TODAY'S NEUROSCIENCE, TOMORROW'S HISTORY

A Video Archive Project

Professor Alan North

Interviewed by Richard Thomas

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Interview transcript

Whether to study physics or medicine?

I grew up in Halifax, in West Yorkshire, and went to the local grammar school. At that time it was called Heath Grammar School. Now it's changed it's name. I think its called Crossley Heath, but it was classic local grammar school, founded in 1596 or something like this. And the major influence on me there, by far, was the physics teacher, and he was … we called him 'Nuffa' Morris. I think he had a huge influence in turning me onto quantitative ideas. He was very proud of the fact that he was a Cambridge man, and I think he'd got … he got in his mind that he really wanted me to follow in his footsteps. But my mother had other ideas, and I think that she had thought for a long time, that I would be a doctor. That was her real aspiration, and by the time the A-levels came along, I was still, I suppose, mostly under maternal influence. I applied for medical schools and I was rejected from all of them. So I thought, 'Well, this is great. I'm going to be a physicist. I'll go to Cambridge. I'll do theoretical physics.'

In the summer of 1962, I was in the Alps for probably eight or nine weeks, climbing my way through major peaks, and during that time I'd completely given up on the idea of medical school. I was all set to go to Cambridge, or at least to stay another year at school and take the Cambridge entrance exams. And then I went to a small post office in a tiny village called Blatten at the head of the Lurchental - it's small village in the Bernese Oberland of Switzerland - and picked up a letter that my mother had sent, forwarded from the University of Aberdeen. In early September, they decided that they *could* offer me a place, so I was thinking, oh, you know, 'Medicine, six years. Every summer a long vacation. A degree in medicine - passport to the world's mountains.' So I just changed my mind, and said I would go and do medicine in Aberdeen.

Aberdeen University – a PhD with Hans Kosterlitz

When I went there, I had never done any biology. So, I knew a lot of physics, and maths, and chemistry, and I sat through the first classes, and I remember leaning over to the person beside me and saying, 'What's this thing called the cell?' I had absolutely no idea what the lecturer was talking about, and so the first year I just forgot maths, and I forgot physics, and I picked up some biology. But then there was the opportunity to do … to intercalate a year in the medical programme, so you could do an additional year and finish up with a BSc degree. During that year, I met Hans Kosterlitz, because Hans Kosterlitz at that time was a reader in … or a senior lecturer, in the department of physiology. And so the physiology practical classes were all day classes, where they would start at eight in the morning and go till five or six at night. And that happened twice a week, so you got very intimate connections with the professors or the senior lecturers who were involved in doing the classes with you. So it was during that intercalated year that I first met Kosterlitz. Then, of course, after that, I returned to the clinical work and finished the clinical programmes, did the house jobs, and then immediately returned to join Kosterlitz to do a PhD, because it was very much in my mind.

He was a very, very energetic person, so in 1968 when he was due to retire - he must have been sixty-five-years-old then - that's when he was appointed as Professor of Pharmacology. Previously he'd been in the Department of Physiology. So, he actually took his first chair at the time when most people would be retiring. He put a huge amount of energy into the work. It was a very collegial kind of place to work. There were always big arguments going on, so, in the coffee room or the tea room, major arguments. Hans Kosterlitz just loved to argue with his colleagues, and nobody was afraid to argue with him. He wasn't an overbearing character, but he stimulated argument and discussion. So it was one of these places where the young people around were encouraged to argue and to discuss what was going on, and that's something that's carried with me throughout my career. That's the only environment in which you can really operate.

Searching for the opiate receptor ligand, mid-1970s

So, I suppose during the period when I went back to Kosterlitz as a postdoctoral fellow – so, this was '74-75. Then, sure, I was trying to work out what morphine and opiates did to single nerve cells. So that was a little bit parallel to the main activity in his lab. The main activity in his lab had, for many years, been studying morphine and similar drugs of abuse, on isolated tissue preparations, and he'd been doing this from the late sixties. It was … he'd found a way of predicting opiate-like activity by looking at their actions on various tissues removed from the animal and kept *in vitro*. So, one of these was a piece of the intestine from a guinea pig. Another one was the vas deferens from a mouse, for example, and there were several

pieces of tissues like this that could be maintained alive for several hours *in vitro*. What Kosterlitz had found was that if you take several opiate drugs like morphine, heroin, codeine, pethidine, then their ability to act on these isolated tissues very closely tracked their effects in man to relieve pain. So he had become accepted as the real guru of predicting the activity of opiate-like drugs.

So it was natural to think that if the receptors were so clearly definable on the basis of the actions of a series of drugs on them, then why wouldn't there be a natural ligand for the opiate receptor? When he started to look for that … so, he recruited John Hughes to Aberdeen in about 1970 or '71, and that's when the first ideas were beginning to be discussed about an endogenous ligand. The obvious thing was to simply take the brain, mash up the brain, extract the material from it, and see if it had an effect on these isolated preparations that were similar to that of morphine. The key test depended on the antagonist for all the morphine actions, which is naloxone. It's a morphine analogue, widely used clinically to treat people with heroin overdose because it's a pure antagonist at the opiate receptors. The key test became, if there was any activity in an extract from the brain, which looked on the isolated tissues to be like morphine, then it must be reversed by naloxone. And they had not worked very long before they found material in the extract which inhibited the contraction of the mouse vas deferens, and that was reversed by naloxone. So those findings were made in about 1973, I think. They were published in a *Brain Research* paper in 1974.

There was no identity of the material, but it was quite clear that there was a material, and I think this is what …a material with opiate-like properties. I think this is what really got Sol Snyder excited, and Lars Terenius. This is really … and Eric Simon. This is what really drove them into a frenzy. The fact that there was someone in Aberdeen – they'd already got the activity, biological activity. The group in Aberdeen had one unique advantage: they did their assays for this material on a living piece of tissue. The other people were doing their assays for the material by looking at its ability to displace radioactive morphine or a similar molecule. So it was possible for them to find an artefact. It would be something that would displace the radioactive morphine but it might not have the biological activity. The nice thing about the Aberdeen approach was that it was based on what's called bioassay, that is, they were testing the unknown by seeing its biological effect. Because of Kosterlitz's huge background experience on opiates, he knew that this biological effect resulted from occupation of the opium receptor. I think the period through 1974 and into '75, was really quite frenetic. I moved to the United States in '75, and in May of that year, just after I'd arrived in the States, I attended the meeting of the International Narcotics Research Conference at Airlie House in

Virginia. There was clearly a great undercurrent and nobody was talking very much about how far they were down the road. Lars Terenius - we all thought he was extremely close. Eric Simon - we all thought he was close. But you see, all these people were looking at the displacement of radioactive compounds, and I think that must have been a very slow way to go.

In fact, the Aberdeen group did not know the identity of it at that time. It was only after that, that they took it to the mass … you know, they realised it was a peptide, and they took it to the mass spectrometer, and they discovered that, in fact, it was a mixture of two peptides. And then the paper appeared in the 18th December 1975 - the *Nature* paper. This enkephalin was the beginning of the real era of peptides, which took off in the late '70s and ran for about ten years.

Loyola School of Medicine, Illinois, and electrophysiology – how opiates act on single nerve cells

When I arrived at Loyola, Nishi had already left. I inherited his grant. In fact, I reapplied and lost it, but then on the second time round I got it. So I was the PI (principal investigator) on a grant that was already in its thirteenth year. If fact, I maintained the grant until it was in its twenty-sixth year. It was called, 'Studies on Single Neuron Activity'. So I had the support, I had a PhD student, John Williams. I quickly got a postdoctoral fellow back from Nishi's laboratory – his name was Katayama – and we immediately turned our attention to finding out what opiates did to enteric neurons.

This (graphic) shows the wall of the intestine with its layers peeled apart. The neurons, or nerve cells, lie in these ganglia of the myenteric plexus. Each is less than a millimetre in length. We developed ways to insert the tip of a fine glass micro pipette into the neuron. It could stay in that position for several hours whilst we monitor the potential across the membrane of the cell. So, the cell is about twenty microns - thirty micrometres across. During that time, we can apply opiates and we can find what the opiates are doing. And what we found, to cut a long story short, is that they make … they increase the membrane potential across the cell membrane, and in many subsequent experiments we showed that was because they open channels for potassium ions to pass through. It took many years to show this formally, but now we have, for the first time, a direct, cellular action of opiate drugs on single nerve cells. That might not have got this into *Nature*, but enkephalin was so trendy at the time. Anything with enkephalin in the title was going into *Nature*. So although, actually, we were just as interested in morphine as in enkephalin, we did obviously … we did

experiments with both morphine and enkephalin, and *Nature* would hoover up papers at this time, not just on enkephalin but, in fact, on any peptides.

Opiate tolerance and dependence

Since we had now discovered an action of opiates - whether enkephalin or morphine - on single neurons, the naïve experiment to do next is to put them on for a very long time and see whether they become tolerant to the action of the morphine. That is, does the effect of the opiate to open the potassium channels, does that pass away over a period of time? That would be tolerance. Even more dramatically, if we were to add the antagonist suddenly to displace the morphine or simply just withdraw the morphine quickly, would we have signs of physical dependence? Because, you know, that if you take a human addict who is on morphine and then suddenly withdraw it, then he goes into cold turkey. He has all kinds of physical signs of withdrawal - he pours with sweat, his hair stands on end, his blood pressure falls. We were trying to see if we could see similar effects at the level of a single nerve cell. And, in fact, that's what the paper in *Nature* says: 'Tolerance and Dependence at the Level of a Single Nerve Cell.' Well, we could see the effects, for sure, but it was of course simplistic to think that they completely represented the effects that you see in an intact person. But the holy grail with opiate studies at that time, and still today, is to understand the mechanism by which people become tolerant and dependent, that is, when you remove the opiates they have a very strong reaction to the absence of the opiate.

Understanding the action of opiates

I think the main agenda at that time was, really, to convince ourselves that the primary action of opiates, when they acted on their receptors, was to open potassium channels. About that time, 1978, I think, there was a nice paper out of Gerry Fishbach's group which showed that opiates and some other substances like GABA and noradrenaline, had another action on nerve cells, which was to reduce calcium inflow - the closing of calcium channels. And there was quite a bit of controversy going on at that time, until several years later, we actually worked out that opiates do both these things. They act on the same receptor. In some cells their main effect is to open up potassium channels. In other cells, or even in other parts of the same cell, their main effect is to close down calcium channels. Both these can explain the inhibition or release of acetylcholine or noradrenaline. So they can both contribute to the inhibition of transmitter release.

We defined the receptor on the neurons pretty carefully. We'd got a very good understanding of the potassium channels that were opened when this receptor was activated. We did not know how these two connected together, and the real clue to that came from an experiment

that was originally done in Bertal Hille's lab, not on opiates, but on muscarinic receptors, and they showed that a G-protein was involved in transducing the effects of the muscarinic receptor. We immediately tested the hypothesis that a G-protein was an intermediate between the opiate receptor and the potassium channel, and as soon as we did that experiment, we found obvious positive results. So there were now three players in the act: there was the opiate receptor, there was a potassium channel, and there was a G-protein involved in transducing between them.

So, the late seventies and the early eighties were a major advance to start taking slices of brain tissue and just treating them in the same way that we treated the enteric neurons - keep them alive for ten to twelve hours, so that we could record from the cells one by one, and so that we could apply known concentrations of antagonists. This allowed us to characterise the receptors on brain neurons in a way which had never been done before. So we just translated everything we'd been doing in the peripheral cells to pieces of tissue in the brain. The first one was the locus ceruleus, but then we moved to other places: the spinal cord, moved off to more relevant places - places that are more likely to be involved in understanding opiate action. The locus ceruleus has no special role in opiates, but when you go to the dopamine cells in the ventral tegmental area, then they are likely to have a much bigger role in terms of understanding opiate action. I think, by the early eighties, we were in a position, we could take the brain and record from virtually any part of the central nervous system by taking a slice of tissue and keeping it alive in an *in vitro* situation.

Massachusetts Institute of Technology – disbelief at our studies of the spinal cord *in vitro*

So when we were convinced that opiates acted by opening potassium channels, we clearly wanted to move out of the enteric nervous system or even the locus ceruleus, into parts of the brain where there was clear relevance to this. And the first place that we moved was into the spinal cord and the dorsal horn of the spinal cord because these are the nerve cells that receive the incoming pain signals. So, they are the afferent or sensory fibres that get activated when you have a painful stimulus. They terminate - they end, in the dorsal horn of the spinal cord, so we developed ways of taking the spinal cord, making a slice, and recording from it. And we found that the key neurons there, were indeed hyperpolarised by opiates, or by enkephalin. That's one of the *Nature* papers that, I think, was in 1982. In fact, this was the first time that anyone had taken a spinal cord and kept it alive in slices. There's an interesting story around that because I wrote to NIH (National Institutes of Health) for a grant to support this work, and the grant application was disapproved. Not a low score, below the bottom of a low score, and the reason for that was because nobody believed that you

could study the spinal cord *in vitro*. It had to be intact in the animal. So I then … we did the experiments. In terms of preliminary data, we had a paper in *Nature* that we'd submitted. We had a paper in the *Journal of Physiology*. We sent the grant back again, and this time it got a score of 3.4. It was just at the very bottom of the scoring range. Absolutely no hope of getting any money at all. So, there was not a lot of belief around at that time that you could take pieces of the nervous system and keep them alive *in vitro*. Fortunately, we still had money coming from other sources, in fact, particularly from the National Institute for Drug Abuse because it was the drug abuse connection that was really driving us forward, and it was absolutely fundamental to understand the actions of opiates on single nerve cells if we were to understand why people liked to take opiates.

Dopamine cells and drug-seeking behaviour

It was becoming clear that the substantia nigra - the dopamine cells of the substantia nigra, and the ventral tegmental area, were intimately involved in drug-seeking behaviour. There was quite a lot of evidence for that. I mean, you could actually stimulate the axons coming out of the ventral tegmental area and produce the same kind of rewarding behaviour. The nerve cells shown in red in this diagram are the dopamine neurons with their cell bodies in the ventral tegmental area, and projections to higher brain regions. The nucleus accumbens, the striatum, and the prefrontal cortex. The area of the nucleus accumbens is shown in blue. Activity of these neurons was known to be critical for drug-seeking behaviour whether for opiates, cocaine or nicotine.

So, we transferred out techniques from the locus ceruleus into the dopamine regions - the substantia nigra and the ventral tegmental area. As it turned out, there was an interesting twist there, because the dopamine cells, themselves, are not sensitive to opiates. The first thing we did was to distinguish two kinds of cell within this nucleus. There is a dopamine containing cell, and these cells run up and project to the nucleus accumbens. These cells are known to be involved in reward and in drug-seeking behaviour. But there was another kind of cell in the ventral tegmental area that had quite different properties, and these cells are GABA-containing interneurons. The GABA-containing interneurons inhibit the dopamine cells. When we studied opiates we found that opiates had no effect on the dopamine cells, but the opiates open the potassium channels and inhibited the GABA-containing interneurons. If you inhibit an inhibitory cell then you excite the follower cell, and this is why opiates excite the dopamine cells. They get more activity - more activity goes to the nucleus accumbens, and this is the essence of the drug seeking behaviour.

Now, the first one we talked about was the heroin or morphine. That increases activity of the cell. It causes more cell firing and it does so by this indirect action - by acting on an inhibitory neuron. The second is cocaine. The dopamine release is normal but the uptake of the dopamine is blocked, so the dopamine action is greater. And then the third one to consider is nicotine. So, we found that when we recorded from the dopamine content in cells of the VTA, the dopamine cells themselves were sensitive to nicotine, and when you apply nicotine, it excites them. So, because the dopamine cells are directly excited by nicotine they put out more dopamine. So, three fundamentally different cellular mechanisms - they all produce, essentially, the same effect. They all produce more dopamine action in the target area of the nucleus accumbens.

Potential treatment for drug-seeking behaviour

I think what we've learnt over the twenty to thirty years that we were studying the cellular basis of drug abuse is that it's very unlikely that you'll find a cellular solution to it. Nicotine is probably the best target, if you like, because it may be that the receptors on the dopamine cells on which nicotine acts, can been distinguished from other nicotinic receptors everywhere else. So, it could be that you would find a molecule - a drug - which would block only the nicotinic receptors on the dopamine cells because if you block nicotinic receptors anywhere else, you stop breathing, you stop thinking. So it has to be highly selective, and because there are so many different subtypes of nicotinic receptor, that is a possible way to move forward. This would be very useful in terms of treating drug-seeking behaviour for nicotine, which is, essentially, smoking.

I think similar approaches for cocaine and opiates are going to be much harder to come by, because there's no … even blocking opiate receptors, of course, stops people from wishing to take opiates because they don't get any effect from it, and of course that's been known for a long time. Naloxone is one of the best therapies for drug abuse, but the only people that will take naloxone to cure their drug-seeking behaviour are very highly motivated people like physicians. Anyone else will simply stop taking it after a few days or a few weeks, and that's true even for long-acting preparations. It's possible to get naloxone subcutaneously which will act for a month and it will stop you taking … you will get no reward from opiate drugs during that period of time, but when the month is up, people often relapse to drug-seeking behaviour.

Vollum Institute, Portland, Oregon: molecular physiology – classifying nerve cells on the basis of channel and receptor expression

You could go into any part of the nervous system and put in an electrode and start to define the cell, not only by its sets of channels and whether it fires quickly or fires slowly, but also by its receptivity to drugs that you put on, that is to say, which receptors it expresses. And if you're able to do that in a quantitative way - to define the receptors on an individual nerve cell, then we were in the position of saying, 'Not this cell response to acetylcholine, but this cell expresses M2 receptors.' Whereas another cell next to it, or in the next part of the brain, we found might have M3 receptors. The response is the same, but the receptors are different. And so we spread out to track through different parts of the nervous system, to classify cells on the basis of what channels they express and what receptors they express. And it very quickly became clear that there were lots and lots of transmitters out there, but only a few channels.

Convergence and divergence in transmitter action

Convergence is where several transmitters released from different cells can act on the same postsynaptic cell. In this case, from the submucous plexus of the intestine we have Alpha-2 receptors for noradrenaline, delta receptors for opiates, and also receptors for somatostatin. They all led to the opening of the same potassium channel. If you opened them by activating one receptor, you couldn't open them any more by activating the second or the third receptor, so this was the idea of convergence of many different receptors, or transmitter action onto a single channel. I think that was probably the essence of this 'many transmitters, few channels' paper. Of course, we now know from the molecular basis there are actually many different variants of those channels, but I think, fundamentally still, even with our now molecular insight, we know that many different receptors converge onto a single channel. So many different … there are so many different peptides and transmitters, but they finish up doing the same thing at the cellular level.

In divergence, a single transmitter such as acetylcholine, can act on nicotonic or muscarinic receptors on postsynaptic cells and there are many subtypes of each. Shown here are M2A and M2B as they were known in the 1980s. I think that when it became clear that there were many subtypes of muscarinic receptor, all of which did different things to the postsynaptic cell, then you have a situation where a single transmitter – acetylcholine – can diverge to affect an M1 receptor or an M2 receptor, or an M3. In fact, there are now five muscarinic receptors, I mean. What we were finding was not two different muscarinic receptors on the *same* cell, but the same set of presynaptic nerves could release acetylcholine and activate M1 receptors on one cell, and M2 receptors on another cell. When you activate the M1

receptor, the cell is excited. It's depolarised. When you activate an M2 receptor, the cell is inhibited because potassium channels are opened.

So, I think this is really the essence of the divergence idea that a single transmitter can have a multiplicity of effects on postsynaptic cells, and now that's taken for granted. ATP can act on P2X receptors and P2Y receptors. You often see them on the same postsynaptic cell. Acetylcholine: nicotinic and muscarinic. Noradrenaline and dopamine are interesting because there is no fast ligand-gated ion channel that is opened by noradrenaline and dopamine. They have the slow actions, but not the fast action. But then GABA: for a long time we thought that all that GABA did was directly have a fast action to open chloride channels, but now we know, through GABA-B receptors, that it also has a slower action, to open potassium channels. So, the same transmitter, with the various examples that I've given you, can have a fast synaptic action, and one or more slow synaptic actions on the postsynaptic cell. This was really the essence of the divergence.

Visualising nicotinic receptors and potassium channels

I think we're a little bit limited by restricting our attention of postsynaptic signals to opening and closing ion channels and EPSCs and IPSCs. Of course, that's one thing that these transmitters do, and that's the privilege that you have if you're an electrophysiologist. You can measure that fantastic resolution in time, fantastic resolution in space, but do we really think that's the only thing these transmitters are doing? I think many of them are going through G-proteins, they're changing gene expression, they're having much longer-term effects. From a philosophical point of view, electrophysiology went through a fantastic period in the sixties and seventies because its resolution is absolutely superb. The signals are clear and loud. But we also … we used to think that the channels were … the only important part of the channel was the hole down the middle. In fact, we now know that the transmitter through a receptor can go to huge signalling complexes of proteins, which are associated with the channel. You may change expression of the channel in the membrane. You may signal downstream into the nucleus and change gene transcription. It's just that the ones that we were focussing on for so many years were just the hole down the middle of the channel because we could measure the ions that go through it so easily.

And the thing that broke open the field … I suppose there were two things. One was for the nicotinic receptors, and this was Nuwa's work in Japan - of cloning nicotinic receptor sub-units. Somehow, for the first time in 1983-84, people recognised that you could get a feel for what these proteins looked like, and then later, in about 1987-88, Lily Jan isolated a CDNA for a drosophila potassium channel, and for the first time we had an idea what a potassium

channel was like. As soon as we saw Lily Jan's paper, John Adelman and I immediately cloned the mammalian potassium channel because as soon as you've got one bit of sequence, then you can use this as a basis for homology-based cloning approaches.

Lessons in molecular biology

At that time, I had little idea what A, G, T, and C stood for. I mean, my molecular biology was in a gross state of naiveté. But then John Adelman also had no idea was Ohm's Law was, or the relation between current and voltage. So we had quite a number of sessions where we spent time together, and it was very productive because neither of us felt in the least inhibited about asking the most stupid questions. And I was putting on a course of lectures for postdocs and PhD students about quantitative biophysics. You know, some of it was a little bit heavy-duty mathematics, but some of it was relatively simple. John Adelman, even though he was a staff member at that Vollum Institute at that time, he attended all of the lectures and he did all of the homework, and very quickly he knew more about ion channels than most of my postdocs. So, the converse of that was, at the same time I was learning some molecular biology, and learning how to make libraries and particularly learning a lot about the structure and function of proteins.

Therapeutic potential of blocking and unblocking potassium channels

RBK1 - that we got from rat brain - we expressed it in oocytes and it forms beautiful potassium channels. Of course, there was then a huge slew of similar channels coming out afterwards. Now it turned out, that was not the potassium channel that was opened by opiates or noradrenaline or somatostatin. It was just another kind of potassium channel, actually one that's opened by depolarising the cell, but of course, eventually, it led to the identification of the precise molecular kind of potassium channel that is opened when you activate an opiate receptor or a noradrenaline receptor.

But now we were in a position where we could make very small mutations to a single amino acid at what we thought was the mouth of the potassium channel. It didn't affect the potassium channel function at all, but it reduced the blocking effect of tetraethylammonium. So the power of this approach was really enormous. This figure from 1988 shows how we considered the channel to form from four symmetrical sub-units indicated by the blue outlines. In each sub-unit, the polypeptide chain of the protein crosses the membrane six times, shown here as the cylinders. The potassium ions pass through a central pore. Tetraethylammonium had been used for many years to block the pore of potassium channels. We found that one amino acid at the outer end of one cylinder, a tyrosine residue,

was involved in the blocking by tetraethylammonium. All four such tyrosines were needed for full block.

Therapeutic outcomes from the cloning of potassium channels are with us, so there are several drugs available now which selectively block molecular sub-types of potassium channel. Many in development, but the ones that are currently with us are ones which are used for the treatment of epilepsy. There are many other potassium channels which are targets, particularly in the immune system, but not yet drugs on the market.

Glaxo Institute for Molecular Biology, Geneva – two P2X receptors discovered

I didn't think about purinergic signalling at all, really, until some time after going to Glaxo. Well, about the time we moved there. The reasoning was this. I was fully engaged at the Vollum Institute in potassium channels, and structure and function, but Suprenant who was working at the Vollum at that time, had brought with her from Australia this interest in ATP. She worked with Molly Holman doing her PhD, and Molly Holman was the person who did the recordings with Geoff Burnstock in the 1960s. And then, when we moved to Glaxo in 1992 or 1993, the people in Glaxo had been influenced by Pat Humphries' work. And Pat Humphries, who also was a Glaxo employee, had then been working on ATP – actually, mostly on P2Y receptors. But when *we* arrived at Glaxo, we indicated that it would be very important, particularly because of my background in cloning channels - we said it would be very important to clone the receptor through which ATP was acting, which we called the P2X receptor.

We took the vas deferens out of the mice. I remember, Anne Marie Suprenant killed a lot of mice and I did some mice. We took out the vas deferens, pulled the tissue, and then Gary Buell made the RNA and made a cDNA library, and then we injected the cDNA library into oocytes. Much of this work was done by Soledad Valera, who was the first author of the 1994 paper, but I think the creativity behind the library, the creation of the library, and the selection of pools of the library was largely Gary Buell's. And then, of course, as soon as a single cDNA was isolated, which conferred sensitivity to ATP, then it was mostly Suprenant and Evans who took this and expressed it in a range of cells and showed that this really corresponded to the P2X receptor.

We didn't know what Brake was up to until a few weeks before the papers appeared, and I can't remember how we got the intelligence, but we exchanged manuscripts after they'd been … just about as they were in the final review process, so you're never alone in this world! That's not the first time that I've opened a copy of *Nature* and seen my paper and

seen the one of my competitors alongside it. Sometimes you know about it, more often not. It sounds unlikely that, but you know, there are only a small number of really big questions, so it's not surprising that several groups will converge on the big questions, and don't forget also that *Nature* itself is much more likely to accept a paper if it thinks that there are two or three groups working on it because that gives it the imprimatur of importance and significance. So if you look back through issues of *Nature* over the years, you'll find a lot of doubles or triples, where different groups have submitted the work at about the same time. Fortunately, we finished up with two different P2X receptors, so that was a tremendous advance to the field. We'd not identified the same sub-unit. Of course, we called ours P2X1 and they called theirs – they agreed afterwards – to call theirs P2X2. It doesn't really matter which way round it is, but it was by comparing the sequences of the two receptors that we immediately got a huge increase in our understanding about how these molecules worked.

Establishing receptor types on nerve cells

P2X receptors are trimers formed from three sub-units. Each of these sub-units has two transmembrane domains, shown here as TM1 in red, and TM2 in green, which cross the membrane as helices. Each subunit also has a large extracellular domain shown in black here with some of the critical amino acid residues identified. The permeation pathway for ions forms as a pore between the three subunits indicated in blue. There's nothing there that gives us a clue about which part of the molecule is responsible for binding the ATP, and after about ten years of mutagenesis, and changing amino acids here and there, we're not actually a huge amount further on in terms of knowing which bit really binds the ATP. We've made some progress, but it's very slow progress.

We cloned out all the other ones within a year or two, mostly by using homology based approaches, and using polarised chain reaction, using primers based on the sequence of the P2X1 and P2X2, and they finished up picking up the other five members of the family. The first of those was the P2X3, and the two things that surprised us about that were (a), that its distribution was extraordinarily restricted. It was only in a subset of afferent or sensory neurons. And then (b), its properties didn't correspond to the properties of ATP responses in sensory neurons. So when you put on ATP to a P2X3 receptor you get a certain kind of response, a certain kind of inward current, but when you put it onto a P2X2 receptor, you get another kind of inward current. Neither of those corresponded to the current that you get if you take a real nerve cell from the sensory ganglion and put on ATP. So we thought, 'Well, what's going on?' Either there are some more P2X receptors in the nerve cell, or perhaps these two different kinds come together as a heteromer. So what we did was to take the P2X2 and the P2X3 - take the two different cDNAs - express them together and record the

currents in the cells in which you've expressed them. And hey presto! That looks just like the currents that you see in a sensory neuron. So this was the evidence that the real receptor that's made naturally in a neuron probably has got P2X2 and P2X3 sub-units in it.

The receptor that we're looking at, seems to have two copies of 3 and one copy of 2. Its properties though, are fundamentally different from the 2 alone or the 3 alone. Why is that important? Because the target that the drug companies want to screen against should be the target that's really expressed in sensory neurons. So, in order to show the molecular identity of that target, they can now express that, and they can screen and they can identify drugs which block that particular kind of P2X receptor.

So, the 1995 paper on P2X3 receptors, those two papers were published simultaneously. One was the group from UCL lead by John Wood, then also with Burnstock and Calquhon there, and the other, of course, was our paper from Geneva. The difference there is that I think their paper was actually superior in terms of the localisation of the receptor because frankly they knew … John Wood, in particular, he knows much more about sensory ganglion than we did. But our paper had the additional feature that when you co-expressed the P2X2 and the P2X3, then you get a phenotype or a current, which resembles that in sensory neurons. So they both brought something slightly different.

P2X receptors as a pain target

The organisation in Geneva was set up at that time, essentially, to deliver drug targets. So, when they bought BioGen, originally that was to buy the molecular biology technology. When I arrived there, as I've indicated, it was already full of very good molecular biology technologists. Very good people. So the Institute had sort of transformed itself into a target discovery institute.

So, take pain, for example. They were looking for target molecules in pain. There's a P2X receptor. Now, as it happens, there are ten or a dozen pharmaceutical companies now - as we speak in 2008 - working on P2X receptors as a pain target. But if you go back to 1995, we couldn't get Glaxo to pay any attention to P2X as a pain target because they already had adenosine A1, they had somatostatin, they had Alpha2, they had GammaB, they had Mguar receptors. There were many other potential pain targets with groups working on them, mostly in Stevenage, and so they would obviously favour their own when it came to prioritising them to go into high throughput screening, and so on. So, this is one of the challenges that companies of that type face when they allow small operations to work in the distance. It's really very, very hard to coordinate the activity.

So our original thinking around ATP receptors expressed on sensory nerves, was that when tissue is damaged, ATP is released and it activates P2X receptors on sensory nerves. P2X2/3 receptors would be good candidates because they are the ones that are there. And because the P2X2/3 receptors are activated, the end of the nerve is depolarised, an action potential is set up, and that carries centrally the pain sensation. That's the simple theory behind it, and it works. I mean, if you knock out the P2X3 receptor or if you use an antagonist to block it, then that will prevent pain or nociceptive responses in quite a large number of animals models. So, that clearly works. Of course, being a Glaxo employee I would be the last person to know about what was going on at Abbott or at Roche. I got to know much more about that as soon as I left Glaxo. I think Roche had got a programme from very early on, particularly on P2X3, and Abbott have had a programme which continues, I mean, for the last ten years, on P2X receptors. There are a number of other companies now in the game. They were mostly on P2X3.

I was aware that there was … that Roche was supporting some of the work at UCL for a number of years, and I had rather little to do with Roche. I mean, I have consulted to them over the last three or four years. Certainly, their compounds are very effective in terms of selective blockers, there's no doubt about it, but I don't know what the situation is at the moment with them taking them forwards.

P2X7 and the release of pro-inflammatory cytokines

The P2X7 receptor was pretty unusual. In fact, it was actually a little bit more difficult to clone and we finished up stitching together two halves of the receptor. I think one came from superior cervical ganglion cell library and other one came from the brain, but the most striking things about the P2X7 receptor were, first of all, their structure. Because, unlike all the others it had got a much longer cytoplasmic C terminus, so it had two hundred amino acids more. That's quite unusual compared with the other ones. Clearly, one of the first unusual properties is that when you activate them, the pore down the middle of the receptor, which allows the ions to pass through, progressively gets bigger and bigger. Now, we eventually found a similar property in the P2X2 and P2X4, but it's much more striking for P2X7, and then, of course, the other property, which was already known in the sense that when you activate ATP receptors in a macrophage you can drive interleukin release. That was now known to result from activation of P2X7 receptors. So that tied the P2X7 receptor directly to the interleukin release rather than any of the other P2X receptors.

When you activate P2X7 receptors you obviously set in train a whole host of rearrangements of the cytoskeleton. One of these rearrangements that you see is blebbing, where very large bulging out of the plasma membrane occurs. What that means in normal circumstances for macrophage in tissue, we don't know. Don't forget that these macrophages are isolated. They are free to bleb. It may be if that macrophage was in a tissue, then what we are looking at is an uncoordinated movement of the macrophage through the tissue because you probably know macrophages are very motile cells. So, I think what we're seeing in our isolated environment is probably not what goes on with a resident macrophage, which is in the tissue and surrounded by other cells. But it does certainly indicate that we've got major cytoskeletal rearrangements going on.

Now, the other thing that happens is like blebbing but in a much, much smaller space domain, and that is tiny particles of the membrane – we call them micro vesicles – become shed off the cell. Now, this is an actual loss of membrane, so small particles of membrane, about half a micron in diameter, are budded off and disappear into the solution around the cell. These particles contain interleukin. They contain proinflammatory cytokines, because you can catch the particles and then break them open and show that the cytokines are in there. So this was a novel method of secretion. There's been a lot of controversy about how cytokines are secreted from cells, and it looks now as though they're secreted by this process of micro vesicle shedding, which a number of other people have subsequently shown as well.

P2X2 antagonists, rheumatoid and osteo arthritis

I think the P2X7 research as a whole is going very heavily in the direction of drug discovery. So, there are several companies now that are developing P2X7 antagonists for use in inflammation, and there is one clinical trial result published of a small Phase 2 clinical trial, reported by Astra Zeneca last year at the rheumatology meetings, which shows very positive benefit of P2X7 antagonists in the group of rheumatoid arthritis sufferers. So, I think rheumatoid is one area where it's going. I think osteo arthritis is another area where it's going, and I think neuropathic pain is also ready to be exploited with P2X7 antagonists. And this is not an action on the nerve cells. This is an action on the inflammatory cells on the microglia, which are involved in reorganising nerve pathways during chronic pain.

Exploring P2X4 receptors in the central nervous system

I'm not still involved with the pharmaceutical context of it. So my involvement – I'm actually not doing very much on P2X7 receptors now anyway, because that's mostly being done by the Suprenant lab and some other labs. Most of my current efforts are on P2X2, P2X3, P2X4

sub-units in terms of, first of all, understanding how they function as a receptor. So, we're getting into the minute details of probing the permeation pathway - the pore down the middle - and to do this we're using single channel recordings and measuring the currents through individual channels as they open and close. And then the second area that we're still working hard on is to understand that what P2X4 receptors, in particular, are doing in the central nervous system, particularly in the hippocampus, but also in some other parts of the nervous system. And as I've indicated before, I don't think that's as simple as *direct* synaptic transmission. I think it's something much more subtle that we're teasing out by a whole series of experimental approaches, but mostly comparing wild type mice with mice lacking the P2X4 receptor, and looking for the systematic differences between them.

Astrocytes and microglia are P2X receptors

I mean, the other big area which is now developing in CNS work is astrocytes, which are P2X receptors, and microglia, which are P2X receptors. So, as we speak, there are people in my laboratory recording from astrocytes and also recording from microglia in slices of brain tissue, and that's been made possible because it's now … you can now breed mice in which only the microglia – that's, the macrophages of the brain - only those cells have got a green fluorescent protein in them, so that when you look at the slice of tissue down the microscope, only the microglia are green. You can see them with a fluorescent microscope and you can record from them. When you go back to the days of the locus ceruleus, there's no way that you could have ever seen a microglia in the brain slice, but now it's possible to pick them out.

The role of ATP receptors

One of the difficulties of the central nervous system with ATP-mediated synaptic transmission is that it is pathetically small in magnitude. So, when Francis Edwards first described it in the 1992 paper in the medial hevenula, this is a great advance. But in order to see the ATP component you have to block all the glutamate component, and the ATP component is usually typically less than five per cent of amplitude of the glutamate component. And the same has been shown to be true virtually everywhere else in the central nervous system where ATP is proposed to be a direct synaptic transmitter. So, in strictly electrical terms, it's very hard to know what this tiny current is doing. So you can show it. You can block all the glutamate component, you can block the NMDA receptors, you can block the AMPA and KNA receptors, and there is a bit left which is mediated by ATP, but it's very hard to get excited about it. And that's a clear distinction to transmission from a nerve to the vas deferens or a nerve to the blood vessel, where the electric current is huge and it clearly is the substance that is driving the post-synaptic response.

So, I think that the ATP receptors at the synapse probably have a slightly different role from just allowing electric current to flow. And, of course, the role that we're all testing at the moment is that their high calcium permeability may allow calcium ions to go in through the activated P2X receptor, and if that calcium is delivered inside the postsynaptic cell at exactly the right position, then it will have downstream effects on signalling through other mediators such as glutamate. It's similar in a way to NMDA receptors. NMDA receptors allow calcium to go through. As a result of that, glutamate receptor signalling is enhanced, and this is what gives rise to long term potentiation or LTP. It may well be that ATP acting through P2X receptors, signals by virtue of the calcium going in, whereas our previous thinking had always been it signals by virtue of the big current and the depolarisation and the EPSP. That now seems to be unlikely.

PX2 receptors and 'reverse physiology'

Well, it's certainly true to say that I've focussed my efforts completely on it for the last ten years or so. It's showing no signs of slowing down. The number of citations continues to go up strongly every year. So, as a field, the P2X receptor field is starting to bring in some other significant players from outside, and that's good because it needs it. I mean, there are still really only ten or a dozen groups around the world that are seriously focussing their efforts exclusively on P2X receptors. We need a few more. We need some new thinking and technology. I don't think recording currents in cells is going to give us the answers. Obviously, we need clever people who can measure calcium in very, very small domains of the cells, and we need also, now that we've got the knockout mice, to move into much more behavioural contexts - what these things do to animal behaviour. And, of course, we need further tools - pharmacological tools. Can you imagine a hundred years ago, studying nicotinic receptors without curare? You see, and that's what we … and that's the situation with P2X4 receptors. We don't have a curare – we don't have anything with which to block it. You would never have known about muscarinic receptors without atropine. So, we need these tools and it's good that the industry is starting to deliver these tools. I think … I think, when we have them the field will propel further.

The corollary of this, which is a little bit sad, in a way, is that I'm actually involved in reverse physiology. That is to say, in the old days you had a physiological question and you drove down to the cellular basis. It might be drug abuse, it might be pain. You drive down to the cellular basis, and then the molecular basis to understand that physiological question or even that disease. Therapeutic question. In the case of P2X receptors, we've still got a family of proteins widely expressed in the body and in the brain for which we really don't fully understand the functional role. So, it's reverse physiology. We've cloned the proteins. We

know a lot about the proteins, but as you go further and further up towards the cellular and the systems and the behavioural level, we know less and less and less. It's a bit unsatisfactory still to be left at this reverse physiology stage at which we've not yet focussed our attention exclusively on one particular function or disease in which the P2X receptors are involved. There is a lot happening and beginning to take off, and I've mentioned some of them, but I don't think yet we've got the really big … the big question to go after.