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TTF1, a homeodomain containing transcription factor, contributes to regulating periodic oscillations in GnRH gene expression

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Abstract

Thyroid transcription factor 1 (TTF1), a member of the NK family of transcription factors required for basal forebrain morphogenesis, functions in the postnatal hypothalamus as a transcriptional regulator of genes encoding neuromodulators and hypophysiotrophic peptides. One of these peptides is gonadotropin-releasing hormone (GnRH). Here we show that *Ttf1* mRNA abundance vary in a diurnal and melatonin-dependent fashion in the preoptic area (POA) of the rat, with maximal *Ttf1* expression attained during the dark phase of the light/dark cycle, preceding the nocturnal peak in *GnRH* mRNA content. *GnRH* promoter activity oscillates in a circadian manner in GT1-7 cells, and this pattern is enhanced by TTF1 and blunted by siRNA-mediated *Ttf1* gene silencing. TTF1 trans-activates *GnRH* transcription by binding to two sites in the GnRH promoter. Rat GnRH neurons *in situ* contain key proteins components of the positive (BMAL1, CLOCK) and negative (PER1) limbs of the circadian oscillator, and these proteins repress *Ttf1* promoter activity *in vitro*. In contrast, *Ttf1* transcription is activated by CRY1, a clock component required for circadian rhythmicity. In turn, TTF1 represses transcription of *Rev-erbα*, a heme receptor that controls circadian transcription within the positive limb of the circadian oscillator. These findings suggest that TTF1 is a component of the molecular machinery controlling circadian oscillations in *GnRH* gene transcription.

Keywords

circadian rhythm; GnRH gene expression; clock genes; TTF1; transcriptional control

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Introduction

Thyroid transcription factor 1 (TTF1), also known as Nkx 2.1 and T/ebp (1,2), is a member of the Nkx family of homeobox genes (3,4) that is expressed in the embryonic basal forebrain in a temporally restricted manner. In the absence of TTF1, development of the hypothalamus (as well as other structures of the basal forebrain) is severely impaired (1,5,6).

Notwithstanding the critical importance of TTF1 in basal forebrain embryogenesis, selected postmitotic neurons of this region continue to express the *Ttf1* gene indicating that TTF1 not only provides morphogenetic signals to the developing forebrain, but also contributes to regulating mature neuronal functions (2,6,7). After birth, TTF1 can be detected in gonadotropin-releasing hormone (GnRH) neurons of the preoptic area (POA), preproenkephalinergic neurons of the lateral ventromedial nucleus (8,9), kisspeptin neurons of the arcuate nucleus (10), and ependymoglial cells of the third ventricle and median eminence (8,9). In female rats, *Ttf1* mRNA abundance increases in the hypothalamus before the natural onset of puberty, and after puberty-inducing hypothalamic lesions, suggesting an involvement of TTF1 in the control of female puberty (8). Using immortalized GnRH producing GT1-7 cells, TTF1 has been show to regulate genes involved in neuronal morphogenesis and differentiation, as well as genes that participate in the neuroendocrine control of reproductive development (11).

An intriguing feature of the GnRH neurosecretory system is the circadian/diurnal periodicity of GnRH release [reviewed in (12,13)]. Such an oscillatory behavior has been long recognized as a critical component of the process by which the GnRH neuronal network is activated at puberty (14–16) and during the estrous cycle (13). It is also clear that the diurnal changes in GnRH neuronal activity are driven by a hypothalamic pacemaker residing in the suprachiasmatic nucleus (SCN) (17,18). At puberty, GnRH secretion begins to increase in the evening (rodents) or at night (primates, including humans), before comparable increases become apparent during the day (14–16). During the rodent estrous cycle, the preovulatory surge of gonadotrophins, driven by changes in GnRH secretion, only occurs in the evening (12,19). Although it is well established that this rhythm is, to a significant extent, determined by a circadian oscillator located in the suprachiasmatic nucleus (13,20), recent studies have unveiled the presence of a clock mechanism intrinsic to GnRH neurons (21–23).

Whether transcription factors regulating GnRH expression, such as TTF1, do so via mechanisms coupled to or interacting with the circadian clock is not known. Suggesting that this may be the case are the observations that *Ttf1* expression changes diurnally in the rat medial basal hypothalamus (24) and that TTF1 regulates transcription of another hypothalamic peptide, the pituitary adenylate cyclase-activating polypeptide (PACAP), in a circadian fashion (24). It is thus plausible that the trans-activating effect of TTF1 on GnRH promoter activity has a temporal dimension entrained by the circadian clock. The studies we report here support this concept.

Materials and Methods

Animals and tissue preparation

Two-month-old male Sprague-Dawley rats (Daehan Animal Breeding Company, Chungwon, Chungbuk, South Korea) were housed in a room with a conditioned photoperiod (12 h light/12 h darkness, lights on from 0700 – 1900 h) and temperature (23 −25 °C), and allowed ad libitum access to tap water and pelleted rat chow. To determine daily rhythm of mRNA levels, the animals were acclimated by exposing them for a week to dim red light during the night time. Animals were sacrificed by decapitation at 3 h intervals during the

day (0900 h, 1200 h, 1500 h and 1800 h) and night (2100 h, 2400 h, 0300 h and 0600 h). The night experiments were performed under dim red light. Following dissection of the preoptic area (POA) region, the tissues were quickly frozen on dry ice, and stored at −85 °C until RNA isolation. The POA fragments collected consisted of the entire suprachiasmatic region, including the POA proper and the suprachiasmatic nuclei (SCN).

Pinealectomy and administration of melatonin

To remove the pineal gland, the animals were placed in a stereotaxic frame, and the gland was excised with a fine forceps via a 4-mm-wide orifice drilled at the level of the skull's lambda landmark. After surgery, the orifice was covered with dental cement (Stoelting, Wood Dale, IL). Sham-operated animals underwent an identical procedure, but without removing the gland. Two weeks after the operation, some animals were subcutaneously injected with melatonin (25 mg/kg body weight, Sigma-Aldrich. ST. Louis, MO) at 1800 h and sacrificed 6 h later under dim red light. Following dissection of the POA, the tissues were quickly frozen on dry ice, and stored at −85°C until RNA isolation.

RNA extraction

Total RNA was isolated using Trizol reagent (Sigma-Aldrich, St Louis, MO) as previously reported (24,25).

RNase protection assay (RPA)

RPA Probes: A 331-bp BamHI-ApaI cDNA fragment corresponding to the 5' end of the *Ttf1* coding region was excised from a plasmid (kindly provided by R. DiLauro, Stazione Zoologica A. Dohrn, Naples, Italy) containing the entire *Ttf1* coding region (26). The 5' cDNA fragment was cloned into the BamHI-ApaI sites of the riboprobe vector pGEM-11Z(+) and used for transcription of both sense and antisense RNA strands labelled with ^{32}P-UTP. The GnRH probe used for RPAs was described previously (27). The template cDNA (227 bp size) used to transcribe a MT1 cRNA was cloned by reverse transcription-PCR of total RNA from rat POA using primers designed according to the rat *MT1* mRNA sequence (NM_053676). The primers used were: sense, 5'-AAC CTG CTG GTC ATC CTG TCT G-3'; antisense, 5'-CCC GGT GAT GTT GAA TAC CGA G-3'. The PCR product obtained was cloned into pGEM-T vector (Promega, Madison, WI). Antisense and sense RNA probes were generated by T7 and SP6 polymerase-directed transcription, respectively. The cyclophilin probe used was described previously (8,27,28).

The RPA procedure employed has been described in detail elsewhere (8,28). Briefly, sense and antisense ^{32}P-UTP-labeled rat *Ttf1, MT1* and *GnRH* RNA probes were generated and purified using a Fullengther apparatus (Biokey Co., Portland, OR), as reported (8,28). RNA samples (5 μg/tube) or increasing amounts of *in vitro* synthesized *Ttf1, MT1* and *GnRH* sense mRNA (0.03–2 pg/tube, used to generate standard curves) were hybridized to 500,000 cpm of *Ttf1, MT1* and *GnRH* cRNA probes, respectively, for 18–20 h at 45 °C. The tissue RNA samples were simultaneously hybridized to a cyclophilin cRNA probe (5,000 cpm/tube) to correct for procedural losses (8). At the end of the hybridization, the samples were treated with ribonucleases A and T1 to remove unhybridized RNA species. The protected cRNA fragments were separated on a polyacrylamide-urea gel (5% acrylamide, 7 M urea), and the hybridization signals were detected by exposure of the dried gels to X-Omat X-ray film (Amersham Biosciences, Uppsala, Sweden). The intensity of the signals obtained was quantified as reported (28), using the NIH Image J program.

Electrophoretic mobility shift assays (EMSA)

These assays were carried out using a purified TTF1 homeodomain (TTF1-HD) protein. Procedures for expression, purification, and quantitation of the TTF1-HD were described elsewhere (29). Double-stranded oligodeoxynucleotides, 5'-labeled with λ^{32}P-ATP were the probes used for gel-retardation assays. Their sequences were: *GnRH* enhancer site 1: 5'-ACA-AAT-TGG-GTT-TAA-GTA-ATT-GGG-CTT-3', *GnRH* promoter site 2: 5'- GAT-TTT-AAT-GAC-CAA-GTT-TAA-GAA-AAT-3'. Binding assays (30 min incubation) employed different concentrations (15–150 nM) of TTF1-HD protein and 20,000 cpm of DNA probe in a buffer containing 20 mM, Tris-HCl pH 7.6, 75 mM KCl, 0.25 mg/ml bovine serum albumin (BSA), 5mM dithiothreitol (DTT), 12.5 μg/ml calf thymus DNA, and 10% glycerol. Protein-DNA complexes were separated from unbound DNA on native 7.5% polyacrylamide gels using 0.5× TBE as the running buffer (1.5 h at 4 °C). Gels were fixed and subjected to phosphoimaging analysis using a Bio-Rad GS525 instrument. Protein-DNA complex signals were quantitated using Multi-analyst software (Bio-Rad Laboratories, Hercules, CA). Binding of TTF1-HD to oligodeoxynucleotides derived from the GnRH promoters was expressed as percentage of the TTF1 bound to oligonucleotide C, which contains the core binding motif and flanking sequence of the proximal TTF1 binding site in the thyroglobulin promoter (30).

Cell culture

GT1-7 cells were grown in high glucose DMEM (4.5 mg/ml glucose, Hyclone Laboratories, Inc., Logan UT) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Inc., Logan UT), and 100 U/ml penicillin/streptomycin (Thermo Fisher Scientific, Inc., Pittsburgh, PA), and maintained at 37°C in 5% CO_2. Cells were seeded in 6 well plates (500,000 cells/well) and synchronised by incubation in a serum-free medium for 3 h followed by incubation in DMEM containing 10% FBS (T=00h). Cells were then harvested for RNA extraction or used in promoter assays.

NIH3T3 cells (passages 5 to 10, ATCC, Manassas, VA) were grown in Dulbecco's modified Eagle medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS, Hyclone), 100 μg/ml penicillin, and 100 μg/ml streptomycin. For promoter assays, the cells were seeded in 12 well plates (200,000 cells/well), and grown in 10% FBS DMEM without antibiotics.

Quantitative (q) PCR

GT1-7 cells were cultured as detailed above. After synchronisation, the cells were harvested at 4h intervals for 48 h and total RNA was isolated. Following reverse transcription (50 ng of total RNA in a 15 μl volume) using a Reverse Transcription System (Promega, Madison, WI), segments of *Ttf1, MT2* and *GnRH* mRNA were amplified by real-time PCR using 10 μl of the reverse transcription reaction and the following primer sets: *Ttf1* sense, 5'-CGA GCG GCA TGA ATA TGA GT-3'; Antisense, 5'-GCT GTC CTG CTG CAG TTG TT-3'; *GnRH* sense, 5'-ACT GTG TGT TTG GAA GGC TGC-3'; antisense, 5'-TTC CAG AGC TCC TCG CAG ATC-3'; *Mt2* sense, 5'-AGG AAG GCA AAG GCT GAG AG-3'; antisense, 5'-TGG CGA AAA CCA CAA ACA CT-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense, 5'-TGT GAA CGG ATT TGG CCG TA-3'; antisense, 5'-ACT TGC CGT GGG TAG AGT CA-3'. Real-time PCR using the QuantiTect SYBR Green PCR Kit (Promega), was carried out in capillaries of a DNA Engine Opticon Continuous Fluorescence Detection System (MJ Research Inc., Waltham, MA) for approximately 40 cycles using the following conditions: at 94°C for 30 sec, 56°C for 30 sec and 72°C for 35 sec.

Secreted alkaline phosphatase (SEAP) assay

To continuously monitor *GnRH* promoter activity under the influence of TTF1, we used an SEAP assay system. The rat *GnRH* promoter (−3002 to +88) conjugated with a SEAP reporter sequence (pGnRH-SEAP) was subcloned into a SEAP vector (pSEAP2-Basic, Clontech, Mountain View, CA) after excising it from the construct pGnRH3.0Luc (provided by J.L. Roberts, University of Texas Health Science Center at San Antonio, USA) using XhoI and HindIII restriction enzymes. Twenty-four hours after seeding in 10% FBS DMEM without antibiotics, GT1-7 cells were transiently transfected with pGnRH-SEAP vectors together with TTF1-pcDNA (24) or Tttf1-shRNA vectors (31) using Lipofectamine as the transfection reagent (Invitrogen, Carlsbad, CA). After synchronisation, the medium was collected at 4 h intervals for 48 h. Chemiluminescence detection of SEAP activity was performed as previously reported (24).

Western blots

TTF1 protein was detected in GT1-7 cells by western blot analysis, as previously reported (24). Briefly, GT1-7 cells were transfected with p*GnRH*-SEAP vectors together with TTF1-pcDNA (32) or Ttf1-shRNA vectors (31) as described above and were harvested for western blot experiment 24 hour after transfection. Thirty ug of protein were loaded on a 10% SDS polyacrylamide gel under denaturing conditions (samples containing a final concentration of 5 % β-mercaptoethanol); after electrophoresis the size-fractionated proteins were transferred to a polyvinylidene difluoride membrane (PVDF, Millipore, Billerica, MA, USA). The membrane was blocked in 5% skim milk and incubated overnight with monoclonal mouse anti-TTF1 antibody (1:1,000, Thermo Fisher Scientific Inc.) or with monoclonal mouse anti-β-actin antibody (1:4,000, Sigma-Aldrich). The membranes were incubated for 2 h with goat anti-mouse IgG-HRP antibodies (1:4,000, Santa Cruz Biotech. Inc., Santa Cruz, CA). The intensity of the signals obtained was quantified using the NIH Image J program.

Site-directed mutagenesis of the GnRH promoter and transcriptional assay

Two putative TTF1-binding sites containing the core recognition site of TTF1 (26) were identified in the promoter region of the m*GnRH* gene using the Transcription Element Search System (TESS; http://www.cbil.upenn.edu/bin/tess/tess). The first one was identified in the enhancer region (−1622 to −1617, 5'-TTAAGT-3') and the second one in the proximal promoter region (−101 to −96, 5'-CCAAGT-3'). These putative binding sites were deleted by site-directed mutagenesis using the The QuickChange® XL Site-Directed Mutagenesis Kit (Stratagene, San Diego, CA) and the previously characterized pGL3-*LHRH*p construct (8), termed here pGL3-GnRHp. As previously described (8,33), GT1-7 cells were plated in 10% FBS DMEM without antibiotics, as reported above, and cells were transiently transfected with the Lipofectamine/PLUS reagent (Invitrogen) following the manufacturer's instruction. Briefly, 1.5 μl Lipofectamine reagent (Invitrogen, Carlsbad, CA) per μg DNA were premixed for 15 min with the intact or mutated luciferase reporter plasmid pGL3-GnRHp (34), in conjunction with various amounts of pcDNA3.1-Zeo-rTTF1 added to the Plus Reagent (Invitrogen) at a ratio of 1.5 μl/ug DNA. The resulting mixture was incubated for an additional 10 min at RT before it was added to the cells. The transfection medium was replaced after 5 h by fresh 10% FBS DMEM without antibiotics. Transfection efficiency was normalized by cotransfection with the control plasmid pCMV•Sport-βgal (20 ng/well, Invitrogen). The DNA amount per well was kept at 250 ng using an empty pcDNA3.1-Zeo plasmid as the equalizer. Cell lysates were assayed for luciferase and β-gal 48 h after transfection (8,33).

TTF1/Clock gene promoter assays

Twenty-four hours after seeding NIH 3T3 cells, 1.5 μl Lipofectamine reagent (Invitrogen, Carlsbad, CA) per μg DNA were premixed for 15 min with the various reporter gene constructs in Opti-MEM medium (Invitrogen) and added to a mixture of Opti-MEM/Plus Reagent (Invitrogen) at a ratio of 1.5 μl/ug DNA. The resulting mixture was incubated for an additional 10 min at RT before it was added to the cells. Transfection efficiency was normalized by co-transfecting cells with 100 ng/well of a TK-β-galactosidase construct (provided by BS Katzenellebogen, University of Illinois at Urbana-Champaign). The total amount of DNA transfected was kept constant at 1.1 μg/well (Experiment 1) or 1.2 μg/well (Experiment 2) by adding the appropriate amount of empty pcDNA3.1 plasmid to each well. The transfection medium was replaced after 5 h by fresh 10% FBS DMEM without antibiotics. The cells were harvested 48 h after transfection and assayed for luciferase and β-galactosidase, as reported (8,35). Each condition was tested using at least 3 wells/experiment, and each experiment was repeated 2–3 times. Promoter assays using GT1-7 cells were performed as described in the previous section.

To generate a plasmid expressing luciferase under the control of the *Bmal1* promoter, we subcloned the *mBmal1* promoter from the pd2EGFP-mBmal1prom1 construct (plasmid expressing eGFP under the *mBmal1* promoter, provided by N. Cermakian, McGill University, Montreal, Canada) into pGL2 Basic (Promega). Briefly, we excised the promoter from pd2EGFP with BglII/XmaI restriction enzymes (New England Biolabs, Ipswich, MA).The 3' ends were blunted using the large Klenow fragment of DNA polymerase I (New England Biolabs), and the DNA fragment was subcloned into pGL2 Basic linearized at the SmaI restriction site using the Stratagene DNA ligation kit (Agilent Technologies, Santa Clara, CA). The correct promoter orientation into this vector was verified by restriction enzyme digestion (BamHI, Promega) before amplification of the plasmid for transfection assays. All plasmid DNA preparations were purified using the Qiagen EndoFree Plasmid Maxi kit (Qiagen, Valencia, CA).

In one series of experiments, the long (5kb) form of the *Ttf1* rat promoter (500ng/well) inserted in the pSVoa-delta luciferase plasmid (36) (provided by S. Kimura, Laboratory of Metabolism, National Cancer Institute, NIH, Bethesda, MD) was co-transfected with 250ng/well of the following expression vectors: mBMAL1-pcDNA3.1 and mCLOCK-pcDNA3.1 (provided by S. Honma, Hokkaido University Graduate School of Medicine, Sapporo, Japan), mREV-ERBα-pcDNA3 (provided by P. Gos, University of Geneva), mPer1-pcDNAV5 and mPer2-pcDNAV5 (provided by H. Okamura, Kyoto University, Kyoto, Japan) and mCry1V5-pcDNA (provided by S. Reppert, University of Massachusetts Medical School, Boston, MA).

In a second series of experiments, increasing concentrations (100, 250, 500 and 1000ng/well) of TTF1-pcDNA (8) were co-transfected with 100ng of the following promoter constructs: *mBmal1*-pGL2 basic and *hREV-ERBα*-pGL2 basic (provided by V. Laudet, University of Lyon, France), *mCry1*-pGL3 basic and *mPer1*-pGL3 basic (from S. Reppert, University of Massachusetts Medical School, Boston, MA). Because REV-ERBα has been shown to repress both the *Bmal1* promoter (37) and its own promoter (38), we used REV-ERBα (mREV-ERBα-pcDNA3; 250ng/well) as a positive control in experiments involving the effect of TTF1 on the activity of these promoters (m*Bmal* pGL2 basic and h*REV-ERBα*-pGL2 basic constructs). BMAL1 (mBMAL1-pcDNA3.1) and CLOCK (mCLOCK-pcDNA3.1), previously shown to trans-activate *Cry1* and *Per1* transcription (39–41) were used (at 250ng each/well) as a positive control when examining the effect of TTF1 on these promoters (m*Cry1*-pGL3 basic and m*Per1*-pGL3basic constructs).

Immunohistofluorescence detection of clock gene products in GnRH neurons

We stained GnRH neurons with mouse monoclonal antibody 4H3 (42), at a 1:3,000 dilution. CLOCK was detected with rabbit polyclonal antibodies (1:2,000; Millipore, Billerica, MA); PER1 also with rabbit polyclonal antibodies (1:2,000; Millipore), and BMAL1 with guinea pig polyclonal antibodies (1:4,000; Millipore). Prepubertal, 28 day-old female rats were perfused via the left ventricle of the heart between 10:00 and 13:00 h with 4% paraformaldehyde-PBS, pH 7.4. Frozen sections (30 μm) of their brains were prepared using a freezing sliding microtome, mounted on Superfrost glass slides and dried for 2 h under an air stream before staining. The sections were incubated for 48 h at 4 °C with the primary antibodies indicated above. At the end of this period, GnRH immunostaining was developed to a red color using Alexa 568-donkey antimouse IgG (1:500; Invitrogen). CLOCK and PER1 staining was developed to a green color by incubating the sections with biotinylated donkey antirabbit gamma globulin (1:250, Invitrogen), followed by Streptavidin-Alexa 488 (1:500, Invitrogen). BMAL1 staining was also developed to a green color by incubating with biotinylated goat antiguinea pig gamma globulin (1:250, Invitrogen), followed by Streptavidin-Alexa 488. Fluorescent images were acquired as reported (43,44), and images derived from a single 0.5 μm optical section were selected for presentation. Specificity of the primary antibodies used has been reported earlier (42,45,46). In addition, for every reaction we included sections incubated without the first antibodies. All negative controls omitting the BMAL1, CLOCK and PER1 antibodies showed only GnRH neurons. The converse was also true, i. e, omitting the GnRH antibodies resulted in the visualization of only BMAL1, CLOCK and PER1 positive cells. We used 2% normal donkey serum for blocking, which in our experience results in minimum background staining.

Statistical analysis

Data were analyzed using SigmaStat sofware (version 3.11; Systat Software Inc., San Jose, CA). Data passing a normality test were analyzed using the two-tailed Student's *t* test to compare two groups or a one-way ANOVA followed by the Student-Newman-Keuls multiple comparison test to compare several groups. When the data failed the normality test, they were analysed using an ANOVA test on ranks followed by the Kruskal-Wallis one-way ANOVA ranks test. A P value of <0.05 was considered statistically significant.

Results

Ttf1 and GnRH mRNA levels vary in a diurnal fashion in the rat POA during the normal day-night cycle

Diurnal changes in *Ttf1* and *GnRH* mRNA levels were determined by RPA in the POA of 2-month-old male rats. Both *Ttf1* and *GnRH* mRNA abundance varied in a diurnal manner (Fig. 1). *Ttf1* mRNA levels were relatively constant during day time, but increased sharply after the lights were turned off at 1900 h, peaking at 2100 h. Levels decreased steadily thereafter, reaching day time values by 0600 h (Fig. 1A). *GnRH* mRNA levels showed a similar pattern of expression, with peak levels occurring at midnight, i.e., 3 h after the peak in *Ttf1* mRNA levels (Fig. 1B). Levels decreased between midnight and 0600 h, but without reaching day time values until later in the day. By 0600 h (1 h before the lights are turned on), *GnRH* mRNA levels were about 2.5-fold higher than at 0900 h. These results indicate that expression of the *Ttf1* and *GnRH* genes in the POA oscillates in a diurnal fashion, and that the changes in *Ttf1* mRNA expression precede those in *GnRH* mRNA levels.

Pinealectomy prevents, and melatonin restores, the diurnal changes of Ttf1 and GnRH mRNA abundance in the POA

One major extracellular input to the neuroendocrine hypothalamus directed by the day-night cycle is melatonin released from pineal gland (47). Melatonin release increases during the night and induces a nocturnal increase of hypothalamic *GnRH* expression in rats (48,49). This effect is in all likelihood mediated by the high-affinity melatonin receptors MT1 and MT2, as these receptors are not only expressed in the rat hypothalamus as a whole (50), but also in GnRH neurons in situ (MT1) (50) and GT1-7 cells in vitro (MT1 and MT2) (51). Based on these observations, we reasoned that the nocturnal increase of *Ttf1* expression in the POA may also be a melatonin-dependent phenomenon. *Mt1* mRNA abundance in the POA was not significantly different between the day (1200 h, L) and night (2400 h, D), but 2 weeks after Px, the mRNA levels (measured at midnight) were strikingly decreased (Fig. 2A). This result is consistent with previous findings showing that *Mt1* mRNA content decreases in both the SCN and pars tuberalis of PX rats (52). In contrast to *Mt1* mRNA, *Mt2* mRNA levels were significantly increased during the night (Fig. 2B), and this increase was obliterated by Px (Fig. 2B). Though expression of *Mt2* in rat GnRH neurons *in vivo* is uncertain (50), the nocturnal increase in *Mt2* mRNA abundance we observed is strikingly similar to that of *Ttf1* and *GnRH* mRNA levels, suggesting that the MT2 receptor may also be involved in the diurnal regulation of *Ttf1* and *GnRH* expression, perhaps indirectly through associated neuronal systems.

As before (Fig. 1A), *Ttf1* mRNA levels in the POA showed a diurnal variation (Fig. 2C), with low values at 1200 h (L) and high values at 2400 h (D); this diurnal fluctuation was significantly attenuated in PX rats (D/PX, Fig. 2C), and enhanced by a single dose of melatonin (25 mg/Kg BW) given 6 h earlier (at 18:00 h). In agreement with earlier observations (49), the diurnal fluctuation in *GnRH* mRNA levels was also prevented by PX, and restored by melatonin (Fig. 2D), as previously shown by others (48,49). Our results indicate that, as is the case of *GnRH* mRNA, the nocturnal increase of *Ttf1* mRNA abundance in the POA appears to be due, to a significant extent, to melatonin released from the pineal gland.

Circadian oscillations of GnRH promoter activity are facilitated by TTF1

To study the effect of TTF1 on the circadian activity of the *GnRH* promoter *in vitro*, we used GT1-7 cells, as they were previously found to express *GnRH* mRNA in a circadian fashion (23). We first determined if GT1-7 cells also display an endogenously circadian rhythm of *Ttf1* mRNA expression. We collected the cells at 4 h intervals for 48 h after synchronising them by serum deprivation, and measured their *Ttf1* and *GnRH* mRNA content. The results showed that, consistent with the *in vivo* oscillation in expression (Fig. 1A), *Ttf1* mRNA levels vary in a circadian fashion in GT1-7 cells during the first 24 h after synchronization (Fig. 3A), with peak values being observed about 4 h before the circadian peak in *GnRH* mRNA values (Fig. 3B). Neither circadian oscillation was sustained during the second 24 h period examined after synchronisation.

We subsequently set out to determine if the diurnal changes of *GnRH* expression observed *in vivo* in the rat POA depend on circadian variations in TTF1 levels. To answer this question, we monitored changes of *GnRH* promoter activity for 2 days in cultured GT1-7 cells that were transfected with a construct in which the coding region of a secreted alkaline phosphatase gene is controlled by the *GnRH* promoter (pGnRH-SEAP). TTF1 was overexpressed by co-transfecting the cells with a TTF1 expression vector; endogenous TTF1 production was reduced by RNA interference using a *Ttf1* siRNA previously described (31). Under the culture conditions employed, *GnRH* promoter activity exhibited an autonomous 24 h rhythm (Fig. 3C). Increasing the cellular content of TTF1 enhanced the peak amplitude

of *GnRH* promoter rhythmic activity, without affecting nadir values of activity. In contrast, shRNA-mediated *Ttf1* knock-down blunted these fluctuations (Fig. 3C). These data suggest that TTF1 facilitates the circadian rhythm of *GnRH* transcription, by increasing the amplitude but not the frequency of the rhythm.

In addition to the increased peak amplitude, and as previously observed with the PACAP promoter (24), total *GnRH* promoter activity was also enhanced by TTF1 (Fig. 3D). Conversely, knocking-down TTF1 expression blunted both basal *GnRH* promoter activity and the TTF1-induced peak amplitude (Fig. 3D). Measurement of TTF1 protein revealed that, as expected, cells transfected with the TTF1 expression vector had increased TTF1 protein levels; *Ttf1* shRNA reduced the increase in TTF1 protein content seen in cells transfected with the TTF1 expression vector (Fig. 3E). It was also evident that *Ttf1* shRNA decreased basal TTF1 levels in cells not transfected with the TTF1 expression vector (Fig. 3E). This finding indicates that, as seen before in rat GnRH neurons *in situ* (8), GT1-7 cells also express the *Ttf1* gene.

TTF1 transactivates the GnRH promoter via specific binding sites

We previously showed that TTF1 increases *GnRH* promoter activity (8). Because the 5' flanking region of the m*GnRH* gene contains sequences that conform the consensus TTF1 binding motif (26), we sought to determine if the purified TTF1 homeodomain (TTF1-HD) could bind to these regions. Computer analysis of the 5' flanking region of the m*GnRH* gene using a search program (www.cbil.upenn.edu.tess/) verified the existence of two putative core TTF1 motifs. One of these sites is located within the proximal *GnRH* promoter (−101 to −96) and the other in the *GnRH* enhancer region (−1622 to −1617). This latter region targets expression of the *GnRH* gene to GnRH neurons in the basal forebrain and enhances *GnRH* promoter activity (53,54).

To determine if TTF1 binds to these sites, we used increasing amounts of the purified TTF1 homedomain (TTF1-HD) protein in EMSAs and 24-mer oligodeoxynucleotide probes containing the putative TTF1-HD binding sites and flanking sequences present in either the enhancer (*GnRHenh*) or proximal promoter (*GnRHp*) region of the m*GnRH* gene (Fig 4A). The oligodeoxynucleotide "C" containing the sequence of a high-affinity TTF1-HD binding site found in the thryroglobulin promoter (30) was used both as a positive control and as a reference to estimate the affinity of TTF1-HD to the TTF1 sites in the GnRH promoter. Cβ is a mutant of the C oligodeoxynucleotide in which the core binding site for TTF1-HD was deleted (30). When comparing the binding affinity of both *GnRH* probes to that of the C probe (considered as 100%), we found that the *GnRH*p and *GnRH*enh probes also bound TTF1-HD, although with less (~35%) affinity (Fig. 4B). As expected, a mutated C probe (Cβ) exhibited no binding (Fig. 4B). Promoter assays using a *GnRH* promoter construct in which the enhancer region is placed directly upstream from the proximal *GnRH* promoter (55) demonstrated that TTF1 trans-activates this construct (Fig. 4C). Neither deletion of the distal TTF1 binding site (TTAAGT) nor the proximal site (CCAAGT) affected the ability of TTF1 to transactivate the promoter. However, deletion of both sites obliterated the TTF1 effect (Fig. 4C). These results indicate that TTF1 binds to specific binding sites in both the *GnRH* enhancer and proximal promoter to stimulate *GnRH* transcription. Furthermore the ability of TTF1 to enhance circadian *GnRH* promoter activity, and the circadian pattern of *Ttf1* mRNA expression in both the POA and GT1-7 cells, suggests the involvement of clock genes in this relationship.

GnRH neurons in situ express core components of the circadian clock

To determine if GnRH neurons *in vivo* contain core clock proteins, we detected these proteins by double immunofluorescent staining. It is known that GT1-7 cells express core

clock proteins (22,23), and that at least three of these proteins (BMAL1, PER1, PER2) can also be detected in mouse GnRH neurons *in vivo* (21,56). By confocal imaging, we found that GnRH neurons of the rat POA express CLOCK, in addition to BMAL1 and PER1, and that all three of them have a nuclear localization (Fig. 5). Although the content of these clock proteins would be expected to display a diurnal variation, no attempts were made to verify this assumption.

Clock proteins and TTF1 trans-activate the GnRH promoter in a non-synergistic, non-additive manner

The *GnRH* 5'-flanking region contains several E-boxes (Supplementary Fig.1A), which are recognition sequences typically mediating the trans-activational effects of CLOCK/BMAL1 heterodimers on clock-controlled genes, like *Per1, Per2, Cry1* and *Cry2* (17,57,58). Promoter assays carried out in GT1-7 cells showed that CLOCK/BMAL1 increase *GnRH* transcription and that this effect is neither additive nor synergistic to that of TTF1 (Supplementary Fig. 1B). These results suggest that core clock genes and TTF1 trans-activate the enhancer/proximal region of the *GnRH* promoter along a common pathway.

The Ttf1 promoter is regulated by core clock proteins and TTF1 protein modulates the promoter activity of hREV-ERBα and mPer1 in vitro

The aforementioned findings and the presence of several E-boxes in the 5'-flanking region of the rat *Ttf1* gene (Supplementary Fig. 2) prompted us to investigate the effect of core clock proteins on *Ttf1* promoter activity using gene reporter assays in GT1-7 (Supplementary Fig 3A and B) and NIH-3T3 cells (Fig 6 and Supplementary Fig. 3 C–F). *Ttf1* promoter activity was slightly reduced by the transient expression of both mBMAL/mCLOCK proteins (Fig 6A, Supplementary Fig 3A) and also by mPER1/mPER2 (Fig 6B). A modest decrease was observed in cells over-expressing the transcriptional repressor REV-ERBα (Fig 6C). In contrast, mCRY1 unambiguously increased *Ttf1* promoter activity (Fig 6D). These results suggest that clock genes mainly affect *Ttf1* transcription via a CRY1-mediated trans-activational effect.

To determine if TTF1 is involved in the transcriptional control of core clock genes, we tested the effect of TTF1 on *Bmal1*, *Per1*, *Cry1* and *REV-ERBα* promoter activities in NIH 3T3 cells. TTF1 had no effect on *mBmal1* promoter activity (Fig 6E), a minimal repressive effect on *mPer1* transcriptional activity (Fig 6F), and no effect on *mCry1* promoter activity (Fig. 6G). In contrast, TTF1 was very effective in repressing the *REV-ERBα* promoter (Fig 6H). These assays were conducted using conditions that allowed us to reproduce known effects of clock proteins on clock gene expression. For instance, REV-ERBα has been reported to repress the *Bmal1* promoter (37), and inhibited *mBmal1* transcription in our study (Fig.6E). CLOCK/BMAL1 transactivate *Per1* (40) and *Cry1* transcription (41) and this is the effect we observed (Fig. 6F, G). Lastly, REV-ERBα repressed transcription of its own gene (Fig. 6H), in agreement with results reported by others (38). The repressive effects of TTF1 on *Per1* and *REV-ERBα* were maximal at the concentration of 100 ng, remaining at this level at concentrations as high as 500–1,000 ng (Supplementary Fig. 3D and F).

Discussion

The present study provides evidence for the concept that TTF1, a transcriptional activator of *GnRH* transcription (8,10), plays a role in facilitating circadian changes in *GnRH* expression. *Ttf1* mRNA abundance in the rat POA area oscillates in a diurnal fashion; peak levels of *Ttf1* mRNA, attained during the early night hours, precede the nocturnal peak in *GnRH* mRNA. The latter rhythm, previously shown by others (21), is confirmed by our observations. Our results show that TTF1 enhances *GnRH* transcription by binding to at

least two recognition sites, one located within the *GnRH* enhancer region and the other in the proximal *GnRH* promoter. The fact that both of these regions are required for cell-type specific expression of the *GnRH* gene (53,55), suggests that TTF1 contributes to controlling circadian *GnRH* expression in the POA by targeting core regulatory components of the GnRH transcriptional machinery for trans-activation.

The nocturnal changes in both *Ttf1* and *GnRH* expression were obliterated by PX, which also reduced drastically *Mt1 and Mt2* receptor mRNA levels in the POA. Conversely, melatonin administration restored the nocturnal increase in both *GnRH* and *Ttf1* mRNA levels. It would appear that this effect is exerted via MT1 receptors, because most actions of melatonin in the CNS are mediated by these receptors (59). Their loss compromises the ability of melatonin to both inhibit SCN neuronal firing (59) and to act as a zeitgeber throughout the brain (47). Previous reports have shown that PX results in decreased MT1 receptor density in the SCN (52) and lowers *GnRH* mRNA levels in GnRH neurons of the rat POA (49). Other authors have shown that melatonin administration increases *GnRH* mRNA abundance in the aging rat brain (48). Our findings indicate that melatonin is not only required for the diurnal oscillation in *GnRH* mRNA levels, but is also essential for the nocturnal increase in *Ttf1* expression in the rat POA. These *in vivo* findings are at odds with *in vitro* results showing that melatonin decreases GnRH mRNA content in GT1-7 cells (51). The discrepancy may be due to heterogeneity among GT1-7 subcultures as Provenzano et al did not find detectable levels of TTF1 in these cells (11) whereas we did (Fig. 3E). It is also possible that GT1-7 cells behave differently from GnRH neurons *in situ* or that melatonin requires cellular networks other than GnRH neurons to facilitate *in vivo* diurnal changes in GnRH expression.

The mechanism(s) underlying this supportive effect of melatonin is unknown. Because MT1 receptors are widely expressed throughout the brain (47), melatonin may support circadian oscillations in *TTF1* and *GnRH* mRNA abundance by not only acting on the SCN pacemaker (59), but also on cellular subsets functionally and anatomically connected to GnRH neurons. Melatonin may also activate MT1 receptors located on GnRH neurons, because MT1 mRNA (but not MT2 mRNA) is expressed in some GnRH neurons in situ (50).

GnRH promoter activity varies in a circadian manner in GT1-7 cells with peak levels attained in mid-subjective night. This pattern of activity closely resembles the *in vivo* changes in *GnRH* mRNA abundance, and its amplitude is enhanced by TTF1. It was, however, surprising that neither the *Ttf1* nor the *GnRH* mRNA rhythm persisted beyond the first 24 h after *in vitro* synchronization. This could be due to the synchronization protocol we used, to the absence of an initial TTF1 peak of sufficient amplitude to sustain the circadian activity of the GnRH promoter, and to the absence of melatonin supplementation to entrain these rhythms. We believe that the most parsimonious explanation resides with the latter two possibilities, because a) Increasing TTF1 levels in GT1-7 cells does result in robust and sustained circadian changes in exogenous GnRH promoter activity (Fig. 3c), and b) *In vivo* melatonin administration restores the diurnal rhythm in hypothalamic *Ttf1* and *GnRH* expression lost after PX (Fig. 2). A requirement for melatonin to sustain the *in vitro* circadian rhythms of both *Ttf1* and *GnRH* expression is also suggested by the results of Roy et al (51) who demonstrated that melatonin does entrain a circadian rhythm in GnRH mRNA levels in GT1-7 cells. Because RNAi-mediated suppression of endogenous *Ttf1* mRNA levels blunts the circadian oscillations in GnRH promoter activity, it is likely that the ability of TTF1 to trans-activate the *GnRH* promoter is tied to a clock mechanism that at least in part operates within the GnRH neuronal network. The presence of CLOCK, BMAL1 and PER1 in the nucleus of GnRH neurons *in situ* indicates that these cells have some of the basic molecular components required for this mechanism to operate *in vivo*.

TTF1 may amplify the circadian peaks of GnRH promoter activity by modifying the expression and/or activity of clock genes known to regulate GnRH circadian output (22,23). Alternatively, *Ttf1* expression itself may be under the control of clock genes. The diurnal pattern of *Ttf1* mRNA abundance observed *in vivo* suggests that *Ttf1* expression is regulated by clock proteins. At least part of this regulation is exerted directly at the transcriptional level because: a) The *Ttf1* 5'flanking region contains eight recognizable E-box sites (Supplementary Fig. 1), and b) in vitro *Ttf1* promoter activity is regulated by clock genes.

Ttf1 transcription was modestly inhibited by CLOCK/BMAL1, two protein components of the circadian oscillator's positive limb (60–62), and also by PER1/PER2, which are components of the negative limb (60–62). It is likely that the CLOCK/BMAL1-mediated *Ttf1* transcriptional inhibition is not a direct effect, but is mediated by an increase in PER1/PER2 transcription as both conditions result in similar inhibitory effects. REV-ERBα, a negative regulator of *Bmal1* expression that controls the period length of rhythms in gene expression within the positive limb of the circadian oscillator (37) had a negligible inhibitory effect. In contrast, *Ttf1* promoter activity was increased by CRY1, a component of the negative feedback loop that inhibits CLOCK/BMAL1-induced transcription (41), but that also enhances *Bmal1* expression (62) by preventing ubiquitination of PER2 (63), a positive regulator of *Bmal1* expression. According to the basic transcriptional mechanisms regulating core clock networks {7283,7250}, it is expected that overexpressing CLOCK/BMAL1 would produce a synchronous increase in PER1/2 and CRY1 which would subsequently affect *Ttf1* transcription in a similar fashion. Although the results we observed are contradictory to these expectations, there are reasonable interpretations that can be entertained. A potential explanation is that overexpression of proteins able to regulate clock controlled genes disrupts the circadian expression of other elements of the clock network, as was reported in the case of CRY1 and REV-ERBα {5331}. It is also possible that the activating effect of CRY1 may be due to direct trans-activation of the *Ttf1* promoter. This latter effect is consistent with the reported stimulatory effect of CRY1 on GnRH release (22), the evolutionary conserved direct transactivation of downstream promoters by Cry1 (64,65), and the emerging concept that different components of the mammalian circadian clock can act directly on clock associated genes to directly activate or repress transcription (66).

TTF1 trans-activates *GnRH* transcription by directly binding to specific recognition sites in the *GnRH* promoter. In a cellular context this effect is manifested as an increase in the amplitude – but not the period – of the diurnal changes in *GnRH* promoter activity. This feature suggests that TTF1 increases *GnRH* transcription by interacting with clock proteins as they bind to E-boxes in the *GnRH* 5'flanking region (Supplementary Fig. 2), and/or by directly modulating clock gene transcription. In the present study we only addressed the later possibility by assessing the ability of TTF1 to affect clock gene transcription. Our results showed that TTF1 markedly represses the *REV-ERBα* promoter without affecting *Bmal1* or *Cry1* promoter activity, and modestly repressing *Per1* transcription. The lack of effect of TTF1 on the *Bmal1* promoter is surprising, because it would be expected that following repression of REV-ERBα, *Bmal1* expression would rise. Such an effect may no longer detected 48 h after TTF1 transfection, or alternatively, it may be discerned only in a circadian context. It is also plausible that it may have been obliterated due to a disruption of circadian gene networks caused by TTF1 overexpression [as previously observed following Cry1 overexpression (58)]. Further studies are required to resolve this issue.

Within a broader context, our results suggest that, instead of acting directly on core components of the primary feedback loop controlling the circadian oscillator (CLOCK/BMAL1), TTF1 modulates clock gene circuit activity by repressing one of the interlocking associated loops, driven by the retinoic acid-related orphan receptor Rev-erbα (37). By

doing so, TTF1 would act in a circadian context to reduce Rev-erbα accumulation, resulting in activation of *Bmal1* expression (37) and/or Cry de-repression (67).

Though complex, these interactions are amenable to interpretations that can be integrated into a transcriptional working model (Fig. 7). According to this model, daily cues such as the light/dark cycle and diurnal fluctuations in melatonin secretion would entrain periodical oscillation in *Ttf1* and *GnRH* expression. *Ttf1* can be considered as a clock-controlled gene that regulates *GnRH* transcription both directly by binding to its promoter and indirectly through interaction with core clock components (Rev-erbα, Cry1, thick lines). Although high-throughput approaches coupled with systems biology strategies have revealed an astounding complexity of circadian oscillators (66), the basic interlocking transcriptional regulatory loops underlying the circadian clock remain firmly established (framed box, Fig. 7). BMAL1 dimerizes with CLOCK to stimulate the transcription of PER1/2, CRY1/2 and REV-ERBα (17,66). While PER1/2 and CRY1/2 negatively regulate CLOCK/BMAL-induced transcription (62), REV-ERBα inhibits the transcription of BMAL1 (37), which subsequently leads to their own transcriptional downregulation. Our data indicate that TTF1 may be part of a secondary regulatory loop involving an increase in *Ttf1* transcription driven by CRY1 and a decrease in *Rev-erbα* transcription (Fig 7, thick lines). This is of particular interest as CRY1 expression, similarly to that of TTF1, peaks at evening phases in the SCN (58,67). In addition, Cry1 is under positive and negative transcriptional regulation by CLOCK/BMAL (41) and REV-ERBα (58,67), respectively. We therefore envision a mechanism by which the nocturnal increase in CRY1 stimulates Ttf1 transcription that will, in turn, decrease REV-ERBα transcription. The latter would delay the transcriptional inhibition of *Bmal1*, lengthening the time during which CRY1 is sufficiently elevated (41) to increase *Ttf1* transcription. This TTF1-mediated regulatory loop would eventually result in increased amplitude of the circadian oscillations in *GnRH* promoter activity, as revealed by secreted alkaline phosphatase assays measuring GnRH promoter-driven transcription. The idea that CRY1 is positively coupled to the circadian control of GnRH secretion is supported by previous studies showing that CRY1 overexpression in GT1-7 cells leads to an increase in peak amplitude of GnRH secretion (22). Our results also show that PER1 has an inhibitory effect on *Ttf1* transcription, which could be part of an inhibitory sub-loop counterbalancing the stimulatory effect of CRY1 on *Ttf1* transcription. It is important to recognize that clock proteins may also act directly on the *GnRH* promoter to modify its activity and elicit GnRH secretion (22,23). The lack of synergism or additivity between TTF1 and CLOCK/BMAL1 effects on *GnRH* promoter activity we observed in our study may not hold true for TTF1 binding sites and E-box recognition sequences present in regions of the GnRH 5'-flanking region other than the enhancer and proximal promoter we examined here.

Altogether our results are compatible with the interpretation that TTF1 is part of a regulatory transcriptional sub-network that, acting within the POA, contributes to the circadian oscillator controlling GnRH secretion. By showing entrainment to the light-dark cycle, dependence on melatonin, periodicity and robustness of its daily fluctuations in expression, trans-regulatory strength, and functional connectivity to the molecular machinery underlying the circadian oscillator controlling GnRH secretion, *Ttf1* emerges as a tissue-specific clock-associated gene. Furthermore, our results indicate that TTF1 is a modifier of circadian amplitude, and not of clock period. As such, it appears to belong to a handful of clock-related genes regulating the amplitude of circadian oscillations (66). Derangements of these circadian interactions may play a role in the genesis of disorders affecting GnRH secretion both at puberty and during adult reproductive life.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Kimura S, Hara Y, Pineau T, Fernandez-Salguero P, Fox CH, Ward JM, Gonzalez FJ. The *T/ebp* null mouse: Thyroid-specific enhancer-binding protein is essential for the organogenesis of the thyroid, lung, ventral forebrain, and pituitary. Genes Dev. 1996; 10:60–69. [PubMed: 8557195]
2. Sussel L, Marin O, Kimura S, Rubenstein JL. Loss of Nkx2.1 homeobox gene function results in a ventral to dorsal molecular respecification within the basal telencephalon: evidence for a transformation of the pallidum into the striatum. Development. 1999; 126:3359–3370. [PubMed: 10393115]
3. Price M, Lazzaro D, Pohl T, Mattei M-G, Rüther U, Olivo J-C, Duboule D, Di Lauro R. Regional expression of the homeobox gene *Nkx-2.2* in the developing mammalian forebrain. Neuron. 1992; 8:241–255. [PubMed: 1346742]
4. Pera EM, Kessel M. Demarcation of ventral territories by the homeobox gene NKX2.1 during early chick development. Dev Genes Evol. 1998; 208:168–171. [PubMed: 9601992]
5. Takuma N, Sheng HZ, Furuta Y, Ward JM, Sharma K, Hogan BL, Pfaff SL, Westphal H, Kimura S, Mahon KA. Formation of Rathke's pouch requires dual induction from the diencephalon. Development. 1998; 125:4835–4840. [PubMed: 9806931]
6. Marin O, Baker J, Puelles L, Rubenstein JL. Patterning of the basal telencephalon and hypothalamus is essential for guidance of cortical projections. Development. 2002; 129:761–773. [PubMed: 11830575]
7. Marin O, Anderson SA, Rubenstein JL. Origin and molecular specification of striatal interneurons. J Neurosci. 2000; 20:6063–6076. [PubMed: 10934256]
8. Lee BJ, Cho GJ, Norgren R, Junier M-P, Hill DF, Tapia V, Costa ME, Ojeda SR. TTF-1, a homeodomain gene required for diencephalic morphogenesis, is postnatally expressed in the neuroendocrine brain in a developmentally regulated and cell-specific fashion. Mol Cell Neurosci. 2001; 17:107–126. [PubMed: 11161473]
9. Davis AM, Seney ML, Stallings NR, Zhao L, Parker KL, Tobet SA. Loss of steroidogenic factor 1 alters cellular topography in the mouse ventromedial nucleus of the hypothalamus. J Neurobiol. 2004; 60:424–436. [PubMed: 15307147]
10. Mastronardi C, Smiley GG, Raber J, Kusakabe T, Kawaguchi A, Matagne V, Dietzel A, Heger S, Mungenast AE, Cabrera R, Kimura S, Ojeda SR. Deletion of the *Ttf1* gene in differentiated neurons disrupts female reproduction without impairing basal ganglia function. J Neurosci. 2006; 26:13167–13179. [PubMed: 17182767]
11. Provenzano C, Pascucci B, Lupari E, Civitareale D. Large scale analysis of transcription factor TTF-1/NKX2.1 target genes in GnRH secreting cell line GT1-7. Mol Cell Endocrinol. 2010; 323:215–223. [PubMed: 20211691]
12. Khan AR, Kauffman AS. The Role of Kisspeptin and RFRP-3 Neurons in the Circadian-Timed Preovulatory Luteinizing Hormone Surge. J Neuroendocrinol. 2011 Accepted Article-
13. Turek, FW.; Van Cauter, E. Rhythms in reproduction. In: Knobil, E.; Neill, JD., editors. The Physiology of Reproduction. 2nd Edition. New York: Raven Press; 1994. p. 487-540.

14. Boyar R, Finkelstein J, Roffwarg H, Kapen S, Weitzman E, Hellman L. Synchronization of augmented luteinizing hormone secretion with sleep during puberty. N Engl J Med. 1972; 287:582–586. [PubMed: 4341276]

15. Terasawa E, Bridson WE, Nass TE, Noonan JJ, Dierschke DJ. Developmental changes in the luteinizing hormone secretory pattern in peripubertal female rhesus monkeys: Comparisons between gonadally intact and ovariectomized animals. Endocrinology. 1984; 115:2233–2240. [PubMed: 6499767]

16. Urbanski HF, Ojeda SR. Gonadal-independent activation of enhanced afternoon luteinizing hormone release during pubertal development in the female rat. Endocrinology. 1987; 121:907–913. [PubMed: 3304982]

17. Hastings MH, Reddy AB, Maywood ES. A clockwork web: circadian timing in brain and periphery, in health and disease. Nat Rev Neurosci. 2003; 4:649–661. [PubMed: 12894240]

18. Saper CB, Lu J, Chou TC, Gooley J. The hypothalamic integrator for circadian rhythms. Trends Neurosci. 2005; 28:152–157. [PubMed: 15749169]

19. Freeman, ME. The neuroendocrine control of the ovarian cycle of the rat. In: Knobil, E.; Neill, JD., editors. The Physiology of Reproduction. 2nd Edition. New York: Raven Press, Ltd.; 1994. p. 613-658.

20. Maywood ES, O'Neill JS, Chesham JE, Hastings MH. Minireview: The circadian clockwork of the suprachiasmatic nuclei--analysis of a cellular oscillator that drives endocrine rhythms. Endocrinology. 2007; 148:5624–5634. [PubMed: 17901233]

21. Hickok JR, Tischkau SA. In vivo circadian rhythms in gonadotropin-releasing hormone neurons. Neuroendocrinology. 2010; 91:110–120. [PubMed: 19786732]

22. Chappell PE, White RS, Mellon PL. Circadian gene expression regulates pulsatile gonadotropin-releasing hormone (GnRH) secretory patterns in the hypothalamic GnRH-secreting GT1-7 cell line. J Neurosci. 2003; 23:11202–11213. [PubMed: 14657179]

23. Gillespie JMA, Chan BPK, Roy D, Cai F, Belsham DD. Expression of circadian rhythm genes in gonadotropin-releasing hormone-secreting GT1-7 neurons. Endocrinology. 2003; 144:5285–5292. [PubMed: 12960008]

24. Kim MS, Hur MK, Son YJ, Park J-I, Chun SY, D'Elia AV, Damante G, Cho S, Kim K, Lee BJ. Regulation of pituitary adenylate cyclase-activating polypeptide gene transcription by TTF-1, a homeodomain-containing transcription factor. J Biol Chem. 2002; 277:36863–36871. [PubMed: 12122016]

25. Chomczynski P. A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. BioTechniques. 1993; 15:532–534. [PubMed: 7692896]

26. Damante G, Fabbro D, Pellizzari L, Civitareale D, Guazzi S, Polycarpou-Schwartz M, Cauci S, Quadrifoglio F, Formisano S, Di Lauro R. Sequence-specific DNA recognition by the thyroid transcription factor-1 homeodomain. Nucleic Acids Res. 1994; 22:3075–3083. [PubMed: 7915030]

27. Junier M-P, Wolff A, Hoffman GE, Ma YJ, Ojeda SR. Effect of hypothalamic lesions that induce precocious puberty on the morphological and functional maturation of the luteinizing hormone-releasing hormone neuronal system. Endocrinology. 1992; 131:787–798. [PubMed: 1639024]

28. Ma YJ, Dissen GA, Rage F, Ojeda SR. RNase protection assay Methods: A Companion to Methods in Enzymology. 1996; 10:273–278.

29. Damante G, Pellizzari L, Esposito G, Fogolari F, Viglino P, Fabbro D, Tell G, Formisano S, Di Lauro R. A molecular code dictates sequence-specific DNA recognition by homeodomains. EMBO J. 1996; 15:4992–5000. [PubMed: 8890172]

30. Civitareale D, Lonigro R, Sinclair AJ, Di Lauro R. A thyroid-specific nuclear protein essential for tissue-specific expression of the thyroglobulin promoter. EMBO J. 1989; 8:2537–2542. [PubMed: 2583123]

31. Kim JG, Park BS, Yun CH, Kim HJ, Kang SS, D'Elia AV, Damante G, Lee KU, Park JW, Kim ES, Namgoong IS, Kim YI, Lee BJ. Thyroid transcription factor-1 regulates feeding behavior via melanocortin pathway in the hypothalamus. Diabetes. 2011; 60:710–719. [PubMed: 21282365]

32. Kim JG, Son YJ, Yun CH, Kim YI, Nam-Goong IS, Park JH, Park SK, Ojeda SR, D'Elia AV, Damante G, Lee BJ. Thyroid transcription factor-1 facilitates cerebrospinal fluid formation by

regulating aquaporin-1 synthesis in the brain. J Biol Chem. 2007; 282:14923–14931. [PubMed: 17371871]

33. Ojeda SR, Hill J, Hill DF, Costa ME, Tapia V, Cornea A, Ma YJ. The Oct-2 POU-domain gene in the neuroendocrine brain: A transcriptional regulator of mammalian puberty. Endocrinology. 1999; 140:3774–3789. [PubMed: 10433239]
34. Guo ZS, Wang L-H, Eisensmith RC, Woo SLC. Evaluation of promoter strength for hepatic ce of autoregulation Endocrinology 1998; 139: 1999–2006. gene expression in vivo following adenovirus-mediated gene transfer. Gene Ther. 1996; 3:802–810. [PubMed: 8875229]
35. Matagne V, Mastronardi C, Shapiro RA, Dorsa DM, Ojeda SR. Hypothalamic expression of *Eap1* is not directly controlled by ovarian steroids. Endocrinology. 2008; 150:1870–1878. [PubMed: 19022886]
36. Oguchi H, Kimura S. Multiple transcripts encoded by the thyroid-specific enhancer-binding protein (T/EBP)/thyroid-specific transcription factor-1 (TTF-1) gene: eviden.
37. Preitner N, Damiola F, Lopez-Molina L, Zakany J, Duboule D, Albrecht U, Schibler U. The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. Cell. 2002; 110:251–260. [PubMed: 12150932]
38. Adelmant G, Begue A, Stehelin D, Laudet V. A functional Rev-erb alpha responsive element located in the human Rev-erb alpha promoter mediates a repressing activity. Proc Natl Acad Sci U S A. 1996; 93:3553–3558. [PubMed: 8622974]
39. Chang DC, Reppert SM. The circadian clocks of mice and men. Neuron. 2001; 29:555–558. [PubMed: 11301014]
40. Gekakis N, Staknis D, Nguyen HB, Davis FC, Wilsbacher LD, King DP, Takahashi JS, Weitz CJ. Role of the CLOCK protein in the mammalian circadian mechanism. Science. 1998; 280:1564–1569. [PubMed: 9616112]
41. Kume K, Zylka MJ, Sriram S, Shearman LP, Weaver DR, Jin X, Maywood ES, Hastings MH, Reppert SM. mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop. Cell. 1999; 98:193–205. [PubMed: 10428031]
42. Urbanski HF. Monoclonal antibodies to luteinizing hormone-releasing hormone: Production, characterization, and immunocytochemical application. Biol Reprod. 1991; 44:681–686. [PubMed: 2043738]
43. DeFazio RA, Heger S, Ojeda SR, Moenter SM. Activation of A-type g-aminobutyric acid receptors excites gonadotropin-releasing hormone neurons. Mol Endocrinol. 2002; 16:2872–2891. [PubMed: 12456806]
44. Heger S, Mastronardi C, Dissen GA, Lomniczi A, Cabrera R, Roth CL, Jung H, Galimi F, Sippell W, Ojeda SR. Enhanced at puberty 1 (EAP1) is a new transcriptional regulator of the female neuroendocrine reproductive axis. J Clin Invest. 2007; 117:2145–2154. [PubMed: 17627301]
45. Lee C, Etchegaray JP, Cagampang FR, Loudon AS, Reppert SM. Posttranslational mechanisms regulate the mammalian circadian clock. Cell. 2001; 107:855–867. [PubMed: 11779462]
46. Hastings MH, Field MD, Maywood ES, Weaver DR, Reppert SM. Differential regulation of mPER1 and mTIM proteins in the mouse suprachiasmatic nuclei: new insights into a core clock mechanism. J Neurosci. 1999; 19:RC11. [PubMed: 10366649]
47. Simonneaux V, Ribelayga C. Generation of the melatonin endocrine message in mammals: a review of the complex regulation of melatonin synthesis by norepinephrine, peptides, and other pineal transmitters. Pharmacol Rev. 2003; 55:325–395. [PubMed: 12773631]
48. Li S, Givalois L, Pelletier G. Effects of aging and melatonin administration on gonadotropin-releasing hormones (GnRH) gene expression in the male and female rat. Peptides. 1997; 18:1023–1028. [PubMed: 9357061]
49. Li S, Pelletier G. Effects of pinealectomy and melatonin on gonadotropin-releasing hormone (GnRH) gene expression in the male rat brain. Endocrine. 1995; 3:533–536. [PubMed: 21153210]
50. Ishii H, Tanaka N, Kobayashi M, Kato M, Sakuma Y. Gene structures, biochemical characterization and distribution of rat melatonin receptors. J Physiol Sci. 2009; 59:37–47. [PubMed: 19340560]

51. Roy D, Angelini NL, Fujieda H, Brown GM, Belsham DD. Cyclical regulation of GnRH gene expression in GT1-7 GnRH-secreting neurons by melatonin. Endocrinology. 2001; 142:4711–4720. [PubMed: 11606436]
52. Gauer F, Masson-Pevet M, Pevet P. Differential regulation of melatonin receptors by short-versus long-term pinealectomy in the rat suprachiasmatic nuclei and pars tuberalis. J Pineal Res. 1994; 16:73–76. [PubMed: 8014826]
53. Whyte DB, Lawson MA, Belsham DD, Eraly SA, Bond CT, Adelman JP, Mellon PL. A neuron-specific enhancer targets expression of the gonadotropin-releasing hormone gene to hypothalamic neurosecretory neurons. Mol Endocrinol. 1995; 9:467–477. [PubMed: 7659090]
54. Lawson MA, MacConell LA, Kim J, Powl BT, Nelson SB, Mellon PL. Neuron-specific expression *in vivo* by defined transcription regulatory elements of the GnRH gene. Endocrinology. 2002; 143:1404–1412. [PubMed: 11897697]
55. Nelson SB, Lawson MA, Kelley CG, Mellon PL. Neuron-specific expression of the rat gonadotropin-releasing hormone gene is conferred by interactions of a defined promoter element with the enhancer in GT1-7 cells. Mol Endocrinol. 2000; 14:1509–1522. [PubMed: 10976927]
56. Olcese J, Domagalski R, Bednorz A, Weaver DR, Urbanski HF, Reuss S, Middendorff R. Expression and regulation of mPer1 in immortalized GnRH neurons. NeuroReport. 2003; 14:613–618. [PubMed: 12657897]
57. Ko CH, Takahashi JS. Molecular components of the mammalian circadian clock. Hum Mol Genet. 2006; 15(Spec No 2):R271–R277. [PubMed: 16987893]
58. Ueda HR, Hayashi S, Chen W, Sano M, Machida M, Shigeyoshi Y, Iino M, Hashimoto S. System-level identification of transcriptional circuits underlying mammalian circadian clocks. Nat Genet. 2005; 37:187–192. [PubMed: 15665827]
59. Liu C, Weaver DR, Jin X, Shearman LP, Pieschi RL, Gribkoff VK, Reppert SM. Molecular dissection of two distinct actions of melatonin on the suprachiasmatic circadian clock. Neuron. 1997; 19:91–102. [PubMed: 9247266]
60. Jin X, Shearman LP, Weaver DR, Zylka MJ, De Vries GJ, Reppert SM. A molecular mechanism regulating rhythmic output from the suprachiasmatic circadian clock. Cell. 1999; 96:57–68. [PubMed: 9989497]
61. Bunger, Mk; Wilsbacher, LD.; Moran, SM.; Clendenin, C.; Radcliffe, LA.; Hogenesch, JB.; Simon, MC.; Takahashi, JS.; Bradfield, CA. *MOP3* is an essential component of the master circadian pacemaker in mammals. Cell. 2000; 103:1009–1017. [PubMed: 11163178]
62. Shearman LP, Sriram S, Weaver DR, Maywood ES, Chaves I, Zheng B, Kume K, Lee CC, van der Horst GT, Hastings MH, Reppert SM. Interacting molecular loops in the mammalian circadian clock. Science. 2000; 288:1013–1019. [PubMed: 10807566]
63. Yagita K, Tamanini F, Yasuda M, Hoeijmakers JH, van der Horst GT, Okamura H. Nucleocytoplasmic shuttling and mCRY-dependent inhibition of ubiquitylation of the mPER2 clock protein. EMBO J. 2002; 21:1301–1314. [PubMed: 11889036]
64. Thum KE, Kim M, Christopher DA, Mullet JE. Cryptochrome 1, cryptochrome 2, and phytochrome a co-activate the chloroplast psbD blue light-responsive promoter. Plant Cell. 2001; 13:2747–2760. [PubMed: 11752385]
65. Bose S, Boockfor FR. Episodes of prolactin gene expression in GH3 cells are dependent on selective promoter binding of multiple circadian elements. Endocrinology. 2010; 151:2287–2296. [PubMed: 20215567]
66. Zhang EE, Kay SA. Clocks not winding down: unravelling circadian networks. Nat Rev Mol Cell Biol. 2010; 11:764–776. [PubMed: 20966970]
67. Ukai-Tadenuma M, Yamada RG, Xu H, Ripperger JA, Liu AC, Ueda HR. Delay in feedback repression by cryptochrome 1 is required for circadian clock function. Cell. 2011; 144:268–281. [PubMed: 21236481]

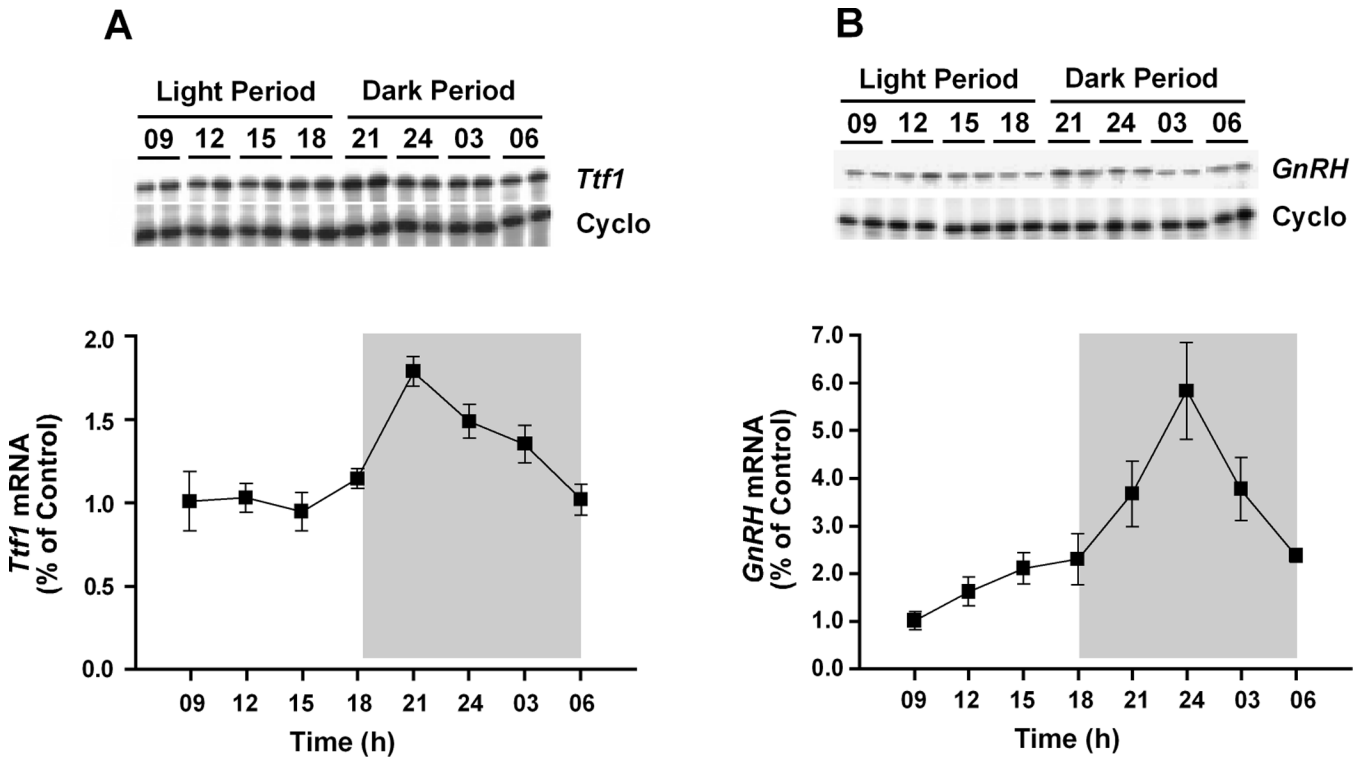

Figure 1. Daily rhythms in *Ttf1* and GnRH mRNA levels in the rat POA as assessed by RPA

A, *Diurnal changes in Ttf1 mRNA abundance*; A representative autoradiogram illustrating these changes is shown on top of a graph depicting changes in mean *Ttf1* mRNA levels (n = 8/time point). **B**, Diurnal changes in GnRH mRNA abundance (n = 6/time point). The shaded areas denote the dark period of the day. Each value represents the mean ± SEM for each time period and is calculated using individual values normalized according to the cyclophillin (Cyclo) mRNA content detected in each sample. Values at 0900 h are used as the reference point to express subsequent fluctuations in mRNA levels.

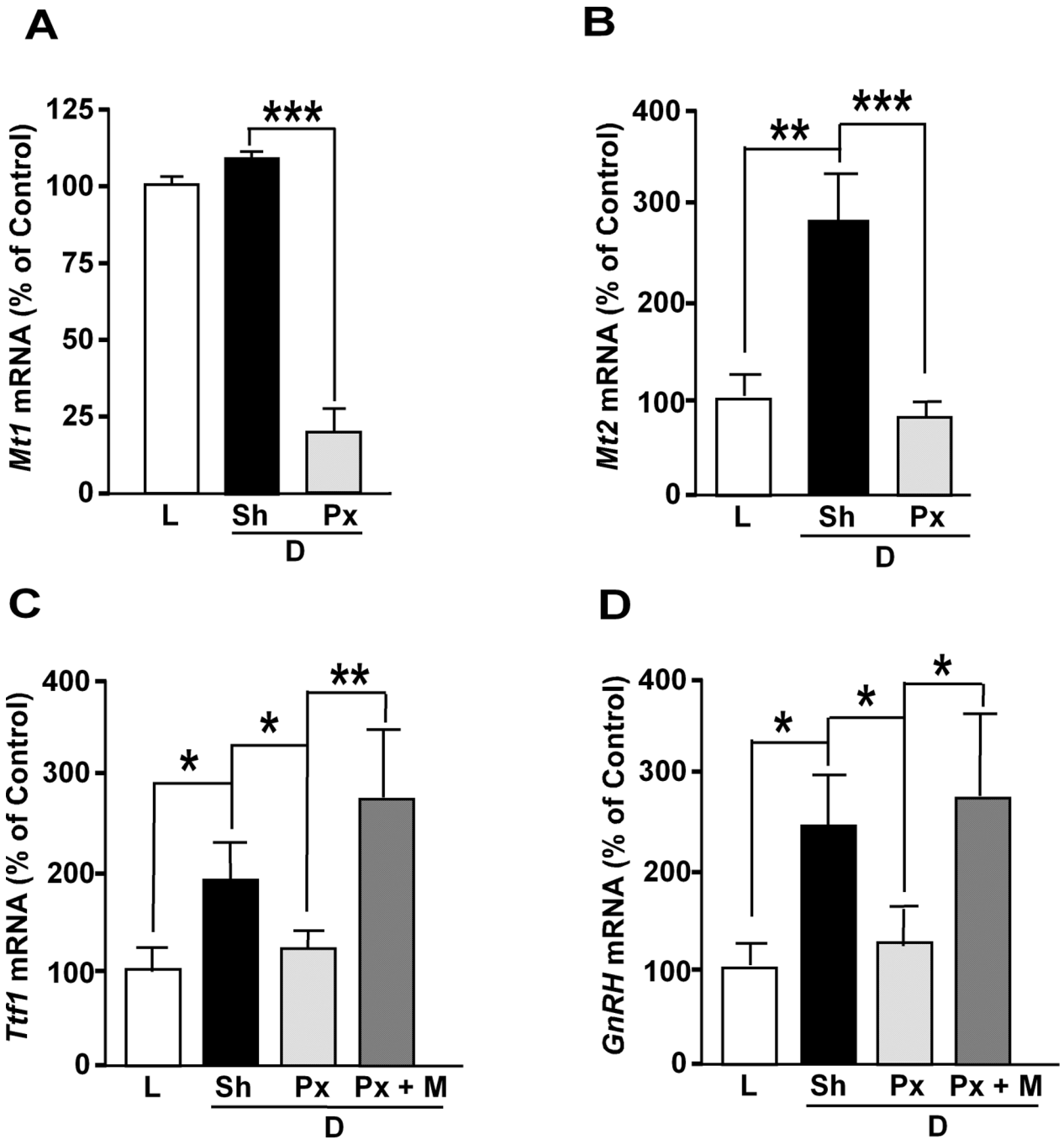

Figure 2. Effect of pinealectomy and melatonin on the diurnal change of *Ttf1* and *GnRH* mRNA levels in the rat POA

Two-month old male rats were pinealectomized (Px) or sham-operated (Sh) and kept under a normal light and dark cycle (12 h light/12 h dark, lights on 0700 – 1900 h) for 2 weeks. Then, groups of animals were euthanized at either 1200 h or 2400 h to determine effect of light (L) and dark (D) on *Mt1* mRNA with RNase protection assays and *Mt2*, *Ttf1* and *GnRH* mRNA levels by using qPCR. Some animals were subcutaneoulsy injected with melatonin (M) 6 h before sacrifice. **A**, *Mt1* mRNA abundance is slightly greater at midnight (Sh/D) than at noon (L), and decreased markedly after Px, in animals sacrificed at 2400 h (Px/D). **B**, *Mt2* mRNA content significantly increases at midnight (Sh/D) as compared to

noon (L), and decreases after Px (Px/D). **C**, *Ttf1* mRNA abundance increases significantly at midnight (Sh/D) as compared to noon (L), and Px obliterates this increase (Px/D); in contrast, melatonin increases *Ttf1* mRNA abundance (Px+M/D). **D**, *GnRH* mRNA content changes in a similar fashion. Bars are mean ± SEM (n=4–6/group). One-way ANOVA followed by Student-Newman-Keuls multiple comparison test * = P<0.05, ** = P<0.01, *** = P<0.001.

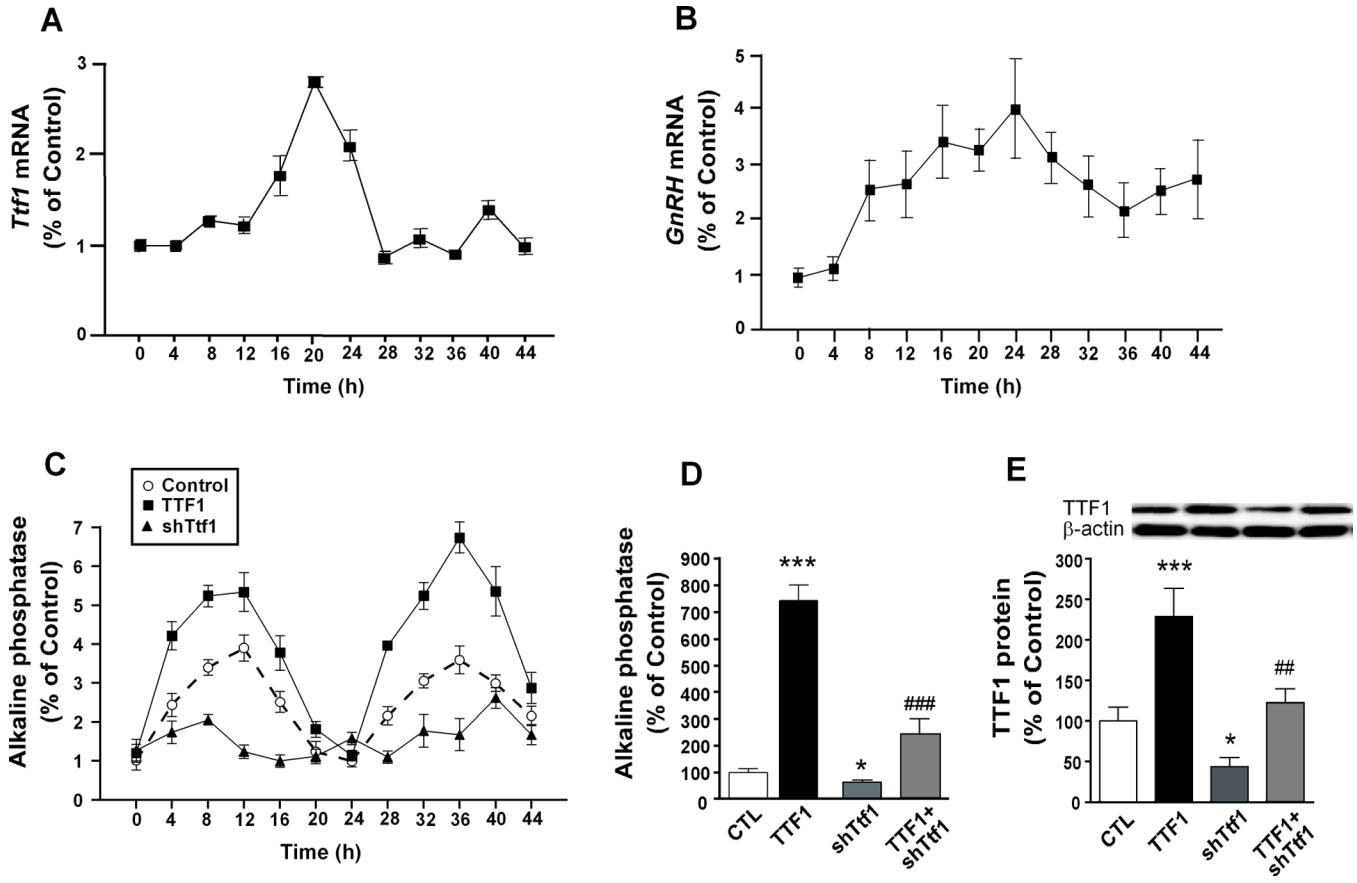

Figure 3. *Ttf1* mRNA abundance varies in a circadian fashion in GT1-7 cells and TTF1 enhances the circadian increase in *GnRH* promoter activity as assessed by a SEAP reporter assay

A, *Ttf1* mRNA levels measured by real-time PCR fluctuate in a circadian manner in GT1-7 cells after synchronisation of the cells with serum-free medium. **B**, *GnRH* mRNA content also varies in a circadian manner in GT1-7 cells. **C**, Amplification of the diurnal changes in *GnRH* promoter activity by TTF1 and blunting of these changes by shRNA-mediated *Ttf1* knock-down. An SEAP reporter vector containing a 3 kb-long *GnRH* promoter was cotransfected (500 ng/well) into GT1-7 cells along with a TTF1 expression vector (TTF1, 500 ng) or a Ttf1 shRNA vector (shTtf1, 500 ng). Starting 24 h after transfection, the medium was collected for SEAP assay at 4 h intervals for 48 h. Each point represents the mean ± SEM (n=6 for control and TTF1; n=4 for shTtf1). **D**, Quantification and statistical analysis of the peak values achieved by each group shown in C; bars are mean ± SEM. **E**, Changes in the content of TTF1 protein after transfecting GT1-7 cells with either the TTF1 expression vector or shTtf1, or both, as determined by Western blotting. β-actin was used as a normalizing control. One-way ANOVA followed by Student-Newman-Keuls multiple comparison test * = P<0.05, *** = P<0.001 vs. control; ## = P<0.01, ### = P<0.001 vs. TTF1.

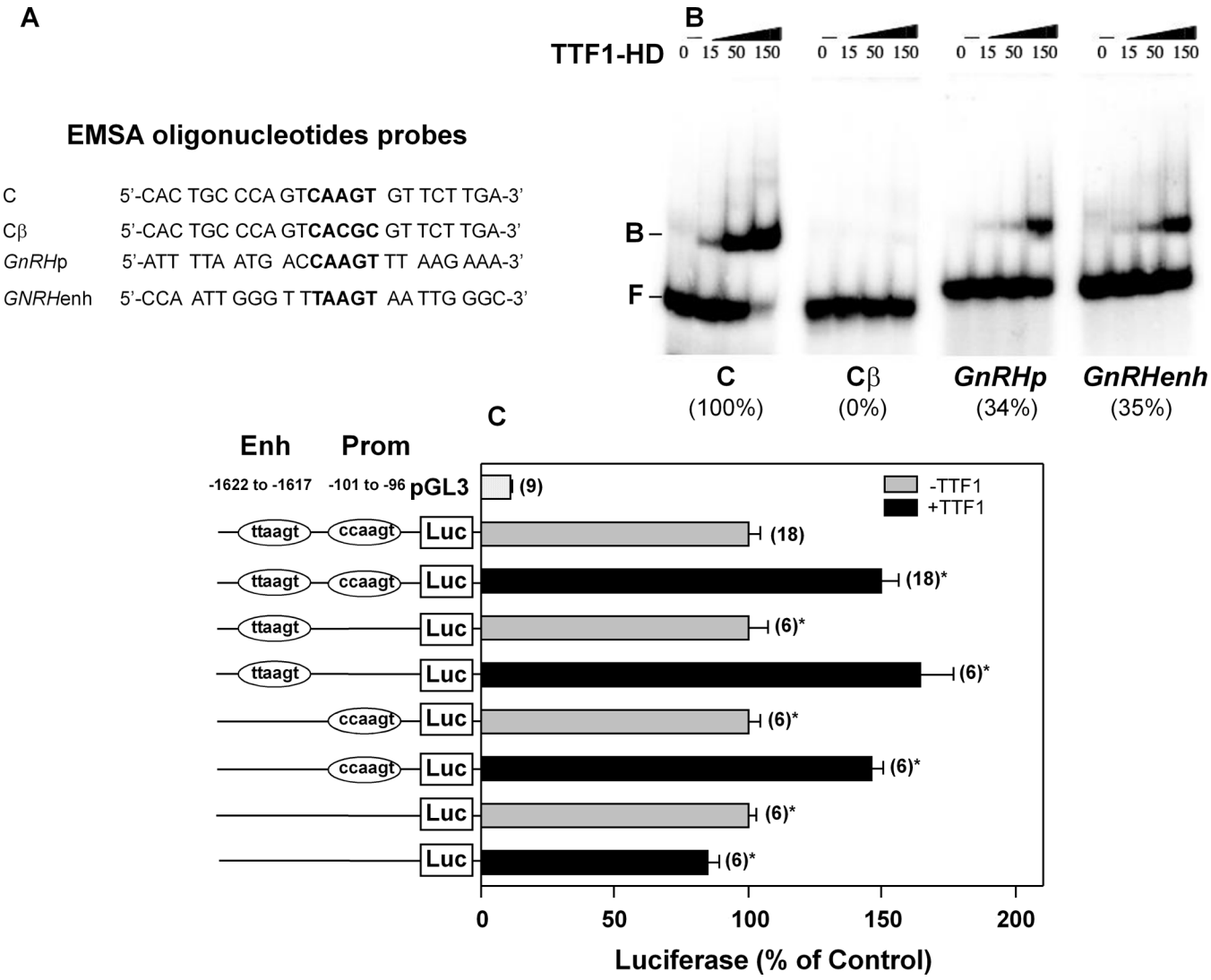

Figure 4. TTF1-HD binds to putative TTF1 binding sites in the *GnRH* promoter and trans-activates *GnRH* transcription

A, Sequences of the oligodeoxynucleotides used for EMSA. **B**, Autoradiograms of electrophoretic mobility shift assays (EMSA) demonstrating that increasing concentrations of TTF1-HD (15, 50 and 150 nM) results in increasing TTF1-HD binding to the indicated oligodeoxynucleotides described in A. The relative affinity of each binding site for TTF1-HD is expressed as percentage of the binding intensity of TTF1-HD to the canonical TTF1 recognition site in the positive control thyroglobulin promoter (C, considered as 100%) (30). Cβ, a mutant form in which the core binding site for TTF1-HD was deleted (26), was used as a negative control. F = free DNA; B= protein-bound DNA. **C**, A pGL-3 plasmid carrying the intact enhancer/promoter regions of the *GnRH* gene (Enh/Prom) or DNA fragments bearing either 6 bp deletions of TTF1 core binding sites present in each of these regions (Del-*GnRH* enh, Del *GnRH* prom) or a double deletion (Del-GnHRH enh/prom) were cotransfected (250 ng/well) into GT1-7 cells with 100 ng/well pcDNA-Zeo-rTTF-1 and the resulting changes in luciferase activity were measured 48 h later. The positions of the deleted TTF-1 binding sites (enh −1622 to −1617; prom −101 to −96) are indicated. Bars represent mean ± SEM and figures above bars are number of wells per group. Student t test, * = P<0.05 vs. groups not treated with TTF1.

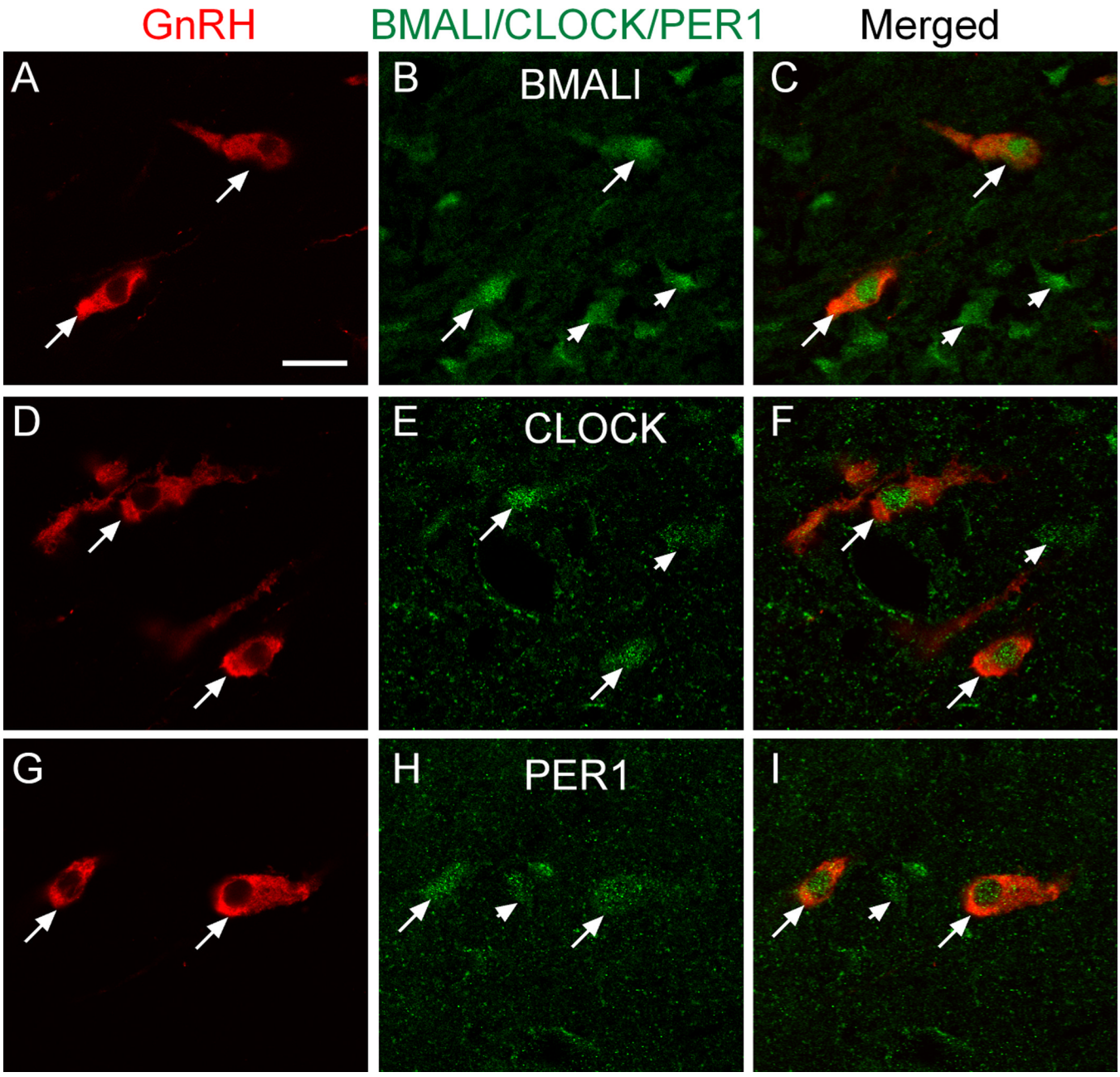

Figure 5. GnRH neurons of the rat POA contain the core clock proteins BMAL1, CLOCK and PER1 as detected by double immunohistofluorescence

A–C, BMAL1; **D–F**, CLOCK; **G–I**, PER1. Arrows point to examples of colocalization. Arrowhead in **H** points to a PER1 positive cell lacking GnRH immunoreactivity. Notice the nuclear localization of BMAL1, CLOCK and PER1. Bar = 10 μm.

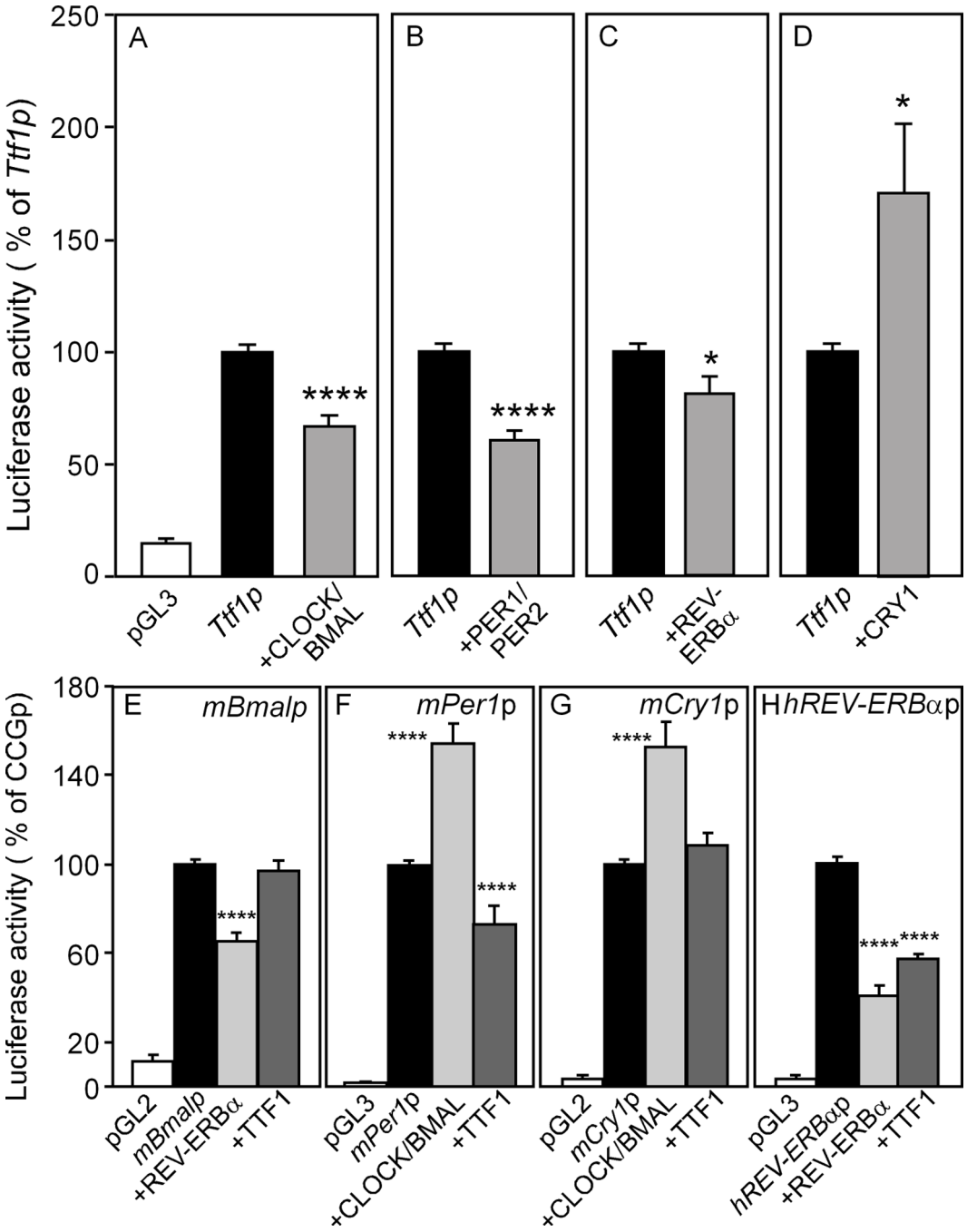

Figure 6. Effect of clock proteins on *Ttf1* promoter activity and effect of TTF1 on the transcription of core clock genes (CCGs) in NHI 3T3 cells

A–D, mBMAL/mCLOCK, mPER1/mPER2 and mREV-ERBα repress the *Ttf1* promoter, but CRY1 increases *Ttf1* transcription. The rat *Ttf1* promoter was used at 500ng/well. The expression vectors (mBMAL1-pcDNA3.1, mCLOCK-pcDNA3, mREV-ERBα-pcDNA3, mPer1-pcDNAV5, mPer2-pcDNAV5 and mCry1V5-pcDNA) were used at 250ng/well. The total DNA amount/well was kept constant at 1.1 μg/well by adding the appropriate amount of pcDNA3.1 to each well. Results (mean±SEM) are expressed as percentage of *Ttf1* promoter-driven luciferase activity alone. T test, Ttf1p vs CCG, *, p<0.05; ****, p<0.0001. n=16/condition (3 different experiments). **E–H**, TTF1 represses the *Per1* and *Rev-erbα*

promoters (F,H), without affecting the transcriptional activity of *Bmal1* and *Cry1* (E,G). The responsiveness of each CCG gene promoter (*CCG*p) was verified by co-transfecting plasmids encoding clock proteins known to modify the transcriptional activity of each promoter being tested, i.e. mREV-ERBα for the m*Bmal* and h*Rev-erbα* promoters (E, H) and mBMAL/mCLOCK for the m*Cry1* and m*Per1* promoters (F, G). Results (mean±SEM) are expressed as percentage of each promoter-driven luciferase activity alone. One-way ANOVA, experimental condition vs control (*CCG*p) **, $p<0.01$; ***, $p<0.001$;. n=18–24/condition (4–7 different experiments) except for (F), n=6–12, (2 different experiments). TTF1-pcDNA was used at 500 ng/well; *mBmal1*-pGL2 basic, *hREV-ERBα*-pGL2, *mCry1*-pGL3 and *mPer1*-pGL3 were used at 100ng/well; mREV-ERBα-pcDNA3, mBMAL1-pcDNA3.1 and mCLOCK-pcDNA3.1 at 250ng/well. Total DNA amount/well was kept constant at 1.2 μg/well by adding the appropriate amount of pcDNA3.1 to each well.

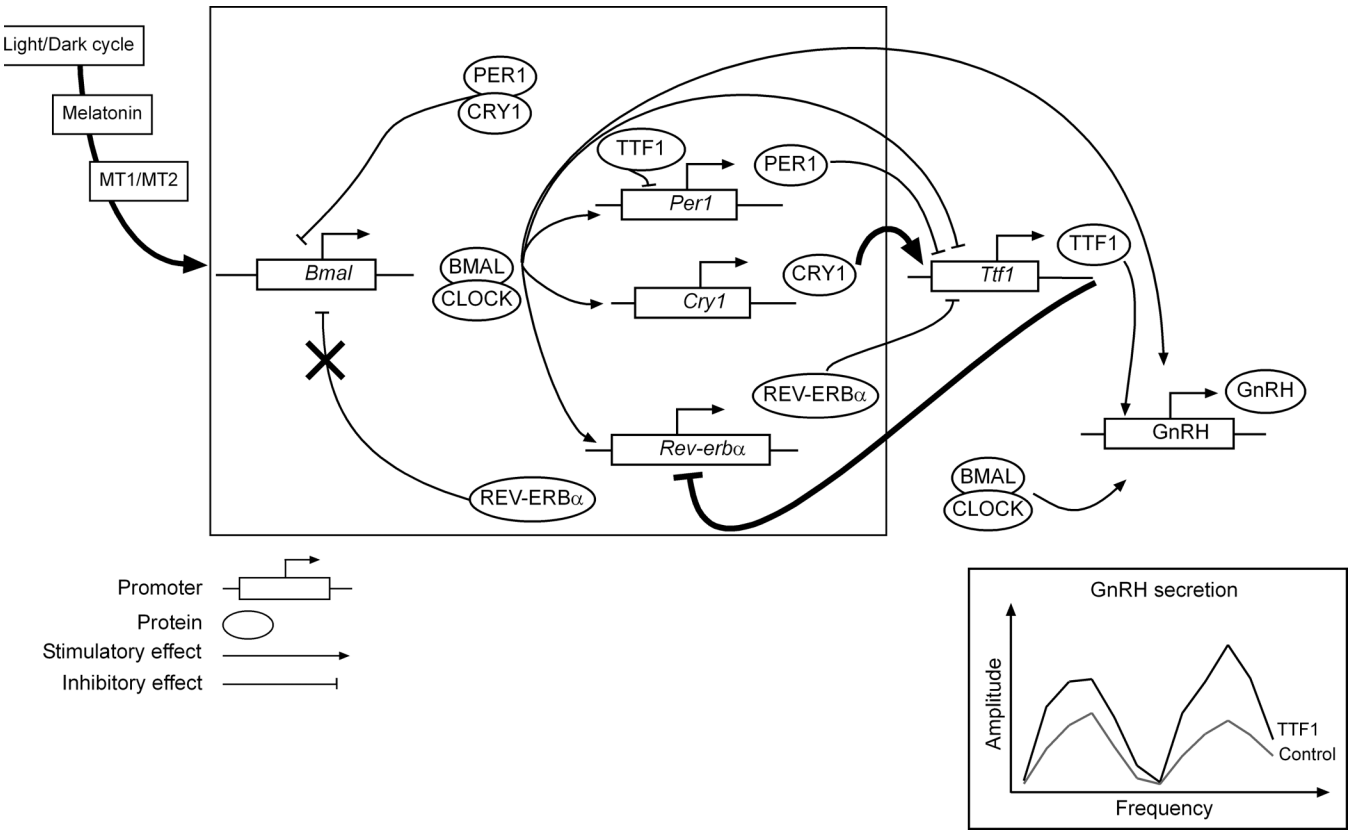

Figure 7. A draft model of TTF1 interactions with core clock genes to regulate circadian changes in GnRH promoter activity within GnRH neurons

The (simplified) transcriptional model includes the well established rhythmic secretion of BMAL1 that, upon dimerization with CLOCK, stimulates the transcription of REV-ERBα PER1/2 and CRY1/2. For the sake of clarity, and according to our own experiments, only *Per1* and *Cry1* promoters are represented). In turn, these factors inhibit the transcription of BMAL1, which then leads to their own transcriptional downregulation in what is known as the inhibitory limb of the circadian clock (17,66). In GnRH neurons, this rhythmic expression of clock gene is thought to be involved in the manifestation of circadian GnRH secretion (21–23). Our data indicate that daily changes in melatonin secretion entrain the diurnal expression of *Ttf1* and *GnRH* mRNA, acting via MT1 and perhaps also MT2 receptors. In addition of a direct effect of TTF1 and CLOCK/BMAL on GnRH transcription, TTF1 may be part of a secondary regulatory interloop (thick lines) in which, on the one hand, CRY1 stimulates the transcription of Ttf1 while, on the other hand, TTF1 inhibits REV-ERBα transcription. It is speculated that this regulatory loop underlies the ability of TTF1 to increase GnRH transcription in a circadian fashion by increasing the amplitude, and not the period, of clock-driven circadian GnRH promoter activity.