A non-circadian role for clock-genes in sleep homeostasis: a strain comparison

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Abstract

Background: We have previously reported that the expression of circadian clock-genes increases in the cerebral cortex after sleep deprivation (SD) and that the sleep rebound following SD is attenuated in mice deficient for one or more clock-genes. We hypothesized that besides generating circadian rhythms, clock-genes also play a role in the homeostatic regulation of sleep. Here we follow the time course of the forebrain changes in the expression of the clock-genes period (per)-1, per2, and of the clock-controlled gene albumin D-binding protein (dbp) during a 6 h SD and subsequent recovery sleep in three inbred strains of mice for which the homeostatic sleep rebound following SD differs. We reasoned that if clock genes are functionally implicated in sleep homeostasis then the SD-induced changes in gene expression should vary according to the genotypic differences in the sleep rebound.

Results: In all three strains per expression was increased when animals were kept awake but the rate of increase during the SD as well as the relative increase in per after 6 h SD were highest in the strain for which the sleep rebound was smallest; i.e., DBA/2J (D2). Moreover, whereas in the other two strains per1 and per2 reverted to control levels with recovery sleep, per2 expression specifically, remained elevated in D2 mice. dbp expression increased during the light period both during baseline and during SD although levels were reduced during the latter condition compared to baseline. In contrast to per2, dbp expression reverted to control levels with recovery sleep in D2 only, whereas in the two other strains expression remained decreased.

Conclusion: These findings support and extend our previous findings that clock genes in the forebrain are implicated in the homeostatic regulation of sleep and suggest that sustained, high levels of per2 expression may negatively impact recovery sleep.

Background

At the cellular level, circadian rhythms are thought to be generated by transcriptional-translational feedback loops made up of positively and negatively acting transcriptional regulators [1]. In mammals, the core positive elements are CLOCK and NPAS2 and their obligate dimerization partner BMAL1. These transcription factors drive per and cry transcription. PER and CRY proteins, in turn, interact with CLOCK/NPAS2:BMAL1 heterodimers to inhibit their own transcription, thus constituting the
negative feedback elements. The circuity is clearly more complex as additional feedback loops and post-translational modifications are involved [1]. Clock genes underlie circadian rhythm generation in many tissues, but the circadian rhythm in the suprachiasmatic nucleus (SCN) is required for the manifestation of overt physiological and behavioral rhythms, and is therefore considered the master circadian pacemaker [1,2].

Although the role of clock genes in generating circadian rhythms is firmly established, we and others have found that they also play a role in the homeostatic regulation of non-REM sleep (NREMS) [3-5]. This notion is based on the observation that mice deficient for one or more clock genes show an altered response to sleep deprivation (SD) and on the observation that clock-gene expression in brain areas outside the SCN, notably the cerebral cortex, is influenced by the prior occurrence of sleep and wake. For instance, the typical responses in NREMS to SD were reduced or absent in mice lacking the cry1 and -2 (cry1,2/-) [4] or bmall1 (bmall1/-) [6] genes compared to wild-type controls. The diminished NREMS variables included duration, consolidation, and EEG delta power. EEG delta power quantifies the prevalence and amplitude of delta oscillations and is widely used to index the homeostatic need for NREMS [7]. Sleep under undisturbed, baseline oscillations and is widely used to index the homeostatic need for NREMS [7]. Sleep under undisturbed, baseline conditions was also altered in mutant mice. clock-mutant (clock−/−) [8] and npas2−/− [3] mice spent less time in NREMS whereas cry1,2−/− [4] and bmal1−/− [6] mice slept more. NREMS consolidation was increased in cry1,2−/− mice while the opposite was observed in clock−/− [8] and bmal1−/− [6] mice and in mice lacking albumin D-binding protein (dbp−/−) [9]; a gene under the transcriptional control of CLOCK [10] and that also has been implicated in circadian rhythm generation [11]. Average levels of EEG delta power were reduced in clock−/− [8], dbp−/− [9], and bmal1−/− [6] mice and increased in cry1,2−/− [4] mice.

Clock-gene expression in the brain, notably the cerebral cortex, depends on the sleep-wake (or rest-activity) distribution. Cortical levels of per are high at times when sleep drive is high, irrespective of the phase at which the circadian expression of per peaks in the SCN. Thus, in both nocturnal and diurnal species, per expression in the cortex is maximal in conjunction with the major waking episode [12-14]. Under conditions where the phase (methamphetamine administration, restricted feeding) or distribution (circadian splitting) of locomotor activity is altered, per expression in the cortex parallels the overt rhythm of wakefulness, while per expression in the SCN remains unaffected [13,15-17]. Consistent with these observations, our data demonstrate that per expression increases when mice are kept awake [3,4]. The lack or alteration of particular clock genes in the various mouse models mentioned above, changes the expression levels of other clock genes in the circuitry. For instance, basal per expression levels are decreased in clock−/− mice [10,18] and increased in cry1,2−/− mice [4,19-21] which corresponds to their opposing sleep phenotypes (see above). Also, the SD-induced increase in per expression is reduced in npas2−/− mice [3] which suggest that NPAS2 is implicated in linking per2 expression to the sleep-wake distribution. Likewise, npas2−/− mice lack circadian rhythms in cortical per2 expression despite an intact circadian organization of sleep and waking [12].

The finding that NPAS2 couples wakefulness to clock gene expression is of particular interest given the evidence that NPAS2 (and CLOCK) mediated transcription is redox sensitive [22]. Redox sensitivity suggests that this clock gene network may be linked to cellular energy metabolism [23]. Since restoration of an optimal neural energy state has long been considered a possible function of sleep [24], this gene network might underlie a fundamental aspect of sleep homeostasis. If so, another critical gene in this network is likely to be ldha (lactate dehydrogenase A). ldha can be induced in cell culture by NPAS2, has multiple E-box sites, is a critical enzyme for neuronal energy metabolism, and utilizes NADH as a co-factor [12,22]. Therefore, we decided to investigate gene expression in the forebrain in greater detail over the course of a SD and after recovery sleep for the CLOCK and NPAS2 target genes per1, per2, dbp, and ldha. Furthermore, we undertook this examination of the sleep-wake dependent changes in gene expression in three inbred strains of mice known to differ in the rate at which sleep need, quantified as EEG delta power, increases during wakefulness [25]. We reasoned that if clock genes are implicated in sleep homeostasis, then the time course of changes in gene expression during a SD in these strains should vary according to the published differences in EEG delta power dynamics.

Results

Control conditions

Under undisturbed control conditions, forebrain levels of per1, per2, and dbp expression varied both as a function of time-of-day and of genotype (2-way ANOVA factors ‘genotype’ and ‘ZT’ P < 0.0001). Generally, all three genes reached highest expression levels at ZT18 (post-hoc Tukey; P < 0.05; Fig.1), but there were time-course differences. The values for dbp expression were lowest at ZT1 and ZT3 and then increased throughout the light period. In contrast, the expression of per1 and per2 first decreased from ZT1 reaching lowest levels at ZT3 or -6 before increasing. The time courses observed here match well those previously published for most brain areas outside the SCN and for certain other peripheral tissues [16,26-28] with lowest mRNA values observed early (dbp) or mid (subjective) day (per1, per2) and peak levels being reached around ZT8 for dbp or around ZT12-14 for per1 and per2 (not assessed
No systematic variation in \( ldha \) expression levels was observed across the 4 time points in any strain.

Although the time-dependent changes in gene expression did not differ among the 3 inbred strains, genetic background did affect overall expression levels within the control group. With few exceptions, expression of \( per1 \), \( per2 \), and \( dbp \) was higher in D2 compared to AK and B6 mice at all four time points (post-hoc Tukey; \( P < 0.05 \); Fig. 1, summarized in Fig. 2A). Elevated expression levels for D2 were maintained even during SD-conditions (Fig. 2A). Genotype did not affect the level of \( ldha \) expression.

**Sleep deprivation**

Against this varying background the effects of enforced wakefulness on the expression of clock and clock-controlled genes was evaluated. Confirming our previous results in B6 mice [3,4], 6 h SD increased \( per1 \) and \( per2 \) expression and decreased \( dbp \) expression in comparison to time controls (Fig. 1). The present experiment allowed us to follow the time course of gene expression during the 6 h SD and also enabled us to assess and compare the dynamics among mice of different genetic backgrounds.

SD changed the expression of these genes in the same direction in all three strains, however, significant genotype differences were observed in the dynamics and the magnitude of these changes (Figs. 1 and 2B). In AK mice \( per1 \) and \( per2 \) expression was significantly increased after only 1 h of SD compared to control levels at ZT1. In D2 mice only \( per1 \) was increased after 1 h of SD, and in B6 mice neither \( per1 \) nor \( per2 \) were increased by 1 h of SD. However, \( per1 \) and \( per2 \) mRNA in AK mice did not increase further as SD progressed whereas in D2 mice (as well as in B6 for \( per2 \)) a progressive increase was observed from 1- to 3- to 6 h SD (Figs. 1 and 2B). Forebrain mRNA levels of \( dbp \) increased over the 6 h of the experiment in both control and SD groups of D2 and B6 mice, but the SD values tended to be lower than the control values after 3 and 6 h SD. The increase of \( per1 \), \( per2 \), and \( dbp \) over the course of the SD was quantified by linear regression analysis which demonstrated that the rate of increase was significantly greater in D2 mice compared to AK mice (Figs. 1 and 2B). The largest changes (both increases and decreases) in mRNA levels were observed after 6 h SD. For \( per1 \) and \( per2 \) expression the increase at this time point differed significantly among inbred strains (2-way ANOVA interaction ‘genotype’-'SD': \( per1 \) \( P = 0.0001 \), factor ‘ZT’: \( per1 \) \( P = 0.0002 \), \( per2 \) \( P = 0.004 \), \( dbp \) \( P < 0.0001 \)). For both \( per \) genes the SD effect differed among genotypes (‘SD’-’genotype’ interaction: \( per1 \) \( P = 0.039 \); \( per2 \) \( P = 0.006 \)). Linear regression lines (red) describe the time course of mRNA changes during SD (ZT1 through ZT6; see Fig. 2). Asterisks and crosses mark times when SD differed from controls (\( P < 0.05 \) and \( P < 0.10 \), respectively; post-hoc t-tests). Symbols ‘a’ (> AK) and ‘b’ (> B6) mark strain differences for each time point and condition (\( P < 0.05 \); post-hoc Tukey).

The genotype specific changes in clock-gene expression during SD (Fig. 2B), the genotype differences in the aver-
age expression levels during both control and SD (Fig. 2A), and the larger relative increase in per1 and per2 after 6 h SD are all opposite to the strain differences we previously reported in the dynamics of the homeostatic regulation of sleep [25]. For this phenotype the rate of increase in sleep need during wakefulness (quantified as EEG delta power during NREMS) was fastest in AK mice and slowest in D2 mice (Fig. 2B). This suggests a negative correlation between the magnitude of the increase in these clock genes and the homeostatic regulation of sleep. To facilitate a direct comparison between our present and our previous study we made every effort to follow the same experimental protocol and conditions including the LD cycle, light intensity, ambient temperature, animal provider, sex and age of the mice, acclimatization time, start time and method of sleep deprivation.

Recovery sleep
Two hours of recovery sleep affected per1 and per2 forebrain mRNA levels in a strain specific manner (Fig. 1). per1 expression sharply decreased from the levels reached at the end of the 6 h SD and in AK mice values reached at ZT8 even fell below control levels. A similar recovery pattern was observed for per2 expression with the exception of D2; in this strain, recovery sleep could not lower per2 mRNA levels and values remained significantly elevated compared to control. Conversely, only in D2 mice did the decrease in dbp expression revert to control levels.

Correlations
The expression of all four genes studied here are regulated by CLOCK and NPAS2 through E-boxes [22,29-31] although other cis-acting elements are known to be involved; e.g., cAMP-response and DBP-binding elements

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**Figure 2**

A) Average mRNA levels under both control (mean ZT1-6; ± 1SEM; n = 12/strain; grey) and SD (mean ZT1-6; red symbols; n = 12/strain) conditions were generally higher in D2 mice compared to AK and B6 mice [*a* (> AK), *b* (> B6) *P* < 0.05; post-hoc Tukey]. No genotype differences were observed for ldha expression (not shown).

B) Mean (± 1SEM; n = 12/genotype) slope of the regression lines quantifying the relationship between SD duration (1-, 3-, and 6 h) and clock-gene expression (fraction/h; see Fig. 1) in the forebrain of AK, B6, and D2 mice. Changes in expression were affected by genotype (2-way ANOVA: 'genotype'-'ZT' interaction: per1: *P* = 0.0002; per2: *P* = 0.032; dbp: *P* = 0.0002). Slopes were steepest for D2 mice and the strain distribution pattern for this phenotype seemed to negatively correlate with the increase of EEG delta power (%/h) during wakefulness (i.e., 'Process S' in lower right panel; modified from [25]; with permission).
[11,26,29,32,33]. Given the likelihood of shared transcriptional regulation through E-boxes, we tested expression patterns for similarities among these four genes and how these relationships were affected by the SD (Fig.3). Under both control and SD conditions the two period genes were strongly correlated sharing 63 and 42% of the variance, respectively (R²; linear regression, \( P < 0.0001 \); Fig.3). Other significant correlations observed during control conditions were between per1 and dbp (R² = 0.28; \( P < 0.0001 \)) and between dbp and ldha (R² = 0.24; \( P = 0.0004 \)) expression. These relationships changed dramatically with SD; per2 expression, which was affected the most by SD, now became correlated with that of the other 3 genes while the per1-db and dbp-ldha correlations no longer reached significance levels and the per2-db correlation, that was not significant during control, became equally important as the per1-per2 correlation (R² = 0.44 and 0.42 respectively, \( P < 0.0001 \); Fig.3). The SD-induced increase in the correlation coefficient between per2 and dbp expression (-0.15 vs. 0.50) and the decrease observed for the per1-db correlation (0.45 vs. -0.12) were significant (\( P < 0.005 \); 2-sided; \( n = 48/\)condition). Within strains, the same relationships were observed, although they did not always reach significance levels (analyses not shown).

**Discussion**

We previously found that cortical mRNA levels of the clock genes *per1* and *per2* were increased after SD in rats and B6 mice [4]. Here we have extended that observation in two additional inbred strains; AK and D2. Furthermore, by following the time course of these changes during SD and recovery sleep, we established that *per* expression changes as a function of the time-spent-awake and asleep. Such a time course is consistent with a role for these genes in the homeostatic regulation of sleep since the need for sleep increases with duration of wake and decreases with

![Figure 4](http://www.biomedcentral.com/1471-2202/8/87/figure4)

**In situ hybridization**

To gain insight into which forebrain regions contributed to the SD-induced changes in *per1* mRNA levels reported above, we performed *in situ* hybridizations. During control conditions, forebrain expression levels were low, especially in the cerebral cortex, while expression in the cerebellum was high (Fig.4), matching previously published studies [12,13,16,34]. Following 6 h SD, *per1* expression increased throughout the brain (Fig.4). This increase was especially pronounced in the cerebral cortex, consistent with our qPCR data (Fig.1). Within the cortex, increases were especially notable in the frontal cortex and in and around the cingulate cortex. Expression in the cerebellum was also importantly increased. Expression patterns and effects of SD described here for *per1* match those of *per1* and *per2* publicly available from the Allen Institute for Brain Science website [35] (Fig.5).

**Figure 3**

Significant correlations among forebrain expression levels of the four genes examined during control (left; \( n = 48 \)) and sleep deprivation (SD; right; \( n = 48 \)) conditions. Only those correlations between gene pairs are indicated that remained significant after partialing out the influence of the other two. In blue the Pearson’s correlation coefficients, in red the partial correlation coefficients. Line thickness corresponds to correlation strength.

**Figure 4**

Representative *in situ* hybridizations for *per1* expression in two horizontal brain sections from a sleep deprived (SD, lower panels) and a control (upper panels) mouse. Following 6 h SD *per1* mRNA increased throughout the brain. This increase was especially pronounced in the cerebral cortex and cerebellum (CB), consistent with our qPCR data (Fig.1). Note the very low cortical expression in control mice which, at ZT6, spent most of the preceding 6 h sleeping. Within the cortex, increases were especially notable in the frontal cortex (FC) and in and around the cingulate cortex. Caudate-Putamen (CP) and hippocampus (HP) are labeled for orientation.
duration of sleep. A novel aspect of this study is the comparison of the SD-induced changes in gene expression among three inbred strains for which the homeostatic regulation of sleep differs [25]. During both spontaneous and enforced periods of wakefulness the need for sleep, indexed by the level of EEG delta power, increased fastest in AK, intermediate in B6, and slowest in D2 mice. If indeed per expression would simply parallel EEG delta power we would expect to find the largest increase in per expression after SD in AK mice and the smallest in D2. Here we found evidence for the opposite; although per expression was consistently increased with SD, this increase was modulated by genotype and was found to be highest in D2 and lowest in AK. In addition, per2 expression remained elevated during recovery sleep in D2 mice only. Thus high, sustained levels of per2 expression might negatively impact the sleep recovery process and/or expression of EEG delta oscillations during NREMS.

Figure 5
In situ hybridizations for per1 (upper) and per2 (middle panels) expression in four sagittal brain sections from sleep deprived (SD, right) and control (left panels) mice. All sections were taken close to mid-line (see lower panel; red vertical line in coronal section indicates the plane of the sagittal sections). per was expressed mostly in cortex and cerebellum and SD induced an increase in both these areas, confirming our own per1 expression data (Fig. 4). These data were taken, with permission, from the publicly available in situ studies of the Allen Institute for Brain Science [60], done in collaboration with Drs. T.S. Kilduff and J.P. Wisor of SRI International. The experimental protocol was identical to that of the 6 h SD in the present study; both SD and control animals were sacrificed at ZT6. ID numbers of sections: per1 control: 145-0202054795 at 3625; SD: 153-0202055093 at 3825; per2 control: 140-0205056163 at 3500; SD: 132-0205058042 at 3300. Lower right panel is the histological view (Nissl stain) of the upper right panel (per1-SD) with cerebral cortex (CTX), thalamus (TH), hypothalamus (HY), and cerebellum (CB). The presence of EEG electrodes in some mice caused cortical damage during brain removal.
The in situ hybridization studies indicated that SD induced pronounced changes in per expression in cerebellum and cerebral cortex. This is in line with our previous findings using qPCR showing that per expression was increased in the cerebral cortex but not in brainstem and hypothalamus [4] and with the per2 increase observed after SD both in cerebellum and cerebral cortex [44]. While the in situ data are limited, we observed some of the highest expression and largest increases in per1 mRNA in the most frontal area of the cortex. EEG delta power is generally higher and its increase after SD more pronounced in frontal cortex relative to other cortical areas such as occipital regions [45-47] in accordance with the concept that frontal areas in particular are affected by SD [48]. In mice lacking a functional PER1 protein (per1∆/∆) EEG delta power was decreased in the frontal derivation specifically and the SD-induced increase in EEG delta power was smaller in per1∆/∆ and per2∆/∆ mice also only in the frontal derivation [49].

Already clear from the present data is that not all changes in forebrain per expression can be attributed to changes in the sleep-wake distribution: expression increases during the latter part of the light period while animals remain mostly asleep. This increase precedes dark onset after which maximal per expression levels are reached when mice are the most active [16,27]. A major adaptive advantage provided by circadian rhythms is that it allows organisms to anticipate and prepare for predictable periodic changes in the environment [50]. The anticipatory increase in per expression might be part of a molecular repertoire preparing the animal for the active period. Some types of anticipatory behavior, however, don’t require an intact circadian system, as food-restriction studies have shown [51]. Moreover, clock-genes in the forebrain seem to play a permissive role in such anticipatory behaviors. For example, npas2−/− mice, which have intact circadian rhythms in overt behavior, have difficulty adapting to and anticipating altered feeding schedules [17]. Besides food intake, other homeostatically regulated behaviors such as sleep seem to be similarly affected [3].

Thus, the same molecular network of transcriptional regulators that sets internal time-of-day in the SCN might, in the forebrain, track and signal time without sleep (or food). Conceptually the distinction between homeostatic and circadian processes at the molecular level thus becomes unclear. Mechanistically we proposed that changes in clock-gene expression could be tied directly to cellular energy state based mainly on the observation that CLOCK and NPAS2 mediated transcription is sensitive to redox state [22,23]. In this respect the lack of an effect of SD on ldha expression is disappointing. Although ldha has a canonical E-box and has been shown to be activated by both NPAS2 and CLOCK in a neuroblastoma cell line [22], mRNA levels of this gene were surprisingly stable.
across time-of-day, experimental condition, and genetic background.

**Conclusion**
We found that the expression of three circadian clock genes changes as a function of time-spent-awake and that these changes were influenced by strain. These results further indicate that circadian clock genes play a role in the homeostatic regulation of sleep. Additional evidence in support of our hypothesis has recently been obtained in humans homozygous for one of two known polymorphisms in hPer3 [52]. Based on SCN lesion experiments in rats [53] and forced desynchrony studies in humans [54] it is generally thought that homeostatic and circadian processes operate separately and have different neurophysiological substrates. Our current results underscore that, at least at a molecular level, these two processes are difficult to separate. Recent data also show that at the neuro-physiological level, SD can affect the activity of SCN neurons previously thought to be dedicated exclusively to setting internal time-of-day[55]. Finally, the present results might be of relevance for research on the biology of mood disorders. In this field there is increasing interest in the relation between clock-genes and e.g. seasonal affective disorders [56] or bipolar disorders [57]. The effects of sleep deprivation on clock-gene expression demonstrated here combined with the fact that sleep deprivation is a well known antidepressant add a molecular component to the intricate relationship between mood, circadian rhythms, and sleep (reviewed in [58,59]).

**Methods**

**Animals and Experimental Protocol**
A total of 102 mice contributed to this study. Of those, 96 were used for quantitative PCR (qPCR) analyses of per1, per2, dbp, and ldha expression in AKR/J (AK), C57BL/6j (B6), or DBA2/j (D2) mice (n = 32/genotype). The remaining 6 B6 mice were used for in situ hybridization studies of per1. All mice were 11-week old males which were kept under a 12 h light/dark cycle for at least 14 days before the experiments. For the qPCR experiment half of the mice (the SD-group) were sleep deprived by gentle handling during the light phase which is their usual rest phase. The other half served as controls and were left undisturbed so they could sleep *ad lib*. SD started at light-onset (Zeitgeber time, ZT0). Mice of the SD-group were sacrificed after 1-, 3-, or 6 h SD (i.e., at ZT1, -3, or -6). A 4th group was allowed 2 h of recovery sleep after 6 h SD (ZT8). At each time point 4 mice per genotype were taken. An equal number of control group mice were sacrificed at these 4 time points (n = 4/time-point/group/genotype). For in situ hybridization studies 3 mice were sleep deprived for 6 h starting at ZT0; the remaining 3 served as controls. Animals of both groups were sacrificed at ZT6.

**qPCR**
Brains were rapidly removed, dissected, and frozen at -70°C. Dissection involved removal of the cerebellum and pons-medulla, followed by a single mid-sagittal cut through the remaining brain (referred to as forebrain) producing a left and a right hemisphere. Total RNA from mouse forebrains was extracted and purified using TRIzol® Reagent (Invitrogen). RNA pellets were resuspended and incubated in RNase-Free™ (Ambion). Prior to cDNA synthesis, RNA samples were treated with Turbo DNA-free™ (Ambion) to remove genomic DNA contamination, and three micrograms of total RNA were converted into first strand cDNA with Oligo(dT)12-18 and Superscript II™ (Invitrogen).

To determine gene expression profiles, cDNA of individual mice was diluted 1:4 and 2 µl was aliquots were amplified in a 50 µl reaction mix containing 25 µl of iQ™ SYBR® Green Supermix (BioRad) and 0.2 µM each of forward and reverse gene-specific primers. Samples were placed into 96-well 0.2 thin-wall PCR plates (BioRad), covered with optical-quality sealing tape (Biorad), and subjected to 90 cycles of 15s at 94°C, 60s at 60°C, and 1 min at 72°C in an iCycler iQ™ real-time Detection System (BioRad). After PCR, products were subjected to a melt curve protocol to ensure that primer-dimers and non-specific products are not present. Briefly, PCR reaction products were melted at 95°C for 15s, then the temperature was lowered to 60°C for 1 min and then the temperature increased in increments of 0.5°C per 10s over 80 cycles. SYBR binds to the double-stranded PCR product until the Tm of the product is reached. Then, a sharp decline in fluorescence occurs resulting in a single peak as long as only one PCR product was amplified (gel electrophoresis of PCR products also confirmed a single band for all primer sets). The following forward and reverse primers used in this experiment were designed with Primer3, synthesized (Sigma-Genosys), and utilized as described above: per1 forward 5'-CTTGATGATGTCAGTCCTATT-3', reverse 5'-AGCTGGGGCAGTT-TCCATT-3', per2 forward 5'-ATCTCCAGCCGTTGTGA AG-3', reverse 5'-AGGGTATCCTGTCGGCCTCTT-3', dbp forward 5'-AAGGCAAGGAAGTCAGATG-3', reverse 5'- TGGGACAAGGAAGTAATGG-3', ldha forward 5'-CTGGTGTTGGATGTGTG-3', reverse 5'-TCGAGCTTTGGA CAGTGAAGT-3', gapdh forward 5'-CCATCAACGAC CCTCTTCATT-3', reverse 5'-TCTCGTGGTTCACCCATC-3', actb forward 5'-GCCGGGACCTGAGACTAC-3', reverse 5'-ATGGTGCTAGGAGCCAGAC-3'.

Relative abundance of each gene was normalized to both *gapdh* and *actb* determined by the relative standard curve method in which undiluted cDNA of all samples in each experiment were pooled and serial dilutions of the pool (stock, 1:5, 1:10, 1:100, and 1:1000) were run simultane-
ous, acetylcholine, and 50 μM GTP. Each reaction was incubated for 1 h at 37°C, followed by treatment with neutralization solution (50 mM DTT, 5% acetic acid, 100 mM sodium acetate).

Sections were thawed to room temperature, rehydrated, and incubated with 20 g/ml proteinase K, and treated with RNase A (Boehringer Mannheim, Indianapolis, IN); RNasin (Promega, Madison, WI); 35S-UTP (> 1,000 Ci/mmol; NEN, Boston, MA). Probes were incubated with hydrolysis solution (50 mM DTT, 40 mM NaHCO3, 60 mM Na2CO3) for 1 h at 37°C, followed by treatment with neutralization solution (50 mM DTT, 5% acetic acid, 100 mM sodium acetate). Probes were then precipitated and stored at -70°C.

Sections were hybridized for 12 h at 60°C with 5 × 10^6 cpm/ml 35S-labeled per1 RNA anti-sense probe in hybridization buffer [50% formamide, 300 mM NaCl, 20 mM Tris HCl (pH 7.4), 5 mM EDTA, 10 mM NaPO4 (pH 8.0), 100 mM DTT, 10% dextran sulfate 1 × Denhardt, 50 g/ml total yeast RNA] under siliconized coverslips. Coverslips were floated off in 4 × SSC at room temperature, followed by four washes in 2 × SSC, DTT. Slides were rinsed in washing buffer [400 mM NaCl, 10 mM Tris HCl (pH 7.5), 5 mM EDTA] and incubated with 200 μl RNAase A in washing buffer at 37°C, followed by washes in 2 × SSC and 1.0 × SSC. Slides were dehydrated and placed on autoradiography film (Kodak Hyperfilm MP) for direct imaging to reveal areas of RNA expression resulting from annealed 35S-labeled anti-sense probes. Film was developed after 18-day autoradiography film exposure.

**Authors’ contributions**

PF initiated and designed experiments, analyzed data, and wrote manuscript. RT conducted experiments, developed methods, and analyzed data. HCH was involved in experimental design, lab direction, and editing manuscript. BFO, along with PF, initiated and designed experiments, analyzed data, directed all experimental work by RT, and co-wrote manuscript. All authors have read and approved the final article.

**Acknowledgements**

We thank Tom Kilduff and Jonathan Wisor of SRI International and Ed Lein and Carol Thompson of the Allen Institute for Brain Sciences for explanations and use of their publicly available gene expression studies presented in Figure 5. We thank Vinh Cao for technical assistance. This work was supported by National Institute of Mental Health Grant MH67752, and in part by a NSF Functional Genomics Infrastructure Award EPS-0132295.

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