Circadian transcription depends on limiting amounts of the transcription co-activator nejire/CBP

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Running title: nej/CBP co-activates CLK/CYC-dependent transcription

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The circadian clock orchestrates physiological and behavioral activities, including metabolism, neuronal activity and cell proliferation in synchrony with the environmental cycle of day and night. Here we show that the Drosophila ortholog of the CBP/p300 family of transcription co-activators, nejire (nej), is an intrinsic component of the circadian clock that performs regulatory functions for circadian controlled transcription. Screening of over-expression mutants revealed that gain of nej function is associated with a loss of behavioral and molecular rhythms. Overexpression of NEJ suppresses the long period phenotype of a mutation in the clock gene period (per). NEJ physically interacts through two binding sites with CLOCK and the CLOCK/CYCLE (CLK/CYC) complex. Induction of CLK/CYC-dependent transcripts upon induction of nej expression from a heat shock-promoter showed that NEJ is limiting. Reduced CLK/CYC-mediated transcription in a nej hypomorphic mutant indicates an essential function of NEJ/CBP for CLK/CYC-activity and a regulation of circadian transcription by availability of the co-activator. Competition for recruitment of NEJ/CBP provides a potential mechanism for cross-talk between circadian transcription and other CBP-dependent physiological processes.

The circadian clock controls genome wide transcription of many key regulatory components in a diverse selection of vital pathways (1-3) that ultimately allow a coordination of physiological and behavioral activities and their synchronization with the environmental cycles of day and night. The analogous and homologous clock mechanisms in Drosophila and mammals are based on two interconnected feedback loops (4,5). In Drosophila, the heterodimeric complex of transcription factors CLOCK (CLK) and CYCLE (CYC) (BMAL1 in mammals) activates expression of its own inhibitors PERIOD (PER) and TIMELESS (TIM) forming the first feedback loop. This loop is interconnected with CLK/CYC-mediated expression of the transcription repressor vrille (vri) and the activator par-domain protein 1 (pdp1). VRI and PDP1 control the rhythmic transcription of Clk and contribute to the robustness of molecular oscillations (6,7).

Oscillations in cyclic nucleotide, calcium and MAPK signaling (8-10) likely contribute to a circadian control of physiological processes such as cell proliferation (11) and the sleep/wake cycle, which is important for memory formation (12). However, these pathways also feed back on the molecular oscillator at least in part through control of CLK/CYC activity (13). Cross-talk between circadian and cell signaling may increase the robustness of circadian oscillations and allow a coordination of circadian transcription with physiological requirements. Previous studies showed that recruitment of the CREB-binding-protein (CBP) from a limiting cellular pool mediates cross-talk between the transcription factors E2F, JAK/STAT, AP1 and nuclear hormone receptors (14-16) that control e.g. entry into the cell cycle and the immune response. Here we show that CLK/CYC-mediated transcription is also dependent on CBP, and importantly circadian transcription responds to changes in limiting levels of the co-activator. These findings suggest a novel mechanism for cross-talk between CLK/CYC and other CBP-dependent transcription factors.

EXPERIMENTAL PROCEDURES

Fly stocks - w[ ]; P[w+[mC]=hs-nej[+]]1 (hs-nej) and y[1] nej(Q7) v[1] f[1] Dp(1;Y)FF1, y[+]/C(1)DX, y[1] w[1] f[1] (nej57) were obtained from Bloomington Drosophila stock center and EP(X)950, EP(X)1410, EP(X)1149 and EP(X)1179 were obtained from Szeged Drosophila stock center. nej57 flies were crossed with Canton-S flies to obtain +/Dp(1;Y)FF1, y[+] flies (Dp(1;Y)FF1). hs-nej and
Dp(1;Y)FF1 males were used as nej overexpressing flies together with Clk\textsuperscript{b/b} for arrhythmic and wild-type Canton-S and w\textsuperscript{118} for rhythmic controls. The BGlac reporter gene (17) that expresses luciferase from a per-promoter was crossed into these backgrounds to generate BGlac and BGlac\textsuperscript{b/b} as well as BGlac/Dp(1;Y)FF1 and BGlac\textsuperscript{b/b}; hs-nej flies. EP-lines were crossed with tim-GAL4 flies to generate EP(X)/Y; tim-GAL4/+ as shown in figures.

**Locomotor activity assays** - Flies were entrained during eclosion for four days in 12 hours light and 12 hours dark cycles (LD) at 20°C or 22°C. One to four days old flies were analyzed in locomotor activity assays using the DAM system IV (TriKinetics, Waltham, MA, USA) for 3 days in LD cycles, with or without application of a 37°C heat-shock during the last hour in darkness, and subsequently for 7 days in constant darkness at 20°C or 22°C. Data from the first five days in constant darkness was analyzed using Clocklab (Actimetrics, Wilmette, IL, USA) and LandA software (17) to identify rhythmic flies and determine period estimates.

**Real-time bioluminescence measurements** - Bioluminescence from luciferase reporter gene expression in live flies was determined for 3 days in constant darkness as described previously (17) after entrainment of flies for four days in 12 hours light and 12 hours dark cycles (LD). Rhythmic flies were identified by analysis of the first three days in constant darkness with LandA software (17).

**Quantitative Real-time PCR (RT-PCR)** - 1-7 days old flies were harvested after incubation for at least 48 hours in constant light at either constant 20°C or after application of a 30 min heat shock at 37°C. Heads were isolated and total RNA was purified by homogenization in peqGOLD TriFast (PEQLAB, Erlangen, Germany) following manufacturers’ instructions. 1 µg of total RNA was reverse transcribed with random hexamer primers using the Quantitect Reverse Transription Kit (QIAGEN, Hilden, Germany) following manufacturers’ instructions. cDNA products were amplified in an ABI PRISM 7000 (Applied Biosystems, Foster City, CA, USA). Forward (fwd) and reverse (rev) primers and probes for TaqMan quantitative RT-PCR were designed with the ABI PRISM Primer Express software (Applied Biosystems) as follows: n-syb (fwd: GGC GGC GTG TAA GCA ATC; rev: CCC GCT GAA GGA GCA CAC TA; probe: 6-FAM-CGC TGC CAG GAC GAA AGT TTC TCG A-TAMRA), nej (fwd: GGT GCA AGT TCC ACG TCA TC; rev: AGT CGA TAC CGA GCT GAG TGG T; probe: 6-FAM-TCC TCG GGC GGC TCG GGT-TAMRA), pdp1 (fwd: CTT GGT CTT GGC CAC ATA ACC; rev: GGT TCG CGG ATC AAA GTC A; probe: 6-FAM-CGG CCG AGT CAA CAT TTT CGT TCG-TAMRA), per (fwd: CCA ATG GCA CCA ACA TGC T; rev: TGT GGC GTA TGG CGA ACT T; probe: 6-FAM-AGC AGC TAC AAG GTT CCC GAC GAG ATT C-TAMRA), tim (fwd: CTG GCT GCA GTT GGT CAT G; rev: TGG CTG CAC TGA TGG ACT TG; probe: 6-FAM-TCC CAG CGT TGT CAT TGG CTC CT-TAMRA), vri (fwd: CGT CCG GCT ATC CAA TAT ATC G; rev: GGA CAA CGG ATG CAA GTT AGA AG; probe: 6-FAM-TCC ATG AAC GGC AGC TCC AAC GA-TAMRA). n-syb mRNA levels were determined as a constitutively expressed internal control. mRNA levels for individual clock genes were normalized towards n-syb transcript levels and quantified by the \(2^{-\Delta\Delta Ct} \) method as described (18,19) and according to manufacturers’ instructions (Applied Biosystems).

**Western blotting** - CLK protein levels were determined by western blot analysis as detailed in the legend of Fig. 4 using an antibody that was raised in rabbit against a peptide composed of the C-terminal 15 amino-acids of CLK.

**Immunocytochemistry** - Third instar larvae were entrained for 3 days in cycles of 12 hours light and 12 hours darkness (LD) and the brains were dissected during the first day of constant darkness at times indicated in the figures. Circadian time 0 (CT0) marks time of subjective ‘lights on’ and CT12 marks time of subjective ‘lights off’. The experiments were repeated twice and for each time-point at least 8 brains were dissected. Brains were fixed in 4 % formaldehyde in PBM buffer (100 mM Pipes pH 6.9, 1 mM EGTA, 2 mM Mg-sulfate) for 2 hours, blocked with 10 % normal goat serum (NGS) in PT buffer (PBS plus 0.3 % Triton X-100) for 2 hours at room temperature and subsequently incubated for 48 hours at 4°C in 50 µl of primary antiserum solution containing 1:200-diluted rabbit anti-PER (Alpha Diagnostics, San Antonio, TX, USA) and 1:1000-diluted guinea pig anti-PAP (20) in PT buffer with 10 % NGS. Brains were rinsed with PT and PT plus 5 % NGS for 20 minutes each and then incubated at room temperature for 2 hours in secondary antiserum solution containing 1:200-diluted TRITC-conjugated donkey anti-guinea pig (Jackson Immunoresearch, West Grove, PA, USA) and 1:200-diluted FITC-conjugated goat anti-rabbit (Calbiochem, San Diego, CA, USA) in PT with 10 % NGS. After incubation, brains were rinsed with PT and PT plus 5 % NGS for 20 minutes each and mounted in the mounting medium (50 mM Tris-Cl, pH8, 90 % glycerol, 2.5 % DABGO (Sigma, St Louis, MO, USA)).
Confocal microscopy and quantification of staining - Optical sections of larval lateral neurons (LNs) were imaged on a Carl Zeiss LSM 510 META confocal microscope. For each CT, LNs from at least 16 brain hemispheres per fly strain were scanned. For each LN sample, PAP staining was used to identify and select an optical section, which was then scanned for PER immunoreactivity. Three images were taken per LN with single laser at 488 nm, 543 nm and with both laser together. Images taken with single 488 nm laser were imported to NIH Image J 1.34S and the localization of neuronal PER staining was determined by the double-laser images. Total pixel intensity of PER staining was measured and the means ± SEM of all brains at a particular CT are reported in the graphs.

Expression constructs and co-transfection assays - Clk, E1A(12S) and a E1A(12S) construct that carries a deletion of the first 72 amino-acids (E1A(ΔN)) were cloned into Xho I and Pme I sites of pAct5.1/V5-HisA vector (Invitrogen, Carlsbad, CA, USA) for expression in Drosophila S2 cells. pHT-control vector, reporter-plasmids pRLcopia for Renilla-luciferase control and pGL3-(4-per-E-box)hs::luc+, pGL3-per::luc+, and pGL3-tim::luc+ for expression of firefly-luciferase from a minimal heat-shock promoter with four per-E-box elements, a per-promoter and a tim-promoter respectively were described previously (13,21). Co-transfection assays were performed as described previously (13) with either 200 ng of pHT control, pAc-E1A(12S) or pAc-E1A(ΔN) vectors. Averages of at least three independent experiments are shown as percent of control. Control is CLK/CYC-activated LUC-activity set to 100 %.

Expression levels of E1A constructs were determined by western blot analysis using an anti-E1A(12S) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

For dsRNA production approximately 500 bp long DNA fragments of the coding region of target genes Clk, cyc, nej and EGFP control were generated by PCR using primers that contained a 5' T7 RNA-polymerase binding site followed by gene specific sequences. Primers were as follows: Clk (fwd: TAA TAC GAC TCA CTA TAG GGG GTG TCT GCA CCC ATA AGG; rev: TAA TAC GAC TCA CTA TAG GGC GTG CAT GTG CAT GGA CT), cyc (fwd: TAA TAC GAC TCA CTA TAG GGT GGG TGT TGG TGA CCG AGG AC; rev: TAA TAC GAC TCA CTA TAG GGA GTT GGC AAC GTT GGG C), nej (fwd: TAA TAC GAC TCA CTA TAG GGC AAT CTG ACG GGT CTG GTA GTG CAT; rev: TAA TAC GAC TCA CTA TAG GGT GGG TTG CTG CTG TGT TTG CTG ATG), EGFP (fwd: TAA TAC GAC TCA TAG GGC ACA TGA AGC AGC ACT T; rev: TAA TAC GAC TCA CTA TAG GGA CTG GGT GCT CAG GTA GTG G). Purified DNA fragments were in vitro transcribed using the T7 Ribonuclease-Express Large Scale RNA Production System (Promega, Madison, WI). The RNA products were isolated by using the SV Total RNA Isolation System (Promega) and annealed to dsRNA by incubation at 65°C for 30 min and slow cooling to room temperature.

To test effects of dsRNAs on reporter gene expression in co-transfection assays, 250 μl Drosophila S2 cells (2 x 10⁶ cells/ml) were incubated in serum-free Schneider’s insect medium (Sigma) with 7.5 μg of dsRNA for 45 min prior to addition of 250 μl of Schneider’s medium containing 20 % FBS. Cells were incubated for 24 hours and co-transfection assays were performed essentially as described previously (13) with the difference that another 7.5 μg of dsRNA were added 45 min prior to the addition of 20 % FBS. Averages of at least three independent experiments are shown as percent of control. Control is CLK/CYC-activated LUC-activity in the absence of dsRNA set to 100 %.

Co-immunoprecipitation experiments - Flag-tagged CLK or CLKJrk as well as CYC and domains of NEJ were expressed from a pAc5.1 vector, which carried a SP6 promoter insertion in the Kpn I site, in reticulocyte lysate using the SP6 quick coupled transcription/translation system (Promega) as instructed by the manufacturer. Flag-tagged CLK or CLKJrk was expressed with unlabelled methionine to reduce background, while CYC and NEJ fragments were labeled with [³⁵S]-methionine. The immunoprecipitation was performed as previously described (22). In brief, 10 μl of NEJ-expressing lysate was pre-incubated with 10 μl of anti-Flag-M2-conjugated agarose (Sigma) in 150 μl IB solution (5 mM Tris-Cl, 10 mM HEPES [pH7.5], 10 % glycerol, 50 mM KCl, 0.05 % Triton X-100, 1 mM EDTA, 1 mM dithiothreitol, 1X Complete protease inhibitor (Roche Diagnostics, Mannheim, Germany)) to eliminate non-specific interaction. The agarose was then removed from the lysate. The NEJ lysate was added to different interaction reactions either with or without 10 μl of CLK or CLKJrk lysate in the absence or presence of 10 μl CYC lysate (as indicated in Fig. 2) and the final volume of the interaction mixture was adjusted to 500 μl with IB solution. The protein mixture was incubated at 25°C with gentle rotation for 30 minutes. Subsequently, 10 μl of anti-FLAG-M2-conjugated agarose, previously pre-blocked in IB solution with 1 % milk, was added and incubated for 2 hours at 25°C. The beads were then collected and
RESULTS AND DISCUSSION

We identified a circadian function of *nejire* (*nej*), which is the CBP ortholog in *Drosophila*, by screening of duplication mutants for phenotypes in circadian behavioral rhythms. A duplication of the X-chromosomal *nej* locus on the Y chromosome in Dp(1;Y)FF1 caused a large increase in the number of arrhythmic flies (Fig. 1A, Table 1). Specific overexpression of *nej* from a heat-shock promoter (hs-*nej*) resulted in a similar phenotype. Interestingly even a moderate increase in *nej* levels by about 45%, as observed in *hs-nej* flies in the absence of heat shock (Fig. 1B), was associated with a loss of behavioral rhythms in 71% of the flies (Table 1, Fig. 1A, and Fig. S1). The remaining rhythmic flies displayed a wild-type period length. This phenotype was reminiscent to the effects observed for the overexpression of *Clk*, which also increased the number of arrhythmic flies without affecting period length (23). A similar increase in arrhythmicity of *nej* overexpressing flies was observed after entrainment in temperature cycles under subsequent free-running conditions (Table 1). This finding indicated that *nej* does not affect a specific entrainment pathway but the circadian clock itself. To further test specificity of the behavioral phenotype, we overexpressed *nej* from a UAS-enhancer after transcription of a GAL4 driver from a *tim*-promoter (tim-GAL4). Several EP-lines carry an insertion of the UAS-enhancer in the 5’-UTR of *nej* (24). EP(X)1179 overexpressed *nej* by the tim-GAL4 driver, while two lines, EP(X)950 and EP(X)1410, carry the UAS-enhancer in the inverse orientation, serving as a control that lacks *nej* overexpression (Fig. 1C, D). Overexpression of *nej* by tim-GAL4 in EP(X)1179 resulted in an increase of behavioral arrhythmicity, without significant effects on general activity levels (Table 1). In contrast, EP(X)950 and EP(X)1410 showed wild-type rhythms. These results demonstrate that *nej* gain of function causes an increase in the number of behaviorally arrhythmic flies.

To analyze whether *nej* has a role in the molecular oscillator itself, we investigated the circadian profile of clock gene expression by real-time monitoring of CLK/CYC-dependent expression of a luciferase reporter from a per-promoter in individual BGluc flies (25). After entrainment of BGluc flies in cycles of 12 hours light and 12 hours dark CLK/CYC-dependent luciferase bioluminescence was monitored for 3 days in constant darkness. Luciferase expression oscillated in BGluc control flies, while the antimorphic *Clk<sup>hs</sup>* mutation caused molecular arrhythmicity (Fig. 2A). Already a moderate overexpression of *nej* from the heat-shock promoter at 20°C induced arrhythmicity in 40% of *hs-nej* flies (Fig. 2A). Similarly, duplication of the *nej* locus caused molecular arrhythmicity in 64% of the flies (Fig. 2A). These results show that overexpression of *nej* affects the ability to maintain free running molecular rhythms, consistent with the observed increase of behaviorally arrhythmic flies.

We also analyzed PER protein expression in PDF positive pacemaker lateral neurons of larval brains from EP lines that carried a tim-GAL4 driver (Fig. 2B, C, and data not shown). While EP(X)950 and EP(X)1410 control flies displayed normal PER oscillations with trough levels at CT 10 and a peak at CT 24, the EP(X)1179 line showed a strongly reduced amplitude of PER protein oscillations. PER staining intensities in *nej* overexpressing EP(X)1179; tim-GAL4 flies were for both time points at about trough levels similar to the EP(X)950; tim-GAL4 control line at CT10 (Fig. S2). In summary the data show that gain of *nej* function is associated with a strong increase in molecular and behavioral arrhythmicity.

In contrast to gain of function, a *nej* loss of function cannot be analyzed due to embryonic lethality. Two heterozygous *nej* loss of function mutants (*nej<sup>3</sup> and *nej<sup>Q7</sup>*) did not display a phenotype due to the remaining wild type allele (data not shown). The lack of a period phenotype for *nej* partial loss of function is reminiscent to the effects of decreased CLK/CYC activity in heterozygous *Clk* loss of function flies, which show a reduced amplitude but wild type period length of circadian oscillations (26). However, the insertion of the UAS-enhancer in the 5’-UTR of *nej* in EP(X)1149 flies has also been shown to cause a *nej* partial loss of function, when investigated in the absence of a GAL4 driver (24). We observed a severe long period phenotype in EP(X)1149 flies (Table 1). However, this phenotype did not segregate with the EP-element insertion. Sequencing revealed that this line carries an additional mutation in the per locus that substitutes serine 45 to tyrosine and has previously been coined *per<sup>SLIH</sup>* (27). The *per<sup>SLIH</sup>* mutation causes a temperature-sensitive phenotype with a long period oscillation of about 27.6 hours at low temperature and a shorter period length at higher temperatures. Since *nej* can be overexpressed in EP(X)1149 flies by a tim-GAL4 driver (Fig. 1D), we analyzed the effects of *nej* overexpression on the *per<sup>SLIH</sup>* phenotype. In the presence of the tim-GAL4 driver, overexpression of *nej* in EP(X)1149 caused a

washed three times with 500 μl IB for 10 minutes each. Proteins were eluted with 30 μl of 2X sodium dodecyl-sulfate (SDS) sample buffer and radio-labeled proteins were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

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strong increase in arrhythmic flies, similar to the observations in EP(X)1179 (Fig. 1A, Table 1). The remaining rhythmic flies showed however a wild type period length at low temperatures demonstrating that nej overexpression suppresses the temperature sensitive effect of the perSLIH mutation. The genetic interaction between per and nej supports an intrinsic clock function of nej as a co-activator for CLK/CYC-dependent transcription.

To directly test this hypothesis we first analyzed the effects of decreased nej activity on CLK/CYC function in cell culture. CLK/CYC-dependent luciferase expression from a four per-E-box containing reporter in cell culture (21) was strongly reduced by co-expression of the adenoviral E1A(12S) protein (Fig. 3A), a well characterized inhibitor of CBP/p300 (28). Specificity of NEJ/CBP inhibition was verified by co-expression of E1A(ΔN), a truncated construct that lacked the N-terminal CBP/p300 binding domain, which showed no inhibition of CLK/CYC activity. The same results were obtained using a reporter construct that expressed luciferase from a genomic fragment of the tim- or per-promoter (Fig. 3B and data not shown). Both E1A(12S) and E1A(ΔN) were expressed to similar levels (Fig. 3C). To confirm the specificity of reduced CLK/CYC function by inhibition of nej, we analyzed the effects of a knock-down of nej expression by small interfering RNAs (siRNA) in the co-transfection assay (Fig. 3D). siRNA targeting either CLK or cyc expression strongly reduced CLK/CYC activity as expected. In contrast, control siRNA targeting the enhanced-green-fluorescent-protein (EGFP) gene had no effect on CLK/CYC function. When siRNA targeting nej transcription was included in the reporter assay, CLK/CYC activity was significantly reduced. The decrease in CLK/CYC-dependent transcription was about proportional to the reduction in nej mRNA levels (compare Fig. 3D and Fig. S3). An almost complete inhibition of CLK/CYC activity (less than 10%) was observed after reduction of nej transcription by siRNA and additional inhibition of residual NEJ by E1A(12S) co-expression (Fig. S3). These results strongly suggest that nej plays an important role for CLK/CYC-dependent transcription.

A direct interaction between NEJ and the CLK/CYC complex would be expected, if NEJ acts as a transcription co-activator for CLK/CYC-dependent transcription. Yeast-two-hybrid assays indicated a binding between CLK and the N-terminus of NEJ (data not shown). In order to map the interaction between Drosophila NEJ and either CLK or CLK/CYC, we performed co-immunoprecipitation experiments of reticulocyte lysate expressed domains of NEJ (Fig. 3E) with FLAG-tagged CLK in the presence or absence of CYC (Fig. 3F). The intensity of the CYC signal served as a control for the efficiency of co-immunoprecipitation with CLK, since binding of CYC was specific (Fig. 3G). We identified two domains of NEJ that bound to CLK as well as to CLK/CYC complexes (Fig. 3H), the N-terminal fragment from amino acid 1 to 898 (NEJ 1) that also interacts with nuclear hormone receptors such as the retinoic acid (RAR, RXR), oestrogen, progesterone, thyroid hormone and glucocorticoid receptors (29), as well as the middle domain of NEJ from amino acid 1751 to 2741 (NEJ 3) that contains the histone acetyl transferase (HAT) activity and the zinc finger domains C/H2 and C/H3. Binding of the N-terminal NEJ 1 fragment to CLK was independent of CYC, while the interaction of the middle domain NEJ 3 to CLK appeared to be enhanced by the presence of CYC (Fig. 3H). The interaction was independent of the glutamine-rich domain of CLK, since both fragments of NEJ bound to the C-terminally truncated CLKJrk protein that lacks this region (Fig. 3I). The antimorphic nature of the CLKJrk mutation may be due to effects on the histone acetyl transferase activity of CLK that has previously been identified in mammals (30). Our results show that NEJ physically interacts through two binding sites, the N-terminus and a middle domain, with an N-terminal part of CLK, which is consistent with a role of NEJ as a co-activator for CLK/CYC-dependent transcription in Drosophila. The interaction of the mammalian CLK/BMAL1 complex with CBP and p300 family members suggests that this function is conserved between the invertebrate and vertebrate clock (31-33), although mutant phenotypes await to be investigated in a mammalian model.

We next addressed the question of whether recruitment of NEJ to the CLK/CYC complex is a constituent of the basal transcription machinery or NEJ is a regulatory component of the circadian clock. NEJ as well as its’ mammalian ortholog CBP affects transcription through different modes of action: 1) through histone acetylation, 2) by mediating interactions between transcription factors and the basal transcription machinery (coactivation), 3) by mediating cross-talk between transcription factors (29). Previous studies in mammals showed that the cellular concentration of CBP is limiting and CBP-dependent transcription factors such as AP1, nuclear hormone receptors, the JAK/STAT pathway and E2F compete for recruitment of CBP, thereby establishing a CBP-mediated crosstalk between these signaling pathways (14-16). The molecular arrhythmicity induced by overexpression of NEJ in flies suggests
that NEJ is a limiting factor for CLK/CYC activity as well. To further analyze a co-activator function of NEJ for CLK/CYC-mediated transcription in vivo and in order to test whether trans-activation by CLK/CYC responds to changes in nej levels, we assayed constitutive CLK/CYC-dependent transcription in wild-type and nej mutant flies under constant light conditions, when the Drosophila circadian clock does not oscillate. Under such conditions at least the per/tim-feedback loop is not functional due to degradation of PER and TIM proteins in light. Therefore feedback mechanisms that may compensate for an increase or decrease of CLK/CYC activity are at least partially non-functional, which allows to monitor effects on CLK/CYC activity in flies more directly. tim and pdpl transcript levels were decreased in the nej partial loss of function mutant EP(X)1149 compared to EP(X)950 control, consistent with a co-activator function of nej for CLK/CYC-mediated transcription (Fig. 4A, C, E). hs-nej flies showed an induction of tim and pdpl mRNA levels upon heat shock, while wild-type flies displayed no significant changes or rather decreased transcript levels (Fig. 4B, D, F). Similar results were obtained for per and vri expression (Fig. S4). CLK levels were not significantly different in these fly strains and conditions as determined by western blotting (Fig. 4G, H). These results show that overexpression of nej increases CLK/CYC-dependent transcription by approximately 50% suggesting that under wild-type conditions about one third of CLK/CYC complexes lack NEJ in order to reach maximal activity. The decrease in CLK/CYC-dependent transcripts in EP(X)1149 flies is proportional to the decrease in nej levels (Fig. 4A, C, E). These experiments further demonstrate a co-activator function of NEJ for CLK/CYC activity and they show that trans-activation by CLK/CYC responds to changes in limiting cellular levels of NEJ.

Consistent with our results, a parallel study by Lim et al. (34) found a role for nej in the Drosophila circadian clock. From the finding of low expression levels of CLK/CYC-dependent genes after constitutive overexpression of nej it was concluded that nej acts as a repressor on CLK/CYC function. We show however that a reduction in nej levels results in a proportional decrease in CLK/CYC activity in flies as well as in cell culture and induction of nej expression in flies induces CLK/CYC-dependent transcripts, which demonstrates that nej acts as a co-activator for CLK/CYC function. Reduced levels of CLK/CYC-dependent genes observed after constitutive overexpression of nej (see also PER levels in Fig. 2 and Fig. S2) are likely due to circadian feedback regulation that compensates for hyper-activation of CLK/CYC by nej.

The involvement of NEJ in the functional organization of the Drosophila circadian clock adds additional modes to the regulation of circadian transcription. The finding that moderately reduced or increased levels of NEJ, decrease or enhance CLK/CYC-dependent transcription respectively, demonstrates that CLK/CYC activity is sensitive to limiting levels of NEJ and availability of NEJ regulates circadian transcription (Fig. 1, 4). Studies on transcriptional activation by AP1, nuclear hormone receptors, the JAK/STAT pathway and E2F have shown that multiple transcription factors compete for recruitment of the mammalian ortholog CBP from a limiting pool, thereby establishing negative cross-talk between these pathways (14-16). Our results suggest that CLK/CYC joins this competition for recruitment of NEJ (or CBP in mammals) allowing a cross-talk between circadian transcription and NEJ/CBP-dependent transcriptional regulation of metabolism, development, cell proliferation, memory formation and other physiological processes. In addition it has been shown that a number of signaling pathways such as cyclic nucleotides, calcium and Ras/MAPK regulate NEJ-dependent transcription through both, direct phosphorylation of NEJ as well as phosphorylation dependent recruitment of NEJ to target transcription factors (35). The regulation of CLK/CYC-dependent transcription by these signaling pathways is, in addition to direct phosphorylation of CLK (13), likely mediated through the control of NEJ co-activator function. NEJ thereby provides an interface for cross-talk between circadian transcription and vital physiological processes, which likely assists the circadian orchestration of life.
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Figure 1. Overexpression of *nej* increases behavioral arrhythmicity. A) Average locomotor activity over all flies of indicated genotypes for the last day in light:dark cycles (LD) and five days in constant darkness (DD). (B-D) Overexpression of *nej* by heat-shock induction or the GAL4/UAS system. B) *nej* transcript levels, determined by quantitative RT-PCR, in *w1118* control and *hs-nej* flies either after incubation at constant 20°C (black bars) or after application of a 30 min heat shock at 37°C (grey bars). Average *nej* mRNA levels ± SEM from at least eight independent experiments per genotype are shown with *nej* mRNA levels in *w1118* flies set to 100. C) Representation of different EP-element insertions in the 5′-UTR of *nej* that either allow overexpression of *nej* from the UAS-promoter (EP(X)1149 and EP(X)1179) or that contain an inverted insertion of the UAS-promoter not allowing overexpression of *nej* (EP(X)950 and EP(X)1410). D) *nej* transcript levels, determined by quantitative RT-PCR, in EP-lines with a tim-GAL4 driver. Average *nej* mRNA levels ± SEM from at least four independent experiments per genotype are shown with *nej* mRNA levels in *w1118* flies set to 100.

Figure 2. Overexpression of *nej* increases molecular arrhythmicity. A) Average real-time luciferase bioluminescence rhythms over all rhythmic or arrhythmic BGluC flies of indicated genotypes for the last day in LD and three days in DD. Number of rhythmic (upper panels) or arrhythmic flies (lower panels) vs. total number of flies is given in brackets together with percentage. B) PER immunocytochemistry in PDF expressing lateral neurons of larval brains harvested at CT 10 and CT 24 during the first day in constant darkness after entrainment in LD (CT 0 is subjective time of lights on, CT 12 is subjective time of light off) from genotypes as indicated in the figure. The PER signal from double stained brains is shown in green and the signal from cytoplasmic PDF-precursor PAP in red, yellow indicates co-localization of PER and PAP. C) Mean ± SEM staining intensity of PER in immunocytochemistry images as shown in B) for at least 14 brain hemispheres per genotype and time-point.

Figure 3. NEJ co-activates CLK/CYC-dependent transcription and physically interacts with CLK and CLK/CYC complexes. CLK/CYC-dependent luciferase reporter gene expression in *Drosophila* Schneider 2 cells expressing luciferase either from a four *per*-E-box containing promoter (A, D) or a genomic fragment of the *tim* promoter (B). A, B) CLK/CYC-dependent luciferase reporter gene expression in the absence (control) or presence of co-expressed E1A(12S) or E1A(ΔN). C) Western-blot analysis of E1A(12S) and E1A(ΔN) expression in S2 cells after transfection of 2 μg for each expression construct or for untransfected control. Results shown are from the same blot and exposure. D) CLK/CYC-dependent luciferase reporter gene expression in the absence (control) or presence of small interfering RNAs (siRNA) targeting either Clk, cyc, EGFP or *nej*. Values are mean ± SEM LUC activities expressed as a percentage of the control value (set to 100 %) in the absence of *nej* inhibitors or siRNAs. E) Representation of NEJ fragments that were cloned and expressed in reticulocyte lysate for interaction studies (cysteine/histidine rich zinc finger domains: C/H1, C/H2 and CH3; CREB binding domain: CBD; histone acetyl transferase domain: HAT). F-I) Autoradiograph images from interaction studies with 35S-methionine labeled proteins. F) Input (15 %) controls for 35S-methionine labeled FLAG-CLK (lane 1), FLAG-CLKΔk (lane 2) and CYC (lane 3) as well as precipitation of FLAG-CLK (lane 4) or FLAG-CLKΔk (lane 5) with anti-FLAG-antibody. G) Co-immunoprecipitation of 35S-CYC (lane 1 input) with anti-FLAG beads alone (lane 2) or with non-radiolabeled FLAG-CLK (lane 3) or FLAG-CLKΔk (lane 4). H) Co-immunoprecipitation of 35S-labelled domains of NEJ with non-labeled FLAG-tagged CLK with or without 35S-CYC by anti-FLAG antibody (lane 1, 5, 9, 13 input controls for NEJ fragments; lane 2, 6, 10, 14 control pull-down in the absence of CLK; lane 3, 7, 11, 15 pull-down in the presence of CLK; lane 4, 8, 12, 16 pull-down in the presence of CLK and 35S-CYC). I) Co-immunoprecipitation of NEJ1 and NEJ3 fragments with non-radiolabeled FLAG-tagged CLK or CLKΔk in the presence or absence of 35S-CYC as in H) (lane 1 and 7 input controls for NEJ fragments; lane 2 and 8 pull-down in the absence of CLK or CLKΔk; lane 3 and 9 pull-down with CLK; lane 4 and 10 pull-down with CLK and 35S-CYC; lane 5 and 11 pull-down with CLKΔk; lane 6 and 12 pull-down with CLKΔk and 35S-CYC).

Figure 4. CLK/CYC-dependent transcription is sensitive to cellular levels of NEJ in flies. A-F) Results from quantitative RT-PCR for transcript levels of *nej* (A, B), *tim* (C, D) and *pdp1* (E, F) from fly heads of indicated genotypes. Flies were previously incubated in constant light for at least 48 hours and harvested either at 20°C (black bars) or after application of a 30 min heat shock at 37°C (grey bars). Average mRNA levels ± SEM from at least five independent experiments per genotype are shown with mRNA levels in EP(X)950 (A, C, E) or in the
absence of heat shock (B, D, F) set to 100. G, H) Western-blot analysis of CLK levels for genotypes and conditions as in A-F) showed no effect of NEJ levels on CLK expression. Representative western-blot results are shown with indicated CLK and an asterisk marking an unspecific band detected by the anti-CLK antibody, which served as a loading control. Mean CLK levels ± SEM from two or three experiments are shown below with CLK levels in EP(X)950 (G) or in the absence of heat shock (H) set to 100.
Table 1: Results of free-running locomotor activity measurements

| Genotype            | #rhythmic/#total | % rhythmic | period length ±SEM |
|---------------------|------------------|------------|--------------------|
| **Analysis of locomotor activity after entrainment in cycles of 12 hrs light and 12 hrs dark** |
| Oregon R            | 35/42            | 83         | 24.4 ± 0.1         |
| w^{1118}            | 45/46            | 98         | 23.7 ± 0.1         |
| Canton S - hs       | 120/147          | 82         | 23.7 ± 0.1         |
| Canton S + hs       | 45/58            | 78         | 24.1 ± 0.1         |
| Hs-nej * - hs       | 11/146           | 8          | 23.9 ± 0.2         |
| Hs-nej + hs         | 13/60            | 22         | 24.4 ± 0.3         |
| Dp(1;Y)FF1 - hs     | 35/111           | 32         | 23.5 ± 0.1         |
| Dp(1;Y)FF1 + hs     | 3/57             | 5          | 23.9 ± 0.4         |
| ClkJrk - hs         | 2/96             | 2          | NA                 |
| ClkJrk + hs         | 3/58             | 5          | NA                 |
| EP(X)950            | 38/46            | 83         | 24.1 ± 0.2         |
| EP(X)950; tim-GAL4/+| 81/91            | 89         | 24.9 ± 0.1         |
| EP(X)1410           | 31/51            | 61         | 23.4 ± 0.2         |
| EP(X)1410; tim-GAL4/+| 77/94           | 82         | 24.1 ± 0.1         |
| EP(X)1179           | 30/43            | 70         | 24.1 ± 0.3         |
| EP(X)1179; tim-GAL4/+| 33/89           | 37         | 24.9 ± 0.2         |
| EP(X)1149           | 27/46            | 59         | 27.6 ± 0.4         |
| EP(X)1149; tim-GAL4/+| 16/90           | 18         | 24.3 ± 0.2         |

| Genotype            | #rhythmic/#total | % rhythmic | period length ±SEM |
|---------------------|------------------|------------|--------------------|
| **Analysis of locomotor activity after entrainment in cycles of 12 hrs 18°C and 12 hrs 28°C** |
| Canton S            | 29/39            | 74         | 22.7 ± 0.1         |
| Hs-nej              | 6/37             | 16         | 23.5 ± 0.4         |
| Dp(1;Y)FF1          | 3/32             | 9          | 22.7 ± 0.7         |
| ClkJrk              | 0/37             | 0          | NA                 |

hs: locomotor activity without (-hs) or with (+hs) application of a 37°C heat shock for the last hour of darkness during prior entrainment in light dark cycles
NA: not analyzed
*A detailed statistical analysis indicated that within the group of arrhythmic flies, about 33% of Dp(1;Y)FF1 and 21% of hs-nej flies maintain weak behavioral rhythms (Fig. S1).*
Figure 1, Hung et al. 2007

A

Average locomotor activity [counts/min]

Canton S  
Dp(1;Y)FF1  
Clk\textsuperscript{Jrk}  
w\textsuperscript{1118}  
hs-nej  
EP(X)\textsuperscript{950}; tim-GAL4/+  
EP(X)\textsuperscript{1149}; tim-GAL4/+  

Time [days]

B

nej mRNA level [Arbitrary Units]

20°C  
37°C  
hs  
w\textsuperscript{1118}  
hs-nej

C

EP(X)\textsuperscript{950}  
EP(X)\textsuperscript{1149}  
EP(X)\textsuperscript{1410}  
EP(X)\textsuperscript{1179}

D

nej mRNA level [Arbitrary Units]

20°C  
37°C  
hs  
w\textsuperscript{1118}  
EP(X)\textsuperscript{950}  
EP(X)\textsuperscript{1149}  
EP(X)\textsuperscript{1410}  
EP(X)\textsuperscript{1179}  
lim-GAL4  
lim-GAL4  
lim-GAL4  
lim-GAL4
Figure 2, Hung et al. 2007
Figure 4, Hung et al. 2007

A) nej mRNA level (Arbitrary Units)

B) nej mRNA level (Arbitrary Units)

C) tim mRNA level (Arbitrary Units)

D) tim mRNA level (Arbitrary Units)

E) pdp1 mRNA level (Arbitrary Units)

F) pdp1 mRNA level (Arbitrary Units)

G) CLK protein level (Arbitrary Units)

H) CLK protein level (Arbitrary Units)
Circadian transcription depends on limiting amounts of the transcription co-activator nejire/CBP
Hsiu-Cheng Hung, Christian Maurer, Steve A. Kay and Frank Weber

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