999; 30:1490–1497. [PubMed: 10573529]

Roussa E, Oehlke O, Rahhal B, Heermann S, Heidrich S, Wiehle M, et al. Transforming growth factor beta cooperates with persephin for dopaminergic phenotype induction. Stem Cells. 2008; 26:1683–1694. [PubMed: 18420832]

Sánchez E, Charli JL, Morales C, Corkidi G, Seidah NG, Joseph-Bravo P, et al. Expression of the proprotein convertases PC1 and PC2 mRNAs in thyrotropin releasing hormone neurons of the rat paraventricular nucleus of hypothalamus. Brain Res. 1997; 761:77–86. [PubMed: 9247068]

Seo S, Lomberk G, Mathison A, Buttar N, Podratz J, Calvo E, et al. Kruppel-like factor 11 differentially couples to histone acetyltransferase and histone methyltransferase chromatin remodeling pathways to transcriptionally regulate dopamine D2 receptor in neuronal cells. J Biol Chem. 2012; 287:12723–12735. [PubMed: 22375010]

Sevarino KA, Wu P, Jackson IM, Roos BA, Mandel G, Goodman RH. Biosynthesis of thyrotropin-releasing hormone by a rat medullary thyroid carcinoma cell line. J Biol Chem. 1988; 263:620–623. [PubMed: 3121619]

Shi Y, Massague J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell. 2003; 113:685–700. [PubMed: 12809600]

Spittau G, Happel N, Behrendt M, Chao TI, Krieglstein K, Spittau B. Tieg1/Klf10 is upregulated by NGF and attenuates cell cycle progression in the pheochromocytoma cell line PC12. J Neurosci Res. 2010; 88:2017–2025. [PubMed: 20155803]

Subramaniam M, Harris SA, Oursler MJ, Rasmussen K, Riggs BL, Spelsberg TC. Identification of a novel TGF-beta-regulated gene encoding a putative zinc finger protein in human osteoblasts. Nucleic Acids Res. 1995; 23:4907–4912. [PubMed: 8532536]

Subramaniam M, Hefferan TE, Tau K, Peus D, Pittelkow M, Jalal S, et al. Tissue, cell type, and breast cancer stage-specific expression of a TGF-beta inducible early transcription factor gene. J Cell Biochem. 1998; 68:226–236. [PubMed: 9443078]

Subramaniam M, Gorny G, Johnsen SA, Monroe DG, Evans GL, Fraser DG, et al. TIEG1 null mouse-derived osteoblasts are defective in mineralization and in support of osteoclast differentiation in vitro. Mol Cell Biol. 2005; 25:1191–1199. [PubMed: 15657444]

Tachibana I, Imoto M, Adjei PN, Gores GJ, Subramaniam M, Spelsberg TC, et al. Overexpression of the TGFbeta-regulated zinc finger encoding gene, TIEG, induces apoptosis in pancreatic epithelial cells. J Clin Invest. 1997; 99:2365–2374. [PubMed: 9153278]

Vogel T, Ahrens S, Buttner N, Krieglstein K. Transforming growth factor beta promotes neuronal cell fate of mouse cortical and hippocampal progenitors in vitro and in vivo: identification of Nedd9 as an essential signaling component. Cereb Cortex. 2010; 20:661–671. [PubMed: 19587023]

Wibrand K, Messaoudi E, Havik B, Steenslid V, Lovlie R, Steen VM, et al. Identification of genes co-upregulated with Arc during BDNF-induced long-term potentiation in adult rat dentate gyrus in vivo. Eur J Neurosci. 2006; 23:1501–1511. [PubMed: 16553613]

Xiong Y, Khanna S, Grzenda AL, Sarmento OF, Svingen PA, Lomberk GA, et al. Polycomb antagonizes p300/CREB-binding protein-associated factor to silence FOXP3 in a Kruppel-like factor-dependent manner. J Biol Chem. 2012; 287:34372–34385. [PubMed: 22896699]

Yajima S, Lammers CH, Lee SH, Hara Y, Mizuno K, Mouradian MM. Cloning and characterization of murine glial cell-derived neurotrophic factor inducible transcription factor (MGIF). J Neurosci. 1997; 17:8657–8666. [PubMed: 9348334]

Yin X, Wolford CC, Chang YS, McConoughey SJ, Ramsey SA, Aderem A, et al. ATF3, an adaptive-response gene, enhances TGF{beta} signaling and cancer-initiating cell features in breast cancer cells. J Cell Sci. 2010; 123:3558– 3565. [PubMed: 20930144]

Zhang JS, Moncrieffe MC, Kaczynski J, Ellenrieder V, Prendergast FG, Urrutia R. A conserved alpha-helical motif mediates the interaction of Sp1-like transcriptional repressors with the corepressor mSin3A. Mol Cell Biol. 2001; 21:5041–5049. [PubMed: 11438660]

Zhang W, Shields JM, Sogawa K, Fujii-Kuriyama Y, Yang VW. The gut-enriched Kruppel-like factor suppresses the activity of the CYP1A1 promoter in an Sp1-dependent fashion. J Biol Chem. 1998; 273:17917–17925. [PubMed: 9651398]

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.mce.2014.10.021.

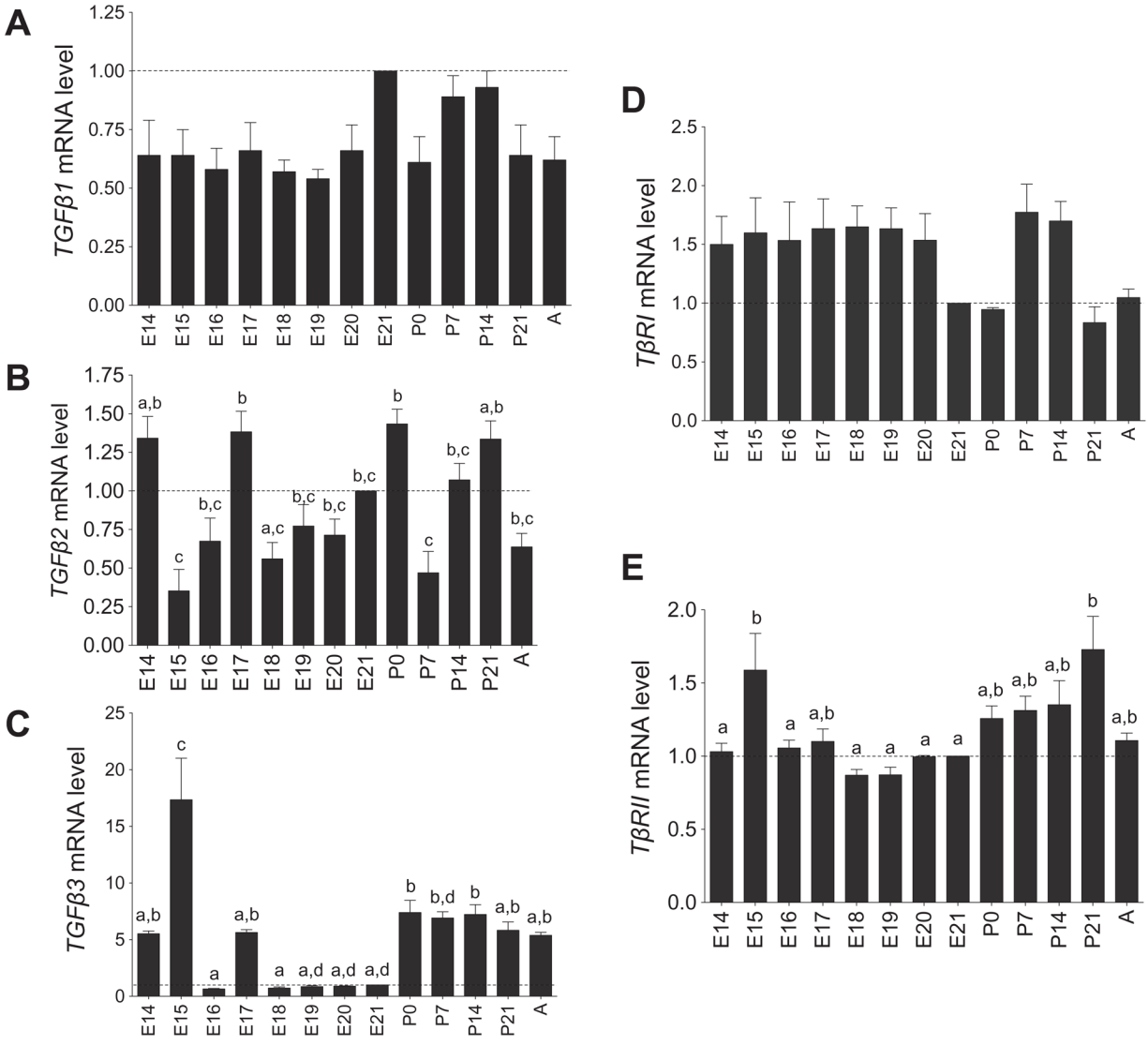

Fig. 1.

TGFβ isoforms and receptors are expressed during hypothalamic development. Developmental profile of *TGFβ1* (A), *TGFβ2* (B), *TGFβ3* (C), *TβRI* (D) *and TβRII* (E) mRNA levels. At the indicated developmental stages, hypothalami were processed for RT-PCR assays. Data represent the ratio of *TGFβ* (isoform or receptor) cDNA to *Gapdh* cDNA (internal control) signal normalized to embryonic (E) day 21 taken as one. Results represent the mean ± SEM of at least three independent experiments each in triplicate. An identical letter denotes an absence of a statistically significant difference. Data shown for *TGFβ3* and *RII* correspond to $P < 0.05$; for *TGFβ2* $P < 0.001$ by post-hoc Fisher analysis. E: embryonic day; P: postnatal day; A: adult.

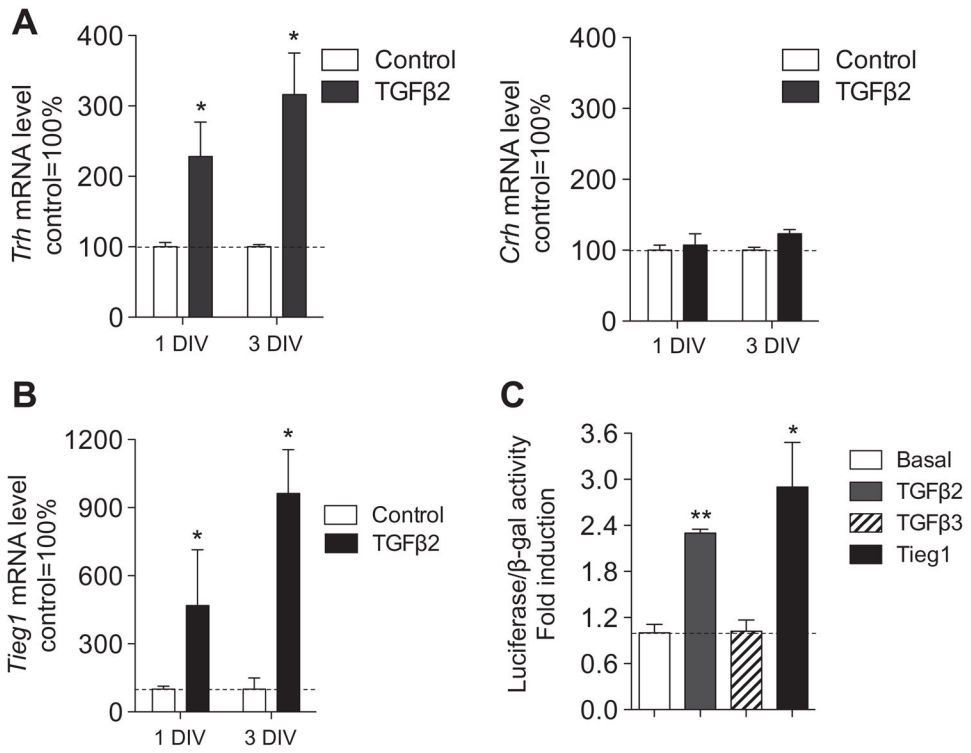

Fig. 2.

TGFβ2 regulates *Trh and TIEG1* expression. Primary cultures of fetal (E17) hypothalamic cells maintained in serum-free medium were treated with 2.5 ng/ml *TGFβ2* for 1 or 3 DIV (*A*,*B*). Relative *Trh* (*A*, left panel), *Crh* (*A*, right panel) or *TIEG1* (*B*) mRNA levels were determined by RT-PCR, and calculated as the ratio of *Trh*, *Crh* or *TIEG1* cDNA/*Gapdh* cDNA signal; values were expressed as percentage of control. (*C*) Fetal hypothalamic cells were transfected with Trh-Luc together with either the pcDNA4/TO-TIEG1 expression vector or the equivalent concentration of empty pcDNA vector in the presence or absence (basal) of 2.5 ng/ml of either TGFβ2 *or* TGFβ3. All cultures were co-transfected with an SV40 promoter-driven β-galactosidase expression vector. Seventy-two hours after transfection the luciferase activity was determined and normalized to β-galactosidase activity. Fold induction was calculated relative to Trh-Luc transfected cells. Bars represent the mean ± SEM of three replicates in three independent experiments. **P* < 0.05; ***P* < 0.01 *vs.* basal by post-hoc Fisher analysis.

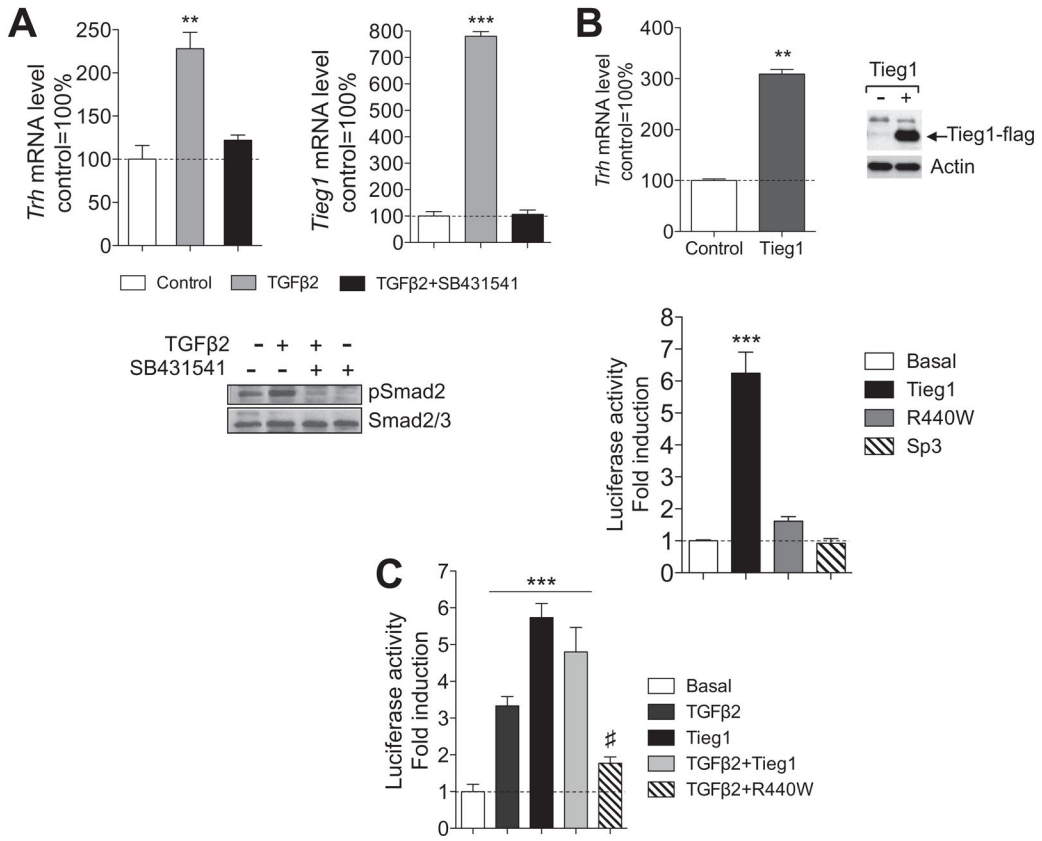

Fig. 3.

TGFβ2 regulates *Trh* expression via a TIEG1 dependent mechanism. (*A*) CA77 cells cultured in 0.2% fetal bovine serum were treated with 5 ng/ml of TGFβ2 for 2 DIV. When indicated the inhibitor SB431542 (10 μM) was added to the culture 30 min before TGFβ2 treatment. *Trh* or *TIEG1* mRNA levels were determined by RT-PCR and calculated as the ratio of *Trh or TIEG1* cDNA/*Gapdh* cDNA signal; values were expressed as percentage of control (upper panel). The levels of phosphorylated Smad2 (p-Smad2) and total Smad (Smad2/3) were detected by immunoblot (lower panel). (*B*) CA77 cells were transfected with the pcDNA4/TO-TIEG1 expression vector or empty vector and *Trh* mRNA levels determined by RT-PCR 4 days after transfection (upper left panel). TIEG1 expression was confirmed by immunoblot using an anti-Flag antibody; actin levels were used as a loading control. The arrow indicates the flagged TIEG1 band (TIEG1-flag) (upper right panel). CA77 cells were transfected with the minimal *Trh* promoter fused to the firefly luciferase reporter gene (Trh-Luc) together with either the pcDNA4/TO-TIEG1 or a mutant version of TIEG1 (R440W), or the pcDNA-Sp3 expression vector or the equivalent concentration of empty pcDNA vector (basal). Forty-eight hours after transfection the firefly luciferase activity was determined and normalized to *Renilla* luciferase values (lower panel).(*C*) CA77 cells were transfected with Trh-Luc together with the pcDNA4/TO-TIEG1 or a mutant version of TIEG1 (R440W) in the presence or absence of 5 ng/ml of TGFβ2. Basal denotes cells transfected with Trh-Luc together with a concentration of empty pcDNA vector identical to that used to express TIEG1. Forty-eight hours after transfection the luciferase activity was determined and normalized to *Renilla* luciferase values. The results were

plotted and expressed as relative fold change compared to basal values. Bars represent the mean ± SEM of three replicates in three independent experiments. $^{**}P < 0.01$; $^{***}P < 0.001$ *vs.* basal; $^{\#}P < 0.001$ *vs. TGFβ2* by post-hoc Fisher analysis.

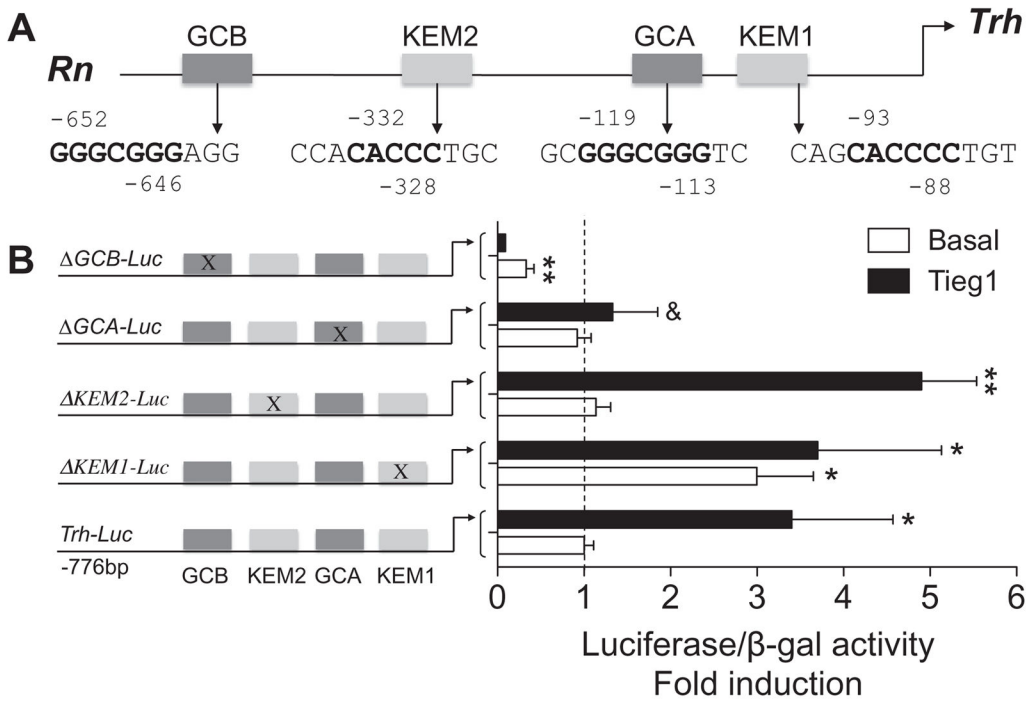

Fig. 4.

A GC response element within the *Trh* promoter mediates TIEG1 action on *Trh* gene transcription. (*A*) Schematic representation of the *Trh* gene promoter region of the *Rattus norvegicus* (Rn) indicating the positions of the CACCC and GC motifs relative to the transcription start site. (*B*) Primary cultures of fetal (E17) hypothalamic cells were transfected with either the wild-type *Trh* promoter fused to the firefly luciferase reporter gene (Trh-Luc), or the KEM1 (ΔKEM1-Luc), or the KEM2 (ΔKEM2-Luc), or GCA (ΔGCA-Luc), or the GCB (ΔKEM1-Luc) mutated versions of Trh-Luc together with the pcDNA4/TO-TIEG1 expression vector or the equivalent concentration of empty pcDNA vector (basal). X indicates a mutation. Firefly luciferase activity was normalized to β-galactosidase activity. Fold induction was calculated relative to wild-type Trh-Luc transfected cells. Bars represent the mean ± SEM of three replicates in three independent experiments. **P* < 0.05 and ***P* < 0.001 *vs.* Trh-Luc (basal); &: *P* < 0.05 *vs.* wild-type Trh-Luc/TIEG1.

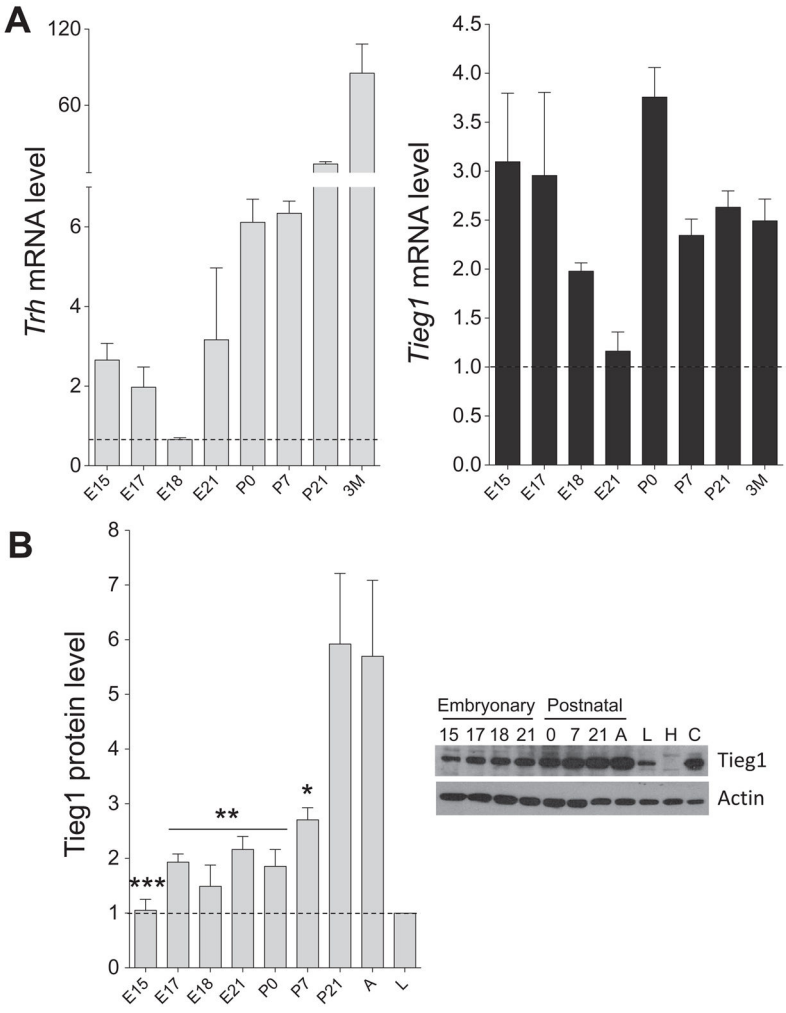

Fig. 5.

TIEG1 and *Trh* l expression profiles during rat hypothalamic development. (*A*) Developmental profile of *TIEG1* (left panel) and *Trh* (right panel) mRNA levels. At the indicated developmental stages, hypothalami were processed for RT-qPCR assays. Relative expression levels were determined using actin as a constitutive expression gene, and the embryonic (E) day 21 values used for normalization. Data represent the mean ± SEM of three independent experiments each in triplicate. E: embryonic day; P: postnatal day; 3M: 3 month old. *: $P < 0.001$ *vs.* all developmental stages by post-hoc Fisher analysis. (*B*) TIEG1 protein levels during hypothalamus ontogeny. At the indicated developmental stages, nuclear extracts were used for Western-blot assays. Actin was used as a loading control. Data represent the mean ± SEM of three independent experiments. E: embryonic day; P: postnatal day; A: adult (3 month old); L: lung; C: CA77 cells; H: HEK-293 cells used as negative control. ***$P < 0.001$; **$P < 0.01$ and *$P < 0.05$ *vs.* P21 and A by post-hoc Fisher analysis. A representative immunoblot is shown.

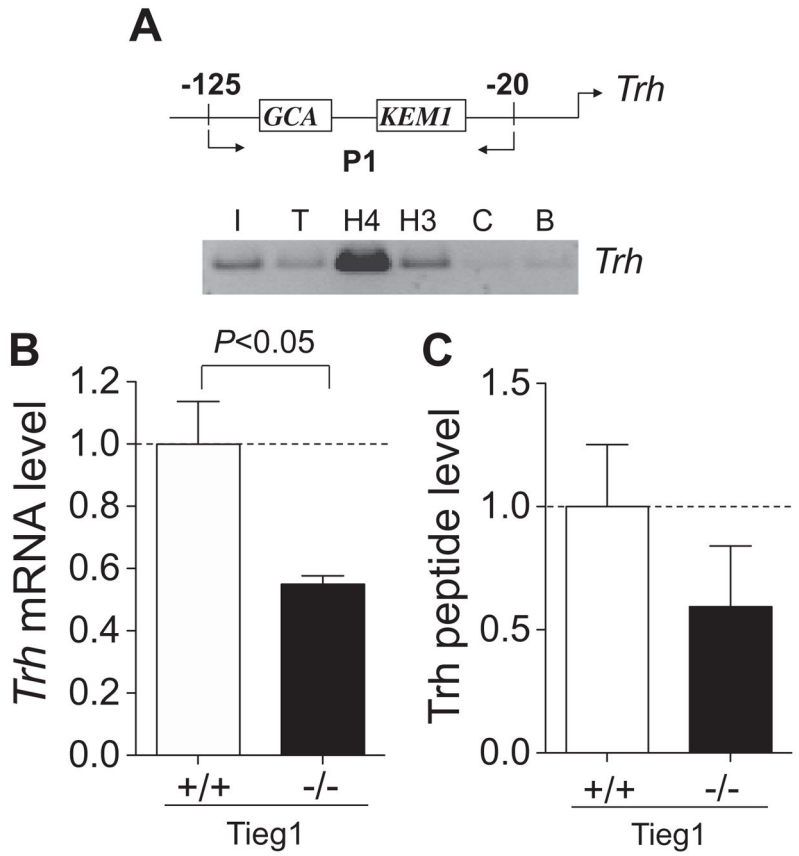

Fig. 6.

TIEG1 transcription factor binds to the *Trh* promoter in the fetal rat hypothalamus, and is necessary for proper *Trh* gene expression in the fetal mouse hypothalamus. (*A*) TIEG1 is recruited to the rat *Trh* gene promoter *in vivo.* Upper panel: Diagram indicating the relative positions (left and right arrows) of the PCR primers (P1) on the rat *Trh* promoter region. Lower panel: PCR product derived from ChIP-enriched genomic DNA from E17 hypothalamus. This experiment is a representative of 3 replicates in three independent experiments. I: input; T: anti-TIEG1 antibody; H4: anti-acetyl-histone H4 (K16) antibody; H3: anti-histone H3 (tri-methyl K4); C: IgG immunoprecipitation; B: beads. *Trh* mRNA and peptide levels were determined by qRT-PCR (*B*) and radioimmunoassay (*C*), respectively. Total RNA or peptide extracts were prepared from 17-day-old embryo hypothalamus derived from wild-type (+/+) or knockout (−/−) mice. For RT-qPCR, relative expression levels were determined using actin as a constitutive expression gene. For RIA, data are expressed as pg of TRH per mg of total protein, and normalized to mean value in TIEG+/+ animals. Results represent the mean ± SEM of three (RT-qPCR) or two (radioimmunoassay) independent experiments, each in triplicate. $P < 0.05$ *vs.* wild-type by post-hoc Fisher analysis.