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Flies and Fish: Birds of a Feather

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Abstract

The identification of specific clock-containing structures has been a major endeavour of the circadian field for many years. This has lead to the identification of many key components of the circadian system, including the suprachiasmatic nucleus in mammals, and the eyes and pineal glands in lower vertebrates. However, the idea that these structures represent the only clocks in animals has been challenged by the discovery of peripheral pacemakers in most organs and tissues, and even a number of cell lines. In *Drosophila*, and vertebrates such as the zebrafish, these peripheral clocks appear to be highly autonomous, being set directly by the environmental light/dark cycle. However, a hierarchy of clocks may still exist in mammals. In this review, we examine some of the current views regarding peripheral clocks, their organization and how they are entrained.

A major focus of the clock field for over 30 years has been to identify the specific structures that contain the central or 'master' circadian clock. This includes the discovery of the suprachiasmatic nucleus (SCN) in the mammalian hypothalamus in 1972 (1, 2), and many subsequent studies (3). In addition, the demonstration of independent clocks in the eye and pineal gland of lower vertebrates, including reptiles and several species of bird and fish, is well known (4). The same is also true for clock structures in invertebrates, such as the eyes of marine molluscs, *Aplysia* and *Bulla*, and the lateral neurones in *Drosophila* (5, 6). The situation appeared to be clear: circadian clocks were highly localized cellular processes and were usually found in neural or neuronal structures. In the lower vertebrates, these central clocks can be entrained directly due to the light-responsive nature of cells in the retina and pineal gland. In mammals, specific photoreceptor-types in the retina and neuronal pathways are required to set the 'master' SCN clock (3).

However, over the last 6 years, our understanding of circadian clock organization has changed dramatically. The first 'small cracks' began to appear when Tosini and Menaker demonstrated that an endogenous circadian clock resided within the cultured rodent retina, as measured by rhythmic melatonin release (7). One year later, in 1997, Plautz and colleagues went further and, using a *period-luciferase* transgenic *Drosophila*, showed that a rhythm in gene expression could be detected in a variety of isolated body parts (8). In fact, 8 years previously, Giebultowicz and colleagues had laid the groundwork for this by demonstrating the existence of an autonomous circadian clock in the insect testis, controlling the timing of sperm release (9). Subsequently, in 1998, the potential for generating circadian rhythms in clock gene expression in mammalian cell lines, following serum manipulation, was shown, as well as the existence of independent circadian clocks in a number of cultured zebrafish organs (Fig. 1) (10, 11). By the time that tissue clocks were shown to exist also in rat organs in 2000, again by use of a *period-luciferase* transgenic animal, we had reached a point where a decentralized circadian system was known to exist in a wide range of species, from *Drosophila* to zebrafish and on to mammals (12).

The extent of this 'decentralization' will be discussed later in this review, but what was the cause for this apparently sudden explosion in the discovery of peripheral clocks? Clearly, one major reason had been the discovery, over a similar timeframe, of genes involved in the central clock mechanism. The cloning of *period* in *Drosophila*, of course, occurred sometime ago (13), but with the identification of the *clock* gene in the mouse (and then a homologue in zebrafish), it became possible to look for the expression patterns of clock components directly (11, 13, 14). It was their wide tissue distribution, and the oscillations in transcript levels, that were critical for the discovery of autonomous peripheral clocks. Before the discovery of these genes, clock-function could only be inferred by measuring oscillations in clock outputs or downstream clock-regulated processes. Wheel running was an excellent assay for circadian function in rodents, but what is a relevant clock-output in the fish gill or fin?

It is probably important to note at this point that clock gene expression is not the only cyclic event and, in reality, one should

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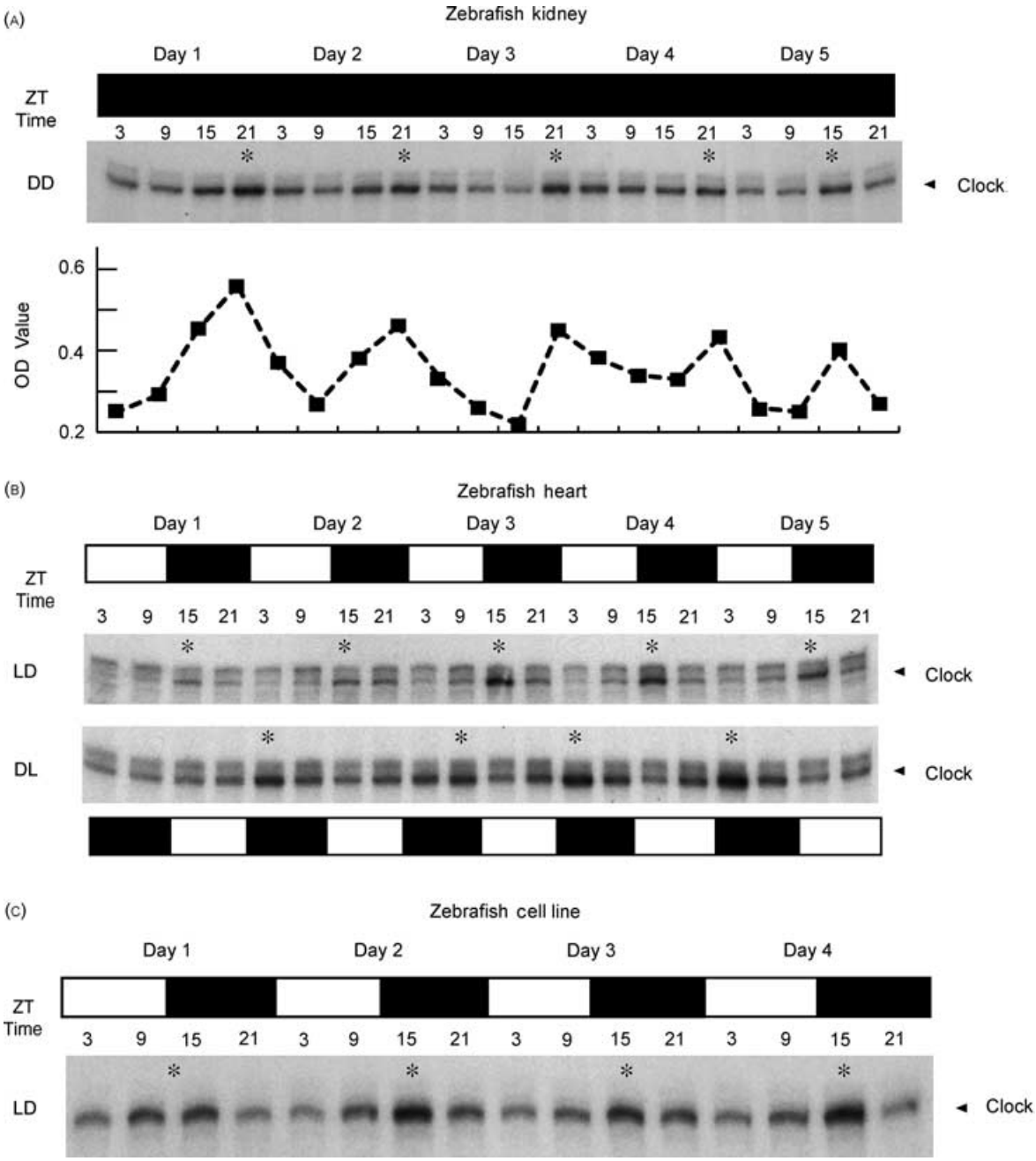

FIG. 1. Showing the circadian oscillation in *clock* gene transcript levels in a variety of zebrafish tissues. (A) Showing *clock* oscillations in cultured zebrafish kidneys in constant darkness over a 5-day period. (B) Showing re-entrainment of cultured hearts placed on alternate light/dark cycles for 5 days. Hearts were dissected from an identical group of fish, and then placed onto out-of-phase light/dark cycles in 'side-by-side' incubators, illuminated with a fibre optic light source. Note that hearts in the 'DL' reversed cycle re-entrain after only 1 day in the new lighting regime. (C) Showing *clock* oscillations in a zebrafish cell line when placed on a light-dark cycle (11, 20).

say that peripheral clocks have been 'rediscovered'. If a search of the literature is made before the discovery of the SCN in the early 1970s, the number of publications describing circadian clocks in tissues and even cell lines is remarkable. These include well-known data describing circadian rhythms in corticosteroid release from cultured rodent adrenal glands, and many other systems (15). In part, perhaps due to the dramatic behavioural data obtained in SCN-lesion experiments and a general move to a greater 'neuro-centric' view of physiology in the 1970s, these early data on peripheral clocks 'fell from favour'. Nevertheless, it might be interesting to revisit some of these old observations, but employing some of the more recent molecular and imaging techniques that have evolved over the subsequent 30–40 years.

Flies and fish see the light

An obvious and major question regarding peripheral clocks relates to the issue of how these oscillators are set or entrained. In the same study that demonstrated tissue clocks in *Drosophila*, by use of a *period-luciferase* transgenic animal, Plautz and colleagues

were able to show that these oscillators could be reset directly by changes in the lighting regime (8). In other words, clocks in the fly antennae, wing, thorax, etc., must contain photopigments and signalling pathways essential for clock entrainment. This was in contrast to the classical view in *Drosophila* that saw the lateral neurones, a small group of neurones residing between the central brain and optic lobes of the fly, as the 'master' clock; a kind of 'SCN of the fly' (16). There is no question that these neurones are essential for regulating many aspects of rhythmic behaviour, but do they function as a 'master' pacemaker with peripheral clocks acting as downstream 'slaves'? Giebultowicz and colleagues have addressed this issue extensively, and a range of data fails to support this hierarchical view (16–18). Instead, the data argue for a highly decentralized collection of independent, and locally entrained, peripheral pacemakers. For one, clocks found in *Drosophila* Malpighian tubules phase shift with near identical kinetics in intact flies as in flies lacking a head (19). The lateral neurones do not appear necessary for resetting. Furthermore, the phase or timing of specific gene expression in lateral neurones and peripheral tissues is similar. If the 'master' lateral neurones were driving downstream gene expression in body tissues, then a phase delay in these peripheral clocks would be predicted.

Could the hormonal environment within the fly be important for regulating these peripheral clocks? Apparently not, because, during fly development, clocks in particular tissues appear to start at different times. For example, the rectal clock starts before eclosion, but the Malpighian tubule clock begins on, or just after, eclosion (18). It is hard to explain such results by the action of a single activating/entraining hormonal signal. The 'nail-in-the-coffin' for a hormonal model, however, comes from an experiment using transplantation of Malpighian tubules. These tissues were transplanted into a host fly that had been maintained on an opposite light/dark cycle to the donor animal. Even though present in a new, 'reversed' humoural environment, the transplanted tubules maintained their original phase, cycling out of phase with the surrounding host tissues (17). Clearly, humoural signals were unable to reset the clocks in this transplantation situation. Therefore, the current model of peripheral clock organization in *Drosophila* at this time is that these oscillators exist in a highly decentralized and autonomous fashion. Tissue clocks can be entrained without the need for a central, 'master' clock, and probably act in an independent manner to regulate local clock outputs and rhythmic physiology (18).

What about zebrafish?

In 2000, Whitmore and colleagues showed that, as in *Drosophila*, the peripheral clocks in zebrafish could be directly entrained by light (20). Hearts and kidneys from the same group of fish were dissected and immediately placed on to opposite light/dark cycles in 'side-by-side' incubators. The cultured tissues rapidly adopted an out-of-phase relationship within a circadian cycle. Consequently, these tissues must also contain the photopigments and signalling pathways essential for clock resetting; a vertebrate with light-sensitive tissues. At the same time, a number of zebrafish cell lines were also shown to have the same properties of light-detection and rapid entrainment (20). These cells contrast to the mammalian cell lines examined so far, in not requiring any serum or hormonal pretreatment to exhibit clock function (10).

It is our conclusion from these, and other unpublished data, that most, if not all, cells within the zebrafish contain a circadian clock that is capable of direct light entrainment. The nature of the photopigment is of some interest, and a recent study suggested a possible role for an ultraviolet-detecting pigment or cryptochrome (21). However, these experiments were performed on detached cells, a situation which disrupts normal clock function and gene expression in cells normally requiring a substrate (unpublished observations). Therefore, further studies are required to identify and functionally test the circadian photopigment in the fish. It is also worth noting that, although these cell lines are a useful tool, it is essential to confirm that any aspects of clock function revealed in the cells are also present in the wild-type fish, if any arguments about biological relevance are to be made.

Unfortunately, the kind of transplant experiments performed in *Drosophila* are not so straightforward in zebrafish, and so less information is available about the role of hormonal cues on peripheral clock entrainment. However, to date, we have no evidence for a possible role of melatonin in tissue entrainment (Whitmore and Foulkes, unpublished data). A role for feeding time on adjustment of clock phase cannot be ruled out and is likely, as we have observed that these animals are capable of taking advantage of environmental cues other than light, to entrain their peripheral pacemakers (unpublished data). Nevertheless, the fact that these organ clocks can be rapidly entrained *in vitro*, and the additional fact that there appears to be no 'time-lag' between pacemakers in the brain and the periphery (the same result as in *Drosophila*), leads us to believe that zebrafish clocks also exist with a high degree of autonomy. At this time, there are no data indicating the existence of a central, 'master' clock in the zebrafish brain, and the presence of a functional SCN-specific clock has yet to be tested.

In a phylogenetic sense, how widespread is the existence of such highly decentralized peripheral circadian clocks? Flies and fish appear to be organized in such a manner, with little evidence for a 'master' clock, but, as we shall see below, this may not be the case for mammals. Unfortunately, few or no data yet exist on peripheral clocks, or their light-responsiveness, in amphibians, such as *Xenopus*, the reptiles or even species of bird. Undoubtedly, this gap in our knowledge will be filled in the near future.

Mammalian peripheral clocks: 'food for thought'

At the same time as the demonstration of clocks in fish tissues, Balsalobre and colleagues demonstrated the potential for circadian oscillations in a range of mammalian cell lines, including rat-1 and H35 hepatoma cells (10). However, it was necessary to provide an unusual regime of serum shock followed by serum starvation in order to reveal a circadian oscillation in genes such as rat *period 1* and *2* (*rper1* and *rper2*). The biological meaning of such data is therefore not clear. Nor is it yet known if this serum treatment is simply synchronizing a population of free-running pacemakers, or activating a *de novo* circadian pacemaker, a technically challenging experiment. However, this was a striking result, which has generated a useful tool for cellular circadian research.

The work of Yamazaki in 2000 finally confirmed the presence of peripheral clocks in the rat (12). Again, the use of a *period1-luciferase* transgenic animal provided the tool with which organs in culture could be examined, and the existence of clock gene oscillations in liver, lung and skeletal muscle were clearly shown.

However, the rhythms in these tissues were seen to dampen out more rapidly than oscillations in cultured SCN. Such data imply that the clocks in the rat periphery may not be as robust as in the central SCN, and may support the view that the SCN is a 'dominant' pacemaker. However, it is clearly difficult to compare oscillations in luminescence between tissues in culture, where factors such as luciferin penetration/metabolism, ATP levels, available oxygen, etc., could just as well generate a wide range of amplitudes and rhythm sustainability.

The fact that the SCN clock shifts phase more rapidly than that in the periphery, and that the SCN clock phase leads gene expression in the periphery by many hours, provides a stronger argument to support the idea of a hierarchy of clock organization in mammals. Certainly, there are no data suggesting light-responsive clocks in mammalian tissues, and our own efforts with mouse embryonic fibroblasts confirm this (unpublished data). Consequently, the idea of a 'master' SCN clock in the mammals still persists. However, it is interesting to note the probable existence of an independent food-entrainable oscillator, which controls a variety of rhythmic physiology. This oscillator continues to run even in animals with lesioned SCN (22). Restricted feeding regimes can set or entrain this oscillator, but where it resides, or even if there is a single anatomical location, is not clear. The role of restricted feeding on the peripheral liver-clock is discussed below.

How are these mammalian organ clocks entrained? In a highly hierarchical model, the SCN would be entrained by light via the retina in the classical manner (3). The entrained SCN would then set the phase and synchronize the timing of clocks in the periphery by the regulated release of some humoural or hormonal factor. Recently, hormone–dependent interactions between the nuclear receptors RAR alpha and RXR alpha, and clock factors such as CLOCK and MOP4, have been described (23). The involvement of these factors and retinoic acid in possible phase shifts of the clock in vascular cells may provide a subcellular mechanism for peripheral clock entrainment, but the significance of these events *in vivo* and in other tissues is yet to be fully tested. A candidate for the hormonal signal has been proposed in the form of the glucocorticoid hormones (24). The glucocorticoid hormone analogue, dexamethasone, can induce expression of a number of clock/clock-related genes (*Per1*, *Per2*, *Per3*, *Cry1*, etc.) when applied to rat-1 fibroblasts, and produces several cycles of oscillation in gene expression, as seen in response to serum treatments (10, 24). Moreover, injection of dexamethasone into mice at a variety of circadian phases generates phase shifts in gene expression within peripheral tissues, particularly the liver, but does not alter the phase of the SCN-clock. In mutant mice lacking a glucocorticoid receptor in the liver ($GR^{AlfpCre}$ mice), no such acute gene induction is seen in response to dexamethasone. The phase response curve (PRC) to dexamethasone in peripheral tissues is unusual in having no dead-zone (phase shifts can be induced at all times of the day), and phase shifts in these tissues appear to be transient. Also, when comparing the phase of gene expression in livers from wild-type and GR-mutant mice, no difference in the timing of peak expression is apparent. Although it is clear that glucocorticoids are capable of altering peripheral clock function, it is likely that other signals exist that synchronize peripheral clocks under normal physiological conditions.

Work by the Schibler and Menaker groups has demonstrated that the timing of clocks in the liver, and other tissues, can be phase shifted when animals are exposed to restricted feeding cycles (25, 26). The rhythm in the liver can be shifted by as much as 10 h within 2 days by such regimes, and yet the phase of the SCN remains locked to that of the light/dark cycle. Entrainment appears to occur most rapidly in the liver, followed by the kidney and heart, but all organs are reset after 1 week of the feeding regime (25). Rather ironically, glucocorticoids appear to slow this entrainment to restricted feeding (27). The rhythm in gene expression in the liver is shifted more rapidly in GR-mutant mice than in the wild-type (the same rate is seen in the kidney where glucocorticoid receptors are present). Clearly, glucocorticoids have a complex role in adjusting the phase, or sensitivity to phase shifting, of peripheral pacemakers.

Entrainment of the liver-clock by restricted feeding also raises the possibility that changes in metabolism or redox state might influence the peripheral circadian clock. This idea is supported by findings of McKnight and colleagues, who showed that the binding of CLOCK/BMAL1 and NPAS2/BMAL1 proteins to DNA (and so their transcriptional activity) is strongly influenced by the redox state of nicotinamide adenine dinucleotide (NAD) cofactors (28, 29). As the CLOCK/BMAL1 dimer is believed to be the positive transcriptional element of the molecular clock, changes in the function or efficacy of this dimer, by redox state changes, might be a reasonable means by which metabolic changes could alter clock phase. Thus, changes in feeding time, or restricted feeding, could provide a zeitgeber or entraining signal through basic changes in cellular redox state, especially in the case of peripheral clocks in tissues such as the liver. However, to date, the influence of reduced or oxidized NAD(H) and NADP(H) on clock factors has only been demonstrated in purified systems, and the relevance of these changes *in vivo*, and under natural physiological conditions, has yet to be shown. Furthermore, the situation is made more complicated by the fact that redox state may be part of an output pathway from the clock, as well as a possible input or entraining signal. However, aspects of metabolism, redox state, electron transport, and so on, are such fundamental and core elements of basic cell function, that it is appealing to believe that they also play a critical role in another apparently ubiquitous phenomena, the circadian clock.

The fact that restricted feeding regimes can alter the phase or timing of the clocks in the periphery, but not the SCN, is potentially revealing about the organization of the circadian system within mammals. It is clearly possible to separate the timing of central, 'master' clocks from their supposed peripheral 'slaves'. This suggests that the periphery is not totally dependent upon entraining signals from the brain, but can be influenced more directly by changes in environmental conditions, such as the timing of food availability (26). A rigid hierarchy of clocks, with the SCN 'at the top', may not be completely true in mammals, and a system of coupled, but more autonomous pacemakers, as in *Drosophila* and zebrafish, may be a more accurate model. However, it is not easy to draw conclusions as, under natural conditions, it is likely that the SCN controls rhythmic behaviour, such as feeding, and may influence peripheral pacemakers by such behavioural entrainment. Not surprisingly, the entrainment and synchronization of the circadian system in mammals probably uses a variety of cues, which may even change depending upon the conditions in which an animal finds itself.

What cellular/physiological processes do these peripheral organ clocks control? The abundance of oligonucleotide arrays, or 'DNA-chips', in the mouse has provided the technology for an

TABLE 1. A Selection of Rhythmic Genes Identified Using DNA Microarrays ('DNA-chips') in Both the Suprachiasmatic Nucleus (SCN) and Liver (30, 32). There is a Wide Range of Oscillating Transcripts in Both Tissues, Involving Many Cellular Processes, Including Vesicle Trafficking, Energy Metabolism and Production, Protein Folding and Degradation, as Well As Some Structural Components.

	SCN	Liver	Liver and SCN
Structural genes		Tubulin monomers: Tubb3/4/5.	Tubulin, beta 5 (Tubb5)
Protein folding and degradation:	Ubiquitin-like 5, ubiquitin-like 3, f-box only protein 3/8, ubiquitin-conjugating enzyme E2D, proteosome subunits	Ubiquitin specific protease 2 (Usp 2), proteosome subunits	Proteosome subunits (different specific genes between liver and SCN)
Solute transporters	Solute carrier family 25 (Slc25a5)	Slc12a2, Slc16a1, Slc19a1, Slc25a11	
Glycolysis/energy metabolism	ATP synthase transporter complexes (various), cytochrome c oxidase subunits, NADH dehydrogenase subcomplexes (various), Slc25a5	6-phosphofructokinase, aldolase (Aldo 1 and 3, glucose phosphate isomerase, pyruvate kinase, glucagons receptor, Slc27a2	Adenylate kinase 4
Vesicle trafficking and transport	Septin 4, somatostatin, syntaxin binding protein 1 and 3, synapsin1, adaptor-related protein complexes (Ap4m1/s1)	Adaptor-related protein complexes AP-1 (Ap1b1, Ap1m1, Ap1s1)	General adaptor-related protein complexes (different genes between liver/SCN)
Cholesterol biosynthesis		Niemann pick type C1, ferredoxin 1, cytochrome P450 (Cyp2a12, Cyp7a1), Slc7a2, Slc22a1	Adipose differentiation related protein (Adfp)?
Other	Cytochrome c oxidase subunits (Cox 4a, 7b, 7c, 6c, etc.), hexokinase 1, mitochondrial malate dehydrogenase	Procollagen types, interferon regulatory factors, murinoglobulin 1 and 2, heat shock protein 60, 84, 86	Arginine vasopressin receptor 1 A, protein tyrosine phosphatase 4a1, period 2, Rev-erb-beta

initial characterization of oscillating gene expression in peripheral tissues on a large scale (30–32). This technology allows the examination of expression profiles for many thousands of genes simultaneously. Such studies have shown that in the order of 10% of all genes, for example, within the mouse liver are under circadian clock regulation. These genes encode proteins involved in energy metabolism, steroid synthesis and even immune function (Table 1). Not surprisingly, considering the differences in physiology and function, there are many differences in the genes that oscillate between heart and liver. However, there is apparently a core of around 37 similar oscillating genes (32). These may represent common clock outputs, perhaps involved in 'housekeeping' or common cellular processes, or they may include new central clock components. Such data provide an excellent substrate for future proteomics efforts, where it will be interesting to discover how many of these transcript oscillations are converted into rhythmic protein changes. It will also be interesting to see how many physiological processes, previously unidentified, are under clock control in these tissues.

Future efforts

Peripheral organ or tissue clocks have now been described in a wide range of species. In the fish and fly, these peripheral clocks appear to have a high degree of autonomy, being directly entrained by the environmental light/dark cycle. The importance of the SCN in the mammals as a central clock cannot be denied, but whether it can be viewed as a dominant, 'master' pacemaker on top of a hierarchy of peripheral pacemakers is open to debate. Future experiments in mammals will certainly focus on the nature of hormonal cues capable of setting these clocks. This will be a complex task, not least due to the likelihood that there are a wide range of potential zeitgebers that have not yet been explored or are only just beginning to be understood. These include obvious events such as seasonal changes, reproductive status and subsequent hormonal changes, age, social status and cues, and so on. However, the topic of photoperiodism and seasonal reproduction is undergoing a 'resurrection', with interesting new research exploring the role of clock genes in regulating photoperiodic time measurement, as well as the significance of the pars tuberalis as a target of signals arising from the central clock (33–35). At the molecular level, the identification of rhythmic physiological processes will be important, as will the identification of drugs that can specifically shift the phase of particular tissues. The clinical benefits of such compounds and approaches are obviously far-reaching.

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