Cellular/Molecular

p75 Neurotrophin Receptor Is a Clock Gene That Regulates Oscillatory Components of Circadian and Metabolic Networks

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The p75 neurotrophin receptor (p75^{NTR}) is a member of the tumor necrosis factor receptor superfamily with a widespread pattern of expression in tissues such as the brain, liver, lung, and muscle. The mechanisms that regulate *p75^{NTR}* transcription in the nervous system and its expression in other tissues remain largely unknown. Here we show that *p75^{NTR}* is an oscillating gene regulated by the helix-loop-helix transcription factors CLOCK and BMAL1. The *p75^{NTR}* promoter contains evolutionarily conserved noncanonical E-box enhancers. Deletion mutagenesis of the *p75^{NTR}*-luciferase reporter identified the −1039 conserved E-box necessary for the regulation of *p75^{NTR}* by CLOCK and BMAL1. Accordingly, gel-shift assays confirmed the binding of CLOCK and BMAL1 to the *p75$^{NTR-1039}$* E-box. Studies in mice revealed that *p75^{NTR}* transcription oscillates during dark and light cycles not only in the suprachiasmatic nucleus (SCN), but also in peripheral tissues including the liver. Oscillation of *p75^{NTR}* is disrupted in Clock-deficient and mutant mice, is E-box dependent, and is in phase with clock genes, such as *Per1* and *Per2*. Intriguingly, *p75^{NTR}* is required for circadian clock oscillation, since loss of *p75^{NTR}* alters the circadian oscillation of clock genes in the SCN, liver, and fibroblasts. Consistent with this, *Per2::Luc/p75$^{NTR-/-}$* liver explants showed reduced circadian oscillation amplitude compared with those of *Per2::Luc/p75$^{NTR+/+}$*. Moreover, deletion of *p75^{NTR}* also alters the circadian oscillation of glucose and lipid homeostasis genes. Overall, our findings reveal that the transcriptional activation of *p75^{NTR}* is under circadian regulation in the nervous system and peripheral tissues, and plays an important role in the maintenance of clock and metabolic gene oscillation.

Introduction

The p75 neurotrophin receptor (p75^{NTR}), a member of the tumor necrosis factor receptor (TNFR) superfamily, is expressed in the nervous system and many peripheral tissues (Lomen-Hoerth and Shooter, 1995) such as white adipose tissue (Peeraully et al., 2004), skeletal muscle (Deponti et al., 2009), and liver (Passino et al., 2007). p75^{NTR} participates in multiple intracellular signaling pathways to regulate a wide range of biologic functions, including sensory neuron development (Lee et al., 1992), liver and muscle regeneration (Passino et al., 2007; Deponti et al., 2009), extracellular matrix remodeling (Sachs et al., 2007), hypoxia and angiogenesis (Le Moan et al., 2011), and glucose metabolism (Baeza-Raja et al., 2012). Although p75^{NTR} has a wide range of expression profiles and biological functions, the physiologic mechanisms of its transcriptional regulation in the brain and peripheral tissues are still unknown. Previous studies of the *p75^{NTR}* promoter revealed the presence of multiple GC elements (Sehgal et al., 1988) that can be activated by Specificity protein 1 (Sp1) transcription factors (Poukka et al., 1996). Sp1 binding induces *p75^{NTR}* expression after osmotic swelling (Peterson and Bogenmann, 2003; Ramos et al., 2007). In addition to Sp1, early growth response factors 1 and 3 have been identified as direct modulators of p75^{NTR} (Gao et al., 2007). However, regulation by these transcription factors alone is not sufficient to explain the pleiotropic expression pattern of p75^{NTR} in central and peripheral tissues.

We recently demonstrated that p75^{NTR} is a unique regulator of metabolic functions, including glucose homeostasis and insulin sensitivity (Baeza-Raja et al., 2012). A common denominator of genes regulating metabolic functions is their regulation by circadian clocks, and conversely metabolic dysfunction is strongly associated with the disruption of circadian clocks (Dibner et al., 2010; Asher and Schibler, 2011; Solt et al., 2012). Similar to *p75$^{NTR-/-}$* mice, mutant mice of the main circadian regulators, *Clock* and *Bmal1*, display increased insulin sensitivity and

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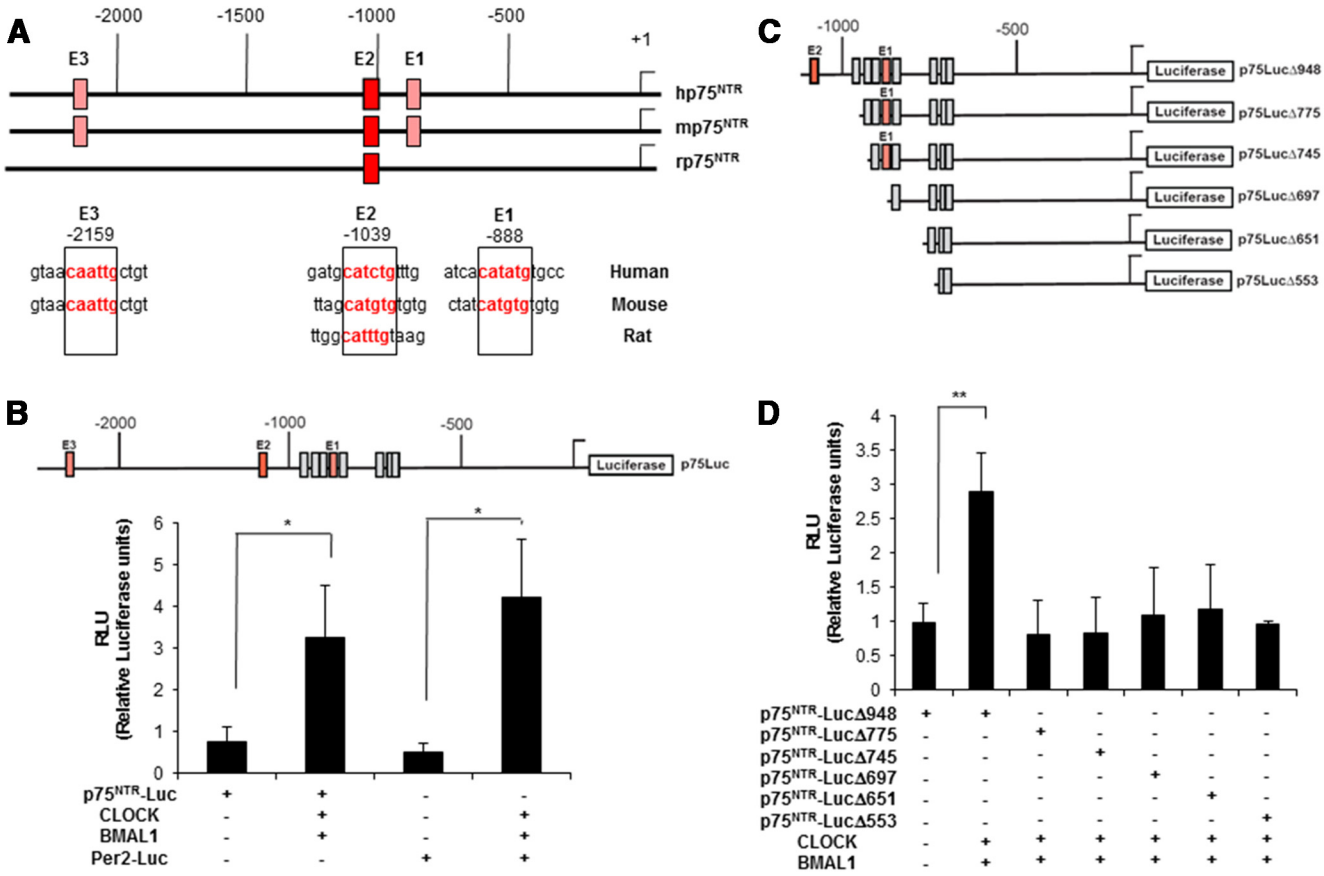

Figure 1. The 5′ regulatory region of human, mouse, and rat p75 ^{NTR} contains evolutionarily conserved E-boxes that are activated by CLOCK and BMAL1. ***A***, Top, Schematic representation of the 2.5 kb upstream region of the human, mouse, and rat p75 ^{NTR} promoter. Putative evolutionarily conserved E-box positions are shown with red boxes. Bottom, Aligned nucleotide sequences of the evolutionarily conserved human, mouse, and rat E-boxes. Alignment of the p75 ^{NTR} promoter was performed using the Clustal 2.0.3 multiple sequence alignment program. ***B***, Top, Schematic representation of the 2.2 kb p75 ^{NTR} promoter cloned in the luciferase reporter vector pGL4.17. Red indicates the evolutionarily conserved E-boxes, while the gray boxes identify other nonconserved E-boxes. Bottom, Histogram of luciferase activity in HEK293 cells transfected with pGL4-p75 ^{NTR}-Luc or Per2-luc in the presence or absence of CLOCK and BMAL1 expression vectors. Luciferase activities are expressed relative to the activity of the reporter constructs alone, which were given a value of 1 (*$p < 0.05$, *t* test). ***C***, Schematic representation of the deletion constructs generated from the 2.2 kb hp75 ^{NTR} promoter and cloned into the luciferase reporter vector pGL4.17. ***D***, Histogram of luciferase activity in HEK293 cells transfected with the different constructs of the hp75 ^{NTR} promoter in the presence of CLOCK and BMAL1 expression vectors. Luciferase activities are expressed relative to the activity of the reporter constructs alone, which were given a value of 1. Results of at least three independent experiments with duplicate measurements are shown. Error bars represent the SEM of the mean value. Statistical significance denoted by asterisks is relative to respective control (*$p < 0.05$; **$p < 0.01$, *t* test).

altered gluconeogenesis (Rudic et al., 2004), while mice lacking *Bmal1*, specifically in liver, show hypoglycemia and increased glucose tolerance (Lamia et al., 2008). Moreover, knock-out mice for *Clock* and *Bmal1* target genes *Cry1* and *Cry2* also show impaired liver regeneration (Matsuo et al., 2003). Because p75 ^{NTR} is widely expressed, and regulates glucose homeostasis and insulin sensitivity—metabolic processes highly influenced by circadian clocks—we hypothesized that the transcriptional regulation of p75 ^{NTR} might be controlled by circadian oscillators.

In this study, we show that *p75^{NTR}* is an oscillating gene regulated by the circadian regulators CLOCK and BMAL1. The 5′ regulatory region of p75 ^{NTR} contains evolutionarily conserved noncanonical E-boxes to which the CLOCK/BMAL1 complex binds and induces p75 ^{NTR} transcriptional activity. We find that p75 ^{NTR} RNA expression oscillates, in an E-box-dependent manner, not only in fibroblasts after serum shock, but also *in vivo* in the suprachiasmatic nucleus (SCN) and liver. In accordance, we show that p75 ^{NTR} oscillation is disrupted in the SCN and liver from *Clock*$^{-/-}$ (DeBruyne et al., 2006) and *Clock*$^{\Delta 19}$ (Vitaterna et al., 1994) mutant mice. Although genetic loss of p75 ^{NTR} does not affect locomotor activity rhythms, it disrupts the expression of circadian clock genes in fibroblasts, SCN, and liver, and also affects the oscillation of genes involved in glucose and lipid homeostasis. Overall, our study identifies p75 ^{NTR} as a first-order clock-controlled gene that oscillates in central and peripheral tissues, and its expression is necessary to maintain circadian and metabolic gene oscillation.

Materials and Methods

Animals. Wild-type (WT) C57BL/6J, *p75$^{NTR-/-}$* (Lee et al., 1992), and *Per2::Luc* (Yoo et al., 2004) mice were obtained from The Jackson Laboratory. *p75$^{NTR-/-}$* and *Per2::Luc* mice were in C57BL/6 background. *Clock*$^{-/-}$ (DeBruyne et al., 2006) and *Clock*$^{\Delta 19}$ (Vitaterna et al., 1994) mice in C57BL/6 and BALB/cJ background, respectively, were also used. Crossings between *Per2::Luc* and *p75$^{NTR-/-}$* mice were performed to generate *Per2::Luc/p75$^{NTR-/-}$* and their littermate controls *Per2::Luc/p75$^{NTR+/+}$*. *In vivo* studies were performed using male mice between 8 and 12 weeks of age. Mice were housed under a 12 h light/dark cycle, were fed standard chow, and had access to food and water *ad libitum*. All animal experiments were performed under the guidelines set by the University of California San Francisco and Irvine Institutional Animal Care and Use Committees and are in accordance with those set by the National Institutes of Health.

Cell culture. HEK293 cells and primary mouse embryonic fibroblasts (MEFs) derived from WT and *p75$^{NTR-/-}$* embryos were cultured in

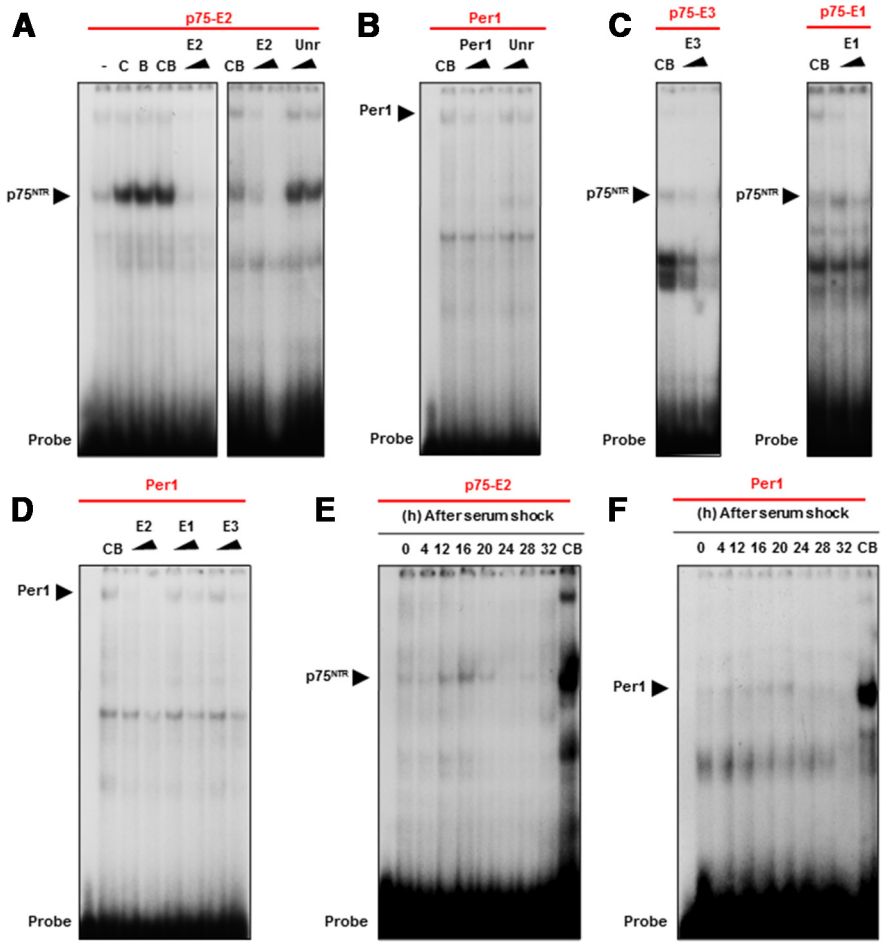

Figure 2. CLOCK/BMAL1 heterodimers bind to the E-boxes of the p75 ^{NTR} promoter *in vitro*. ***A–F***, EMSAs were performed using nuclear extracts of HEK293 cells cotransfected with CLOCK and BMAL1 (***A–D***) or MEFs derived from WT mice (***E***, ***F***) on the different E-boxes (−2159, −1039 and −888 bp) from the human p75 ^{NTR} and Per1 promoter as probes. CB means CLOCK and BMAL1 (CB) coexpression, a triangle indicates increasing amount of competitor (twofold or 10-fold) of the relevant E-box as indicated by the triangles, and a specific complex is indicated by black arrow. ***A***, EMSAs performed on the E-box located at −1039 bp (E2) in the human p75 ^{NTR} promoter as probe. Nuclear extracts from HEK293 cells transfected with an empty vector (-), CLOCK (C), BMAL1 (B) and CLOCK/BMAL1 (CB) expression vectors were used. ***B***, EMSA performed on the E-box from the human Per1 promoter as probe. ***C***, EMSAs performed on the E-boxes −2159 (E3) and −888 bp (E1) from the human p75 ^{NTR} promoter as probe. ***D***, EMSA performed on the E-box from the human Per1 promoter as probe. ***E***, EMSA performed on the E-box located at −1039 bp (E2) in the human p75 ^{NTR} promoter as probe. Nuclear extracts from WT MEFs were collected at the indicated times (h) after serum treatment. ***F***, EMSA performed on the E-box from the human Per1 promoter as probe. Nuclear extracts from WT MEFs derived from WT mice were collected at the indicated times (h) after serum treatment.

DMEM supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin/streptomycin (Invitrogen). Isolation of MEFs was performed as described previously (Russell et al., 2002). Briefly, embryos were isolated at E12–E14, the heads and internal organs were removed, and the remaining embryo was minced with scissors and kept on ice in PBS. Minced embryos were centrifuged for 5 min at 500 × *g* and trypsinized for 20 min at 37°C with occasional shaking with 1× Trypsin-EDTA. Trypsin was deactivated by the addition of DMEM with 10% FBS. MEFs were then pelleted by centrifugation at 500 × *g* for 5 min. Pellets were resuspended in DMEM with 10% FBS and 1% penicillin/streptomycin and then poured through a 70 μm filter onto a 10 cm culture plate.

DNA transfection and luciferase assays. HEK293 cells were transiently transfected with LipofectAMINE (Invitrogen) according to the manufacturer's protocol. After transfection, either with luciferase reporters with or without expression plasmids or with expression plasmids alone, the transfection medium was replaced by growth medium for 24 h. All transfections included constant amounts of β-galactosidase reporter plasmid as a control for transfection efficiency. The total amount of DNA for each transfection was kept constant using an empty expression vector. Cells were then harvested to measure luciferase activity (Promega). Luciferase activities were normalized by dividing luciferase activity by β-galactosidase activity. For each experimental group, a minimum of three independent transfections (in triplicate) was performed.

DNA constructs. The human p75 ^{NTR} promoter was subcloned into pGL4.17 (Promega) luciferase reporter vector to generate pGL4-p75Luc after PCR using oligonucleotides containing enzymatic restriction sites for XhoI and HindIII. Deletion constructs (pGL4-p75LucΔ948, pGL4-p75LucΔ775, pGL4-p75LucΔ745, pGL4-p75LucΔ697, pGL4-p75LucΔ651, and pGL4-p75LucΔ553) were also generated by PCR using oligonucleotides containing XhoI and HindIII restriction sites. The luciferase reporter vector containing the human Per2 promoter and the expression vectors for the human CLOCK and BMAL1 were used.

Serum shock treatment. Circadian induction of MEFs from WT and *p75* $^{NTR-/-}$ mice was performed on confluent cells as previously described (Balsalobre et al., 1998). Briefly, 50% of horse serum was added to the cells for 2 h and after the cells were incubated in DMEM without serum and collected at the indicated time points. Cells were then used for total RNA and nuclear extract isolation.

RNA isolation and RT-qPCR. Total RNA was isolated from MEFs, SCN, and liver using the standard RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA was reverse transcribed to cDNA using the GeneAmp RNA PCR Core Kit (Applied Biosystems) with random hexamers as primers according to the manufacturer's instructions. Real-time PCR was performed on a StepOnePlus real-time PCR system (Applied Biosystems) using an SYBR green PCR master mix (Applied Biosystems). Real-time PCR experiments were performed with the following primers: Per1, 5′-TGACACTGATGCAAACAGCA-3′ (forward) and 5′-AGCCACTGGTAGATGGGTTG-3′ (reverse); Per2, 5′-GGTGGACAGCCTTTCGATTA-3′ (forward) and 5′-AGGGCCTACCCTGACTTTGT-3′ (reverse); Clock, 5′-CACAGGGCACCACCAATAAT-3′ (forward) and 5′-CATATCCACTGCTGGCCTTT-3′ (reverse); Rorα, 5′-ACGCCCACCTACAACATCTC-3′ (forward) and 5′-TCACATATGGGTTCGGGTTT-3′ (reverse); Bmal1, 5′-CGAAGACAATGAGCCAGACA-3′ (forward) and 5′-AAATAGCTGTCGCCCTCTGA-3′ (reverse); reverse erythroblastosis virus-α (Rev-Erbα), 5′-CAGCTTCCAGTCCCTGACTC-3′ (forward) and 5′-GGAGGAGGAGGATGAAGAGG-3′ (reverse); Gapdh, 5′-CAAGGCCGAGAATGGGAAG-3′ (forward) and 5′-GGCCTCACCCCATTTGATGT-3′ (reverse); Pepck, 5′-CTTCTCTGCCAAGGTCATCC-3′ (forward) and 5′-AGTGAGAGCCAGCCAACAGT-3′ (reverse); Fas, 5′-TTGCTGGCACTACAGAATGC-3′ (forward) and 5′-AACAGCCTCAGAGCGACAAT-3′ (reverse); Glut4, 5′-GTGACTGGAACACTGGTCCTA-3′ (forward) and 5′-CCAGCCACGTTGCATTGTAG-3′ (reverse); D-site albumin promoter-binding protein (Dbp), 5′-ACCGTGGAGGTGCTAATGAC-3′ (forward) and 5′-TGGCTGCTTCATTGTTCTTG-3′ (reverse); nerve growth factor (NGF), 5′-CATGGGGGAGTTCTCAGTGT-3′ (forward) and 5′-GCACCCACTCTCAACAGGAT-3′ (reverse); brain-derived neurotrophic factor (BDNF), 5′-GCGGCAGATAAAAAGACTGC-3′ (forward) and 5′-CTTATGAATCGCCAGCCAAT-3′ (reverse); tropomyosin-related kinase A (TrkA), 5′-AGGTCTTTCTCGCTGAGTGC-3′ (forward) and 5′-GGTGCAGACTCCAAAGAAGC-3′ (reverse); TrkB, 5′-GACCTGATCCTGACGGGTAA-3′ (forward) and

5′-TTCCTCCACGGTGAGGTTAG-3′ (reverse); TrkC, 5′-CTTCGGGAAT TGAGACTGGA-3′ (forward) and 5′-TGGCTCACACTGATCTCTGG-3′ (reverse), FAS-TNFα, 5′-TGTGAACATGGAACCCTTGA-3′ (forward) and 5′-TTCAGGGTCATCCTGTCTCC-3′ (reverse), and $p75^{NTR}$ was determined using oligonucleotides obtained from SABiosciences. PCR efficiencies of the primers were calculated by serial dilution of the template, and no significant differences in efficiency were found between the target genes and the housekeeping genes. Results were analyzed with the Opticon 2 Software using the comparative C_T method, as described previously (Livak and Schmittgen, 2001). Data were expressed as $2^{-\Delta\Delta C_T}$ for the experimental gene of interest normalized against the housekeeping gene and presented as the fold change versus the relevant control. Each real-time PCR was performed in triplicate and repeated at least three times.

Electrophoretic mobility shift assays. Nuclear extracts were obtained from HEK293 cell lines transfected with various plasmids and MEFS derived from WT mice after serum treatment. Nuclear protein extraction was performed as described previously (De Cesare et al., 1995). Briefly, cells were washed twice in cold PBS and scraped, and the cellular pellet was resuspended in 10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA, and 0.5 mM dithiothreitol (DTT) on ice. Cells were passed five times through a 26-gauge needle and centrifuged to collect nuclei, which were subsequently resuspended in an equal volume of 10 mM HEPES, pH 7.9, 0.4 M NaCl, 1.5 mM MgCl2, 0.1 mM EGTA, 0.5 mM DTT, and 5% glycerol, to allow elution of nuclear proteins by gentle shaking at 4°C for 30 min. Nuclei were pelleted at 14,000 rpm for 5 min at 4°C, and the supernatant was aliquoted, snap frozen in liquid nitrogen, and stored at −80°C until use. All solutions contained protease and phosphatase inhibitors (Calbiochem). A protein assay (Bio-Rad) was used to determine protein concentration. For electrophoretic mobility shift assays, 10 μg of nuclear extracts were incubated in 50 mM Tris-HCl, pH 7.9, 12.5 mM MgCl2, 1 mM EDTA, 1 mM DTT, 20% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 2 μg of polydI-dC for 10 min at room temperature to titrate out nonspecific binding before the addition of a 15,000–20,000 cpm labeled oligonucleotide. The reaction was then further incubated for 20 min at room temperature. When unlabeled competing oligonucleotides were added, nuclear extracts were preincubated for 30 min or 1 h at room temperature before addition of the labeled probe. Samples were loaded onto a prerun polyacrilamide gel (29:1 in 0.25× Tris borate-EDTA) and electrophoresed at 200 V. Gels were dried and autoradiographed at −80°C. For control electrophoretic mobility shift assay (EMSA) probes, we used the huPer1 E-box probe 5′-CAC CCA CCG GTC ACA CGT GGA CCC TTA ACT GT-3′ and the unrelated probe NF-κB probe: 5′-AGT TGA GGG GGA CTT TCC CAG GC-3′. For the $hup75^{NTR}$ E-box EMSA probes, we designed probes for E3, 5′-CTA ATA ATA GTA ACA ATT GCT GTT TGT AAT TG-3′; E2, 5′-ATT GTT GAA GAT GCA TCT GTT TGT TTG TTG AT-3′; E1, 5′-AAT GTC CGG ATC ACA TAT GTG CCC GTG TGC AT-3′; and E_{NC}, 5′-TGC ACT GTG TGC ACA CCT GTG ACC CCT TCA AA-3′.

Per2::Luc *bioluminescence experiments and data analysis.* SCN and liver explants from *Per2::Luc/*$p75^{NTR+/+}$ and *Per2::Luc/*$p75^{NTR-/-}$ mice were cultured as described previously (Yamazaki and Takahashi, 2005). Briefly, mice were killed 1 h into the light phase, and the livers were isolated and dissected into small pieces. Luciferase activity from sealed cultures maintained at 37°C was monitored for several days with a 32-channel Luminometer (Actimetrics). Period lengths and amplitude were determined in plates with good oscillation using Lumicycle Analysis software. All explants from each animal were monitored simultaneously, and all experiments included explants from *Per2::Luc/*$p75^{NTR+/+}$ and *Per2::Luc/*$p75^{NTR-/-}$ mice to allow for direct comparisons between genotypes.

Mouse behavioral analysis. Wheel-running activity monitoring was performed as described previously (Xu et al., 2005). Briefly, 3- to 4-month-old mice were housed individually in cages equipped with running wheels, and exposed to a 12 h light/dark cycle for >14 d before being released into constant darkness. Using ClockLab (Actimetrics) software, the period and amplitude of activity rhythms was calculated from χ^2 periodogram.

Statistics. Statistical significance was calculated using JMP2 Software by unpaired *t* test for isolated pairs or by one-way ANOVA for multiple comparisons followed by Bonferroni's correction for comparisons of means. Data are shown as the mean ± SEM.

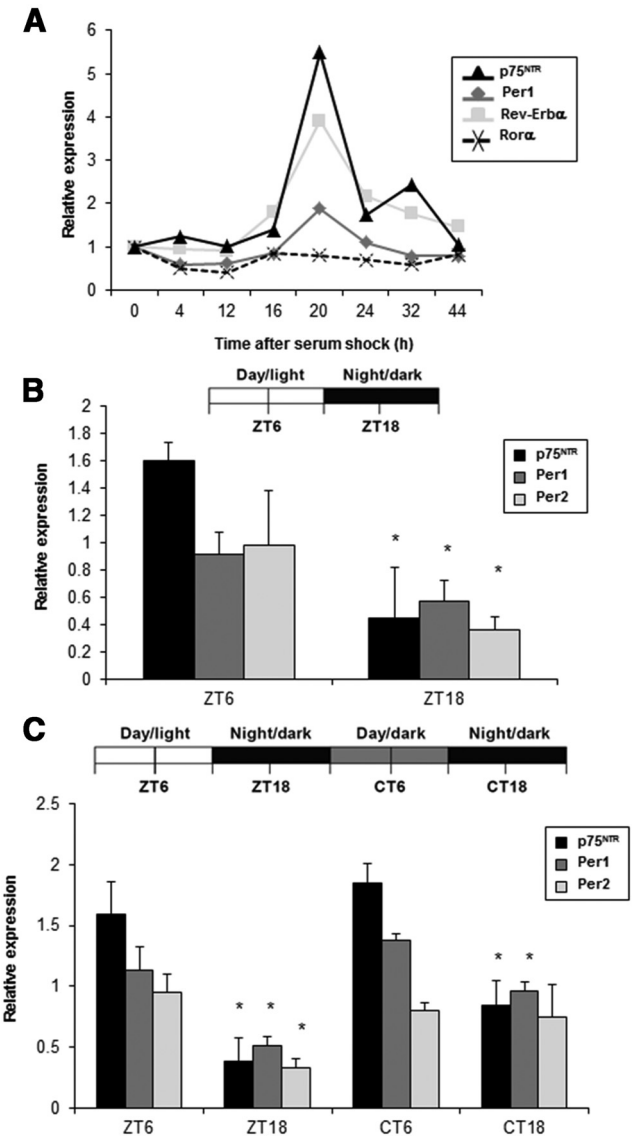

Figure 3. *$p75^{NTR}$* RNA oscillates *in vitro* and *in vivo*. ***A***, Serum shock treatment was performed on MEFs derived from WT mice, and cells were collected at the indicated times (h). *$p75^{NTR}$*, *Per1*, *Rev-Erbα*, and *RORα* mRNA levels were analyzed by real-time PCR. Data are presented as the relative expression level with respect to time 0, which was given a value of 1. ***B***, Top, Schematic representation of the light schedule. Mice were entrained in a 12 h light/dark cycle and killed at ZT6 and ZT18 ($n = 5$ per group). Bottom, At the indicated times, the SCN was dissected; RNA was extracted; and *$p75^{NTR}$*, *Per1*, and *Per2* levels analyzed by real-time PCR. ***C***, Top, Schematic representation of the light schedule. Mice entrained in a 12 h light/dark cycle were transferred to DD. Mice were killed at ZT6, ZT18, CT6, and CT18 ($n = 5$ per group). Bottom, At the indicated times, the SCN was dissected, mRNA extracted and *$p75^{NTR}$*, *Per1*, and *Per2* levels analyzed by real-time PCR. Results of at least three independent experiments with duplicate measurements are shown. Error bars represent the SEM of the mean value. Statistical significance denoted by asterisks is relative to the expression obtained during the day/subjective day (*$p < 0.05$, *t* test). ZT, Zeitgeber time; CT, circadian time.

Results

The 5′ regulatory region of $p75^{NTR}$ contains evolutionarily conserved noncanonical E-boxes that are activated by CLOCK and BMAL1

To examine the physiologic mechanisms that regulate $p75^{NTR}$, we analyzed the *$p75^{NTR}$* promoter using MatInspector 7.7.3, a program for the analysis and prediction of transcription factor

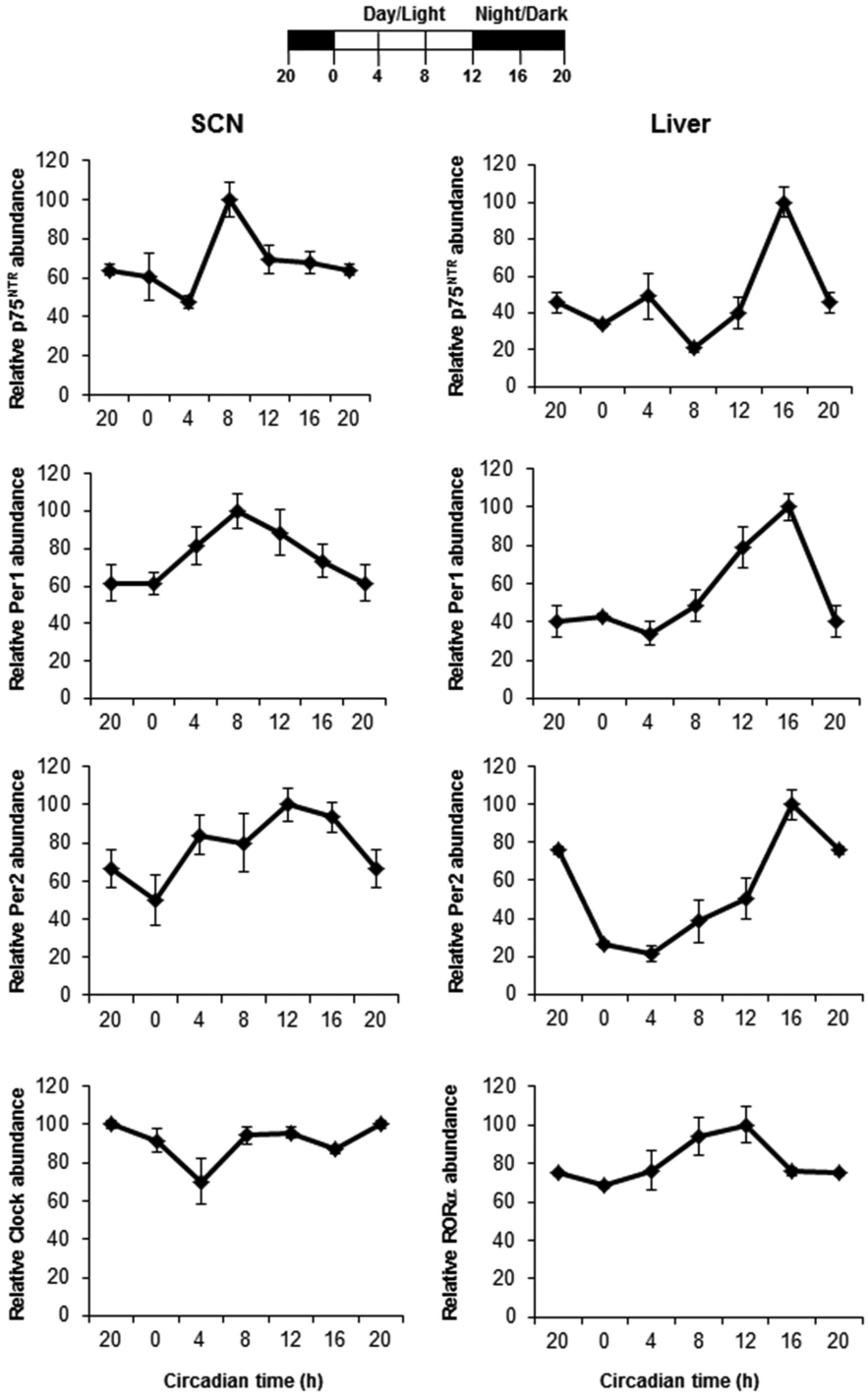

Figure 4. *p75^{NTR}* RNA oscillates in a circadian manner in the SCN and liver. Top, Schematic representation of the light schedule. Mice were entrained in a 12 h light/dark cycle and were killed at 4 h intervals ($n = 4$ per group). Bottom, At the indicated times, the SCN (left) and liver (right) were dissected; RNA was extracted; and the levels of *p75^{NTR}*, *Per1*, and *Per2*, together with nonoscillating control genes such as *Clock* for the SCN and *Rorα* for the liver, were analyzed by real-time PCR. The results of at least three independent experiments with duplicate measurements are shown. Data from CT20 are double plotted. Error bars represent the SEM of the mean, where the maximum RNA amount was set to 100. CT, Circadian time.

binding sites. Computational analysis revealed the presence of several putative E-boxes (5′-CANNTG-3′) in the human promoter sequence of *p75^{NTR}*. Using the multiple sequence alignment program Clustal 2.0.3, alignment of the *p75^{NTR}* promoter sequences from human, mouse, and rat identified three noncanonical E-boxes that were conserved at base pair positions −888 (E1), −1039 (E2), and −2159 (E3) in the human *p75^{NTR}* promoter region (Fig. 1*A*). E-boxes E1 and E3 were conserved between human and mouse, while E-box E2 at position −1039 was the only one conserved in human, mouse, and rat (Fig. 1*A*).

E-boxes are 5′-CANNTG-3′ DNA core sequences that bind transcription factors of the basic helix-loop-helix (bHLH) family. Binding of the bHLH transcription factors CLOCK and BMAL1 to E-box elements in the promoters of clock genes drives their rhythmic transcription (Gekakis et al., 1998; Travnickova-Bendova et al., 2002; Yoo et al., 2005). Since the *p75^{NTR}* promoter contains three putative, evolutionarily conserved noncanonical E-boxes, we hypothesized that p75^{NTR} could be under circadian regulation. To investigate this further, we generated a *p75^{NTR}* promoter–luciferase reporter construct, which contains both conserved and nonconserved noncanonical E-boxes (*p75-Luc*). To examine whether CLOCK/BMAL1 activates the *p75^{NTR}* promoter, cells were transfected with *p75^{NTR}-Luc* and cotransfected with CLOCK and BMAL1 expression vectors (Fig. 1*B*). A *Per2* promoter-luciferase reporter (*Per2-Luc*) was used as a positive control. As expected, cotransfection with CLOCK and BMAL1 activated the circadian oscillating gene *Per2* (Fig. 1*B*). Strikingly, cotransfection of CLOCK and BMAL1 increased transcription of the *p75^{NTR}* reporter (Fig. 1*B*). To identify which E-boxes contribute to the regulation of *p75^{NTR}* transcription by CLOCK/BMAL1, we generated luciferase–reporter deletion constructs of the *p75^{NTR}* promoter (Fig. 1*C*). CLOCK- and BMAL1-induced transcription of the full-length *p75^{NTR}*-luciferase reporter that contains the −1039 E2-box, but not the deletion constructs that contain either the −888 E1-box or the nonconserved E-boxes (Fig. 1*D*). These results suggest that the evolutionarily conserved noncanonical E2-box is necessary for the activation of *p75^{NTR}* by the transcription factors CLOCK and BMAL1.

The CLOCK/BMAL1 heterodimer binds to the E2-box of the p75^{NTR} promoter

To determine whether CLOCK and BMAL1 bind to the *p75^{NTR}* E2-box, we generated oligonucleotide probes corresponding to the E2 region and performed EMSAs using nuclear extracts of cells transfected with CLOCK and BMAL1. CLOCK/BMAL1 heterodimers showed strong binding to the *p75^{NTR}* E2-box probe (Fig. 2*A*). Binding was reduced by the addition of a molar excess of unlabeled *p75^{NTR}* E2-box probe but not by an unrelated probe, thereby confirming specificity (Fig. 2*A*). Also, CLOCK/BMAL1 heterodimers showed binding to the *Per1* E-box probe (Fig. 2*B*), and the binding was

reduced by the addition of a molar excess of unlabeled *Per1* E-box probe, but not by exposure to an unrelated probe (Fig. 2*B*). In contrast, CLOCK/BMAL1 heterodimers bound weakly to $p75^{NTR}$ E1-box and E3-box probes (Fig. 2*C*). Moreover, unlabeled $p75^{NTR}$ E2-box probe was sufficient to compete for the binding of CLOCK/BMAL1 to a *Per1* E-box probe (Fig. 2*D*).

MEFs are commonly used for studies of mammalian circadian gene regulation, as serum shock treatment induces oscillatory expression of clock genes in these cells (Balsalobre et al., 1998). The $p75^{NTR}$ E2-box probe bound strongly to nuclear extracts of primary MEFs after serum shock (Fig. 2*E*). Strikingly, the $p75^{NTR}$ E2-box showed an oscillating binding pattern with the strongest signal observed 16 h after serum shock (Fig. 2*E*). The oscillating binding pattern of the $p75^{NTR}$ E2-box probe showed similar temporal regulation as the *Per1* E-box probe in MEFs (Fig. 2*F*). Altogether, these results demonstrate that CLOCK and BMAL1 bind to the evolutionarily conserved $p75^{NTR}$ E2-box in an oscillating manner.

$p75^{NTR}$ oscillates in the SCN and peripheral tissues

CLOCK/BMAL1 heterodimer regulates the oscillatory transcription of clock genes, such as *Per1*, *Per2*, and *Rev-erbα* (Darlington et al., 1998; Gekakis et al., 1998). Since the $p75^{NTR}$ E2-box probe showed an oscillating binding pattern in MEFs, we examined whether $p75^{NTR}$ expression oscillates. We induced clock gene oscillation in MEFs by serum shock and performed real-time PCR at different time points. Indeed, $p75^{NTR}$ showed an oscillating transcription pattern in phase with the clock genes *Per1* and *Rev-erbα* (Fig. 3*A*). As expected, the expression of *Rorα* showed no circadian oscillation after serum shock (Fig. 3*A*).

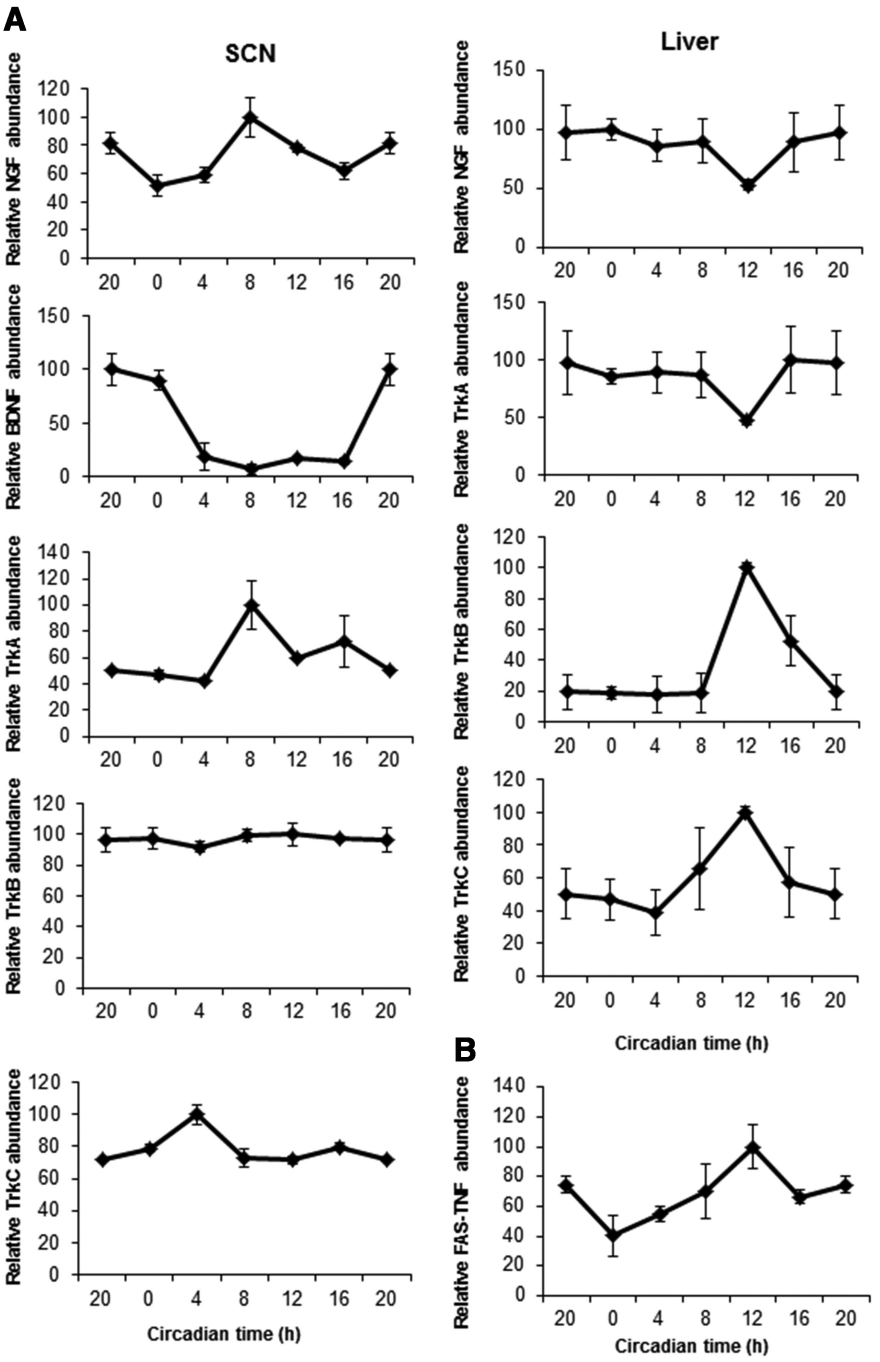

Figure 5. *NGF* and *TrkA* RNA oscillate in a circadian manner in the SCN and *TrkB* and *TrkC* in the liver. Mice were entrained in a 12 h light/dark cycle and were killed at 4 h intervals ($n = 4$ per group and genotype). ***A***, At the indicated times, the SCN (left) and liver (right) were dissected; RNA was extracted; and the levels of *NGF*, *BDNF*, *TrkA*, *TrkB*, and *TrkC* were analyzed by real-time PCR. ***B***, RNA from SCN was extracted and the level of *FAS-TNFα* was analyzed by real-time PCR at the indicated times. Results of at least three independent experiments with duplicate measurements are shown. Data from CT20 are double plotted. Error bars represent the SEM of the mean, where the maximum RNA amount was set to 100. CT, Circadian time.

In mammals, the circadian timing system has a hierarchical architecture composed of a central clock in the SCN and subsidiary oscillators in virtually all cells of the body (Dibner et al., 2010). External cues like light signals stimulate and synchronize clock gene expression in the SCN (Quintero et al., 2003). Since $p75^{NTR}$ is expressed in the SCN (Moga, 1998a,b), we examined whether $p75^{NTR}$ oscillates in a light/dark cycle. Expression of $p75^{NTR}$ was higher during the light period and lower during the dark period, oscillating in phase with the clock genes *Per1* and *Per2* (Fig. 3*B*). To test whether the observed oscillation is driven by the endogenous clock system and not by exposure to light signals, $p75^{NTR}$ expression was analyzed in mice under constant darkness condition. $p75^{NTR}$ transcript levels displayed a robust circadian oscillation that was high during the subjective day and low during the subjective night (Fig. 3*C*), suggesting that $p75^{NTR}$ oscillation in the SCN is E-box dependent, and not light dependent in accordance with previous studies (Beaulé and Amir, 2001, 2002). As expected, similar results were obtained with E-box-dependent clock genes *Per1* and *Per2* (Fig. 3*C*).

Clock genes are not only expressed in the brain, but also in peripheral tissues (Schibler and Sassone-Corsi, 2002; Schibler, 2009). $p75^{NTR}$ is known to be expressed in several peripheral

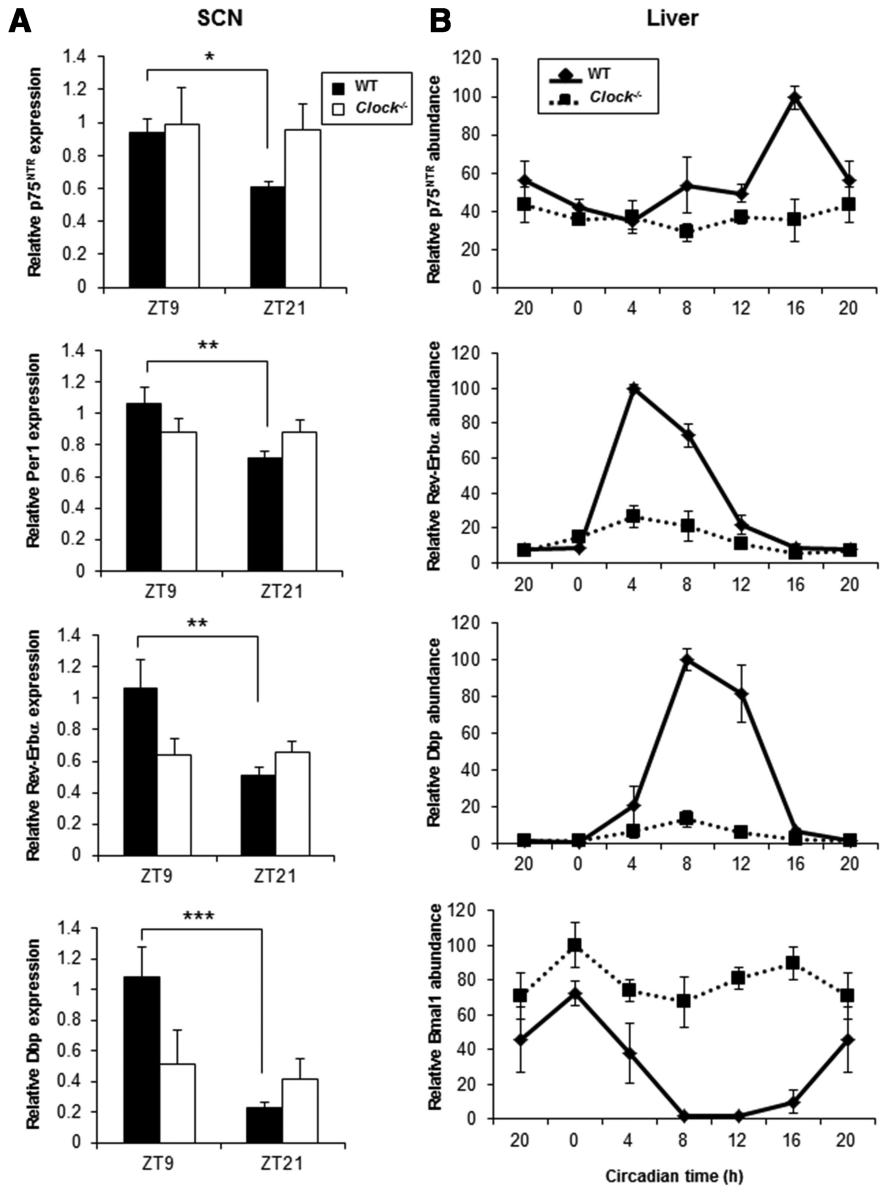

Figure 6. Genetic deletion of *Clock* disrupts $p75^{NTR}$ expression in the SCN and liver. ***A***, Wild-type mice ($Clock^{+/+}$) and $Clock^{-/-}$ mice were entrained in a 12 h light/dark cycle and killed at ZT9 and ZT21 ($n = 4$ per group and genotype). SCN was dissected; RNA was extracted; and $p75^{NTR}$, *Per1*, *Rev-Erbα*, and *Dbp* levels were analyzed by real-time PCR. ***B***, Wild-type mice ($Clock^{+/+}$) and $Clock^{-/-}$ mice were entrained in a 12 h light/dark cycle and killed at 4 h intervals ($n = 3$ per group and genotype). At the indicated times, liver was dissected; RNA was extracted; and $p75^{NTR}$, *Rev-Erbα*, *Dbp*, and *Bmal1* levels were analyzed by real-time PCR. Data from CT20 are double plotted. Error bars represent the SEM, where the maximum RNA amount was set to 100. Statistical significance denoted by asterisks is relative to the expression obtained during the day/light phase ZT9 (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, one-way ANOVA). ZT, Zeitgeber time; CT, circadian time.

tissues such as the liver (Trim et al., 2000; Cassiman et al., 2001; Passino et al., 2007), lung (Sachs et al., 2007), and kidney (Wheeler and Bothwell, 1992). To examine whether $p75^{NTR}$ oscillates in the periphery, we compared the expression of $p75^{NTR}$ and clock genes in the liver and SCN every 4 h during the light/dark cycle. In the SCN, $p75^{NTR}$ oscillates in phase with *Per1* (Fig. 4). Similar to the SCN, oscillation of $p75^{NTR}$ in liver was in phase with both *Per1* and *Per2* (Fig. 4). As expected, *Clock* in the SCN and *Rorα* in the liver did not oscillate (Fig. 4). These results indicate that $p75^{NTR}$ oscillates in a circadian time-specific manner in MEFs after serum shock as well as in the SCN and liver.

Oscillation of $p75^{NTR}$ is in phase with NGF and TrkA oscillations in the SCN

Since $p75^{NTR}$ binds to neurotrophin ligands (Roux and Barker, 2002; Chao, 2003; Reichardt, 2006; Underwood and Coulson, 2008), we examined the expression of NGF, BDNF, and their tropomyosin-related kinase (Trk) receptors A–C in the SCN and liver every 4 h during the light/dark cycle. Interestingly, in the SCN, *NGF* and *TrkA* oscillate in phase with $p75^{NTR}$ and *Per1* (Figs. 4, 5*A*), while *TrkB* and *TrkC* did not oscillate (Fig. 5*A*). In accordance with previous studies (Liang et al., 1998), *BDNF* expression in the SCN showed an oscillatory pattern with higher expression during the subjective night and lower during the subjective day (Fig. 5*A*). On the other hand, *NGF* and *TrkA* expression in liver did not oscillate (Fig. 5*A*), *BDNF* was undetectable, and *TrkB* and *TrkC* expression showed an oscillatory pattern with similar phase to *Per1* and *Per2* clock genes, peaking at 12 h (Fig. 5*A*), suggesting diverse oscillatory patterns for neurotrophins and their Trk receptors in the SCN and peripheral tissues. Since $p75^{NTR}$ is a member of the TNFR superfamily, we also examined whether the expression of other TNF receptors such as TNFR member 6 or FAS, and TNFR1B oscillate in the SCN. *FAS* expression showed an oscillatory pattern peaking at 12 h, while *TNFR1B* was undetectable (Fig. 5*B*), suggesting that oscillation of $p75^{NTR}$ is not in phase with other members of the TNFR superfamily. Overall, these results show that while neurotrophin ligands and Trk receptors differentially oscillate in the SCN and liver, $p75^{NTR}$ is unique among neurotrophin receptors in oscillating in phase with clock genes in both central and peripheral tissues.

Genetic deletion of *Clock* disrupts $p75^{NTR}$ oscillation in the SCN and liver

To study whether CLOCK/BMAL1 heterodimer is required for the circadian oscillation of $p75^{NTR}$, we analyzed $p75^{NTR}$ expression in SCN and liver from $Clock^{-/-}$ (DeBruyne et al., 2006) and $Clock^{\Delta 19}$ (Vitaterna et al., 1994) mice during the light/dark cycle. Consistent with binding of CLOCK/BMAL1 heterodimer to the $p75^{NTR}$ promoter (Fig. 2), $p75^{NTR}$ oscillation was disrupted in the SCN and liver from $Clock^{-/-}$ mice (Fig. 6*A*,*B*), suggesting that CLOCK is a critical regulator of $p75^{NTR}$ transcriptional regulation. We also tested known CLOCK-regulated genes as positive controls. In accordance with previous studies in $Clock^{-/-}$ mice (Preitner et al., 2002; DeBruyne et al., 2006), in the SCN *Per1*, *Rev-Erbα*, and *Dbp* showed reduced expression and disrupted oscillation (Fig. 6*A*), while in the liver *Per1* and *Rev-Erbα* were reduced and *Bmal1* was increased compared with WT mice (Fig. 6*B*). Although $Clock^{-/-}$

mice have defects in the amplitude of rhythmic gene expression of a subset of oscillatory genes (DeBruyne, 2008), $Clock^{\Delta 19}$ mutant mice show global deficits in circadian gene oscillation (Jin et al., 1999; Kume et al., 1999; Ripperger et al., 2000) because it functions as a dominant negative due to impaired $CLOCK^{\Delta 19}$/BMAL1 heterodimers (DeBruyne, 2008). Therefore, we also analyzed whether $p75^{NTR}$ expression and oscillation in SCN and liver from $Clock^{\Delta 19}$ mice were disrupted. Similar to the $Clock^{-/-}$ mice, $p75^{NTR}$ expression and oscillation was disrupted in the SCN and liver from $Clock^{\Delta 19}$ mice (Fig. 7). As expected, *Per1*, *Rev-Erbα*, and *Dbp* expression and oscillations were also disrupted (Fig. 7). Together, these results demonstrate that $p75^{NTR}$ is an oscillatory gene regulated by CLOCK/BMAL1 heterodimer in the SCN and liver.

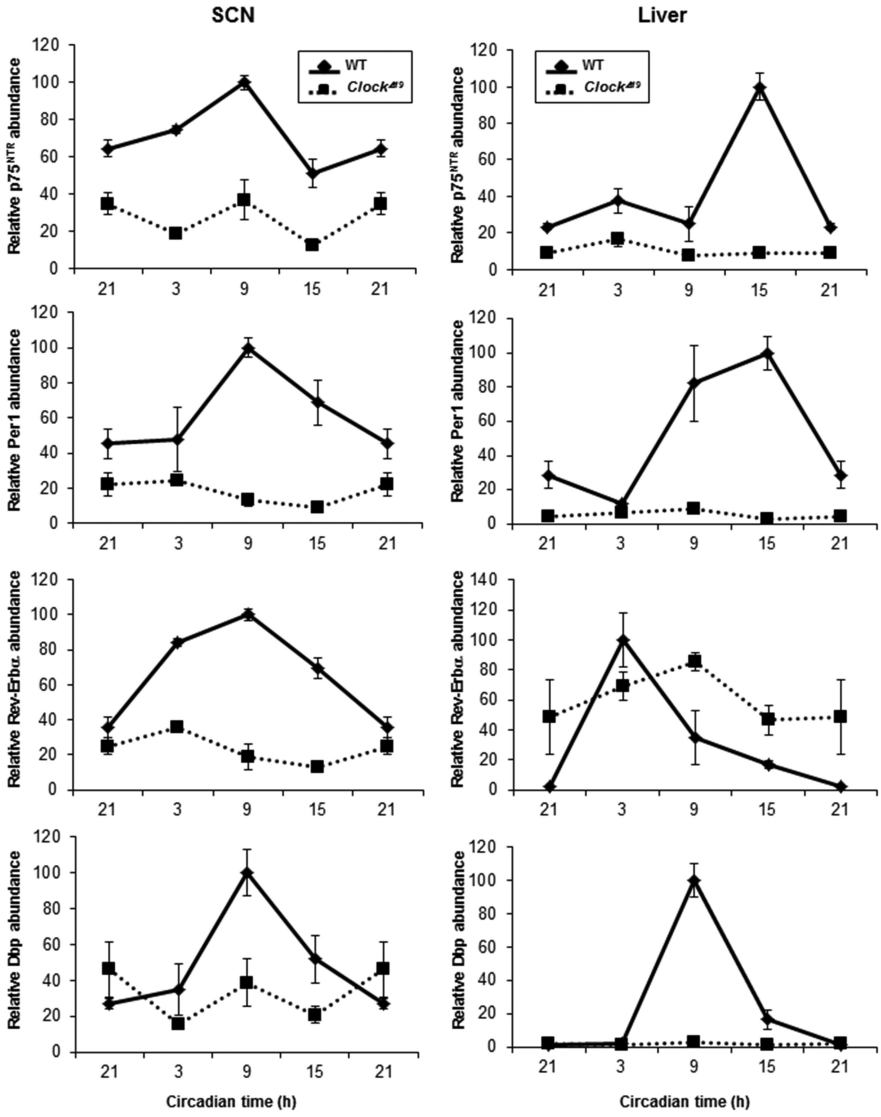

Figure 7. Genetic mutation of *Clock* disrupts $p75^{NTR}$ expression in the SCN and liver. Wild-type mice and $Clock^{\Delta 19}$ mice were entrained in a 12 h light/dark cycle and killed at 6 h intervals ($n = 3$ per group and genotype). At the indicated times, SCN (left) and liver (right) were dissected; RNA was extracted; and $p75^{NTR}$, *Per1*, *Rev-Erbα*, and *Dbp* levels were analyzed by real-time PCR. Data from CT21 are double plotted. Error bars represent the SEM of the mean, where the maximum RNA amount was set to 100. CT, Circadian time.

Loss of p75 ^{NTR} disrupts clock gene oscillation in the SCN, liver, and MEFs after serum shock

Although gene expression profiles obtained from microarray studies have revealed that 10–15% of all transcripts in different tissues display circadian oscillation (Akhtar et al., 2002; Duffield et al., 2002; Panda et al., 2002; Storch et al., 2002), only a subset of genes under circadian control are directly regulated by the binding of CLOCK/BMAL1 to their promoters. Therefore, the finding that $p75^{NTR}$ is an oscillatory gene regulated by CLOCK and BMAL1 suggests that $p75^{NTR}$ might play critical roles in circadian rhythms, either as a core clock protein or transducer of core oscillation. To examine the role of $p75^{NTR}$ in the regulation of circadian rhythms, we compared the expression of several clock genes in the SCN and liver of WT and $p75^{NTR-/-}$ mice every 4 h during the light/dark cycle, and also in MEFs derived from these mice after serum shock. Intriguingly, genetic deletion of $p75^{NTR}$ disrupted the transcriptional oscillations of clock genes such as *Per1* and *Per2* not only in the SCN (Fig. 8*A*), but also in liver (Fig. 8*B*). Consistent with these results, transcriptional clock gene oscillation of *Bmal1*, *Per1*, *Per2*, and *Rev-Erbα* in serum-shocked $p75^{NTR-/-}$ MEFs also caused a loss of the transcriptional oscillatory patterns (Fig. 8*C*), suggesting that $p75^{NTR}$ might be involved in the maintenance of the central and peripheral transcriptional oscillation of clock genes.

p75 $^{NTR-/-}$ mice maintain robust locomotor activity rhythms

The SCN is responsible for regulating locomotor activity rhythms (Reppert and Weaver, 2002; Lowrey and Takahashi, 2004). Mice with disrupted clock gene expression, such as $Clock^{\Delta 19}$ and $Bmal1^{-/-}$ show changes in period and amplitude in locomotor activity rhythms (Vitaterna et al., 1994; Bunger et al., 2000). On the other hand, several other mutant mice, such as $Clock^{-/-}$ and $NPAS2^{-/-}$ [neuronal Per-Arnt-Sim (PAS) domain containing protein 2], despite changes in gene expression, do not exhibit locomotor activity deficits (Dudley et al., 2003; DeBruyne et al., 2006). To determine the potential effects of p75 ^{NTR} deficiency on behavioral rhythmicity, we monitored wheel-running activity under constant darkness (DD) for ~2 weeks, following an initial 2 weeks in a 12 h light/dark cycle. $p75^{NTR-/-}$ mice displayed normal circadian patterns of behavior, with periodicities of 23.88 ± 0.03 (mean ± SEM), similar to those of WT mice (23.80 ± 0.02) (Fig. 9*A*,*B*). Despite the fact that $p75^{NTR-/-}$ mice showed increased activity levels, these differences were not statistically significant (Fig. 9*B*). Thus, similar to other clock mutants, such as $Clock^{-/-}$ and $NPAS2^{-/-}$, p75 ^{NTR} is not required for regulating locomotor activity rhythms.

Loss of p75 ^{NTR} decreases Per2 amplitude in the liver, but not the SCN

The circadian system is composed of several endogenous clocks, and the SCN is the main clock setting the phase of cell-autonomous and self-sustained cellular oscillators in peripheral

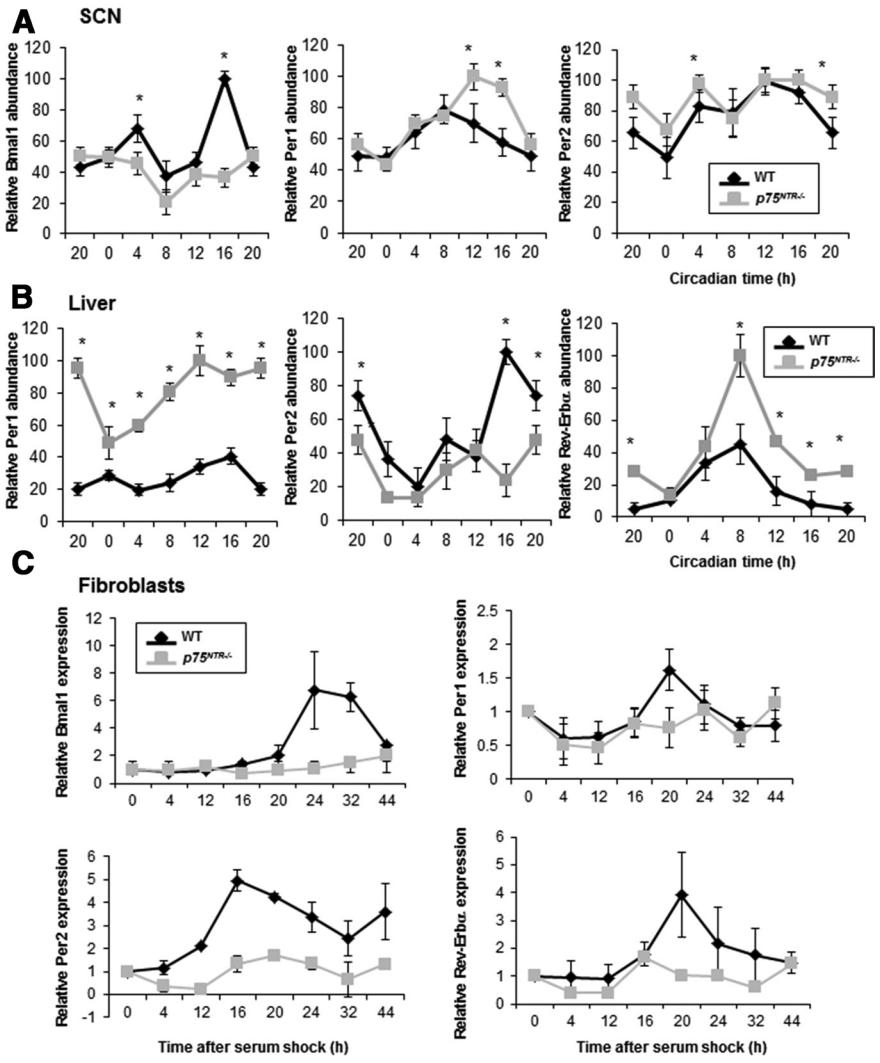

Figure 8. Genetic deletion of p75 ^{NTR} disrupts clock genes in SCN, liver, and fibroblasts. ***A***, Top, Schematic representation of the circadian entrainment of the mice. Mice were entrained in a 12 h light/dark cycle and were killed at 4 h intervals ($n = 4$ per group and genotype). Bottom, At the indicated times, the SCN was dissected; RNA was extracted; and *Bmal1*, *Per1*, and *Per2* levels were analyzed by real-time PCR. Error bars represent the SEM, where the maximum RNA amount was set to 100. ***B***, Mice were entrained in a 12 h light/dark cycle and killed at 4 h intervals. At the indicated times, the liver was dissected; RNA was extracted; and *Per1*, *Per2*, and *Rev-Erbα* levels were analyzed by real-time PCR. Error bars represent the SEM, where the maximum RNA amount was set to 100. ***C***, Serum shock treatment was performed on MEFs derived from WT and $p75^{NTR-/-}$ mice; cells were collected at the indicated times (h); and *Bmal1*, *Per1*, *Per2*, and *Rev-Erbα* mRNA levels were analyzed by real-time PCR. Data are presented as the relative expression level with respect to time 0, which was given a value of 1. Results of at least three independent experiments with duplicate measurements are shown. Data from CT20 are double plotted. Statistical significance denoted by asterisks is relative to the respective WT time point (*$p < 0.05$, one-way ANOVA). CT, Circadian time.

tissues (Asher and Schibler, 2011). To examine the dynamic effects of the p75 ^{NTR} deletion on circadian rhythms, $p75^{NTR-/-}$ mice were crossed with the *Per2::Luc* reporter mice (Yoo et al., 2004), which express Per2::Luciferase fusion protein and allow the recording of oscillatory pattern in real time. Interestingly, the amplitude of Per2 oscillation from $Per2::Luc/p75^{NTR-/-}$ liver explants was significantly decreased compared with that observed in $Per2::Luc/p75^{NTR+/+}$ littermates (Fig. 10*A*). No differences in period were observed between $Per2::Luc/p75^{NTR+/+}$ and $Per2::Luc/p75^{NTR-/-}$ liver explants (Fig. 10*A*). In accordance with the robust locomotor activity rhythms in the $p75^{NTR-/-}$ mice (Fig. 9), $Per2::Luc/p75^{NTR-/-}$ SCN explants exhibited robust circadian oscillations of bioluminescence, and no significant differences were observed in period or amplitude (Fig. 10*B*). Altogether, these results indicate that p75 ^{NTR} is necessary to maintain *Per2* oscillation in the liver, suggesting that p75 ^{NTR} plays an important role in clock gene expression.

Loss of p75 ^{NTR} alters oscillation of glucose homeostasis genes in liver

Previous studies have reported that alterations in the period, phase, or amplitude of clock gene expression can trigger metabolic disorders (Bass and Takahashi, 2010). Since $Per2::Luc/p75^{NTR-/-}$ liver explants—and the livers of $p75^{NTR-/-}$ mice *in vivo*—showed reduced amplitude and altered transcriptional oscillation of clock genes, we analyzed whether the lack of p75 ^{NTR} altered the oscillatory transcriptional expression of genes involved in glucose and lipid homeostasis. Gene expression analysis in the livers of $p75^{NTR-/-}$ mice showed reduced expression of the gluconeogenic gene phosphoenolpyruvate carboxykinase (*Pepck*) and the lipogenesis gene fatty-acid synthase (*Fas*), but increased expression of the glucose transporter 4 (*Glut4*) (Fig. 10*C*). These results show that deletion of p75 ^{NTR} alters the circadian expression of genes involved in glucose and lipid metabolism in liver. Together, our results suggest that circadian regulation of $p75^{NTR}$ by CLOCK and BMAL1 might play an important role in the circadian expression of genes involved in glucose and lipid homeostasis in liver.

Discussion

In this study, we report a novel mechanism for the transcriptional regulation of p75 ^{NTR} by investigating the molecular events that regulate its expression in the CNS and peripheral tissues. Our results demonstrated that p75 ^{NTR} expression oscillates via the direct binding of CLOCK/BMAL1 to noncanonical E-box elements present in the p75 ^{NTR} promoter. CLOCK and BMAL1 transcription factors are expressed not only in the SCN and other brain regions, but also in all peripheral tissues such as liver, heart, kidneys, lungs, and skeletal muscle (Schibler and Sassone-Corsi, 2002; Dibner et al., 2010). The widespread expression pattern of clock genes correlates with the variety of cell types and different organs that express p75 ^{NTR} in the brain and peripheral tissues, suggesting that transcriptional regulation of p75 ^{NTR} by CLOCK/BMAL1 could be a general mechanism to regulate $p75^{NTR}$ expression. Therefore, it is likely that CLOCK/BMAL1 is involved in the circadian regulation of p75 ^{NTR}, while upon injury or other stressors, transcription factors such as Sp1 could drive the expression of p75 ^{NTR} via binding to different elements of the promoter.

CLOCK and BMAL1 are known to regulate clock gene expression by interacting with a promoter element termed the E-box

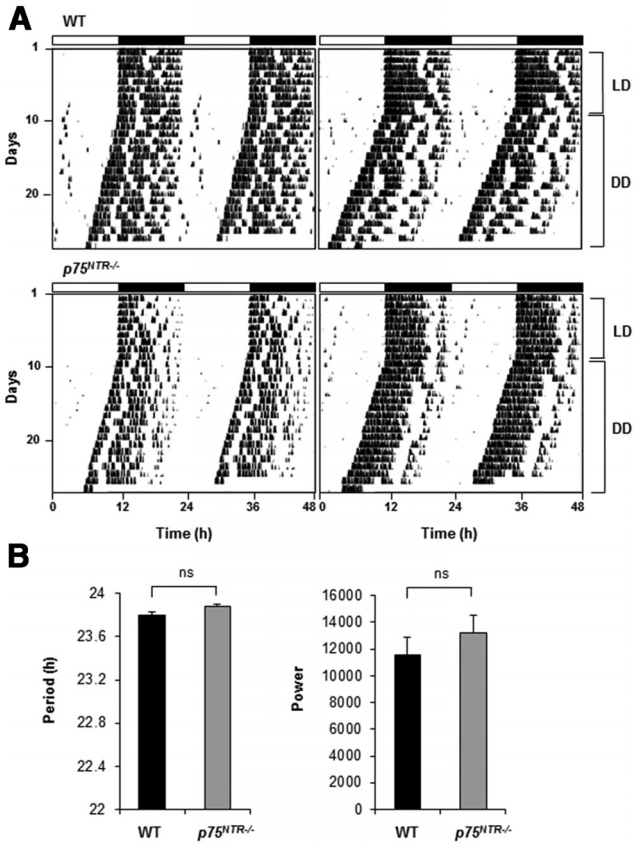

Figure 9. Circadian locomotor activity of $p75^{NTR-/-}$ mice. ***A***, Representative activity records (actograms) of wild-type mice and $p75^{NTR-/-}$ mice are shown in double-plotted format. Each horizontal line represents 48 h. Vertical bars represents periods of voluntary wheel running activity. ***B***, Periodogram estimates of period (h) for each genotype and power corresponding to circadian amplitude (power-significance from periodogram analyses). Each bar is mean × SEM ($n = 14$ per genotype). Statistical comparisons were made with Student's *t* test (ns, not significant).

(CACGTG) (Hogenesch et al., 1998). However, noncanonical E-boxes or E-box-like sequences have also been reported for several clock-controlled genes. For instance, analysis of the gene *Dbp* demonstrated CLOCK and BMAL1 binding at multiple E-boxes, including one noncanonical E-box important for circadian expression (Ripperger and Schibler, 2006). In *Per2*, CLOCK and BMAL1 bind to an E-box-like sequence shown to be sufficient and indispensable for the circadian oscillation of *Per2* expression (Yoo et al., 2005). Therefore, noncanonical E-boxes found in the p75 ^{NTR} promoter are common targets for CLOCK and BMAL1 binding. In addition to *Per* and *Cry* genes (Darlington et al., 1998; Griffin et al., 1999; Kume et al., 1999; Sato et al., 2004), CLOCK/BMAL1 regulates genes that encode essential regulators of hormonal and metabolic control such as *Vasopressin* (Jin et al., 1999), *Dbp* (Ripperger and Schibler, 2006), and the transcription factors *Rev-Erbα* and *Rev-Erbβ* (Preitner et al., 2002). Therefore, the presence of functional noncanonical E-boxes in the $p75^{NTR}$ promoter directly regulated by CLOCK and BMAL1 suggests that p75 ^{NTR} is a clock-controlled gene. Importantly, our results showed altered $p75^{NTR}$ rhythmic expression in the SCN and liver from $Clock^{-/-}$ and $Clock^{\Delta 19}$ mice. Indeed, $Clock^{-/-}$ and $Clock^{\Delta 19}$ mice show altered rhythmic expression of clock genes in the SCN and peripheral tissues (Vitaterna et al., 1994; DeBruyne, 2008). Therefore, the alteration of $p75^{NTR}$ rhythmic expression in the SCN and liver in $Clock^{-/-}$ and $Clock^{\Delta 19}$ mice further supports the identification of p75 ^{NTR} as a novel clock-controlled gene.

Previous studies showed that BDNF and its receptor *TrkB* oscillate in the SCN (Bova et al., 1998; Liang et al., 1998). Indeed, alteration in the expression levels of *BDNF* and *TrkB* into the SCN affects circadian pacemaker responses to light (Liang et al., 2000; Allen et al., 2005). Because p75 ^{NTR} binds all neurotrophins (Chao, 2003), it is possible that neurotrophin and Trk receptor expression could be also regulated by CLOCK/BMAL1. Indeed, our results show that *NGF* and *TrkA* expression oscillate in a similar pattern to clock genes in the SCN, while *TrkB* and *TrkC* oscillate in the liver. Therefore, it is possible that neurotrophins and their receptors might play diverse roles in central and peripheral circadian oscillations. However, p75 ^{NTR} is unique among neurotrophin receptors in oscillating in phase with circadian genes in the SCN and the periphery. This expression pattern is consistent with its pleiotropic functions in peripheral tissues, such as metabolism and liver regeneration (Passino et al., 2007; Baeza-Raja et al., 2012). Expression of Trks can determine the biological outcome of p75 ^{NTR} signaling (Hempstead et al., 1991; Reichardt, 2006). Therefore, it is likely that the regulation of circadian gene oscillation by p75 ^{NTR} could also be modulated by the oscillatory expression of its Trk coreceptors. Future studies will shed light on the contribution of neurotrophins in the regulation of circadian rhythms.

The SCN is important for rhythmic locomotor activity (Lehman et al., 1987). Although $p75^{NTR-/-}$ mice displayed a robust circadian locomotor activity similar to those of WT mice, similar results have been reported for several clock gene-deficient mice. For instance, $Clock^{-/-}$ (DeBruyne et al., 2006) and Per3-null mutant mice (Shearman et al., 2000) also display a robust circadian locomotor activity without substantial differences in period or amplitude. Moreover, single gene mutations in *Per1*, *Per2*, *Cry1*, and *Cry2* have little effect on the locomotor activity period, while only disruption of *Per1* and *Per2* or *Cry1* and *Cry2* genes together causes behavioral and molecular arrhythmicity (Okamura et al., 1999; Vitaterna et al., 1999; Zheng et al., 1999, 2001; Bae et al., 2001; Cermakian et al., 2001). Therefore, it is possible that deletion of p75 ^{NTR} alone is not enough to disrupt circadian locomotor activity in mice. It is also likely that p75 ^{NTR} might be compensated by Trk receptor expression, similar to neuronal PAS domain protein 2 implicated to compensate for loss of Clock (Rutter et al., 2001; DeBruyne et al., 2006). Additionally, locomotor activity is a polygenic trait that varies widely among inbred strains of mice (Flint et al., 1995). Therefore, it is possible that p75 ^{NTR} deletion in other mouse strains could display differences in the locomotor activity.

Clock-controlled genes such as the Per genes (*Per1*, *Per2*, and *Per3*) and cryptochrome genes (*Cry1* and *Cry2*) form a heterodimeric repressor complex that translocates into the nucleus to inhibit CLOCK/BMAL1-mediated activation of clock-controlled genes (Darlington et al., 1998; Griffin et al., 1999; Kume et al., 1999; Sato et al., 2006). Previous studies showed that ubiquitination and proteasomal degradation of PER and CRY proteins play an essential role in the maintenance of clock genes oscillation (Eide et al., 2005; Busino et al., 2007; Godinho et al., 2007; Reischl et al., 2007; Siepka et al., 2007; Yoo et al., 2013). Since our results show altered rhythmic expression of clock genes not only in the SCN and liver from $p75^{NTR-/-}$ mice, but also in serum-shocked $p75^{NTR-/-}$ MEFs, it is possible that p75 ^{NTR} might play a role in the maintenance of clock gene oscillation through the regulation of *Per* and *Cry* proteolytic degradation. Indeed, p75 ^{NTR} directly interacts with E3 ubiquitin ligases (Le Moan et al., 2011), which govern circadian periodicity by degradation of *Cry* (Yoo et al., 2013). Therefore, it is possible that p75 ^{NTR} could regulate pro-

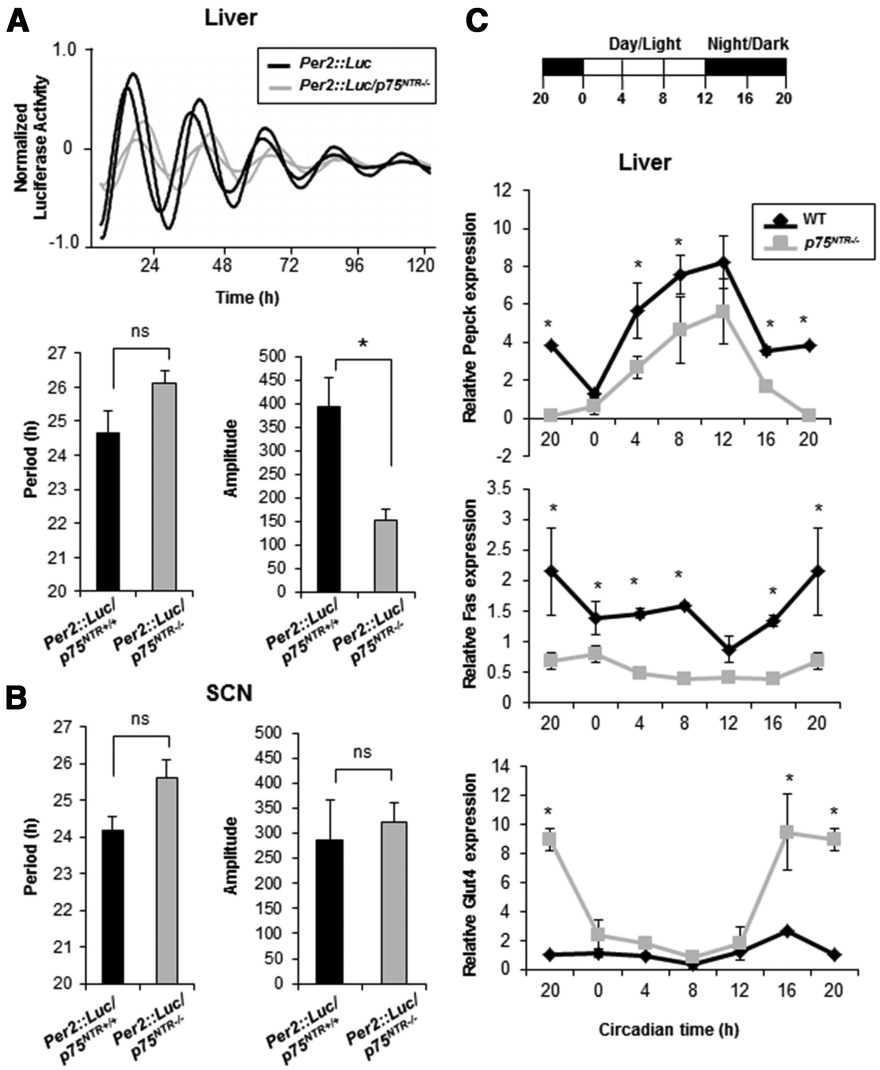

Figure 10. Deletion of p75 ^{NTR} reduces rhythm amplitudes and affects the expression of genes involved in glucose and lipid metabolism in liver. ***A***, Top, Representative bioluminescence profiles of tissue explants from liver. The two representative profiles shown for each genotype (*Per2::Luc p75*$^{NTR+/+}$ and *Per2::Luc p75*$^{NTR-/-}$) are from separate experiments. Bottom, Period and amplitude of the bioluminescence profiles from liver explants. Each bar is the mean ± SEM of *n* (explants)/*n* (animals); *Per2::Luc p75*$^{NTR+/+}$ = 21/7; *Per2::Luc p75*$^{NTR-/-}$ = 21/7 (*$p < 0.05$; *t* test). ***B***, Period and amplitude of the bioluminescence profiles from SCN explants. Each bar is the mean ± SEM of *n* (explants)/*n* (animals); *Per2::Luc p75*$^{NTR+/+}$ = 28/14; *Per2::Luc p75*$^{NTR-/-}$ = 28/14 (ns, not significant). ***C***, Top, Schematic representation of the light schedule. Mice were entrained in a 12:12 h light/dark cycle and were killed at 4 h intervals ($n = 4$ per group). Bottom, At the indicated times, the liver was dissected, RNA extracted, and *Pepck*, *Fas*, and *Glut4* levels analyzed by real-time PCR. Results of at least three independent experiments with duplicate measurements are shown. Data from CT20 are double plotted. Error bars represent the SEM of the mean value, where the maximum RNA amount was set to 100. Statistical significance denoted by asterisks is relative to the respective WT time point (*$p < 0.05$, one-way ANOVA).

teolytic degradation of Per and Cry proteins by regulating E3 ubiquitin ligases. Future studies will determine whether interaction of p75 ^{NTR} with E3 ubiquitin ligases plays a role in the regulation of clock gene oscillation.

Our results show altered rhythmic expression of clock genes such as *Bmal1* and *Per1*, and to a lesser extent *Per2*, in SCN from *p75*$^{NTR-/-}$ mice, suggesting that p75 ^{NTR} might play an important regulatory function for the maintenance of normal clock gene oscillatory patterns in the SCN. Accordingly, analysis of circadian oscillations of *Per2* in SCN explants revealed no differences in period or amplitude. It is possible that the minor differences of *Per2* expression observed in SCN from *p75*$^{NTR-/-}$ were not sufficient to induce changes in period or amplitude. Additionally, factors such as entraining signals or responsiveness to entraining signals, and communication between different areas of the brain or between different peripheral tissues could contribute to the differences in circadian properties of Per2 observed in SCN between explants and at the organismal level (Schibler et al., 2003). Similar to *p75*$^{NTR-/-}$ mice, *Clock*$^{-/-}$ mice show an altered expression of clock genes in the SCN (DeBruyne et al., 2006), although *Per2::Luc/Clock*$^{-/-}$ SCN explants show normal maintenance of Per2::Luc bioluminescence rhythmicity and amplitude (DeBruyne et al., 2007). Future studies will address the specific role of p75 ^{NTR} in the regulation of clock gene oscillation in the SCN and/or other brain regions involved in the maintenance of clock gene expression.

Our study shows that loss of p75 ^{NTR} alters the oscillation of clock and metabolic genes in the periphery, thus raising the question whether the peripheral effects of p75 ^{NTR} are due to its peripheral expression or its effects in the SCN. On one hand, p75 ^{NTR} is expressed in peripheral tissues such as liver (Trim et al., 2000; Cassiman et al., 2001; Passino et al., 2007), lung (Sachs et al., 2007), and kidney (Wheeler and Bothwell, 1992). Moreover, we demonstrated in cell-autonomous systems that p75 ^{NTR} regulates glucose uptake in adipocytes and muscle cells (Baeza-Raja et al., 2012) and hepatic stellate cell differentiation (Passino et al., 2007). Therefore, it is possible that p75 ^{NTR} alters the oscillation of clock and metabolic genes through its expression in the periphery. On the other hand, several studies have suggested an important role of the autonomic nervous system in the circadian regulation of metabolic functions such as glucose homeostasis (Kalsbeek et al., 2010a,b). Indeed, p75 ^{NTR} regulates glucose uptake (Baeza-Raja et al., 2012) and is highly expressed by sensory and sympathetic neurons where it plays an essential role in the regulation of innervation during development (Reichardt, 2006). Interestingly, the SCN not only controls daily circadian rhythms, but also uses its projections to sympathetic preautonomic neurons in the paraventricular nucleus to control hepatic glucose production (Kalsbeek et al., 2004; Cailotto et al., 2005, 2008; Yi et al., 2010). Therefore, it is possible that p75 ^{NTR} might be involved in the communication between the SCN and the periphery to control circadian and metabolic functions. Although p75 ^{NTR} regulates clock gene oscillation in cell-autonomous systems of MEFs, our study does not exclude potential effects of p75 ^{NTR} deletion on circadian regulation due to its major role in nervous system development (Reichardt, 2006). Although loss of p75 ^{NTR} does not affect liver development, as the *p75*$^{NTR-/-}$ mice need to be challenged either by

injury or fibrosis to exhibit defects in liver functions (Passino et al., 2007; Kendall et al., 2009), our study does not exclude a role for $p75^{NTR}$ in the control of peripheral clocks via peripheral innervation. Future studies using tissue-specific deletion of $p75^{NTR}$ will demonstrate the contribution of the central and peripheral expression of $p75^{NTR}$ in the regulation of circadian and metabolic functions.

Our study reveals $p75^{NTR}$ as an important link between the disruption of endogenous clocks in mice and metabolic dysfunction. Circadian clocks participate in the daily regulation of metabolic functions, and this is accomplished by the rhythmic expression of genes encoding regulators and enzymes of various metabolic pathways such as *Fas* and *Pepck* (Froy, 2007). Previous studies showed that CLOCK/BMAL1 directly regulates the expression of the orphan receptor *Rev-Erbα* and the peroxisome proliferator-activated receptors (Preitner et al., 2003; Oishi et al., 2005; Canaple et al., 2006), important regulators of triglyceride mobilization and lipid metabolism, respectively. Furthermore, *Clock* mutant mice present severe metabolic dysfunction including hyperglycemia, hypertriglyceridemia, and hepatic steatosis (Turek et al., 2005). *Clock* mutant and $Bmal1^{-/-}$ mice show increased insulin sensitivity and altered gluconeogenesis (Rudic et al., 2004). Mice with liver-specific deletion of *Bmal1* show hypoglycemia and increased glucose tolerance (Lamia et al., 2008), and liver- and muscle-specific *Clock* mutant mice show impaired glucose homeostasis (Kennaway et al., 2007). In accordance, $p75^{NTR-/-}$ mice show exacerbated hepatic disease (Passino et al., 2007; Kendall et al., 2009) and increased insulin sensitivity (Baeza-Raja et al., 2012). Because $p75^{NTR}$ is an important regulator of glucose homeostasis (Baeza-Raja et al., 2012), it is therefore possible that $p75^{NTR}$ might regulate metabolic processes through the SCN, and by regulating clock genes in the periphery.

Overall, the regulation of $p75^{NTR}$ by CLOCK and BMAL1 identifies a novel first-order clock-controlled gene that oscillates in central and peripheral tissues and is important for the circadian oscillation of clock and metabolic genes. Therefore, these results may reveal $p75^{NTR}$ as a therapeutic target for diseases with altered circadian oscillation such as sleep disorders, neurologic and behavioral disorders, and metabolic diseases.

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