Who ordered that?

By the mid 1930s, physicists thought they had a clear picture of subatomic particles. There were four; the proton, the neutron, the electron and the photon. The world was an ordered place; most of the time, these entities remained that they were and, when they did change, they seemed to do so in a way that produced or absorbed other members of this known family. Then, studying cosmic radiation in a cloud chamber, Anderson and Neddermeyer found compelling evidence for a particle with negative charge and a mass between that of the proton and the electron. On hearing of this particle (now called a mu meson), the physicist Isidor Rabi famously remarked "Who ordered that?".

Rabi's question came to mind earlier today, when I read a pre-print that implies that a 30-year scientific quest has finally come to an end. It is a quest that has a lot of personal meaning for me, because I took the first steps in answering it in my own PhD work. I was at the time very interested in how the nervous system develops. Almost everything that had been written, and certainly everything I had been taught as un undergraduate, was about molecules that formed attractive pathways for growth cones, the navigation structures that can be found at the end of the axons ('wires') of the growing nervous system. For various reasons, I became obsessed with the idea that many aspects of the nervous system would be almost impossible to build with attractive cues but relatively easy to build with repulsive cues. One of my teachers, Dr Mike Bate, encouraged me to read the work of Roger Keynes and to meet him, because he had obtained indirect evidence that repulsion was at work in keeping nerves that leave the spinal cord out of areas that will become the bones of the vertebral column, and had recently published it in *Nature*. Roger had just teamed with with Geoff Cook, a brilliant carbohydrate biochemist and pharmacologist, in the hope of finding the molecule involved. To my immense and undying gratitude, Geoff agreed to take me on as a PhD student, with Roger

My PhD supervisors, Rev Dr Geoffrey MW Cook and Dr Roger Keynes, on a punt on the Cam in 1989. I have no idea whether my current students would recognize the grad' student sitting behind them.

providing anatomical and neurological advice, and Gonville and Caius College gave me the necessary scholarship to pay tuition fees and generally keep body and soul together.

It says a lot for the calm patience of Geoff and Roger that they put up with my glassware-destroying clumsiness and provided all the advice anyone could ask for in my search for the molecule. To shorten a long story, full of long nights spent with columns and scintillation counters, we found that the cells of the areas spinal nerves avoid (P-half-somites, in the jargon), but not the A-half-somites they like, contained some membrane-bound molecule that would cause active growth cones to 'collapse' and stop moving. Moreover, this molecule bound to peanut lectin, and it was possible to use the lectin to purify a protein with a mass of about 56kD. The lectin, or antibodies Geoff raised against the protein, could remove the collapse-inducing activity from extracts of P-half-somites. That is as far as I got in the time available (1986-1989); we knew how big the mystery protein was and that it bound peanut lectin, but we did not know what it was. We wrote the story up and published it in *Neuron*, I went on to a Cancer Research Campaign post-doctoral fellowship in David Garrod's lab, and Geoff and Roger took on a new student to identify the protein.

Identifying proteins, even 30 years ago, was usually reasonably easy but this one was not. One problem was that it could be obtained only from embryonic material, and therefore in very small amounts. Another was that the purified protein, for example recovered from an SDS-PAGE separation gel, seemed not to have any collapse-inducing activity, suggesting it may work only as part of a larger complex. As I built up my own research programme on other things, I watched the Cambridge lab from afar, expecting every year that the mystery molecule would finally be identified. Now, in 2019, a full 30 years later, it has been and, when I read of its identity and mechanism of action, the first thing that came to mind was "Who ordered that?".

Many growth cone-repelling molecules have since been identified in the discovery boom that followed our 1990 *Neuron* paper, and the accompanying papers from two other groups, who demonstrated growth-cone repulsion using two other systems and who published in the same issue (we all knew and helped one another). They are by and large classical signalling proteins, borne on the surface of a non-neural cell and detected by receptor proteins on the growth cone. The receptors trigger internal pathways that modulate the internal protein skeleton of the growth cone. The proteins and receptors are by now well known and appear in all the textbooks of neural development. They include Robo and Slit, Ephs and Ephrins, and Semaphorins. Many are large

families, and I sort-of-assumed that our molecule would turn out to be just another member – maybe even a member already discovered in another context some time in the 90s or early 21st Century.

This month, Geoff, Roger and their colleagues put a manuscript on the pre-print server BioRxiv ('bio-archive' the 'x' is a Greek Chi), which identifies the protein. It is not a classical cell-cell signalling molecule at all, but an enzyme, protein disulphide isomerase, located at the surface of the cell. Eh? An enzyme?? Ridiculous! But the data are rock-solid; mass-spec analyses of the purified peanut-lectin binding protein reveal the amino acid sequences of that enzyme; the enzyme is located where the repulsive activity is; preventing expression of the gene that makes the enzyme makes previously repulsive tissue hospitable to growth-cones; and inhibiting the enzyme with a drug makes previously repulsive tissue hospitable to growth-cones. So far, a clear run of positive data. But, given this, the next part of the manuscript is a surprise: the pure enzyme does not cause growth-cone collapse. This paradox is solved by some outstanding biochemical-pharmacological detective work; it turns out that the enzyme does not directly repel growth cones. Rather, it generates nitric oxide (NO) from any suitable NO donor molecule. NO is already known to be important as a biological signal (Viagra, for example, works by elevating NO and hence, via vasodilation, elevating other things). When Geoff, Roger and colleagues added their protein disulphide isomerase with an NO donor molecule, they saw robust growth cone collapse, though the NO donor had no effect on its own. On the other hand, adding myoglobin, an NO scavenger, inhibits the collapse-inducing actions of the enzyme. NO affects the growth cones by changing their internal protein skeleton, so the end action is not a surprise, merely the chain of events that triggers it.

The manuscript does not just answer the 30-year-old question of what the repulsive molecule really was; it also shows that this enzyme is responsible for most of the growth cone collapse-inducing activity in the fore-brain, activity that might be highly relevant to learning, memory and neural regeneration.

As well as being personally very interesting to me, this story makes a bigger point. Important discoveries are not always made quickly. Some problems are hard, even to very successful labs in places like Cambridge, and are especially likely to be hard when the answer is non-obvious so a lot of time is wasted on the wrong experiments (when we were trying to get pure molecules to work

back in the 80s, it never occurred to anyone to add an NO donor – why would it?). The world of short-term research grants and contracts, and end-of-grant reports that are supposed to say 'Yep, solved as promised' is not suited to questions that are worthwhile but may take years of work, and lateral thinking, to answer.

I sometimes run into Roger (now a Professor, still at Cambridge) at conferences and am looking forward to congratulating him when we next meet. I have not met Geoff for many years, to my regret; indeed, going to congratulate him would make a great excuse for visiting Cambridge for the first time in a quarter of a century.

Jamie Davies
Edinburgh
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Links

The paper on BioRxiv: https://www.biorxiv.org/content/10.1101/838771v1