Keeping time: Circadian clocks

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The clock tower of the Houses of Parliament. Credit: Wellcome Library, London

Our planet was revolving on its axis, turning night into day every 24 hours, for 4.5 billion years - long before any form of life existed here. About a billion years later, the very first simple bacterial cells came into being and evolved into the first animals and eventually - after a billion years or so - the first humans.

Today, of course, the Earth is host to an immense diversity of life: researchers estimate there are around 8.7 million different species on

Earth, most of them insects. One thing nearly every plant, animal and microbe here today has in common is an internal biological clock that ensures all the activities of our cells and organs follow a 24-hour cycle.

That 'circadian' (Latin for 'daily') clock dictates what time of the day or night we feed, when we metabolise food into energy, and when we are active and when we rest. It's responsible for the surge of cortisol that forces us up in the morning, the surge of melatonin that gets us ready for bed at night, and the cycles of hunger, thirst and energy we experience throughout the day.

Although it is intimately tied to the cycle of day and night, our body clock doesn't need external cues to operate. Those cellular activities would follow the same rhythm even if we were stranded at the North Pole in the middle of June or December and exposed to 24 hours of constant light or darkness.

Why we developed those clocks and why, even in the smallest microorganism, they are so intimately connected to the rotation of our planet are questions that have long baffled evolutionary biologists. Their precise mechanism has also been an elusive 'black box' for science.

We do know that disrupting our body clocks - perhaps through shift work or jet lag - throws our cellular rhythms out, putting stress on our bodies over time. Our metabolism gets confused, so we produce less insulin and our blood sugar levels go up, predisposing us to diabetes and obesity. In the brain, our bewildered pineal gland stops producing its usual levels of melatonin, increasing the risk of cancer because melatonin suppresses harmful free radicals and tumours in addition to helping us sleep.

Black box

The crucial part biological rhythms play in our health has made circadian clocks an important field of research. If we can locate their cogs and wheels and understand how they work, we can at least attempt to fix them when they go wrong.

"People have known that there's a biological oscillator keeping 24-hour time all by itself without any external environmental cues since the 1960s," says Dr John O'Neill at Cambridge University. "The big question has always been, how does it work? That's been a black box for science."

Sunrise in Ethiopia. Credit: Sasha Andrews, Wellcome Images

For several decades, we've known that humans and other mammals with brain damage to their hypothalamus lose rhythms in behaviour and physiology. This led researchers to conclude that the circadian rhythms of the cells throughout our bodies are co-ordinated by a 'master clock'

located in our hypothalamus. They dubbed this master clock the 'suprachiasmatic nuclei'.

But exactly how that clock worked - how its own neurons operated, how they influenced cellular activities (including genetic transcription and translation) in other parts of the body, and whether the mechanism was the same in simpler animals and other organisms - remained secrets of the black box.

By the early 1980s, genetics had given us a clue; researchers started to identify and test the first 'clock' genes in flies, fungi and finally, towards the late 1990s, in mice. Deleting (knocking out) those genes from the genome or mutating them changed the duration of that organism's circadian cycles (reducing them from 24 to 21 hours, for example).

Gene switch

These findings led researchers to develop a genetic model of 24-hour circadian timekeeping based on a negative feedback loop. The idea was that a clock gene is switched on around dawn. Once activated, the gene starts churning out mRNA (molecules containing a chemical 'blueprint' for making specific proteins) in a process known as 'transcription'. The mRNA then leaves the nucleus and travels to a cellular factory (ribosome) in the cytoplasm, where the protein specified in the blueprint is assembled in the second part of the protein-making sequence, known as 'translation'.

Levels of the protein in the cell build up and peak around dusk. Then, in one of those jaw-dropping instances of biological ingenuity, the proteins go into the nucleus and neatly turn their own genes off for the night, ready for the cycle to begin again at dawn.

It looked as though the various clock genes people had identified -

including PERIOD, CRYPTOCHROME and CLOCK - were all components of this 24-hour transcription-translational programme. Although the genes varied in structure between different organisms, they all seemed to behave in a similar way, switching themselves on and off at specific times of the day. The level of coordination required throughout the body again seemed to support the idea of a master clock; in mammals, at least, the suprachiasmatic nuclei in the hypothalamus was likely to be the timer governing the on-off switch in all the body's cells.

It was a model of biological timekeeping that successfully accounted for a large body of experimental evidence. There were, however, some inconsistencies. One of these was a 1960s paper describing research on a long, single-celled alga called Acetabularia. The researchers noted that the movement of chloroplasts up and down its stem was circadian regulated. "Even in constant light, even though the alga didn't know at all what time of day it was outside, you still had approximately 24-hour movements of the chloroplasts," says O'Neill.

The really fascinating finding came when they removed its nucleus, which in Acetabularia is contained in a discrete structure, the rhizoid, similar to the root of a plant. Chopping off the rhizoid put a stop to all genetic activity - yet the researchers could still see the same circadian rhythms in chloroplast migration up and down the alga's stem.

The discovery was brushed under the carpet for want of an explanation. "We hadn't found the clock genes then, but once we did, no one wanted to revisit the Acetabularia problem because it didn't fit with the transcriptional-translation explanation for timekeeping, which requires daily changes in gene activity," says O'Neill.

Out-of-body clock

Four decades later, two studies published in 2005 underscored

discrepancies so glaring it was no longer possible to ignore them.

Several groups had demonstrated the cyclical, 24-hour nature of gene activity by inserting the gene that makes luciferase - the protein that causes fireflies to flash at night - into a mouse genome. By fusing LUCIFERASE to a 'clock' gene, they got it to switch on and off at the same time as the clock gene did. LUCIFERASE acted as a 'reporter' for the clock gene, visibly lighting up the cells to indicate when it was switched on and dimming again when it was switched off, over the daily cycle.

Excitingly, in 2004, using LUCIFERASE as a reporter, researchers at the Howard Hughes Medical Institute found evidence of circadian rhythms of gene activity in cultured mouse cells. Since these cells were outside the body and had no way of communicating with the mouse 'master clock' in the brain, it looked as if another circadian mechanism was at work within the cells themselves.

Could that also be the case for humans? Combining the two findings - and taking them one step further - Steve Brown, a young postdoctoral student in Geneva, created a virus system that could insert clock-gene-LUCIFERASE 'reporters' into the genome of human cells. He and his colleagues then went on to measure the circadian rhythm in isolated human skin cells kept in either constant darkness or constant light.

To their excitement, they saw circadian rhythms continuing in the skin cells - despite the fact that, like the mouse cells, they were completely cut off from the master clock in their donor's brain. The 24-hour cycles of activity continued in the test-tube cells without any external environmental cues to tell the cells what time of day it was. Intriguingly, the rhythms very closely matched circadian rhythms in the living body of the person who had donated the cells; rhythms varied more between different people than they did between the skin cells and whole body of

each individual.

The research in mouse and human cells turned the master-clock theory on its head. If individual cells can keep time outside the body, they must have some kind of intrinsic timekeeping mechanism, which doesn't necessarily need to be regulated by the suprachiasmatic nuclei.

Subsequent research has shown that, in fact, the suprachiasmatic nuclei do play an important part in synchronising all our individual cellular clocks, although they don't depend on it to function. It acts like a biological Greenwich Meridian, keeping all of our cellular clocks in what O'Neill calls a 'stable phase-relationship' with each other. "That's why we get jet lag, because the SCN can't immediately adapt to the new lighting environment. In the three to four days it takes you to re-entrain, all of the different cellular clocks in your different organs - whether your guts or liver or lungs - aren't getting have any coherent information from the SCN, so they just free run. That's why you feel hungry and wake up at odd times."

Back in 2005, O'Neill was fascinated by the Geneva group's finding - the idea that you could take a cell from any part of the body and put it in a dish, and it would still have an intrinsic idea of external time even if it couldn't see the sun.

He began to think that circadian clock research had conflated two separate questions: how does timekeeping work at the whole-organism level of physiology and behaviour, and how does it work at the cellular level? He became convinced that to understand the mechanism underlying circadian rhythms, it was essential to look at what happens in individual cells rather than the whole body.

Other researchers shared his doubts, fuelled in part by the fact that the transcription-translation model of timekeeping had grown up over time

by correlation and had never been directly shown to be essential at the cellular level. "It's a very hard model to test, because if you shut down transcription in any cell, the cell will die - and dead cells don't have circadian rhythms," he points out with unassailable logic.

Clock without genes

Research by a group led by Professor Takeo Kondo in Japan and published in *Science* the same year sent even bigger shockwaves through the field.

The group had been looking at Synechococcus elongatus, a species of photosynthetic cyanobacteria. S. elongatus is a prokaryote (meaning it doesn't have a nucleus), which split from our own eukaryotic line of evolution around 3 billion years ago (humans are eukaryotes, meaning the DNA in our cells is contained in a nucleus). Some years previously, Kondo's group had shown that despite not having a nucleus, this particular cyanobacterium had circadian timekeeping in transcription, photosynthesis and other metabolic processes.

Although they don't have a nucleus, cyanobacteria do still have a DNA genome, but it is found in the cytoplasm. Kondo and team identified three clock genes - KaiA, KaiB and KaiC - in the bacterial genome, which appeared to have a similar function to the clock genes found in mammals: deleting or mutating them disrupted rhythms in gene expression.

The really dramatic finding came when they purified the proteins these genes made and put them in a test tube with adenosine triphosphate (the universal currency of cellular energy). The proteins continued to alter their function as they would in a cyanobacterium by adding or removing phosphate groups from ATP onto KaiC (phosphorylation). And, just like in a cyanobacterial cell, they did so in a 24-hour circadian rhythm -

despite the absence of any genetic (transcriptional-translational) activity.

"That was unbelievable - it completely blew everybody away because it opened the doors for completely non-transcriptional mechanisms of timekeeping," says O'Neill. "A lot of people assumed that it couldn't possibly be the case in more complicated organisms like humans or mice. But a few of us thought that if it were true in a cyanobacterium, it could be true in any organism."

In light of the Japanese study - and the fact that there was no strong, direct evidence indicating that transcription-translation loops were essential to timekeeping - he began to seriously consider the possibility that the cellular timekeeping mechanism might be biochemical rather than genetic.

Could a 'transcriptional-translational' model of timekeeping really allow for enough complexity to enable a single cell to keep 24-hour time on its own? Perhaps the transcriptional-translational components were an output of the clock - extremely important for facilitating rhythmic physiology but not necessarily the clock itself. "I didn't think the existing data were wrong, just that things might be more complicated."

Red blood cells

Previous work he had done at the University of Edinburgh, with Professor Andrew Millar, on another single-celled green alga, Ostreococcus tauri, supported the idea. The alga goes into hibernation when kept in the dark and stops transcribing and translating genes into proteins. Yet O'Neill and colleagues still found evidence (albeit indirect evidence) of a circadian metabolic oscillation persisting even in the absence of genetic activity.

In 2009, with Wellcome Trust funding, he moved to Cambridge to hunt

for biochemical non-transcriptional timekeeping mechanisms with Wellcome Trust Clinician Scientist, Dr Akhilesh Reddy. "We hit on the idea of using human red blood cells to look for direct evidence of timekeeping in the absence of transcription," he says. Red blood cells are the only living human cells that don't contain any DNA; they lack a nucleus and other organelles. "They don't die if you inhibit transcription because they don't need to transcribe to survive, so evolution basically handed us a perfect system in which to test whether non-transcriptional mechanisms are sufficient to keep 24-hour rhythms."

First, they had to identify a molecule that could act as a 'reporter' of cellular rhythms in the absence of transcription. Fortuitously, they quickly realised they already had what they needed, courtesy of their work on a previous screen for mouse liver proteins. That screen had revealed that a set of very common enzymes called peroxiredoxins, which are found in every living organism, were modified after they had been 'assembled' (i.e. after translation).

Peroxiredoxins help control the level of harmful reactive oxygen species such as hydrogen peroxide that are generated by cellular respiration, and they become oxidised themselves in the process. The oxidation changes their structure (the modification O'Neill and colleagues noted) in ways that can be easily detected by Western blot, an analytic technique that uses antibodies to identify specific proteins. Follow-up work revealed that the peroxiredoxins were oxidised and reduced again in a 24-hour circadian cycle.

Armed with their biomarker, the researchers measured oxidation in human red blood cells cultured in test tubes and kept in constant darkness. Western blot analyses revealed that although the peroxiredoxin proteins themselves remained at a constant level (as expected, because there were no genes to make new ones), they still continued to be oxidised and reduced in a 24-hour rhythm. That study provided the first

direct proof - and in a human system - that a non-transcriptional timekeeping mechanism must exist.

To follow up, O'Neill and Reddy deleted (knocked out) a clock gene (CRYPTOCHROME) from mouse cells and examined the peroxiredoxin oxidation and reduction within those cells. "When the clock gene was knocked out, we still saw peroxiredoxin oxidation and reduction rhythms, but they weren't completely normal," says O'Neill. "That suggests that if the transcriptional-translational feedback loop is intact, then it must couple very tightly with this metabolic oscillation. But if the transcriptional oscillation is absent, then the metabolic oscillation still persists."

A billion years old

O'Neill returned to his old friend Ostreococcus tauri - the algae that hibernates in the dark - and used the same antibody, in the same Western blot test, to measure peroxiredoxin oxidation and reduction in the algae. Lo and behold, even in the dark, with all genetic activity disabled, they found peroxiredoxin was oxidised and reduced in a circadian cycle.

"That was an unbelievable moment when we found that out," O'Neill recalls, "because Ostreococcus tauri split from the human line of evolution more than a billion years ago. Yet, amazingly, the same antibody works in both systems, and we could see the same oxidation cycle in both." Now they had direct evidence for timekeeping, in two very different systems (the human red blood cell and algae), in the absence of transcription. The findings were published in two *Nature* papers in 2011.

The startling conservation of peroxiredoxin proteins in humans and algae, across a billion years, prompted them to look for it in a range of different organisms - including Archaea, cyanobacteria, fungi, flies,

plants and mice - known to have circadian rhythms. In all of them, metabolic oscillations persisted even when the clock gene circuitry was turned off, suggesting peroxiredoxin oxidation rhythms may be a universal feature of circadian timekeeping.

There are, says O'Neill, a couple of caveats. He and Reddy don't believe that peroxiredoxin itself is the timekeeping mechanism, because interfering with it produces only very subtle effects. "Peroxiredoxin may not be a core regulator driving the 24-hour rhythms of the cell; it may just be the hands of the clock, not the cogs." They have yet to identify the biochemical timekeeping mechanism, although O'Neill believes it is likely to have its basis in hydrogen peroxide synthesis.

The next step will be to unpick that mechanism and try to understand how it couples with transcriptional control - and how varying oxidation rhythms in our red blood cells affect our health. "Clearly, there will be consequences if the level of oxidative stress faced by your red blood cells varies over circadian time. And there may well be significant consequences for understanding oxygen-carrying capacity in the blood and how that varies over time."

The great oxidation event

The ubiquity of peroxiredoxins across plants, microorganisms and animals - and the fact that they undergo 24-hour cycles of oxidation-reduction in every species - may also shed light on a question that has baffled evolutionary biologists: how did circadian rhythms evolve originally? To date, no one has proposed a particularly convincing argument to explain the molecular origins of cellular rhythms, and the (contrasting) differences in clock genes between different species have further muddied the picture.

O'Neill and Reddy were struck by the fact that peroxiredoxin proteins

are known to have evolved about 2.5 billion years ago - coinciding with the 'great oxidation event' we know took place at about that time.

Until then, almost every organism was anaerobic, meaning it did not use oxygen to convert food into energy. Then, 2.5 billion years ago, some bacteria (probably cyanobacteria) evolved the capacity to photosynthesise in water, creating oxygen as a waste product. That led to a rapid increase in the levels of oxygen in the Earth's atmosphere: they rose from 0.1 per cent of atmospheric content to present-day levels of 21 per cent in only a few million years.

Oxygen was toxic to anaerobic life, which underwent a catastrophic decline. Today, anaerobic microorganisms can only survive in extreme environments such as the sulphur-smoking fissures of the ocean floor or the harsh waters of the Dead Sea at the lowest point of the world.

Organisms that survived the great oxidation event had to develop the ability to live in the presence of oxygen, use it in cellular respiration, and disable the reactive oxygen species generated as a by-product - the principal cellular role of peroxiredoxins. Peroxiredoxin proteins may have evolved to enable these organisms to anticipate and cope with daily fluctuating cycles of oxidative stress.

Thus, as O'Neill and colleagues suggest in a 2012 *Nature paper*, metabolic timekeeping in the early aerobes (organisms using oxygen to metabolise food) may have evolved as a direct result of the great oxidation event, not merely alongside it.

Twenty-four-hour peroxiredoxin oxidation and reduction may have been the very first timekeeping mechanism - explaining why it's so well conserved across so many different species. Other cellular timekeeping mechanisms, such as the clock genes and transcription factors, would have been bolted on later, after species diverged from one another,

explaining why different plants and animals have different clock genes.

"We couldn't prove it without a time machine, but it at least provides a coherent framework - a possibly unifying idea - for understanding why circadian rhythms and metabolism are so well conserved across nearly all the different organisms you see on this planet," says O'Neill.

It's a breath-taking idea. That all of our lives - that of every person, plant, animal and microbe on this planet that uses oxygen to survive - are governed by the same ancient clockwork mechanism. That almost every cell in our bodies is still silently ticking away to the same 4.5-billion-year-old metronome, keeping time with Earth as it rolls towards the sun, morning after morning after morning.

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