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In vitro circadian rhythms: imaging and electrophysiology

Christian Beaulé, **Daniel Granados-Fuentes**, **Luciano Marpegan**, and **Erik D. Herzog**[1]
Department of Biology, Washington University in Saint Louis, 1 Brookings Drive, P.O. Box 1137, Saint Louis, MO, 63130-4899, U.S.A.

Abstract

In vitro assays have localized circadian pacemakers to individual cells, revealed genetic determinants of rhythm generation, identified molecular players in cell-cell synchronization and determined physiological events regulated by circadian clocks. Although they allow strict control of experimental conditions and reduce the number of variables compared with *in vivo* studies, they also lack many of the conditions in which cellular circadian oscillators normally function. The present review highlights methods to study circadian timing in cultured mammalian cells and how they have shaped the hypothesis that all cells are capable of circadian rhythmicity.

Introduction

The ability to isolate and study biological systems in a dish has been instrumental in localizing circadian pacemakers and their molecular underpinnings. This *in vitro* approach has been particularly successful when applied to mammalian circadian rhythms, beginning in the 1960s with circadian adrenocorticotropic activity from acute cultures of adrenal and pituitary glands [1] and in the 1970s with daily changes in cell-cycle parameters (nucleus size, DNA synthesis) from cultured human embryonic fibroblasts, hepatoma cells and liver cells [2]. Although hinting at the endogenous, cellular, and self-sustaining properties of circadian clocks, these early studies were largely forgotten until recent advances allowed real-time measurements of molecular oscillations over multiple days.

Three discoveries define the explosion of *ex vivo* analyses of mammalian circadian rhythms: (i) the establishment of the hypothalamic SCN (suprachiasmatic nuclei) as a central pacemaker [3]; (ii) the characterization of canonical clock genes underlying circadian oscillations [4]; and (iii) the confirmation that these genes are ubiquitous and drive circadian rhythms in many tissues [5,6]. These discoveries, coupled with reporters of gene expression or cellular function, enabled scientists to show circadian oscillations in nearly every cell type that has been examined. This chapter surveys *in vitro* techniques currently in use in the study of mammalian circadian functions and emphasizes the significant insights gained.

Advantages and disadvantages of *in vitro* research

One of the main strengths of *in vitro* techniques is also its greatest weakness: it is a simplified system. *In vitro* isolation of a sample allows for rapid and precise control over experimental conditions, including temperature, light and the chemical environment. Furthermore, the availability of immortalized, homogeneous and highly characterized cell lines allows for high-throughput, reproducible, analyses without the need to kill an organism. On the other hand, these isolated tissues and cell lines are reduced preparations that can lack essential signals from elsewhere in the body or acquire traits as a result of their

[1]To whom correspondence should be addressed (herzog@wustl.edu)..

life in a dish. For example, because of the strong link between circadian rhythms and cancer [7], immortalized cell lines or cells from primary tumours can provide insight into how a dysregulated circadian clock could cause pathological cell divisions; however, the results may not relate to how circadian rhythms are generated in post-mitotic cells like neurons.

Although experiments *in vitro* allow for controlled delivery of active molecules, it can be challenging to administer anything without artefacts. For example, circadian rhythms in cultured astrocytes and neurons can be reset and entrained by relatively small changes in temperature. In addition, cultured cells are sensitive to mechanical and ionic perturbations associated with changing culture medium. Minimizing temperature changes can be accomplished by including a buffer (e.g. sealed jugs of water or bricks) inside the temperature-controlled incubator, warming reagents to culture temperature and keeping the incubator door closed as much as possible. Mechanical and ionic perturbations can be minimized by reducing treatment volume relative to the volume of the culture medium and using culture medium in the control treatment. With careful planning and execution, the effects of control treatments can be diminished.

Techniques for real-time measurements of circadian rhythms *in vitro*

Optical imaging

The discovery of clock genes and of their organization into transcription–translation feedback loops has motivated the development of ways to monitor mRNA and protein levels over time (Table 1). Starting in the early 1990s, luciferases and, later, modified versions of GFP (green fluorescent protein) were incorporated into transgenic models and used as an indicator of clock function [8]. Recording luciferase (Luc) activity is rapid and easily mastered. A transgenic sample is placed or grown in a dish with culture medium containing d-luciferin, the substrate for luciferase. A photomultiplier tube collects the light emitted by the oxidation of luciferin catalysed by luciferase. With advances in detection technologies, such as intensified CCD (charge-coupled device) cameras, it is now possible to monitor gene expression in near real-time with cellular resolution. In mammalian circadian research, firefly luciferase has been used typically under the control of promoter elements from the *Per1* (*Period1*), *Per2* or *Bmal1* (*brain and muscle arnt-like 1*) genes [8], but the principle has been applied successfully to other promoter fragments, such as the transcription factor *c-Fos* [9]. Depending largely on the strength of the promoter, these reporters can generate a signal that can be detected within seconds to 60 min. The availability of lines of transgenic mice and rats has popularized further this relatively inexpensive and reliable method for recording circadian changes in transcription [10]. In one case, the technique has been modified to monitor the abundance of a circadian protein (PER2) by replacing the native mouse gene with a functional copy fused to the firefly luciferase gene (mPER2::Luc knockin mice) [6].

Fluorescence detection uses a fluorophore that emits light after being excited by light of a shorter wavelength. The fluorescence signal increases with excitation intensity. This has been used for *in vitro* circadian biology, with fragments of the *Per1* and *Per2* promoters driving expression of variants of GFP [11,12]. Fluorescent reporters have the advantage over existing luciferase constructs of being brighter and allowing for electrophysiological studies of fluorescently identified cells and simultaneous monitoring of multiple genes (e.g. *Per1* and *Per2*) using two fluorophores that can be discriminated with optical filters. Because of its brightness, fluorescence detection offers higher spatial resolution and can be used to probe anatomical relationships between cells. Fluorescent and bioluminescent reporters have previously been used to reveal circadian modulation of intracellular calcium, cAMP and extracellular ATP release in mammalian cells [13-16]. These reporters hold tremendous promise for future dissection of the cellular events involved in circadian rhythm generation and transmission to other cellular events and cells.

A potential limitation in real-time monitoring of circadian reporters is that circadian promoters can have low activity, leading to extremely low signals. Recording fluorescence or bioluminescence for circadian studies requires a stable environment best achieved with an incubation chamber that eliminates environmental light contamination and temperature fluctuations. Fluorescence recording also requires an excitation source of constant intensity that can remain illuminated for several days. Light-emitting diodes are a promising solution to this problem. Repeatedly flashing UV light on fluorescent reporters can also lead to phototoxicity or photobleaching. One should also keep in mind that reporters of gene transcription may have different temporal profiles and responses to stimulation compared with a reporter of protein translation. Thus the choice between a bioluminescent and a fluorescent reporter will depend on the characteristics of the experiment and the spatial and temporal resolutions desired.

Electrophysiology

The study of circadian changes in electrical activity *in vitro* has included intracellular patch- or voltage-clamp recordings from single neurons for up to 1 h and extracellular recording of single to multi-unit APs (action potentials) for up to 30 days [17]. As with optical methods, electrophysiology can reveal circadian rhythms with single-cell resolution in tissue explants or cell dispersals shortly after removal from the animal or after weeks to months in culture.

The first attempts to record electrical activity from SCN neurons *in vitro* used extracellular metal (usually stainless steel, tungsten or platinum-iridium) or glass electrodes of low impedance. Electrodes with a tip size of ~3-10 μm enable the discrimination of APs from single neurons.

To allow stable recordings from the same neuron for multiple days in a sterile environment, Welsh et al. [18] pioneered the use of planar MEAs (multi-electrode arrays) to record from neurons dispersed from the neonatal SCN. Electrode arrays are now commercially available with different electrode patterns. Typical MEAs used for circadian research have 60, 10 or 30 μm electrode tips arranged in an 8×8 grid with electrodes missing from the four corners. Dissociated cells or tissue slices are then cultured on top of the array, allowing cells to make contact with the electrodes. These arrays can stimulate the recorded neurons electrically at discrete electrode locations and could be used in conjunction with optical recording of circadian rhythms. Extracellular recording cannot, however, reveal the synaptic potentials or ion-channel openings that are revealed with intracellular electrodes.

Patch-clamping and intracellular recordings allow precise characterization of the conductances that determine MP (membrane potential). Patch-clamping allows the identification of ion channels involved in generating APs and daily changes in MP. Intracellular recordings allow the measurements of changes in MP throughout the circadian cycle and during AP firing. Whereas MEA recordings can follow the firing patterns of many neurons for many days, patch-clamping and intracellular recordings provide information about the biophysical basis for changes in firing by comparing data from cells recorded at different times of day. For example, these methods have identified specific potassium currents that are closed during the day to depolarize SCN neurons and augment firing. Electrophysiology has thus provided circadian biologists with the ability to study both the electrical properties of the AP of SCN neurons as well as the temporal organization of firing frequencies (Figure 1) In addition, electrophysiological techniques helped characterize the ion channels involved in the circadian changes in MP and in firing frequencies [19].

Circadian properties of *in vitro* models

Circadian rhythms in mammalian SCN neurons *in vitro*

The generation and regulation of circadian rhythms in neurons of the mammalian SCN have been studied extensively *in vitro*. In SCN explants, circadian rhythmicity can be monitored for several days in culture without apparent loss of circadian amplitude or variability in the free-running period. Interestingly, the SCN *in vitro* retains many of its *in vivo* properties. For example, the firing frequency from SCN neurons *in vitro* peaks at projected CT6-8 (circadian time 6-8; approx. 4-6 h before locomotor activity would have started *in vivo*). This *in vivo*-to-*in vitro* analysis has enabled pharmacology to implicate multiple signalling pathways in the entrainment of the SCN to local time, including glutamate, nitric oxide and acetylcholine [20,21]. The circadian fluctuation in firing frequencies varies between neurons, with most neurons increasing their frequency by about 4 Hz. Clock-gene-driven bioluminescence in SCN neurons is also circadian, peaking at times depending on the promoter (e.g. *Bmal1::Luc* at CT4-8 and *Per1* at CT7-10). The bioluminescence of the PER2::LUC fusion-protein-driven luciferase reaction peaks at CT12-14. (Figures 2A-2D and Figure 3)

Luciferase expression has also been placed under the control of the *c-Fos* transcription factor [9]. This mouse was developed to study the temporal dynamics of AP-1 (activator protein 1) transcription, events associated with resetting of the circadian clock. *In vitro* results from the *c-Fos::Luc* SCN indicate circadian regulation of *c-Fos* and rapid induction by serum, potassium chloride or a convulsant drug, similar to results seen *in vivo* [9].

The use of calcium-sensitive dyes such as Fura2, Fluo-AM and chameleon has been instrumental for the characterization of neurotransmitters implicated in SCN activation. SCN neurons display rapid cytosolic calcium oscillations that are stimulated by glutamate and serotonin and inhibited by GABA (γ-aminobutyric acid) [15]. Calcium levels measured from the cell body display circadian oscillations with peak levels during the day, when SCN neurons show high firing frequencies, and low levels at night, when neuronal firing is low. In addition, a different reporter revealed circadian oscillations in cytosolic, but not nuclear, calcium concentrations. This oscillation in calcium concentration peaked approx. 4 h before the daily peak in electrical activity in SCN neurons [13]. This suggests that oscillations in cytosolic calcium concentrations could be involved in circadian firing of SCN neurons.

The *in vitro* preparation also enables co-culture experiments in which the SCN is cultured with another cell type. Using co-cultures, it was shown that the SCN was able to entrain and sustain circadian oscillations of *Period* expression in astrocytes that would otherwise rapidly damp [22] (see the subsection Circadian rhythms in mammalian astrocytes for a discussion of astrocyte rhythms). Importantly, sections from the cortex were unable to entrain and sustain astrocyte rhythms, confirming the rather unique pacemaking ability of the SCN [22]. The co-culture of an SCN with NIH-3T3 cells was also capable of inducing high oscillations in *Rev-Erbα* and *Per1* mRNA in the NIH-3T3 cells, oscillations that were not present when a slice of cortex was added [23].

In vitro experiments have also revealed some surprises. Dual fluorescence driven by the *Per1* and *Per2* genes revealed that some neurons within the SCN express both *Per1* and *Per2*, but that some cells express only one of the fluorescent constructs [11]. The authors concluded that not all SCN cells have the same underlying molecular clock mechanisms. Real-time measurements should be able to determine whether *Per1* and *Per2* differ in their levels or timing of expression in individual cells. Recent studies have shown also that isolated SCN neurons are circadian [24]. These intrinsically pacemaking cells do not fall into a single class based on their neuropeptide expression and, without network interactions,

could start and stop oscillating *in vitro*. Future studies could determine whether single circadian cells retain information about the environment (e.g. photoperiod or light intensity), the age of the animal or other *in vivo* experiences.

Perhaps the biggest advances in our understanding of circadian rhythm regulation came from cases where single cells *in vitro* differed in their properties from *in vivo* behaviour. When mice lacking VIP (vasoactive intestinal polypeptide) or its cognate receptor in the SCN (VPAC2R) were found to be arrhythmic in constant darkness, it was postulated that VIP signalling was required for rhythm generation [25,26]. However, subsequent analysis of the SCN *in vitro* from multiple laboratories revealed that individual cells could express rhythms. Like isolated cells, SCN cells lacking VIP signalling failed to synchronize to each other and showed greater instability in their daily cycling [27]. Another surprise came when dispersed SCN neurons cultured from animals lacking either *Per1* or *Cry1* showed no rhythms in PER2::Luc bioluminescence [28]. This is in sharp contrast with the robust rhythms observed in whole SCN in culture or from locomotor behaviour of mice with the same genotypes [28]. This suggests that intercellular communication, or coupling, can compensate for mutations that would render individual neurons arrhythmic. This result was further validated by a mathematical modelling experiment that demonstrated that network interaction among arrhythmic cells could lead to population rhythms [28]. Future studies can begin to identify the mechanisms by which intercellular communication between SCN neurons synchronizes their circadian periods, establishes their phase relationships and reduces their cycle-to-cycle variability [18,29].

Circadian rhythms in primary and immortalized fibroblasts

In 1996, the first clear evidence of circadian oscillations outside of the SCN was reported in cultured retinas, where circadian melatonin synthesis persisted for at least 5 days [30]. This was followed by the surprising discovery that immortalized Rat-1 fibroblasts and H35 hepatoma cells display circadian rhythms in transcript levels of genes including *Per1, Per2, Rev-Erbα*, *Dbp* (*albumin D-box binding protein*) and *Tef* (*thyrotrophic embryonic factor*) for several days following stimulation with high concentrations of serum [5]. This raised the possibility that many, if not all, cells can generate daily rhythms. The liver, lungs, testes, adrenal glands and olfactory bulbs are some of the structures that have been found to be rhythmic. Cultured fibroblasts are now used as a model of choice for the study of ‘peripheral’ circadian oscillators.

Bioluminescence assays using primary or immortalized fibroblasts have revealed that single fibroblasts are robust circadian oscillators, but that population rhythms damp gradually over time. This damping is attributed to a lack of synchrony between individual fibroblasts due to a lack of intercellular coupling, in contrast with self-synchronizing SCN neurons [31].

Immortalized mouse embryonic fibroblast cells, such as NIH-3T3 cells, are homogeneous and well characterized cell lines that have been instrumental in the identification of molecular and genetic players involved in the generation of circadian oscillations. For example, inhibition of CK2 (casein kinase 2) in NIH-3T3 cells was shown to inhibit PER2 protein degradation, leading to circadian rhythms with reduced amplitude and lengthened periods [32]. In addition, immortalized fibroblasts were used to identify genes expressed rhythmically and compare them with those expressed in SCN-derived oscillators [33]. Interestingly, except for core canonical clock genes, fibroblasts and SCN cells have only few rhythmic genes in common [33].

Circadian rhythms in mammalian astrocytes

Astrocytes are a class of glial cells in the brain. Real-time monitoring of *Period*-driven bioluminescence has revealed that cultured astroglia from the cerebral cortex express damped circadian rhythms *in vitro* (Figures 2E and 2F, and Figure 4). It is unclear whether damping results from individual astrocytes losing rhythmicity or synchrony from each other. These rhythms are entrained, however, when co-cultured with SCN or by daily application of VIP [22,34].

The relevance of *in vitro* circadian oscillations to *in vivo* behaviour remains a holy grail. Circadian rhythms in extracellular ATP accumulation in cultured astrocytes suggest that they may use ATP to send timing information to each other or to other cells [16]. In contrast, there is no evidence for circadian regulation of glutamate uptake or melatonin release by cultured astrocytes, so not all functions are under clock control [35]. Surprisingly, mutations in the *Per2, Clk* (*Clock*) or *Npas2* (neuronal *pas-2*) genes alter astrocytic glutamate uptake, indicating roles for these clock genes outside circadian regulation in these cells [35].

Circadian rhythms in SCN-derived cell lines and human-derived osteosarcoma cell lines

SCN2.2 cells developed by Earnest and collaborators [35a] are adenoviral, E1A-immortalized, neural cells derived from the rat SCN. This cell line is composed of a combination of neurons and glial cells. Characterization of SCN2.2 cells has shown that these cells share many circadian properties with the intact SCN, including entrainment of fibroblasts and re-establishment of circadian rhythms when implanted into SCN-lesioned and arrhythmic rats [36,37]. In addition, SCN2.2 cells were shown to display circadian rhythms in extracellular ATP accumulation, implicating ATP as a potential output signal [16]. Previously, a new line of immortalized cells of SCN origin was generated from rat embryos bearing the *Per1::luc* transgene [38]. These cells appear to be a faithful model for *in vivo* circadian pacemakers and offer the advantage of having a built-in bioluminescent reporter of clock function.

In vitro circadian research has benefited greatly from the use of established and highly characterized human cell lines. Human U2OS osteosarcoma cells have been used in large screens to identify modulators of canonical clock-gene expression and circadian rhythms. U2OS cells were used to identify new genes that regulate circadian period and amplitude by interacting with the canonical transcription-translation feedback loop [39]. These cells were also used to identify enzymes critical for the 24 h profile of clock-gene oscillation. U2OS cells established the enzymes CK1ε and CK1δ as potent regulators of period length. Chemicals that lengthen the period of circadian rhythms do so by inhibiting CK1ε- and CK1δ-mediated PER2 phosphorylation [40]. This finding was also confirmed for NIH-3T3, mouse embryonic fibroblasts and cultured SCN slices, thus validating the role played by these enzymes. In addition, studies in U2OS cells identified CK2 as an enzyme critical for nuclear localization of PER2 protein, a step required for the circadian oscillation [41].

Such large-scale pharmacological and genetic manipulations in U2OS and other highly characterized cell lines is very promising, but caution should be taken when interpreting these experiments and extrapolating them to other systems and to *in vivo* situations. A major concern is that almost all high-throughput studies are short (about three cycles) and circadian oscillations are induced by a serum shock, dexamethasone or similar treatments, leading to the possibility that some of the findings could be relevant only under such conditions. Immortalized cell lines are one tool among many available for *in vitro* analysis of circadian rhythms.

Circadian rhythms outside the SCN

In vitro studies also established the presence of peripheral oscillators in tissues and organs other than the central pacemaker in the SCN [6]. For example, the majority of mammalian organs and tissues tested display circadian oscillations in PERIOD2-driven luciferase [6]. However, these cultures, once isolated from SCN inputs, display endogenous periods and phases that are intrinsic to each structure with a wide range of phases and periods. Such variability highlights the role of the SCN as a central pacemaker responsible for the co-ordination of circadian functions throughout the body. When various brain structures were analysed, a majority displayed damping circadian oscillation in clock-gene expression. The olfactory bulb, however, was in sharp contrast with other structures and was shown to contain a competent, self-sustained, circadian oscillator [10]. Circadian rhythms in electrical activity and *Period1* expression in the olfactory bulb persist *in vitro* for several days and reflect circadian changes in olfactory sensitivity [42]. These results highlight the potential for cell- and tissue-specific circadian properties.

When *in vitro* and *in vivo* results disagree

The information obtained from *in vitro* studies of circadian rhythms has largely been consistent between laboratories and with circadian phenotypes observed *in vivo*. However, a recent example highlights how *in vitro* observations can raise surprising questions. The recent finding that $Per1^{-/-}$ mice show a shortened behavioural period, but their SCN fail to show rhythms *in vitro* [43], stands in contrast with the rhythmicity reported from $Per1^{-/-}$ SCN cells by Liu et al. [28]. Does this reveal a clandestine effect of genetic background on circadian cycling? Could it be that the mutation renders the SCN sensitive to particular culture conditions? Perhaps a small number of rhythmic SCN neurons are sufficient for driving circadian locomotor rhythms? Or are there extra-SCN factors that sustain rhythms *in vivo*? Ultimately, a clock in a dish is a timely tool that must be placed in the context of its role in the organism.

Conclusions

The ability to isolate and study circadian properties of mammalian cells and tissues *in vitro* has provided scientists with a wealth of information regarding the molecular and genetic determinants of circadian clocks. *In vitro* analysis also allowed the identification of differences in oscillatory and pacemaking ability between central and peripheral oscillators. By using large-scale screens in characterized cell lines, *in vitro* research has uncovered a vast number of genes and molecules playing a critical role in determining the phase, period and pacemaking ability of cells. This information establishes a solid base upon which future circadian scientists will build to further characterize circadian clocks.

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Summary

- *Circadian rhythms are observed* in vitro *in a wide variety of mammalian cells and tissues.*
- In vitro studies have established the cell-autonomous nature of circadian oscillators.
- In vitro analysis of circadian clocks has helped uncover the molecular mechanism driving circadian rhythms.
- SCN neurons and SCN2.2 cells are the only cells shown to be able to entrain other cells.
- Fibroblasts were the first cell-autonomous peripheral oscillators described in detail.

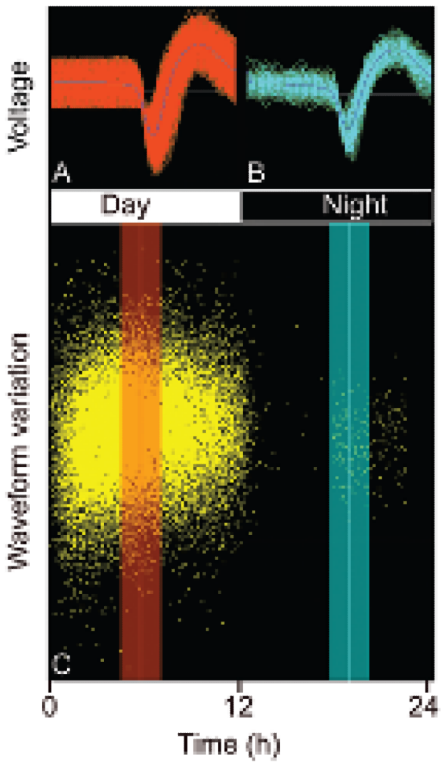

Figure 1. Electrical activity in SCN neurons is higher during daytime

(**A**) Superimposed APs from a single neuron recorded on an MEA during a 3 h period in the subjective day. (**B**) Electrical activity of the same neuron as in (**A**) during 3 h in the subjective night. Note that there are fewer traces due to the lower firing rate at night compared with the daytime. (**C**) Electrical activity of the same neuron over a 24 h interval. Each dot represents one AP plotted as its principal component (defined as waveform variation here) at the time it was recorded. The coloured vertical bars represent the 3 h intervals used in (**A**) and (**B**).

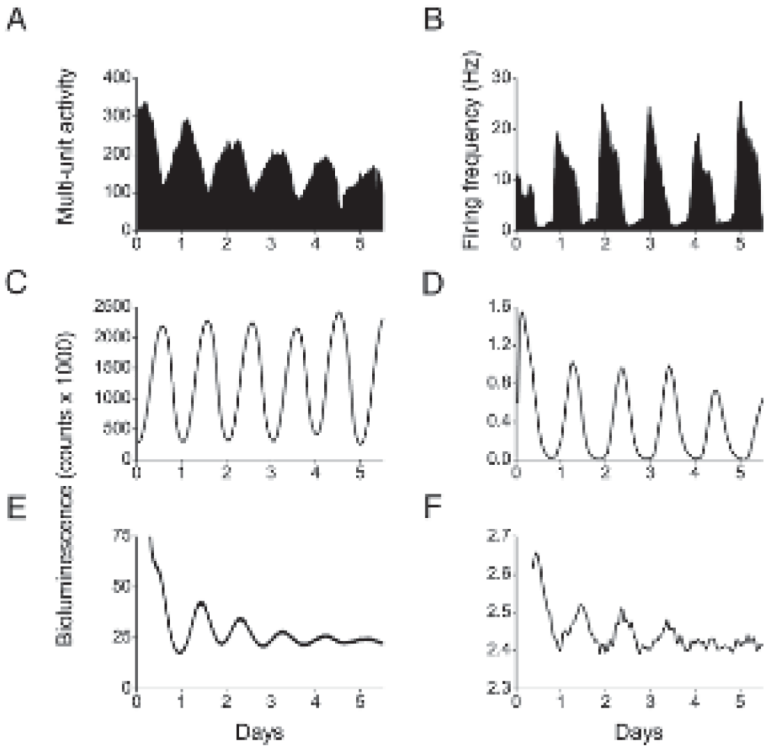

Figure 2. Mammalian cells display circadian rhythms *in vitro*

Representative traces of activity from populations of cells (**A**, **C** and **E**) and single cells (**B**, **D** and **F**) show the diverse outputs that are regulated by the circadian clock in different cell types. (**A**) Summed multi-unit firing rates of 42 SCN neurons recorded on an MEA. (**B**) Spontaneous firing rate of a single SCN neuron. Note that the cell is nearly silent for several hours each day and then rapidly increases its discharge frequency to 10-20 Hz. (**C**) Bioluminescence driven by the PER2-LUCIFERASE knockin reporter shows self-sustained daily cycling in the explanted SCN. (**D**) Bioluminescence from the PER2::LUC reporter recorded in a single SCN neuron within the slice shown in (**C**). (**E**) PER2 expression in a culture of primary astrocytes shows a damped circadian pattern. (**F**) An individual astrocyte shows circadian expression that varies in period and amplitude over 5 days of recording.

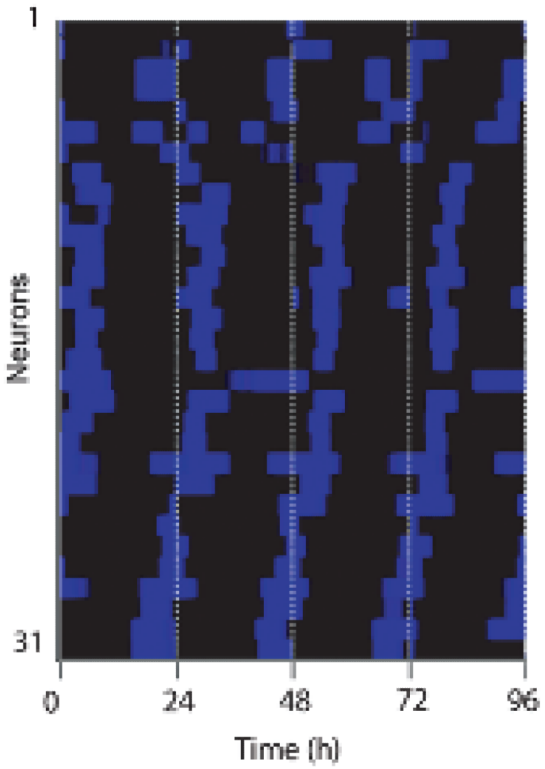

Figure 3. Circadian firing-rate rhythms in individual neurons from SCN dispersals

This raster plot (heat map) displays the normalized firing rate of one neuron on each row. SCN cells were dispersed, plated on to an MEA and recorded after 2 weeks in culture. Note the daily peaks (blue) and troughs (black) in the number of APs per 10 min, indicating that SCN neurons show sustained rhythms.

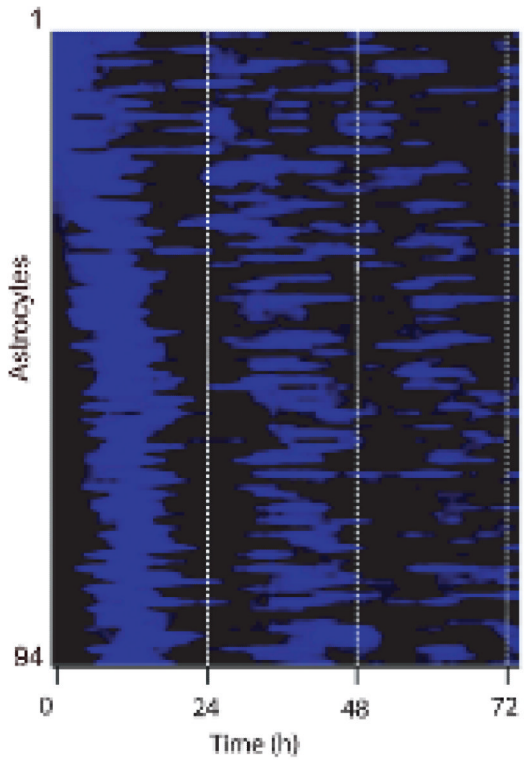

Figure 4. Circadian gene-expression rhythms in astrocytes from mouse cortex

This raster plot (heat map) displays the normalized PER2::LUC bioluminescence emitted by an individual astrocyte on each row with daily peaks (blue) and troughs (black). In contrast with SCN neurons, most astrocytes show gradual damping of their bioluminescence rhythms and less period stability.

Table 1

Optical imaging reporters commonly used *in vitro*

Imaging method	Reporter	What is measured
Bioluminescence	*Bmal1::Luc*	*Bmal1* transcription
	Per1::Luc	*Per1* transcription
	Per2::Luc	*Per2* transcription
	PER2::Luc	PER2 translation
	c-Fos::Luc	*c-Fos* transcription
	Luciferase–luciferin reaction	Extracellular ATP
Fluorescence	*Per1*–GFP	Per1 transcription
	Per1–Venus	*Per1* transcription
	Per2-DsRED	*Per2* transcription
	Cameleon	Intracellular calcium
	Fluo-2 acetoxymethyl (AM)	Intracellular calcium
	Fura-2	Intracellular calcium