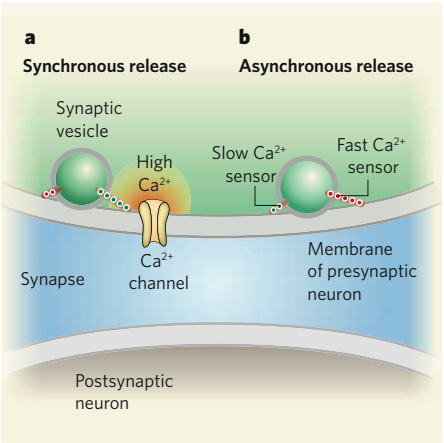

Figure 1 | Two modes of neurotransmitter release. a, Synchronous release is evoked when each of the five calcium-binding sites (green circles) on a fast calcium sensor, such as synaptotagmin-2, is occupied. This happens most readily when a synaptic vesicle is located in close proximity to an open calcium channel. **b**, Sun *et al.*[2] find that asynchronous release is evoked when two binding sites on a slow calcium sensor are occupied (green circles), whereas those of the synchronous sensor are not (red circles). This is most likely to happen in response to a smaller or more sustained increase in calcium levels. The authors propose that occupancy states of the two calcium sensors govern whether release is synchronous or asynchronous.

concentration at presynaptic calcium levels of above 1 micromolar. However, in synapses that lack synaptotagmin-2, the slope of this relationship is markedly reduced. Hints of a shallow relationship for asynchronous release are also apparent in normal synapses[6,7]. Below about 1 micromolar, the curves from normal and synaptotagmin-2-deficient synapses are superimposable.

These fascinating results lead to three important conclusions. First, the calcium sensor for asynchronous release is not only distinct from synaptotagmin-2, but, in the range of calcium concentrations studied, also requires fewer bound calcium ions to trigger release. Second, because release is slow and protracted despite rapid, homogeneous calcium increase, properties intrinsic to this calcium sensor must contribute to asynchronous release. Third, even at normal synapses, release can occur through the asynchronous pathway at low calcium concentrations.

Next, the authors used a computational approach to quantitatively analyse the calcium dependence of the rate of neurotransmitter release. The best fit to the experimental data came from a mathematical model that presupposed a single population of releasable synaptic vesicles containing one calcium sensor for synchronous release and another for asynchronous release (Fig. 1). This assumption is plausible because, according to earlier work[8], the releasable pool of vesicles in the calyx of Held comprises both rapidly and slowly releasing vesicles, corresponding to synchronous and asynchronous modes of secretion. Moreover, Sun and colleagues themselves found that the entire pool of releasable vesicles could be released through the asynchronous pathway in the absence of synaptotagmin-2. The authors' model also presupposes that release could occur spontaneously or be triggered by the binding of calcium ions to either the five low-affinity binding sites on the sensor for synchronous release[1] or the two sites on the sensor for asynchronous release.

The remarkable outcome is that asynchronous release can be triggered by the binding of just two calcium ions to a sensor — rather than the traditional five proposed for synchronous release — and that the calcium affinity of the sensor that mediates this release process is almost identical to that for synchronous release. These findings do not support earlier postulations that asynchronous release must be governed by a high-affinity sensor. Rather, it is the relative speeds with which calcium binds and unbinds the sensors that are important; the simulations suggest that these differ by at least one order of magnitude.

So the properties of the faster, synchronous sensor, synaptotagmin-2, are better suited for triggering release in response to large, rapid increases in calcium concentrations that allow simultaneous occupancy of all five binding sites, whereas the slower, unknown asynchronous sensor described by Sun *et al.*[2] is better suited for triggering release in response to slower, more sustained changes in calcium. These results also indicate that the magnitude and duration of the calcium signal determine which sensor drives release, adding a new dimension to the multiple functions of presynaptic calcium.

In light of the exciting and unexpected findings of Sun *et al.* several questions must be addressed. These include identifying the mysterious calcium sensor that mediates asynchronous release; determining whether this sensor has additional calcium-binding sites, even if not all need to be calcium-bound to trigger fusion[9]; gaining a better understanding of presynaptic calcium dynamics, particularly in the vicinity of the releasable pool of vesicles; and identifying mechanisms that regulate the recruitment and/or coupling of releasable vesicles to presynaptic calcium channels. Such knowledge is vital for a full understanding of the factors that determine the tenor of neurotransmitter release and synaptic signalling[6,10]. ■

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MOLECULAR MOTORS

A step dissected

Zeynep Ökten and Manfred Schliwa

The motor protein kinesin 'walks' by alternately advancing its two motor structural domains. A cutting-edge, single-molecule fluorescence technique reveals further details of this stepping mechanism.

Suppose that walking required energy input in the form of, say, one Gummi bear for every step. In what position would you pop the next Gummi bear into your mouth? When one foot is firmly planted on the ground while the other is lifted up and poised next to it to be thrust forward, or when both feet are on the ground spaced apart at step size? Mori *et al.*[1] (page 750 of this issue) have addressed these questions, not in a person, but in a miniature, biological walking machine — the molecular motor protein kinesin.

The human genome encodes up to 45 kinesins[2], each of which has specialized cellular functions. Common to all kinesins are an evolutionarily conserved structural domain called the head, which acts as a motor, and a variable tail region. One class of kinesin motor, kinesin-1, consists of two identical polypeptide chains linked by a neck region to form a dimer. Kinesin-1 molecules can move great distances on cellular tracks called microtubules (hollow, cylindrical polymers of the protein tubulin), which form an organized web of roadways in the cell. The head region has one binding site for the track and another for kinesin's equivalent of a Gummi bear — an ATP molecule.

Previous work has revealed amazing details about the walking mechanism of kinesin-1 and other motor proteins. Processive (long-distance) movement of kinesin requires the hydrolysis of one ATP molecule per step[3]. The step size is 8 nanometres, which corresponds to the spacing of tubulin subunits[4]. Intriguingly,

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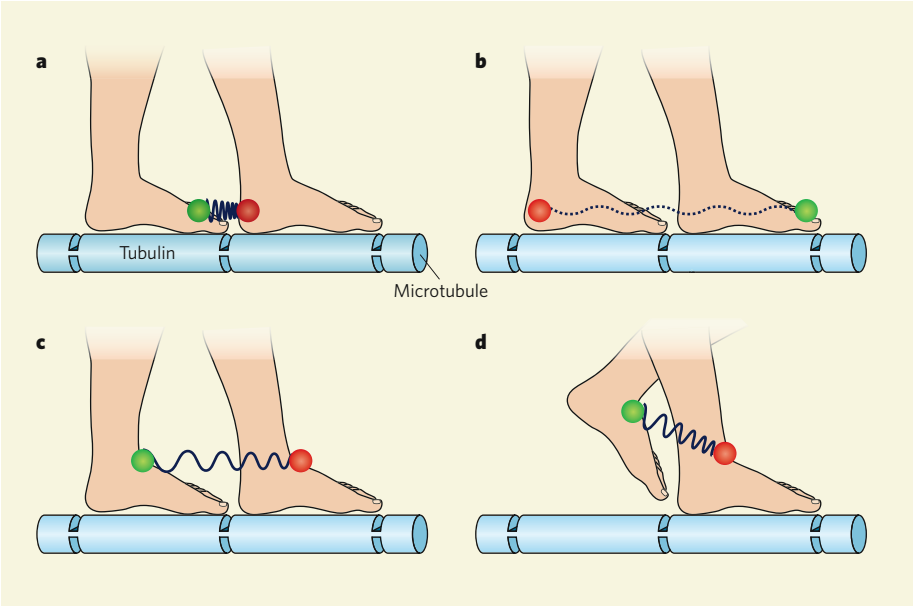

Figure 1 | Positioning of fluorescent sensors on a walking kinesin molecule. If dimeric kinesin-1 is likened to the feet of a walking person, the probes used by Mori *et al.*[1] (green and red balls) for analysis by fluorescence resonance energy transfer (FRET) would be positioned at the toes, heel and near the ankle. Intensity of FRET interaction is indicated by blue waves. The toe–heel pair of sensors yields (**a**) high or (**b**) negligible signals depending on which head leads. The ankle–ankle pair yields (**c**) low signals when both feet are attached and (**d**) a stronger signal when one foot is lifted and poised next to the other. The authors used these position-dependent variations in fluorescence to study the movement of kinesin-1 along microtubules.

kinesin stepping resembles human walking: one head remains firmly bound to the track while the other head moves forward; the two heads alternate in taking steps (the asymmetric hand-over-hand stepping model[5]). During 'walking', the hydrolysis cycles of the ATP molecules on the two heads are kept out of phase[6]. Otherwise, if both heads transit to a detached state synchronously, the motor would lose contact with the microtubule.

Several studies[7–10] also used sophisticated molecular and technical wizardry to answer another question — how the heads of the kinesin-1 dimer are coordinated during processive movement. That is, does the free head pass the microtubule-bound head in one smooth sweep, or is there a characteristic position in which kinesin-1 waits for the next supply of energy? But addressing this question is difficult. Whereas watching a person take a step is easy when you stand nearby, trying to 'see' kinesin move is equivalent to watching a person walk when standing a distance of a kilometre or so away from them.

So how can the gait of a distant walker be figured out? One way would be to attach differently coloured floodlights to the two feet and observe stepping in the dark. From the way the lights add up, mix or become superimposed, one could then draw conclusions about the person's stride.

In a sense, this is the principle Mori *et al.*[1] applied to watch kinesin-1 walk. The first trick they used was to attach molecular floodlights to the two heads of a dimeric kinesin-1. This was done by introducing in desired positions cysteine amino acids to which fluorescent molecules could be attached. If we compare kinesin-1 heads to human feet, informative positions for the floodlights are the toes, heel and ankle. The authors' second trick was to use floodlights of different colours, which could then interact — the third trick — in a process called fluorescence resonance energy transfer (FRET) (Fig. 1).

The FRET technique is based on energy transfer from a short-wavelength donor fluorescent molecule to a longer-wavelength acceptor fluorescent molecule when the two molecules are close together (1–10 nm). This interaction is then detected by monitoring the quenching of the donor molecules' fluorescence or a rise in the fluorescence of the acceptor molecule.

Mori *et al.* applied this technique to kinesin-1 in which the fluorescent molecules were attached to the 'toe' of the rear head region and the 'heel' of the front head; the signal generated from energy transfer between these two molecules would be different if the positions of the two heads were reversed. In the first case (Fig. 1a), the FRET signal would be high because the distance between the fluorescent molecules is only 3 nm; but in the second case, the signal would be negligible because the distance between the donor and acceptor molecules is 13 nm (Fig. 1b). Similarly, when the two fluorescent molecules are attached at positions analogous to the ankle — that is, at neck linkers, one of which emanates from the middle of each of the two kinesin-1 heads — FRET efficiency would be low when the two heads are bound to microtubules (8 nm apart) (Fig. 1c), but higher when one head is detached, hovering near the bound head (Fig. 1d).

By designing experiments with the two fluorescent molecules at different positions on a kinesin-1 dimer, the authors find that when a step is taken in the presence of low levels of ATP, the motor spends most of its time in a one-head-bound state, while the free head lingers next to the bound head. In this case, the free head transits through a short-lived state, when both heads are attached to the next waiting position. But at high ATP levels, an average FRET signal is observed, suggesting that kinesin-1 spends most of its time with both heads bound to the microtubule. These results suggest that, at low ATP levels, the rate-limiting factor for kinesin-1 to take a step is exit from the one-head-bound state. Are these results indicative of two fundamentally different stepping modes of kinesin? Probably not. The basic stepping mechanism is still the same; only some phases may be longer lived than others.

A more precise analysis of the transitions at high ATP levels was impossible because current instrumentation lacks the necessary temporal resolution. This is not surprising. Mori and colleagues' experiments[1] might seem easy on paper, but in practice they are very tricky. The outcome depends not only on the positions of the fluorescent molecules, but also on their relative orientation and the mobility of the head regions. Making these observations at a single-molecule level adds to the technical challenge, and invokes extra caution when interpreting the results. Considering these difficulties, the authors have managed to extract remarkably clear information on the positioning of the two kinesin-1 heads under well-defined experimental conditions. And the use of several engineered kinesins helped them to exclude potential sources of uncertainty.

So despite the big step taken by Mori *et al.*[1], the kinesin walk has not yet given up all of its secrets, as possible transitions and intermediate positions in the fast-stepping mode still remain to be elucidated. Who would have thought that taking a step could be so complex and its study so exciting? ■

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