Module:
Techniques in Neuroscience

Week 1
Understanding the brain:
Who we study, how and why?

Topic 2
Model organisms
– Part 2 of 3

Dr Frank Hirth
Associate Professor and Reader in Evolutionary Neuroscience, Basic and Clinical Neuroscience, King's College London

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So let's go into details now. What I would like to show you first is to illustrate animal models in your science research, a means to address a scientific question and why it is so important to do *in vivo* - as compared to *in vitro* - studies. And to illustrate that, I would like to start with the baker's yeast, which is on the top left corner of the images.

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Baker's yeast, or also called *Saccharomyces cerevisiae*, is obviously not an animal. It's a eukaryote, it's a proper cell. All of the animals are made of eukaryotic cells, but here we would like to illustrate the conceptual framework. What is done in functional studies? On the left-hand side, you see yeast cells and you can see how they bud off- that they form, from one cell, you generate two cells. On the right-hand side, you see an agar plate where different colonies of baker's yeast have been plated. Why is it important to use baker's yeast? Because you can do very, very fast experiments, and an example I illustrated below. Baker's yeast has successfully been used to discover genes and their function, in the regulation of the cell cycle and cell division, like you see on the left-hand side. And later on, it turned out that this is highly conserved. The genes that regulate cell division in yeast, you find homologous genes in mouse, flies, humans. They all regulate cell division.

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Now what we see here is an example of a functional study, using yeast as a means to address a scientific question. In panel A, you see a budding yeast in green. YFP is a protein to illustrate and highlight the cell body, and the nucleus. Then in the middle row, you see TDP-43. This is a gene that has been discovered to be involved in the formation of motor neuron disease. The same is true for FUS (pronounced 'fuss') - again, a protein involved in motor neuron disease. When you compare to the control the YFP, you can see that the green all of the sudden has clumped up in what we call aggregates. Below, you see these yeast colonies plated on an agar plate. 'Glucose on' means a gene has been turned off. 'Galactose' means the gene has been turned on. If you compare the two, you will see that both TDP-43 and FUS inhibit the growth of yeast cultures. This is significant, because it tells you that the aggregate formation has something to do with the viability of yeast. Or, in other words, if these proteins build aggregates, they are pretty toxic. And what these people now have done - this is from the lab of Aaron Gitler - they have used yeast, an expression system which is illustrated in B, to express several RNA-binding proteins. The details about the molecular biology are not so important now, it's more about the logic.

Look underneath about the budding yeast. On the left-hand side, it reads 'cytoplasm diffuse'. The other one reads 'nucleus diffuse', and the other one reads 'cytoplasm aggregates'; and those are the three readouts they have used to screen for genes and proteins that form aggregates.

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What they found is illustrated here. Again in C, you see the YFP control. You see TDP-43 and FUS, and now you see a new one, which is TAF15. As you can see with the glucose and galactose, inactivation or activation, you may appreciate that the expression of TAF15 leads to aggregate formation and has a cytotoxic effect on yeast- that is, the accumulation of this protein seems to cause aggregate formation.

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This is shown here. This is a description in A where you overexpress those proteins. In B, you show the time it takes for them to accumulate, and in C is depicted how they form pellets in an Eppendorf tube, thus means this protein is starting to accumulate and precipitate. Consider like you have crystal formation: it starts with a little crystal, and then it gets bigger, and bigger and bigger, and that is nicely shown in D. You have the example for FUS, TAF15 and TDB-43. From zero minutes to 16 minutes, you can see how they clump together, and that is toxic aggregate formation.

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Why is this significant? These studies have been carried out in baker's yeast, which is a eukaryote. It doesn't even have tissues or a body.

The significance of this finding is shown in this slide, where you see from A to H sections through post-mortem human brain tissue, where these researchers labelled for the human homolog of TAF15, and what they found is quite remarkable. They found that the human homolog of TAF15 also forms aggregates; and as you can see in the depiction, in the control they seem to be no clearer, but in the cases B to H, these are ALS cases, which stands for amyotrophic lateral sclerosis. This is the most common form of motor neuron disease, and as you may appreciate, TAF starts to form aggregates within the nucleus, but sometimes also a little bit outside of the nucleus. So, what started like a screen in yeast ended up with a discovery of a novel disease-related gene in humans.

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So, we have gone now from a gene and its encoded protein, all the way to the relation to disease. All this was done in yeast, and then we made a huge leap into human patients.

Now, the next one we want to look at is the worm.

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The worm that is often use in the lab is *Caenorhabditis elegans*. It has several advantages: it has a simple anatomy, it is only composed of 959 somatic nuclei (of which only 302 are neurons), it's a transparent worm, it is small - it's only one millimetre long, it has a huge number of progeny (250 per generation), it's very easy to cultivate, it eats bacteria, you can grow it on agar plates seeded with bacteria in your laboratory and it has a rapid development. It's life cycle is only three days, at 25 degrees. Now, why would we use the worm to study this? A remarkable discovery that actually led to the Nobel Prize was the discovery of cell lineages and the clonal origin of cell types in tissues.

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Which is illustrated here. This is the finding of John Sulston and colleagues, for whom they later received a Nobel Prize. What you can see here is a pedigree. It's a tree of life, starting at the top from one single cell and all the cells that derive from this. We know now that *C. elegans* is exactly made of 959 cells, and this is remarkable. It somehow reminds a bit on the phylogenetic tree I showed you before, if things are related. That not only accounts for the tree of life, it also accounts for the tree of cells, if you like, for *C. elegans*. We have invariant lineages which give rise to the entire animal, and those invariant lineages, of course, then make it possible for us to study cell fate decisions and how they are determined.

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Another advantage of the worm is its short generation cycle, and that it generates lots of progeny. What you see here is a picture of its lifecycle. It starts with embryonic development, then goes through four larval stages that can be interrupted when there is an overcrowding in the culture or whether there is not much food available, then they could go into what is called dauer larval stage, or otherwise they hatch into the young adults and the adult then can give rise to another egg.

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Now, because of this very short life cycle and because these animals don't get very old, it is the ideal model organism to study the genetic basis of aging, and it was actually Cynthia Kenyon and colleagues in San Francisco at the time where they looked into this more closely.

So here, you have a depiction with different images, but basically the same, where you go from a fertilised egg to the larval stages L1 to L4, and then the adult. As I said, from L1 to L2, you could go into a dauer stage that couldn't be reactivated to go into L4. This is the life cycle of *C. elegans* that made it such a powerful model organism to study aging.

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What Cynthia Kenyon and colleagues in a remarkable study in 1993 found were two genetic mutations that change the lifespan of *C. elegans*. The genes are called daf-2 and daf-16, and what you see here on the left-hand side is the percentage of alive animals, and on the x-axis, you see the days after hatching- 20, 40, 60 days. You can see now from the opened wide squares that the wild-type usually lives around 25 to 30 days- so do other mutations. Now, the remarkable observation was when you look at the daf-2 mutation, these animals can live almost up to 55 days, which is twice as long as they would normally live. That was a remarkable finding.

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What these researchers then found out in subsequent studies was that there was an interaction between daf-2 and daf-16. Actually, the protein daf-2 can inhibit or suppress daf-16, and that plays a very important role in normal lifespan. During development, high levels of daf-2 cause low levels of daf-16, which means you go into L3 stages. If daf-2 is low, as a consequence, you have high levels of daf-16 and the worm goes into a dauer stage. Now during adulthood if you have high levels of daf-2, it causes the suppression of daf-16, which therefore are low, which leads to a normal lifespan- as we said, around 25 days. Now, if by mutation, in adulthood daf-2 is low, it leads to the derepression of daf-16, which then leads to high levels of this protein, and that extends the lifespan.

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Now this remarkable finding in the worm *C. elegans* by the time was it's quite a sensation, and caused quite frantic research and searches into other animals.

As you can see here in this picture, again, things are conserved. In the left-hand side in A you see *C. elegans,* and we talked about daf-2. What daf-2 turned out to be is the insulin receptor, which is a universally conserved receptor molecule involved in nutrition and aging. Daf-2, the insulin receptor, represses daf-16, which codes for FOXO, and those together, as we looked at are involved in longevity. Now, as you can see in B and C, there are homologous genes for that. For the daf-2, it's again the insulin receptor is *Drosophila*. For daf-16, it's also FOXO. The same applies for mice, and, of course, it also applies to us. So, what you see in a nutshell is an evolutionary conserved pathway that is involved in longevity and aging, and, of course, it's of great interest to people whether we can extend our life span by manipulating those studies, but that's a course for another lecture.

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So this was an example where we used the *C. elegans*, the worm, its advantages - like a short life cycle, like a short lifespan - to look into the genetics and biology of aging and longevity.

Now let's have a look at the fruit fly, *Drosophila melanogaster*.

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Here you can see a fruit fly on a pencil tip. This is a very artificial image because all of you know when you're in your kitchen and some fruits have been left to rot, the fruit flies are there, and if you try to catch them, they usually escape in a quite artful manoeuvre. Now, this fly here on a pencil tip wouldn't do so, but it has eaten some drugs, which already tells you what these animals could be useful for. What is interesting here is that *Drosophila melanogaster* was the first animal to be fully sequenced its genome, which turned out to be 180 megabases on only four chromosomes, and people discovered that the genome is made out of 13,600 genes. Now compare this to the 25,000 human genes, which already tells you that the number of genes doesn't necessarily correlate with your size or complexity. What they also found is that of those genes, 65 per cent of them show a structural identity

to human genes that are related to neurological disorders. The fly, even though it's small, it has a complex brain. It shows complex behaviour, including learning and memory. Flies can learn: they can learn complex things and they can remember them. And we have - we call it a sophisticated genetic toolbox available to do molecular, cellular and behavioural studies in vivo, that means in the entire organism.

Where *Drosophila* came to fame was in 1912 in Columbia University in the lab of Thomas Hunt Morgan, where they proved with experiments the chromosomal theory of inheritance. At that time, it wasn't even clear that there are genes and chromosomes, and what Thomas Hunt Morgan and his colleagues showed in very elegant experiments is, genes code for phenotypes and they are inherited.

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The advantage of *Drosophila* is highlighted here - a bit similar to the worm, but, of course, with a much more complex central nervous system. The fruit fly has also a rather short life cycle - it can take up to 10, 12 days at 25 degrees. It goes through an embryonic stage, several larval stages, then it forms a puparium from which the adult hatches. On the right-hand side, you can see what we make advantage of that in a laboratory culture. All of these little tubes are full of a stock of genetically modified animals, and as you can see with the Swiss officer knife next to it, this is rather small. So, you can have various different cultures at the same time, which makes it such a powerful genetic model organism.

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The toolbox that is available for *Drosophila* is illustrated here. One of the toolboxes is that we can make use of a transactivation system, which is shown on the left-hand side. This is the GAL4/UAS system. It derives from a transcriptional activator from yeast, which is called GAL4, that can specifically bind to what is called the UAS, an upstream activating sequence. Now, when you do molecular cloning - we don't have time for going into details but when you do molecular cloning, you can generate transgenic flies that have an enhancer that drives GAL4 expression. That could be, for example, an enhancer that is specific for all the dopaminergic neurons. Now, when all the dopaminergic neurons are active, this enhancer is transcriptionally active, it recognises the abstract activating sequence of a second set. That could for example be green fluorescent protein, which is shown on the right-hand side. What you see there is an adult brain of the fly, on top, and lower is the ventral nerve cord, which corresponds to our spinal cord. In red is shown the CNS, and in green we have visualised cells and circuits in the nervous system using this GAL4/UAS system.

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The power of *Drosophila* is also illustrated by those two pictures here that actually come where I have been involved in this. On the left-hand side, you see a schematic depiction of the human brain, a cross-section through the nerve cord and then the neuromuscular junction. As you can see in the human brain, on top of it, there's what we call an upper neuron that projects into the ventral nerve cord. It then is connected to a lower motor neuron that innervates the muscles. These connections are typically degenerated in people with motor neuron disease. Now, a similar composition you can see in *Drosophila*, even though it's such a small brain, far less complex. You have upper motor neurons that synapse on to lower motor neurons in the nerve cord, that then the lower motor neurons innervate the muscles.

So, the basic principles are the same. They are built according to the same principle, which means an upper motor neuron gives a command to a lower motor neuron, and the lower motor neuron then innervates the muscle in order to conduct movement. On the right-hand side, you now see a rather complex image of a mammalian

brain and an insect brain. I don't want to go into much details, it is meant to illustrate that even at the more complex level that is involved in the regulation of voluntary movements, we can find similar neural circuits in a mammal and in an insect.

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The real power of *Drosophila* comes to its full fruit when we do genetic strings. Because its genome is sequenced, because we have such a good understanding of its phenotype - such as different eye colour, such as wing shape, all the different neural circuits I showed you - what has been established are what is called forward genetics or reverse genetics. Let's first look at forward genetics. People can mutate the genome of *Drosophila*, and then without even knowing what kind of protein or gene is involved, look at the phenotype. These were the classical experiments by Thomas Hunt Morgan. They all of a sudden discovered a fly that has no longer red eyes, but white eyes, and then they tried to isolate the gene, and indeed they found the white gene. So you start with a mutant phenotype, then identify the protein and the gene. You could do the other way around like we did, for example. Our colleagues found a human gene involved in a disease. They told us the gene, we then went into *Drosophila*, looked whether it has a human homolog, or a fly homolog in this case, and then we started to manipulate that gene, and then, we could look, how does that relate to the disease?

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In addition to the powerful genetics in *Drosophila*, we can also look at the behaviour in these flies, and as you can see here on top of it, those are little arenas. These are dishes we use for cell cultures, but here we have converted them into little arenas for flies. So from 1 to 6 you see controlled animals, and from 7 to 12 you see animals that have been induced what we call a Parkinsonism - that is, we have changed their mitochondrion activity which is also defective in Parkinsonian patients. Now, at the bottom you see in A, that we can actually artificially trace the movement of each of the fly. That is a computer programme that does that. In B, you now see the traces of such a fly over three minutes, and you can see that they explore the arena. In C, you now see the movement and behaviour of a fly that has this induced disease, and it's quite obvious, they didn't move very much and it takes them far longer to induce a movement. This is a Parkinsonian-like phenotype in a fly.

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Here are further details of this study. What has been done is mitochondrial dysfunction has been induced in different cell types of the brain of the fly. In A, you see cholinergic neurons, what happens to them; B, dopaminergic; and C, serotonergic.

What these graphs show is the climbing performance of a fly, where the mitochondrial dysfunction has been induced in these different cell types. The climbing performance makes use of an innate behaviour of flies. You put them in a tube, you bang them to the bottom and they naturally climb up towards the light and against the gravitas of the earth. This is an innate behaviour and it's very reproducible, and what you can see already in A is over time, even the controls show a decline of their behaviours. That's normal, you can see it with yourself. When you age, you are less agile than you were with 20. Now, the interesting part is when you compare A, B and C, that only in the dopaminergic inactivation, you see a clear difference between the controls and the ones where mitochondrial dysfunction has been induced in dopaminergic neurons. Now this is very interesting, and it's further illustrated in D, E, F and G. Again, you see one of these arenas, in the control you see them running around, but in the pole G alpha IR, where we have inactivated mitochondria, you can clearly see a movement phenotype. This is further illustrated in E, which shows that the activity of these flies is decreased, the velocity is decreased, and as a result, they walk not as far as the control. So, these experiments already at the behavioural level, clearly

illustrate that when you inactivate mitochondria, they have a much more pronounced effect when inactivated in dopaminergic neurons than compared to other neurons, and that points towards something very, very specific for Parkinson's disease.