Third ACTTION Scientific Workshop
Transformative Strategies-Development of Pain Therapies

June 23, 2014

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THIRD ACTTION SCIENTIFIC WORKSHOP

TRANSFORMATIVE STRATEGIES FOR THE DEVELOPMENT OF PAIN THERAPIES

Monday, June 23, 2014
8:30 a.m. to 5:06 p.m.

FDA White Oak Campus
White Oak Conference Center
Building 31, The Great Room
Silver Spring, Maryland

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P R O C E E D I N G S

(8:30 a.m.)

DR. DWORKIN: All right. It's 8:30. I'm Robert Dworkin from the University of Rochester, and I'd like to welcome you all to this ACTTION scientific workshop.

ACTTION, for those of you who don't know, stands for analgesic, anesthetic and addiction clinical trial translations, innovations, opportunities and networks. It's a public-private partnership with the FDA, and this is our third public scientific workshop. And this one, in distinction to the previous two, is focusing on transformative strategies for the development of new pain therapies.

The objectives of this next two days are on some of the materials you've, I think, received by mail, to discuss progress, prospects and opportunities for genetic, epigenetic, and pharmacogenomic research on pain, review ongoing studies of stem cells, gene therapy and toxins in the treatment of pain. And then tomorrow afternoon,

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examine recent translations of preclinical findings to successful clinical demonstrations of analgesic efficacy.

The steering committee for this meeting are those four individuals, and what I tried to do in the font is indicate the contributions made by those four individuals.

Dr. Basbaum is objecting to the font size. He would like his font size to be larger than Dr. Porreca's, but I think we will leave it to the two of them to settle that at the coffee break.

But as you can see, the important point that the slide makes is that both Dr. Basbaum and Dr. Porreca, who I think many of you know very well, have really done the lion's share of the work for this workshop. And Dennis and I contributed a miniscule amount. Actually, the fonts for our name are probably too large.

The other person I'd like to acknowledge is Dr. Allison Lin here at the FDA, and Allison has really been critically important in arranging the kind of hospitality of the FDA for this meeting.

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And, of course, we couldn't do any of the ACTTION and IMPACT meetings without the help of Valorie and Andrea, who are responsible for all of the logistical arrangements. So thank you, Allison. Thank you, Valorie. Thank you, Andrea.

You should all know that the FDA has required that we tape this meeting, and that's occurring. And both a transcript and video recording will be made available on the ACTTION website. It probably takes three to four weeks for that to happen, and it will be a kind of verbatim transcript and also a video recording of the meeting. Both will become available.

Then most importantly, the agenda for this meeting is really kind of dense and packed with presentations. And we also want to make sure there's enough time for a discussion. So I'd like to beseech the presenters to please, please keep to whatever amount of time is allotted on the program for your talk.

Then if you want to learn anything more about ACTTION, there's a tremendous amount of information

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on the website. We update this every month or two, and the address is ACTTION with two Ts, dot org.

Then finally, before I turn it over, some housekeeping. Because we were a little early this morning, we decided to make the buses a little bit later tomorrow morning. So the buses from the hotel will leave at 7:30 rather than 7:15. There will still be ample time, of course, for breakfast. There will be buses or a bus back to the hotel at 5:00 this afternoon from here, and there will be breakfast again tomorrow as there was today.

We are required to say that the federal government policy prohibits those individuals who work for the government from partaking in the continental breakfast that the rest of you were able to have this morning. I don't know why we're required to say that, but I have discharged my duties to the U.S. government by just saying that bullet.

Then finally, of course, please silence your cell phones.

So it's a great pleasure to introduce Dr. Allan Basbaum, who is going to be starting off.

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He's actually co-chairing the first session of the meeting with Dr. Bill Maixner.

Dr. Basbaum, of course, is a professor at the University of California San Francisco. Dr. Maixner's a professor at the University of North Carolina. And they will be co-chairing -- and neither of them need any introduction at all to this audience. And they will be co-chairing our first session this morning.

Allan.

DR. BASBAUM: Thank you, Bob, and welcome, everyone. We should put this meeting in a little more perspective. There's been so much in the news lately, and you have to be living on another planet if you haven't read it, about the concerns about the translation from preclinical work, whether it's in particularly cancer or pain, into the clinic, whether it's issues of animal models, reproducibility, statistics, power calculations, you name it.

It was becoming a rather intense discussion, and several of us got together and said, well, maybe we need to have a slightly different perspective and

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really look to the future instead of keep constantly looking to the past. And by that, we ended up with this meeting, which the word "transformative" is somewhat overused. But that's really what we're looking for, is where does the future lie. What are some of the new approaches, techniques that might be relevant, that hopefully will be relevant, to the development of useful pharmacotherapies -- I shouldn't even say pharmacotherapies -- for chronic pain?

That's really the rationale for this meeting. It's very fundamental in nature, but hopefully, we'll get into discussions of how this might be translated in the hopefully not too distant future.

So I want to thank everyone for coming. We do encourage discussion. That's what makes these meetings special, and hopefully, we can do that. And it's my pleasure to introduce Steve McMahon, who's not only a good friend but a terrific colleague.

We both trained with Pat Wall many years ago. I'm a little older than Steve, so I was there a little bit before him. He is now Sherrington

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professor -- which is such a wonderful thing to be; I would love to be a Sherrington professor -- of physiology at Kings College. And he's also -- and this is relevant. He's an academic leader of a public/private partnership, which is somewhat analogous to ACTTION, called Europain. And in many respects, their objectives are comparable to those to which ACTTION adheres.

His interests are diverse, whether it's from mechanisms of neurotrophin action. But most recently, his interest turned to epigenetics, and you'll hear more about that. And the title of his talk is the Epigenetics of Pain.

Welcome. Lamarck lives.

(Applause.)

Presentation – Steve McMahon

DR. MCMAHON: Maybe I should start by saying that Pat would hate being at this meeting, I suspect. Genetics, that was not his idea of the way pain research should be.

So I was asked to give a list of potential conflicts of interest, which I do here. I would

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point out that only one of these interactions has got anything to do with epigenetics, and that's a collaboration with Pfizer, who are doing some ChIP-seq analysis with us. I don't know what you might take from that. It says to me one thing, that the pharmaceutical industry to date have not invested a lot in epigenetics in this area.

So that's the title of my talk. Another disclosure is that I'm not an epigeneticist. I'm not a geneticist. I'm a neuroscientist, and I want to give you a perspective on epigenetics from a neuroscientist point of view, and obviously try and draw out some of the relevance to our understanding of painful conditions.

But it's worth saying in the audience we've got a couple of proper card-carrying geneticists who are very good to provide a different gloss in some of the discussion.

So what I thought I would do is to try and structure my talk like so. I'm guessing that many of you don't have a genetics background, and therefore, it's worth spending maybe a little bit of time just

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saying what this epigenetics thing is and how it works. Then I'll say something about what it's meant to do, and then try and get to the main part of my talk of what's the relevance of all this stuff to pain.

I'll finish with a little bit of speculation really or discussion about the opportunities to use this knowledge arising in this field of epigenetics to develop strategies to treat pain. And I'll also try and say a little bit about some of the challenges that might be associated with that.

So let me start with a definition. What is epigenetics? So you can read this while I'm talking. I stole this from a website put out by a new group created last year, the NIH Roadmap Epigenomics program. And you can see what they said. This definition comes in two parts, but the first one is the kind of formal definition.

Epigenetics is an emerging frontier. I mean, the word was coined more than a hundred years ago, but it's still true that it's an emerging frontier in the sense that our understanding is rapidly

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progressing. And the essence of epigenetics is that it's a series of processes that can regulate gene activity without changing DNA sequence. And that sounds like gobbledygook, and I'll come back to it in a second.

The second caveat, I think is interesting and important. So it says for this program, it relates to processes that change gene activity and expression that may be heritable. So, of course, that's in there because lots of the early definitions of epigenetics had as an obligatory nature this idea of heritability. And a lot of debate recently as to whether epigenetics can be anything that's not heritable.

But it turns out that the epigenetic processes that I'll describe to you are at play in postmitotic cells, where the idea of heritability is not really relevant, but this idea is very important; that there may be some cellular memory, some long-term change in cellular function generated by these epigenetic processes. And that's beginning to sound like something that as pain neurologists, we're

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very interested in, long-term stable changes that may be associated with abnormal function.

So that's a formal definition. Schematically, perhaps I can illustrate the point more easily on this slide. So genetics is really how gene function may be modulated by changing DNA sequence, and we'll hear a little bit about that today. But I'm sure that's an idea that many of us are familiar with.

Epigenetics in contrast is a series of ways of altering gene function without changing sequence. And what this slide illustrates is that there are several pretty well established ways in which you can do this, which I show here.

Perhaps the easiest to understand is that DNA, the sequence may be the same, but simply by adding new cell groups to specific sites in DNA, one is able to strongly regulate the expression of genes around those sites. So DNA methylation is a repressive and a very stable form of intervention. And as I've told you for a long time, it's been seen as a kind of permanently stable, although there's a

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lot of evidence emerging now that this DNA methylation status is in some cases reasonably plastic.

Other ways of changing gene expression relate to modifying not DNA itself but the histones with which it's associated in nucleosomes. So many of you will remember from the distant past that DNA is wrapped around octamers of histone proteins, and these stretches of DNA, the functional actions of that DNA are regulated by the status of these histone proteins.

That status can be reflected -- can be changed in a number of ways, which I've told you about. One is that the histones can be post-translationally modified in quite a diverse number of ways. And that changes the way in which those histones interact with DNA, and that in turns changes how the genes, how the genetic material locally, is responsive.

Another mechanism is that in some cells under some times, they may actually synthesize new variants, transcriptionally distinct forms of

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histone, and those histones can be substituted into the nucleosome. And those new histones will have a different kind of interaction with the DNA and again, regulate gene expression by that mechanism.

So it's already starting to sound a little bit complicated. So I'm just going to give you one more layer of complexity about the broad chemistry of how these interactions occur, and then I'll talk about function.

So DNA methylation first, if one imagines a typical gene will have some transcriptional start sites, and around that start site, there is a promoter, which is typically very rich in these dinucleotides, these so-called CpG islands. And that's important because most methylation of DNA occurs at the CpG islands.

The mechanism by which it occurs is understood, and that is that a series of enzymes, methyltransferases, DNA methyltransferases, are able to add methyl groups in this 5 position to cytosines resulting in methylated DNA.

What's been much, I think, more controversial

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until recently is whether or not we have an active series of enzymes, which are able to demethylate methylated DNA. And I think the current evidence is very clear that this is an active process, and we have some good candidates, probably more to be found out about the players that are able to demethylate genes. But obviously, it's an important -- since this methylation mark, it leads to a lot of transcriptional repression, being able to manipulate it is quite an important feature.

The histone modifications, unfortunately, are extremely complicated. And again, the point here is not to kind of convey all the details but to give you the flavor of how this system works. So in this diagram, the boring bit is this gray line. That's the DNA that's wrapped around this octamer of histones. And you can see that these histone proteins have N-terminal tails, which can be post-translationally modified at particular residues in a number of ways.

So for instance, we may acetylate or methylate or phosphorylate, and a variety of other

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changes as well, particular residues. And it's actually a bit more complicated than that. So this residue can be acetylated or not acetylated. It can be monomethylated, dimethylated, trimethylated or unmethylated. And there are many, many sites at which these marks can be made.

In fact, combinatorially, I think there are many billions of potential combinations of all of these histone marks. Of course, it's unlikely that they're all realizable independently, but nonetheless, the point to make is that these histones associated with DNA and able to affect DNA function are remarkably diverse.

If you're looking for a complex way in which you might manipulate DNA function, well, this is it. And that's both a blessing and a curse really. I'd say it's a blessing, but this is a system that has a great power to include many, many different types of information, to memorize lots of information. But it's a curse in the sense that it's obviously rather difficult to unpick what's important.

Now, this is a table that I don't think

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you'll be able to read, but I want to just make a point with it. The issue is, well, what's responsible for making all these epigenetic changes. And in this field, there are several players worth considering.

So on the one hand, we know there are a whole number of enzymes that are able to add these marks that I've been talking about. They're able to add methyl groups to DNA, acetyl groups of histone residues, et cetera.

There are marks that are able to read, to interpret the changes that have taken place in DNA, because of these marks and to execute some effect on gene expression because of that. And then finally, there are a series of proteins that are able to erase some of these marks.

Again, I know you won't be able to see all the details, but it's worth just noting a couple of things. One is that DNA methylation, there aren't many things that methylate DNA that we know of. And as I've told you, at the moment, it looks as if there are some active demethylases, but we're not

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completely confident we understand what they are.

But this actually looks like quite a tractable system, and you're going to hear some more today about DNA methylation. I'll give a couple of examples as well, but there are a finite number of players which gives us some hope that we might be able to understand what they're doing.

With the other, with the histone modifiers, quite a large number of players are involved. I'll point out just one because I'll be saying something about it. And that's this group of enzymes which are together known as HATs, histone acetyltransferases. These are the things that add acetyl groups to histones, and there's conversely a series of HDACs, deacetylases, that are present in many cells.

I'm emphasizing those two classes of families since many of the current pharmacological strategies that we have -- in fact, quite a lot of the data existing to epigenetic change relates to the function of these HATs and HDACs, and I'll come back to that.

But the point here is that there really are lots and lots of players, and so the good news is all

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of these are pharmacological targets. They're all potentially targets that one could manipulate in order to change these epigenetic processes. And you're going to hear later this morning from Chas Bountra who's going to talk about his efforts to make clean chemicals that will offer a clean set of tools that can be used to target some of these players.

So all of this, this complicated set of ideas, give rise to the concept that there's going to be some kind of code, there's going to be a code of epigenetic marking that has a predictable and simple effect on gene function. And from what I've already told you, you'll recognize that that code clearly is going to be very complicated.

But on the other hand, I think it looks to me at least as if a fair bit of progress has been made in trying to understand some of the principles of the code. And obviously, through this complexity, if we're going to manipulate the system, we really would need some confidence in there being a code that we can apply in different circumstances.

So here's one example, I think, of how these

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codes might play out. And what I'm illustrating here is, again, a typical gene with its transcriptional start site, associated promoters, and at some distance some associated enhancer regions, all of which are important in regulating gene function.

From what I've already told you, it's true that if these promoters are heavily methylated, then that's associated with a very repressive environment and a failure of transcription, so a repressive state.

But there are several other states you can imagine these cells being -- that this gene being in. One would be that you lose some of these repressive marks, and you perhaps get some transcription factor activation, some hypermethylation of the DNA itself. And that leaves the cell. It's still not active. The gene is not active, but it's primed. It could be activated under certain circumstances. And this kind of primed inactive state is often associated with this, this monomethylation of this lysine in histone through.

Obviously, there's an active state in which

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transcription is on, and in many genes, that's very strongly associated -- perhaps one of the best correlates is with this mark, this acetylation of this lysine K27 in H3 along with trimethylation of K4 around the transcription site. So there's a little bit of a code here.

But actually, it's also true that there are many intermediate states between primed and active in which the cells are said to be poised. They may be activated by an appropriate series of circumstances. And there may be many different circumstances, many different contingencies which would be specifically be able to active a poised gene. And these poised genes also carry a series of characteristic marks typically.

So I think you get some idea that there may be some simple but some rather reproducible patterns of histone marking that can be -- epigenetic marking that can be used to drive particular patterns of transcription. And those patterns of transcription are not just on and off. They're on, off, and maybe if something else is appropriate.

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So what's it for? So you've got all of these complicated markings. What does it actually do to cellular function? What are the consequences of having this code?

I think again from my perspective, I see sort of three broad areas, which are quite well developed, of more or less interest to us as pain scientists. So one area is in cancer biology. Most tumors show fantastically dramatic alterations in their epigenetic controls. The reasons that tumors are tumorigenic is because of a failure of these epigenetic processes.

So the epigenetic control of the cell cycle is a dramatic example of epigenetics in action of what can go wrong. And whilst that's not of great interest to us as pain biologists, it certainly is true that the knowledge that we have, a great deal of it has derived from study of cancer biology because that's where these processes have been marked out. And I suppose there is a cautionary tale there that if we're going to mess around with epigenetic marks or epigenetic processes in order to treat pain

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patients, for instance, one ought to be careful about the impact on cell cycle.

On the other hand because, as I say, this is a reasonably advanced field, our understanding has come from cancer biology, it's also true that there are a number of drugs in the clinic which are used for the treatment of cancers, which themselves interfere with epigenetic processes. And that's actually quite interesting for us because it means that we have compounds that will be available, potentially available, to test ideas in other systems. So that's one big chunk of epigenetics.

A second chunk is that epigenetics are essential for normal development. As I think I've already alluded to, for a long time epigenetics was seen -- even when the mechanisms were not understood, it was seen as conceptually those processes that led to the orderly development of an organism, that the orderly orchestration of cellular events would take you from an embryo to a differentiated organism.

That includes a variety of processes. Perhaps the most obvious is in lineage determination.

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So these fibroblasts, these Purkinje cells, start off with identical sequence information, genetic information, and yet because of epigenetic processing, they end up expressing different patterns of genes and genes that have completely different forms and functions.

So obviously a lot of work has derived from developmental studies, and again, some of our understanding about epigenetic processes have emerged from this field.

The relevance of these studies to pain may be not so obvious. There are a couple of intersections between these developmental processes and our interest in pain. Perhaps the most obvious is in pediatric pain states. So one of the things that -- I'm not going to talk about today, but it's at least of interest to some people -- is whether or not pain in development in infancy changes the developmental process and leaves the pain signaling system in a distinct state.

That's entirely feasible because I just told you that the orchestration of development requires

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the careful integration of epigenetic processes. And so the impact of painful events on those processes is largely unknown.

But the third category of -- a good example of what epigenetics are good for is listed here. And that is that there is a growing body of evidence that epigenetics is a way in which -- it's many cells when they are activated or stressed or challenged in a number of ways change their function. And that's certainly a concept that's very familiar to pain neurobiologists.

It turns out that that alteration in function is mediated or driven by epigenetic processes in many cases. And so I show one example here that this is using kainic acid. You could actually use a whole number of transmitters and show that hippocampal cells will quite rapidly, within 15 or 30 minutes of exposure to these cellular stimuli, phosphorylate a particular residue. This epigenetic mark is applied. This searing residue is phosphorylated in hippocampus.

So there's acute response, this kind of

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mechanism of plasticity of cells to environmental or cellular challenge. That plasticity is dependent upon epigenetic regulations in many cases, and that's interesting for us.

The other side, though, which I think should be very interesting to us as well, is that these epigenetic processes may provide a kind of cellular memory, a memory of what's happened to those cells in the past. And clearly, a long-term -- so these memories may be very specific for particular types of cells, particular type of stimuli, and obviously for particular genes. That is a very powerful memory mechanism that may have a long-term impact on the functioning of those cells.

There's quite a bit of evidence at least theoretically, this can occur. So here's some data from some twins studies. These are identical twins who were studied either when they were very young or a different cohort who were much older. And the experiment is simply to ask whether or not the methylation status of their genome is similar or different.

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So their methylation status was determined. And for 3-year-old identical twins, perhaps it's not surprising that they have very similar methylation marks across their whole genome. But for the older twins, it turns out that they have increasingly divergent methylation patterns across their genomes and that the longer they've lived apart, the more extreme these patterns are.

Now obviously, we don't know what function of these methylation marks are, but the principle that your cells carry a lifelong history of what's happened to you, I think is enshrined in this simple finding so that these epigenetic marks may determine how you're going to respond to events in the future.

But obviously, from a pain perspective, the idea that an individual may have an epigenetic regulation of how, of their propensity, to develop pain states is an interesting idea that I think we'll talk about some more. It's an interesting idea for us as a community.

So what about epigenetics? What does it actually do in action? So I picked a couple of

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current -- they're not spurious, but an almost random selection of a couple of papers to give you a flavor of the way in which epigenetics is seen to have an impact on nervous system function. And I'll just tell you a couple of very short stories.

So here's quite a nice paper that says, well, how is that we reconsolidate memories, distant memories. And obviously, that's some relevance to treatment of patients with traumatic fear memories. And it turns out that a particular HDAC, one of these deacetylases, is critical for this process of memory reconsolidation. There's quite a bit of literature out there looking at how different epigenetic processes contribute to memory formation and reconsolidation in this case.

Here's another example. There was a nice study a couple of years ago that was looking at the effects of cocaine addiction in an animal model and very nicely demonstrates this idea that one form of life experiences may predispose you to a different form of response to something else later.

So the context here is that repeated -- if

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you make rodents addicted to cocaine and then stress them, it turns out that their responses to stress are very much dependent on whether or not they were prior cocaine addicted.

It's the combination of the two events that are -- the intersection that are very apparent here. And as this paper says, that histone methylation is essential to leave this mark from cocaine addiction, which will then subsequently affect the individual animal's ability to cope with stress.

So the non-addicted animals cope much better to stress than the addicted animals. That's a sort of trivial finding on one level, but a nice epigenetic demonstration of how the memory may operate in practice.

My final example, which is a bit closer to home for many of us, and that's the quite basic processes such as nerve regeneration. So peripheral nerve regeneration, as many of you will know, is an orchestrated event, a coordinated event, of some axon growth program that gets switched on.

Well, this paper makes a nice case that one

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particular epigenetic enzyme, this HDAC5, is important in orchestrating the axonal regeneration program. It's actually important in two ways, which is why I mentioned it. One is that following nerve injury, there is an export of HDAC5 from the nucleus, and that leaves the nucleus at a different transcriptional state because this HDAC5 has moved into the cytoplasm. And that's the nuclear epigenetic part of it.

But it turns out that the HDAC5, I mean, many of these enzymes are able to act on not just histones but on cytoplasmic proteins. And this paper provides some nice evidence that HDAC5 was able to deaceytlate some proteins in this cytoplasm and contribute to axonal growth that way.

So I guess that's just one word of caution that many of the players that we're talking about, yes, we're implying that they have a role in DNA regulation, but they may also have other cytoplasmic roles that we should be careful to unravel.

So there's a reasonable literature that the nervous system, the adult mature nervous system, is

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using these epigenetic processes in a variety of distinct fashions. And so that begs the question of what does this mean from the perspective of pain. And I think there are two topics I'd like to briefly discuss.

Firstly, this one, I think, is always the easiest one, and we have a lot of data on it. I'll show you a little bit of it. And that is that following injury or in some disease states, we're very familiar with the idea that there are pathophysiological consequences that affect many levels of the neuro axons. And those pathophysiological consequences are associated with altered gene expression in many cases.

So what I want to tell you is what do we know about the role of particular epigenetic processes in controlling the cellular responses that arise in the face of injuries and presumably contribute to the hypersensitivity associated with those injuries. So that's one topic.

The other one, I can't show you a lot of data because there isn't a lot of data, but I definitely

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think this is a very intriguing possibility. And it's really the opposite. It's not why do some people get pain after injury. It's really why do some people not get pain; why do most people not get pain after similar injuries.

One answer to that question is that the people who get pain happen to have some vulnerable brain, whereas the people who don't get pain have some resilient brain. And the issue is, are there epigenetic processes, perhaps early in life experiences, that predispose us to being vulnerable or resilient? And as I say, I think it's a very intriguing idea, but one for which, as I told you, we don't have a fantastic amount of data as yet.

But what about this first thing, what's the evidence that these epigenetic things are any good in coordinating responses to injury? So let me start with here's a little bit of data from a post doc of mine, Ana, who did a next generation sequencing study on DRG neurons after a peripheral nerve injury, frequently used as a persistent pain model, spinal nerve transaction.

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What she found was that you get an awful lot of transcriptional disregulation in DRG. There's nearly 4,000 genes out of 80,000 assessed, which is significantly disregulated, either up or down regulated. And that really does beg the question what's controlling this altered gene expression, and there are many things that might control it.

One that I think has been explored quite a lot is this family that I talked about of HDACs and HATs. So this process, we have a bunch of tools -- they're not fantastic tools. But we have a bunch of tools to interfere with this process. And it's probably the single process that's been most extensively investigated in the pain field.

So the idea is that we have a series of enzymes that add acetyl groups to histone residues, and the idea is that that leads to a slightly more relaxed state of chromatin, which will favor transcription. On the other hand, we have a series of enzymes, which deacetylate these histones, leave the chromatin in a more condensed state, and transcriptionally less active in general.

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It's a nice system, and I want to talk about it a bit because we do have a whole bunch of slow molecule inhibitors that are known to inhibit HDACs and thereby prevent this deacetylation to lead, we think, to a more acetylated state and perhaps a more transcriptionally active state.

So here are the little list. We have in this class of HDACs already quite a number of distinct HDACs falling into several subgroups, and we have a number of molecules that are able to target these different HDACs with varying degrees of specificity. And I think it's worth saying that I don't think any of these compounds have ever been formally assessed for their non-HDAC activity, but they almost certainly are not clean specific compounds. But they're what we have. And as it happens, some of these have reached the clinic in the treatment of pain cells.

So we have a number of tools and is one way in which we can ask as pain neuroscientists whether this family of enzymes is important in its ability to affect pain states.

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So we've done a kind of pharmacological experiment in which we've taken groups of animals and subjected them to several different types of neuropathic lesion in several forms, and we've used several different HDAC inhibitors at a couple doses and applied them all intrathecally to see whether or not inhibiting HDACs has any impact on these neuropathic pain behaviors.

The way the experiment is done is that the animals are assessed behaviorally -- their withdrawal -- a measure of withdrawal. It's normalized just to put all the experiments together. We do some baselining. We implant pumps and start delivering HDAC inhibitors, and we give those for a week.

One point is that these inhibitors, they may not be super specific, but they definitely are not horribly toxic. They don't dramatically change the behavior of the animals given intrathecally over this one week.

When the animals then received peripheral nerve injury, this neuropathic lesion, the

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intrathecal-treated animals developed quite a marked hypersensitivity. And one of the things -- I mean, it's not a massive effect, but maybe 40 percent of the hypersensitivity that normally develops is abolished in the presence of these HDAC inhibitors.

We're very, very confident about this result because we've done it so many times. We've done blind, and essentially, it's very, very robust. So I really do believe that whatever the mechanism is, it's a real thing.

We did another experiment with Andrew Rice's group. We developed this model, and it's published now, of d4T-induced neuropathy, a chemical neuropathy rather than a traumatic nerve injury one. And again, pretty much an identical paradigm and an identical result. Again, this was a really blind experiment. The two labs were not in any contact at all.

So again, I think a very nice demonstration that neuropathic conditions may be ameliorated by interfering with HDACs.

We know that the HDACs are doing what they're supposed to do, so in the spinal cord, if we look at

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the level of acetylation of particular residues, we see, as expected from an HDAC inhibitor, a hyper-acetylated state at those residues after treatment with both of these compounds.

Why it produces an analgesia is less clear. In the same experiments, actually, we've done a full transcriptional profile to ask whether or not the HDACs are producing pain relief because they're disturbing the transcriptional profile that would normally occur in the spinal cord. It's a bit of a noisy experiment to do because the transcriptional change is quite small in the spinal cord. But there are many genes that are disregulated in neuropathic conditions when you give HDACs. And if you do a kind of pathway analysis to see what's upstream from all of these genes that are significantly disregulated, you find that there are a couple of apparent upstream events.

One is that HDAC1 is upstream to a large number of genes and highly significantly enriched in this analysis, suggesting that some of the action is to HDAC1. And that's reassuring. The HDACs are

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supposed to inhibit -- inhibitors are supposed to inhibit these things, and this will be the signature that arises.

Again, you can look for signatures of protein, protein interactions, that are disturbed by particular pharmacological agents. This one is one of the HDAC inhibitors that we used, and it too, the signature of disregulated genes fits the known signature of this HDAC inhibitor. So it obviously doesn't tell us why this intervention is analgesic, but it gives us some confidence that we are manipulating in the spinal cord here, particular HDACs that are changing transcriptional profiles that are relevant to the emergence of both pain related but also pain-related behavior.

So that's one example. There's another example I wanted to give from the literature, which I think is a rather nice one and sort of shows maybe the good news of some of these epigenetic processes. So this is quite an old story, but relates to this transcription factor known as REST. And it's a repressor element actually of neuronal genes. It's

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also known as NRSF.
It's an interesting factor that switches off lots of neuronal genes in non-neuronal tissues. So lots of non-neuronal tissues have high levels of REST, and that keeps off a lot of the neuronal genes. And in nervous tissue, there's usually low levels of REST, which allows the transcriptions of these neuronal-specific genes.
What are the neuronal-specific genes? They're genes that have a response element, this RE 1 that REST binds to. And when it binds, it brings to it a whole series of other factors, including HDACs, which lead to this transcriptional inhibition.
What are the genes? Well, again, I know you're not going to read this properly, but there are hundreds of genes that have these RE 1 response elements in it. So it's an attractive idea that there's one transcription factor may be shaping the whole of the neuronal profiling zones.
If you look here actually, these are many of the things that we're very familiar with in the pain field. That's these TRP channels, sodium channels,

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potassium channels, calcium channels. All these channels have REST response elements.
So here's a study from Yoshida & Shoup of a couple of years ago. It's an antisense study, and that may have some problems. But they show a couple of nice things. One is that REST is up regulated in sensory nerves, these DRG neurons, after one of these peripheral traumatic neuropathic pain lesions.
So the REST is up regulated transcriptionally, and for what it's worth, acetylation at H4 around its promoter is also included. They have an antibody that shows the induction of REST, and as far as you can tell, it look as if it becomes induced in nearly all of the cells that are damaged.
What they show on the epigenetic front is that there is a transcriptional effect on a few target genes. And many of the experiments that are done at the moment, you're going to hear some more this morning, have focused on epigenetic controls of particular sort of candidate target genes.
So they picked on a couple of genes here that

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have RE 1 response elements in them. They're all genes that are transcriptionally down regulated in this model with nerve injury. And in this final bar here, they show the antisense to REST reinstates the expression of those transcripts. And they provided a little bit of functional data that the reinstatement of this mu opiate receptor, the MOP here, was associated with the recovery of functional responsiveness to opiates.
So a little bit of data that one element can control many genes, and some of these genes then have a functional impact on the pain response in these animals.
I will give you one other example because I think this is the way a lot of the field is moving, and that's to use a sort of transgenic approach to try and target individual epigenetic players. And we've picked on one, which this HDAC4, and we picked on it for the reasons shown here. It's richly expressed in neurons. There's a little bit of weak human data that it may have something to do with pain, and then some genetic data from a global

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knockout.
So we've done what many people have done, and that's to say, well, maybe we can use pathway analysis to pick on targets and then try to target that in a mouse using mouse genetics.
So one of the things that came out of our next generation sequencing analysis of the transcriptional change in DRG neurons after these neuropathic lesions are several pathways of genes that are disregulated by modal events. And two pathways in particular were very strongly statistically represented. One was a pathway driven by HDAC1, and one was a pathway driven by HDAC4. So again, it makes it quite a logical case. In neuropathic injuries, transcriptional changes do seem to be associated with this HDAC4 pathway.
So to cut a long story short, we've now made two lines of transgenic animals which target HDAC4 in central neurons. One of them is a line in which we use an Advillin-Cre, and in fact, a tamoxifen inducible Advillin-Cre to target essentially all of the central neurons on the DRG and probably some

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sympathetic neurons as well.
The other in a line that the pain field has used quite a lot, and that's to use a Cre that's expressed under NAV 1.8, a sodium channel in most nociceptors, not all, but most nociceptors and unfortunately, some non-nociceptic neurons to try and ablate HDAC4 just from that nociceptor -- from that primary afferent population.
You can see from all of these bar graphs, there are no effects on the basal responses of these animals, but the responses of these animals -- in fact, at neuropathic pain states. But the responses of these animals to inflammatory stimuli are partially attenuated, I mean, significantly attenuated, in both of these transgenic lines. That's to say the blue line is the hypersensitivity we see following CFA in the wild type or littermates. In the proper knockouts, it's attenuated.
Again, we've got a sort of bit of a mechanism going on those mice. We tried -- it looked as if, when we did some transcriptional profiling in this models, it turns out that many of the genes that are

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transcriptionally controlled by NGF seem to be less well controlled in these knockouts. So for those studies, we use a kind of a pain card with about 50 favorite genes. They're all pain genes. Everything looks pain related, but that's because everything on the card is pain related.
But we do see a disregulation -- you can see it in these heat maps here -- of a whole number of transcripts. And this is in the NAV 1.8 Cre given NGF treatment directly. And this is in the Advillin-Cre subjected to CFA.
In both cases, a plausible explanation for the failure of the pain response, the pain abnormality, is that these cells are less able to respond to inflammatory mediators like NGF in the absence of HDAC4.
On the histone variants, there's a little bit of data, and you're going to hear more this morning on deficits associated with DNA methylation. There's one paper here from a couple of years ago that used a chemical inhibitor of DNA methyltransferases, this chemical, 5-azacytidine.

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It's a bit surprising because the way this inhibitor works, it's only supposed to block methylation in dividing cells. So it's not completely clear why it works, but the claim is that these are control animals, these are neuropathic animals, these are animals treated with this methylase inhibitor, and they show a partial recovery of their neuropathic pain behavior.
That would be consistent with the idea that the methylation that takes place following an injury is contributing to the pain behavior. And there's a little bit of evidence to that extent. They looked at global methylation in spinal cord. It goes up a bit with nerve injury, and that's blocked by this inhibitor.
Some of the factors, this MeCP2 is a factor that's associated with methylated DNA. That goes out with nerve injury, and that's also blocked. So again, methylation is likely to play a role in some of these changes.
I should say on the methylation, I think we have some very good tools available now. Here's some

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images from the Allen Brain Atlas. I told you there were three Dnmts, 1, 3A and 3B, that is supposed to regulate methylation. This one is supposed to maintain -- is responsible for the maintenance of DNA methylation, these two for the more kind of plastic, inducible forms of methylation.
You can see these are obviously developing animals, but at P4, the DRG is very rich in Dmnt1 and 3A, and there's almost no 3B. That's consistent with our own sequencing data showing rosy values here. In these neuropathic lesions, this methylase does go up, and that's associated with the increased methylation that's produced.
As I say, I think we've got good tools to study this process now. So you're going to hear later from Chas Bountra, whose group have made very specific DNA methylase inhibitors, Dnmt inhibitors. And here's just some data we've got using their compound. Single intrathecal dose leads to a fairly long-lasting alteration in methylation.
There are also transgenic animals coming along. There's a floxed Dnmt3A/3B animal. There's

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no 3B in the DRG, so if we knock out this construct in DRGs, we can selectively identify the role of Dnmt3A in DRGs. And we've got these animals that are live. Their baselines look normal, so we're very interested to see how this manipulation will impact on their pain responses.

So that's quite a lot of data, and I haven't really been encyclopedic. And I've sort of rather selfishly picked on things that I've been involved with for the most part. And you're going to hear more this morning about other examples of the importance of some of these markers. But the key point is that we have a reasonable case that they are playing a role.

But the last point I really wanted to address scientifically is this one, and that's whether or not these epigenetic processes not just mediate the responses to injury but sort of anticipate them. They're a form of memory that decides how an organism will respond to an injurious condition at some later stage.

It's a great idea, but we don't have a lot of

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data in the pain field that this is actually happening. There's one bit of suggestive evidence that we're pursuing now, and so we've used this intervention of maternal separation. You get some mice or rats, and you separate the pups from their mum for a couple of hours a day over a period of time immediately postnatally. And then they're weaned and allowed to grow up normally.

They do seem to carry the mark of this stressful early event. So one of the things we've done with this group in France, particularly Fabien Marchand, is to ask whether their sensitivity to visceral stimuli is altered later in life as grown-up mice as a consequence of this early maternal separation.

So Fabien has this very nice behavioral system where they can instrument the animals, put a small balloon in their colorectum and distend it and measure abdominally AMG. And they show that the animals that have been separated have a sort of hyper responsiveness to visceral distension.

That's an attractive model for quite a number

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of clinical conditions -- visual sensitivity, irritable bowel, interstitial cystitis -- which have been suggested to be associated with early stressors in people. And so we're very interested in trying to pursue whether or not there are any epigenetic disregulations in this model that may explain this adult behavior, but we don't have it yet.

The only good example we have is one -- a plug really for a talk that is coming later today from Jordana Bell who looked at -- I'm not going to talk about the data, but she looked at the methylation status of identical twins who had different behavioral responses to painful stimuli.

So as far as I know -- I mean, it doesn't prove that this is an action in lots of chronic pain patients, but it's the best data we have that some epigenetic change arising in people has a long-term impact on their pain sensibility. And she'll tell you that story, which I think is conceptually of considerable importance to us to find out whether or not this is an important determinant of long-term pain vulnerability.

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So I've talked quite a lot, and I wanted to just wrap up with a couple of points, which I'll illustrate here. So I think this bit is going very well, trying to ask how -- I mean, in a way, we know that if you've got a lot of gene expression in a nucleus after some insult, that has to involve some epigenetic players. That's what's driving the older transcription.

Understanding what they are, of course, is the important thing, and understanding whether there are particular signatures that may be pain relevant, that's, I guess, the challenge that we have at the moment.

As I've just said, I think this idea that long-term pain vulnerability is established through an epigenetic mechanism I think is one that is really fascinating and one that we ought to pursue whatever the outcome.

One issue, though, is, oh, that's the good news. This is great. But of course, what do you study? Where do you look? And one of the problems is that, essentially, we have one genome, but we have

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hundreds of epigenomes.

So here's a nice paper that appeared last year that simply took a whole bunch of different cell types, and you can see what they are. Some of them were -- this is a tumor cell line. These are primary cells. These are sperm. And they did DNA methylation across the whole genome and then see how these cells clustered.

The important point is a principal component analysis. They're two-class, but they're all very distinct. So the epigenomes of every cell type is likely to be different.

Well, that's a problem because how are we going to -- and where are we going to look? The problem now is which cell are we going to pick now as being pain relevant in our system.

As it happens, just as an aside, a bit of a plug, one of the things we spent the last year doing is trying to play with FACS sorting and magnet sorting to see if we can get pure populations of cells so that we can do some of these epigenetic studies.

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So one example is illustrated here. If you just disassociate DRG neurons and put them in a dish, it turns out that only about 25 percent of the cells are neurons. The rest are satellite cells and other cells. You can purify those with magnetic sorting and get a 99 percent pure DRG population. We can do the same thing with spinal microglia. We can do the same thing with transgenically marked cells that carry fluorescent tanks.

So I do think these approaches will give us an extra lever to try and identify epigenetic processes in a cell-specific way.

So I think from what I've been saying that all of these different mechanisms do look attractive from pain biologists. Of course, these mechanisms are at play in many, many cells and for many different purposes. So as usual, we're going to have a real problem perhaps in identifying those processes that may be the most specific.

On the other hand, I know industry's been very keen to look at biomarkers. You really would think that simple epigenetic evaluations, looking for

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particular marks of particular residues in particular cells, that may be a very powerful way of identifying biomarkers for how people are responding to particular disease states or how they're going to respond to a future disease state.

I have seen that this -- I mean, I thought I'd finish with -- I saw this in the latest edition of Nature Neuroscience. I see what's become very trendy has been to try and combine some of these genome-wide analyses with functional imaging.

So here's a review paper, this is done not with epigenetics but with genetics, looking at efforts to try and combine GWAS studies with functional brain imaging either to ask whether there are particular variants that are associated with particular forms of brain activation pattern or associated with particular structural changes in the brain.

I think that this is an immensely complicated procedure, but there's a real opportunity to do exactly the same with epigenetics and say can we have epigenetic marks that predict differences in

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behavior.

I'd like to finish by just thanking the people that have done the work. This is a group at Guy's Hospital in London. Most of the epigenetic experimental data I've shown was generated by Fran and Maggie. So thank you very much.

(Applause.)

DR. BASBAUM: We have time for some questions.

DR. MCMAHON: It's you he's talking to.

DR. BASBAUM: Can you actually reverse, or would you predict that you can reverse an epigenetic change or if you can prevent --

DR. MCMAHON: So there's a little bit of data there. The Wang study I showed -- yes, and that's whether or not these things, whether you can prevent them, which I think a lot of the interventions have tried to do and done successfully, or whether you can allow a change to become established and then try to reverse it by blocking some epigenetic process.

So that's been done a few times. The Wang study actually produced a nerve injury, allowed some

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transcriptional change to get going for three days, and then gave the methylase inhibitor. So they were able to reverse a partially established state.

We did the same thing with the HDAC inhibitors intrathecally, and we couldn't. So once we established a pain state, then the HDAC inhibitors did nothing in our hands or at least the ones we used.

Actually, it's another reason -- although that's -- it obviously has a bit of an impact for therapy. It also convinced us that these drugs were not just having nonspecific actions on the pain signaling system because the same drug and the same dose was ineffective in those conditions.

DR. GLORIOSO: So I thought it was really a wonderful talk. If you can separate different nerve cell populations for DRG in which there's chronic pain going on, have you tried to look at individual subpopulations to see if particular ones have genetic changes or epigenetic changes and others do not?

DR. MCMAHON: That's the idea. So we struggled a bit to get pure subpopulations. I don't

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think we're alone in this. I think you'll see a whole spate of publications coming out using these techniques.

So yes, you clearly can. You can identify subpopulations, and then you can genotype those. You can epigenotype them. There's a sensitivity issue, but that's -- I think maybe Jordana might say something about the technical difficulties of doing some of these experiments, but theoretically, yes.

DR. GLORIOSO: Well, it raises the possibility that you can deliver a gene like an HDAC directly to the correct subpopulation and perhaps change that population in particular.

DR. MCMAHON: That would be nice. I mean, you're right, targeting agents to particular cells, there's a little bit of precedence for that. But that's non-trivial as well, of course. But yes, theoretically.

DR. MAIXNER: Bill Maixner, UNC. Stephen, is there any emerging knowledge about the type of environmental exposures that will lead to the different regulatory pathways for epigenetics? Are

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there specific environmental factors that may lead to alterations of methylation versus a histone modification?

DR. MCMAHON: Yes, I mean, it's certainly not been done comprehensively. The one manipulation that everyone agrees has a profound effect on brain development in a number of ways is early stress. So stress in different forms is definitely a major dictator. Whether or not -- I mean, it's interesting.

The hypothesis is that that may predispose people to some forms of disorder like fibromyalgia or irritable bowel. That's really to be formally established. But if that was true, then it would imply there was some specificity in the system. But that hasn't been demonstrated to date.

DR. FARRAR: John Farrar, University of Pennsylvania. I'm going to demonstrate my naiveté when it comes to this whole topic area, but it's an amazing system. The question, I guess, is what controls this system and why would certain kinds of -- following up on Bill's question, why would

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certain types of environmental deprivation or other things lead to certain processes being turned on or turned off?

DR. MCMAHON: But they do. I mean, that much we know.

DR. FARRAR: Right.

DR. MCMAHON: So the range of things that we're interested in that can do it are any neurotransmitter, nerve injury, BDNF. I mean, there's lots of the players we're familiar with.

The cocaine story, the suggestion there is that an increased transmission via BDNF being released and acting on TrkB is the trigger for the epigenetic changes.

So many of the common things that we're very familiar with lead through second messenger systems to the recruitment of these epigenetic processes. I didn't really talk much about that, but yes, it's not magic actually, yes.

DR. FARRAR: But I agree. The question is that I would assume that the different stimuli you just spoke about led to slightly different epigenetic

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changes. Is there an underlying process by which certain ones lead to second ones?

I think you're suggesting that there's connections, that there's transmitters that change them in specific ways. But I'm just wondering if there's any work that's looked at, why it is that cocaine exposure leads to a certain set of epigenetic changes.

DR. MCMAHON: So there is -- although I think specificity is going to be an issue, there is quite a lot of specificity in the system. So I showed you one little bit of from the Allen Brain Atlas that one of the DNA methyltransferases is just not expressed in DRG neurons. And we know that some of the other players are tissue specific in their expression.

So we do have a bit of something rolling for us to start with. There's some specificity in the system that we might be able to capitalize on.

But otherwise, no. It's going to be like all of the other things we've been studying. What is the unique combination of events in this patient or under this model at this condition that trigger the change?

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DR. PORRECA: Frank Porreca, University of Arizona. Wonderful lecture, Mac, as always.

I just had really two questions. The first is the -- I wonder if you could comment on the temporal events that might be seen in some of the experimental models. I mean, if you looked at these profiles at a certain time point, is it what you would expect to see many months later when things may be changing? There is some evidence that there are changes that occur quite late that may have potentially stronger correlates to the human condition. That's one question.

The second thing is, any attempts to separate the injury from the so-called pain that might occur. I mean, is that separable? Can you actually identify and link these profiles that you've been looking at in the spinal cord, for example, or in the DRG, to an expression of a pain state, or is it just the consequence of an injury that doesn't necessarily link to the pain?

DR. MCMAHON: So two good questions. The first one, I think Jordana's got some data on

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stability of these changes over time, so maybe she can address that.

I mean, although one of the attractions of this system -- in the pain field for a long time, we've been looking at mobilization of second messages, sensitization over minutes. I mean, these processes are distinct, that they leave a mark that is stable. I mean, it's around for a long period of time, days, weeks, months, potentially years.

So they definitely have a more inherent long-term course of action. How stable they are, there's been a few studies, not so many in pain.

Your other question was?

DR. PORRECA: [Inaudible - off microphone.]

DR. MCMAHON: Oh, to pain. Yes, I know, so I did sort of breeze over a bit. Yes, you're absolutely right. In nearly all of these studies, we don't have causality. We have two lots of phenomena, and we'd really like to demonstrate what's causing what.

I mean, it's possible, but that hasn't really been done to such an -- and I should say, I focus on

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DRG and spinal cord because I know the biology there, but you'll hear later that there -- these epigenetic processes are at play up and down the neuro axis in pain states as well. So it's not just in peripheral tissues.

DR. BASBAUM: Steve, thanks very, very much.

(Applause.)

DR. BASBAUM: Thank you. That was a great start, and it's my pleasure to introduce Laura Stone, who is another good friend for many years.

Laura did her original work at the University of Minnesota, went to OHSU, and then went into industry. And there's a theme here that we're hearing, but then came back to academia and is now on the faculty in the dental school at McGill University.

Laura's original background was largely in pharmacology, spinal cord primary afferents, and then more recently in the last few years, has been working in a really provocative model, which I think is one of the most intractable problems both in the animal and clinical model, namely, back pain. And today,

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she'll tell us about her work on SPARC and back pain. Welcome, Laura.

Presentation – Laura Stone

DR. STONE: So to start with, thank you for inviting me. I don't have any disclosures, but a lot of people I want to thank and a lot of funding agencies to thank listed here. And I want to make a comment about the funding from Pfizer was part of a -- Pfizer Canada has these neuropathic pain research awards, so it's a peer-reviewed open competition. It's not a contract or in any way -- they don't have any say in what we actually do. They just fund it.

So everybody knows that back pain is probably the most common chronic pain condition and one of the hardest to treat. And so much research has been done in chronic back pain, and we've pretty much gotten to this point where we think it might be something about the brain and there might be some stuff happening in the spine. This is the current status of this field.

So we have to start somewhere. Maybe make a little bit more progress than this. The vast

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majority of cases of chronic back pain, we cannot find the cause. It's unknown. It might be something in the spine or in a ligament or in the muscle, but we can't find it.

There are some cases, though, where the back pain seems to be linked to problems in the spine, disk herniations, disk degeneration. So this is at least something that we can study. It certainly doesn't represent all back pain patients, and some of the correlations between back pain and the spinal pathologies are weak. But we can see this. We can measure it. We can induce it. So it's something that we can at least begin to study.

There are many ways that disk degeneration could end up producing pain, and this is just a schematic attempting to outline a couple of these. So here's a healthy normal disk. Here's a degenerating disk, and it's narrower. And then we have some bulging in herniated disks.

A lot of mechanisms are going on during this. So we have local inflammation in and around the area of the injured disk. We have nerve compression, but

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also, there could be compression of DRG neurons or spinal cord. And the compression actually also happens here when there's just degeneration. It does not require herniation.

The inside contents of these disks are just loaded with pro-nociceptive and pro-inflammatory compounds. If you take the inside of a disk and drop it on a nerve, you will induce neuropathic pain symptoms for several weeks in animal models, so just the inside of these disks is really nasty. So if they start to leak or if they herniate and nerves come in contact with these inner contents, that's a very probable source of pain.

Finally, there's now evidence that as disks degenerate, they're normally not really innervated because we don't want nerve fibers to normally be in contact with all these nasty things inside. But as they degenerate, the nerve fibers start to grow in deeper pathologically. So now you have nerve fibers that are inside the disks that are experiencing a chemical environment and mechanical stresses activating nociceptors where they should not be

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there. So normal movement, normal physiology now, might activate nociceptors and cause pain.

This is an example from a disk taken from a back pain patient just showing an example of innervation. This was found quite deep in the disk, and you can see there's a blood vessel. And then this is PGP staining for nerve fibers that were growing deep into the disk along that blood vessel. Sometimes we also see free nerves deep inside disks, but they're not supposed to be there.

The question is how are we going to study the relationships between back pain and disk degeneration and innervation without an animal model because we have some problems dealing with human models. Like, you can't always get the disks. We can't have control disks. Back pain is very complicated. There's all of the emotional and cognitive factors in patients. So we really need an animal model to start to kind of look at the physiology of this.

So we started just looking in the literature to see if there's anything out there, any animal models of disk injury or disk degeneration that we

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could start with. And there was one thing that stood out in the literature, which was there was one report where someone had shown this protein called SPARC, which is -- it's a horrible name, right, secreted protein, acidic, rich in cysteine. It's the worst protein name ever.

It's an extracellular matrix protein, and it had been shown in literature in just one report that as SPARC mice aged, they have disk degeneration and herniation. The SPARC known mice that have this were not developed to study pain or disk degeneration. They were developed by a group that actually studies extracellular matrix and connective tissue; that somebody just had a hypothesis and looked. And sure enough, there was a really big phenotype there. So we contacted Helen Sage, who developed the mice, and she was kind of enough to give us some founders.

So this is basically what we see in terms of degeneration in these animals. What you see here is now a wild-type mouse, a healthy, 6-month-old mouse, and these are the vertebral bones, and these are the disks. And in a healthy disk, we have this nice kind

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of catseye, blue catseye in the middle, surrounded by this sort of orange thing. And this is just some multi-chromatic histological stain that picks up different proteoglycans.

In the SPARC null mice at this age, we start to see some disks degenerated. So this one, you can see there's a clear loss of disk height, and we lose the nice catseye structure in the middle.

By the time the SPARC mice are over a year of age, we start to see now multiple degenerating disks and occasional herniations. So we have a model here of what appears to be a chronic progressive disk degeneration like what we see in humans.

We've also looked at the disks to see if they're innervated as they become more degenerated, and this is an example. So the disk is here, and this is just a herniation that's blown up. And these fibers here are PGP stained nerve fibers.

So this is just an example from a SPARC mouse and the herniation that is innervated. So the contact of these nerve fibers with the contents of this herniation is going to generate pain. It's

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going to activate those fibers, I should say.

So do the mice show any kinds of signs of back pain? But before I answer that question, I just want to draw a distinction between back pain really being two different things. We're talking about axial pain, which is pain in the lower back region, and radiating pain, sometimes called sciatica or radicular pain, which is the pain that might go down one or both legs. And it's thought that these mechanisms are separate, and our model supports that, although I won't get into that today.

So how do you measure axial back pain in mice? When we started this, there was no -- we had to try a bunch of things. And one of the things that we tried that has been very successful was this really old test called the tail suspension assay, and maybe some of you who've been engaged in pharmacology for a long time know this test.

This was developed in the '60s as a test for depression. And what happens is you take the animal upside down for a few minutes. And you watch their behaviors. And the animals have a few choices. They

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could try to rear up and, like, grab the tail or grab the tape. They could try to reach for the floor, or they could kind of give up and go into this posture that's called immobility, where they just kind of hang there.

In the depression literature, the more time you spend in immobility, it's a learned helplessness model, and it's a suggestion you're depressed, basically. And this can be reversed with antidepressants.

So when we tried the tail suspension assay, we thought that the last thing that a mouse that has back pain want to do is an upside down stomach crunch. But as often happens in science, we got exactly the opposite of what we predicted. And what we found instead was that the amount of time that the SPARC null mice spent in immobility where they're just experiencing gravity-inducted stretching along the spine is much reduced. And they struggle more, and occasionally, they vocalize. When they're very old, they vocalize during this test, which mice never do normally during this test.

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So we think that what we're seeing is actually a resistance to having this kind of stretch along the spine.

Measuring radiating pain in one or both of the hind paws is much easier. We have many evoke tests for that. So this is an example of cold sensitivity using the acetone test where null wild-type mice don't respond much to the acetone test, but the SPARC mice have an elevated responsiveness.

This is probably the most consistent phenotype that we see. And it's interesting. It gets worse with age, and at some point, the wild-type mice catch up. And if you're a really old wild-type mouse, you also have cold hypersensitivity. And there's a joke in there about Florida and Canadians.

(Laughter.)

DR. STONE: Having this sort of chronic progress model is going to allow us to do a lot of different types of studies than we would normally do in induced models, where it's sort of an all or nothing thing. And what I'm going to just show you today is one example from an exercise study because

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it really highlights the value of having this sort of progressive model. But there are many other things that we could talk about offline.

So in this experiment, this is the intervention. It's a little plastic wheel. It costs $3. We put it in the home cage, and that's the experiment. Mice love it. They run on it all the time. The control group is the little plastic wheel with a screw placed in it so that it doesn't spin anymore. So they can still play in their hut, and they can still sniff it and chew on it and do what mice do. But it doesn't spin, so they don't get that exercise component.

So what we found is the following. So now I'm just showing the acetone behavior. So this is a radiating pain test, and in wild-type animals, we just had some cages of wild types as controls. And you can see at the baseline, all of the SPARC mice have got elevated cold sensitivity that we talked about.

This dotted line is the SPARC control where their wheels didn't spin. And here's what happens in

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the SPARC mice that had spinning wheels. It takes one to two months, but the cold allodynia completely reversed.

The first question that anyone has when you look at exercise related analgesia is, is this an acute opioid release effect. So we reversed it by just taking away the spinning wheels. In fact, we just put a screw in them so they didn't spin anymore in some of the groups.

What you see here is the ones that continued to have spinning wheels, they kept down there, but it took a little while, actually a couple months, of not exercising before the phenotype completely reversed.

We weren't really sure how far to go out here, so we tested it at one month after the wheels were taken away, and there was still the therapeutic benefit. And we didn't want to publish a paper that says that you only have to exercise like for a couple weeks and then have it last forever. So we kept testing them and testing them, and it did finally reverse.

The amazing thing here -- and I'll get to the

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epigenetic part in a minute -- was that it changed the disk pathology. So looking at the disks afterwards, we can see looking at wild-type control versus the SPARC control, there's a loss of disk height normally -- that's part of the phenotype -- that was partially reversed by the exercise.

In addition, there is a recovery of pathological innervation. So this difference is pathological innervation in SPARC compared to wild-type. This is what happens in the exercise. So we've actually, just by a behavioral modification, reversed some of the pathology that's potentially associated with back pain.

SPARC is a model -- we think it's a model of accelerated aging. But we wanted to kind of get a little bit more how this would relate to normal aging, and the first thing that we did to address this is to ask the question is what happens to SPARC in normal animals as they age because the mice get old enough, they start to have disk degeneration. They start to have signs of pain.

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This is a SPARC mRNA expression, and this is how -- this should be months. This is not weeks. These are our 15-month mice. With time, expression of SPARC goes down as we age. So essentially as we all get older, we're becoming SPARC null mice.

To quantify that, we're able to measure disk height by X-ray. And I just really like this figure because you can see here by histology and herniation. And look at the loss of height in the space here, whereas here's a nice, healthy disk, and there's a nice space in there. So we can measure that space by X-ray and get an index of the degree of degeneration of that disk.

So in SPARC mice, we know there's a loss of disk height, and now also in aging mice, we see at the three ages, and this is also months, that there is a progressive loss of disk height in normal aging. And this is what we see in humans.

We also looked in innervation, and we see a increase in innervation with aging. And that's been quantified. And what's interesting here is -- so the SPARC is the red ones. Eventually, the wild types

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here as they get old enough, they have hyper innervation as well. It just takes longer. So the SPARC really is just accelerating this process.

Finally, the behavior. So this is the SPARC data that I showed you before, and we have very similar phenotype for both the axial pain test and the radiating pain test. Here it's cold. And an interesting thing is that in the SPARC mice, we never see mechanical hypersensitivity. It's only cold. And that's exactly what happened in the aging mice as well. So we have no idea why that is, why we would get cold and not mechanical, but it's really intriguing.

Anyway, so what is the mechanism controlling SPARC expression is the next question. And at the time that we were doing this work, it was already known from the cancer literature that epigenetic modulation of SPARC was important in a lot of different disease conditions, in cancers and a couple of other things, too. And the methylation in particular was very well understood.

So this is a slide from Denk & McMahon

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basically to remind you about epigenetic mechanisms. And I'm going to focus here on DNA methylation specifically as the mechanism. And the way that -- I know we already covered this in the last talk, but just to kind of reiterate, that what happens -- so these are the chromatin, and this is where the transcription factor normally binds.

If you have methyl groups here, your transcription factor can't bind, or you get methyl groups there and a bunch of other stuff binds that causes that chromatin to inactivate. But either way, the net effect is that instead of the gene being made, the gene is not made. So more methylation means less gene expression, as is the general rule.

This is what the mouse SPARC promoter looks like, which is kind of boring. I realize that, but I just wanted to point out that there's multiple sites. This is that CpG island that Dr. McMahon talked about. So there's multiple sites here where we can ask the question is the methylation here changed in aging mouse disks.

We hypothesize that there would be increased

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methylation because we have a decrease in the RNA. So that's basically what we got. So in this graph, each of these triplets is a different methylation site. And the Y axis is the percent methylation at that site, and then within each triplet, we have three different ages.

It's not at all sites, but multiple sites in the CpG island had increased methylation as the mice aged, which is consistent with the reduced expression that we see.

We were curious if we could use a drug to target this and see if that could rescue the reduced SPARC expression. So in this case, we used the drug 5-HDAC, where that was also, fortunately, introduced in the previous talk. So now these are all old mice. They're 1-year-old mice in this case.

In the drug-treated, in several of the sites, the drug did cause some demethylation. So it did that, change RNA expression. And it had a huge effect on RNA expression.

So this is now SPARC expression in the disk. This is untreated, and this is treated. So we can

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rescue this -- by decreasing methylation. We can increase the amount of SPARC. And whether this is going to be a usable drug target or not, do we go after SPARC as the target? Do we go after the epigenetic control of the methylation of SPARC? That, I think we'll talk about a lot over the next two days.

One thing that was interesting, we did not get any significant recovering behavior, but we only tested a few days after the one shot of the drug. So it makes you wonder how long. Maybe we have to give the drug longer. Maybe SPARC expression needs to be elevated for a long period of time before we can see any kind of behavior impact.

But it was interesting that the cold allodynia did actually have a strong correlation with the amount of methylation of the SPARC promoter in these disks. So there's potentially some kind of relationship.

So everything that I've talked about so far has been in mice. And we wanted to know if any of these changes in the SPARC gene could also be seen in

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samples taken from human back pain patients. So we have a cohort of samples from low back pain patients with disk degeneration. They were recruited from a spine clinic before spine surgeries. So they were all thought to have discogenic pain that was serious enough to warrant surgery. And there's a cohort of pain-free controls.

All this is showing you here is that in the back pain patients, there was an increase in pain. There was an increase in the disability measured by a questionnaire, and there was an increase in the amount of disk degeneration. The change in disk degeneration was not that severe compared to pain-free individuals, which is not surprising because one of the biggest mysteries in back pain is why -- as we all age, our disks degenerate to different degrees. But we're all degenerating.

Why do only some people develop disk-degeneration-related pain and others do not? And if we can figure that out, that would be a very important thing to understand.

So anyway, we have disks -- so then these

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people go for surgery, and we go to the OR, and we collect the disks that are removed as part of the surgery. And we tested the methylation of the human promoter now in these human disks from patients. And in this case, I should point out that the control disks are not from the healthy pain-free volunteers, but they're from transplant donors.

But regardless, there was a huge increase in methylation at multiple sites in the CpG island in the genome promoter. So we think that this mechanism that we worked at in mice, where the SPARC gene causes -- is down-regulated with age, it's an epigenetic -- it's regulated through an epigenetic mechanism involving DNA methylation. And it might be contributing to disk degeneration and then back pain.

Should I end now, or can I take a couple more minutes?

DR. BASBAUM: Couple more minutes.

DR. STONE: This is a very short story. I just wanted to end because everything that we've talked about so far is a candidate gene approach. It's one gene and how it's regulated by

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demethylation. And it's not in the CNS. It's in the spine. It's in the actual disk.

So the pain genes that we go after -- we even want to use the term "pain genes" -- might not be in the CNS. They might be the genes that cause the pathology in the periphery that then causes activation of sensory neurons, input into the CNS, and eventually central sensitization.

But we also know from all this extensive literature on back pain that the brain might be involved. So just a few notes on the brain. I believe that Catherine Bushnell is probably get into this much more detailed later, but there's a lot of evidence that the brain changes in chronic pain.

This region, the dorsolateral prefrontal cortex, has this tendency to show up in a lot of the brain imaging studies in humans in all different kinds of chronic pain. It also has been shown in multiple studies now to -- some of the pain-related changes could be reversed if you can make the pain reduced.

So we have this sort of plasticity in this

area, and in order to have these widespread changes in an entire system that could be picked up by functional imaging or by anatomical imaging, a lot of things are changing. So we hypothesized that these might be epigenetically mediated.

So in this experiment, we've now taken mice that they had chronic nerve injury for eight months, so they're really very chronic. And this addresses the question that came up before about the time course.

So we decided to go really chronic because we wanted to know -- we thought really chronic would be the most clinically relevant. So we started there. And what we have to do now, of course, is back up and look at when these changes occur. Is it one day after nerve injury? Is it two weeks after injury? Is it six months after injury?

But I can tell you that eight months after injury in mice, there is a change in global methylation in the frontal cortex. So this is now a big change in the brain that's distant from the original injury that happened in the periphery. It's

distant in both time, and it's distant in space. And we did some transcriptome-wide sequencing and found over 1,000 differentially regulated transcripts in these brains.

So getting back to this theory of exercising and environmental enrichment, one of the things that controls throughout life the epigenetic marks that you have is your lifelong experience, which includes your environment. It is your environment.

So in this experiment that was actually done in collaboration with Dr. Bushnell, we took neuropathic mice, and they were chronically neuropathic. They were at least three months after nerve injury. So this isn't a transition from acute to chronic pain. This is chronic pain.

We took these animals, and they had exactly the same intervention. They got their little running wheels, and this picture is great because this guy is like, "Is it my turn? My turn?" And it took a month, but we do see the same therapeutic benefits of just this voluntary running exercise in this neuropathic model now.

But the real question is, well, what happened to the brain? Did it reverse the pain-related epigenetic changes in the brain? And it really did. So this is the change that you saw before basically in just normal and nerve injured, and here's what happens to that nerve injury group after several months in environmental enrichment.

So we don't know if the genes that were hypomethylated in pain are the ones that regained methylation when we had this therapeutic intervention. We don't know that yet, but there was a global recovery in the pathological change.

So the way that I think we should be thinking about some of these sort of global epigenetic changes is kind of as a landscape. Think about when you're flying in from an airplane and there's all of the city lights, and every single light is either on or off, just like every potential methylation site on the genome is either methylated or not. And depending on the time of day, the lights that are turned on like in office buildings might be different from the ones that are on at night, maybe the street

lights.

Just like in chronic pain conditions, the whole landscape of methylation signatures is going to be different in a healthy individual or in an individual that has whatever the disease condition is. In this case, we're talking about chronic pain, but it could be early childhood adversity leading to problems with stress.

I suppose I'm done.

(Laughter.)

DR. STONE: So I'll skip this slide and just end on two questions. The first question is which direction should this arrow be pointing. Does it start in the spine or the periphery and go to the brain, or does the brain control the output?

Then the second is thinking therapeutically, is it better to have a candidate gene approach? Is it better to look at these global networks that change in chronic conditions, or should we be going after the epigenetic modulators like the enzymes that Dr. McMahon talked about that changed the whole landscape? And that's it. Thank you.

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(Applause.)
DR. BOUNTRA: Chas Bountra. Laura, what other phenotypes does the SPARC null show?
DR. STONE: Okay. So the SPARC null mice has some limitations. And the biggest problem, SPARC is not only expressed in disks. So as they age, they also develop osteopenia. They develop cataracts. They have weird kind of more elastic skin.
We've done every control that we can think of to separate these other potential causes of the behavioral changes from the disks themselves. Probably the most convincing one is showing that if you inject capsaicin in the paws of SPARC null mice, you get a very elevated response. But if you do the same experiment in the face where there is no disks for the nerves to have to pass by to get out, you don't see that phenotype in the face.
So it's not just a weird sensory system gone wrong kind of thing.
Another things that's -- go ahead. Sorry.
DR. BASBAUM: Laura, what do you think the chemistry of the disk is doing normally? You said

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that it's full of inflammatory mediators. Do we know anything about them? Are they released? If there's no innervation normally, what are they targeting? Do we have any idea what they're doing?
DR. STONE: Very little, very little. Some of the things that are happening in disks, there's a lot of signaling going on disks. They're actually quite dynamic. So some of the cytokines, for example, that are in there might be signaling to other cells that it's time to make more extracellular matrix. There's that sort of -- it's quite dynamic.
DR. BOUNTRA: Just a comment, Laura. I mean, Steve touched on it at the end of his lecture. But I think one of the really exciting things about your work is that potentially this could be a biomarker or a diagnostic in a clinic. Have you explored that further? Because that's the big issue, isn't it? People talk about a lot of chronic back pain patients are malingerers or whatever, et cetera. This could be very important.
DR. STONE: There is some potential there as a sort of diagnostic, but we would have to show that

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changes in SPARC are the only thing that cause disk degeneration, and that link is not that clear yet. There's probably a lot of disregulated genes that go into the pathological disk.
So it's certainly something to look into, and as we accumulate more human disk samples, we are going to look at things like that.
DR. BASBAUM: One last question.
DR. PORRECA: Frank Porreca, University of Arizona. Very nice story, Laura.
I was thinking about what you evaluated in the mice, and it was cold allodynia, I guess. But you didn't see mechanical hypersensitivity which is a little puzzling. And then I wondered if you looked at any candidate genes for this. I mean, did you see -- were there are any specific changes that you could identify in TRPM8 or something like this?
Then the second question is this lack of immobility that I think you saw, right, so the animals seem to have an opposite behavior sort of implies that there was no -- if this is actually a model of depression where you have increased

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immobility, those animals were not showing that, which seems almost opposite. So I wonder if you could comment on that.
DR. STONE: Well, the first question was about specific genes, and we have not really looked at that very carefully. We've injected TRPV1 and TRPM8 agonists, and the responses to both of them are elevated in the hind paws, suggesting that they might be involved.
But what would be really interesting is to do in this model a kind of genome-wide screen for changes, like what we heard about earlier and see all the things that are going wrong in the disks and in the sensory neurons.
The second question was about -- what was the second question?
Depression, yes. So the tail suspension test, when it's used in the depression literature, it's longer. I think they go over six minutes, and you only count the last few minutes. We only count the first few minutes and get the opposite phenotype.
But we've done other tests for depression

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like the swimming, and they don't show signs of depression in the other tests. But the tail suspension assay is not the only thing that suggests that there's a resistance to gravity and stretching of the spine.

We've also shown it in the grip test where they have to grab on for their tail. They really don't like having that kind of stretching there, and there's a couple other kind of functional tests that we can talk about offline. So it's not the only evidence for that we have. Thank you.

(Applause.)

DR. BASBAUM: Thank you very much.

DR. MAIXNER: Well, it's my pleasure to introduce our next speaker. Dr. David Clark from San Francisco is a professor in the Department of Anesthesiology at the VA Hospital and at Stanford as well.

I have followed David's work for some time with sort of co-interest in opioid hyperalgesia, some of the underlying mechanisms associated with opioid hyperalgesia. I think David has been a luminary in

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pointing out some of the genetic pathways in this area.

I know David has recently switched into other areas related to the neuroimmune system and neuropathic pain. So he has a wealth of knowledge to pick from today, to speak to us today on this topic of genetic and epigenetic contributors to persistent pain.

David.

Presentation – David Clark

DR. CLARK: Well, thank you very much. I appreciate being asked and invited to speak today. I have no disclosures. I'm not going to be recommending that you use any particular drug for any particular purpose. All of the experiments that I'll display were supported by federal funds, and I have no particular industrial ties to any of this work.

My colleagues and I are interested, in part, in understanding data like these. So what you see here are some data that were collected by a colleague, Ian Carroll. And what he did was he asked patients after their surgeries how long they were

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having pain. So he had a phone bank of students, and they would call every day, even weekends, no kidding, and ask are you having any pain from your surgery. And he did this for months after the operations.

What you see is that you have a gradual decline in how many patients are saying yes. It's probably a little slower than you might have expected, but it does come down.

Two things to point out, though. One is even though most people will experience resolution of their pain after maybe a month or so, many are experiencing pain way out here after many, many months. And if you are a person who happened to have relatively high postoperative pain scores, it's all the more likely you will have that persistent pain.

Persistent pain after surgery, it's a problem. It's a problem for rehabilitation. It leads to very high healthcare expenditures. It's very difficult to deal with in clinics, and we wanted to understand some of the factors that might contribute to data like those. So we have most recently turned to epigenetics as a group of

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mechanisms that might help us understand that problem.

We've had some marvelous discussion of epigenetics. The particular subfield of epigenetics that we chose to look at was histone acetylation, as was very carefully laid out this morning. Basically what you have is a process where histone acetyltransferases are putting acetyl groups on lysine residues on the tails of histone proteins. That leads to a relatively open chromatin structure. There's more transcription. There's more message for the genes around. If you have a deacetylase come along, you have recondensation of that material and less transcriptional activity.

Of course, there are families of enzymes that are governing these processes. And the only thing I'm going to mention is that most of the tools we have available currently to pharmacologically address these are really not so good, especially over here in the area of blockers of histone acetyltransferases. We will use anacardic acid and curcumin as kind of probe compounds, not terribly selective.

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The pharmacology for the deacetylase inhibitors are much better developed and become even more so, probably as was mentioned this morning already, because of the interest in these compounds as being useful in the control of cancers.

So we're interested in incision and operations. So we took our mice, and we made incisions in a hind paw. And we asked what happens if we go messing with histone acetylation in those animals.

So we use -- histone deacetylase inhibitors should prolong the -- it should increase the level of transcription of the genes controlled by histone acetylation in those animals. And sure enough, what we have is a much prolonged period of mechanical sensitivity after those incisions. We're giving the drugs here starting at the time of the incision and continuing for four days after the incisions are made. Thermal sensitization is not touched.

If we do the converse experiment with an acetyltransferases inhibitor, what we have is a tendency to reverse or make more rapid the recovery

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of mechanical sensitization after those incisions are made. Again, thermal sensitivity isn't touched.

I see Dr. Porreca in the audience. He has gotten us all to think a little more about spontaneous pain and the ongoing aversive quality of the incision that might best be looked at using something like conditioned place preference. And though I didn't bring the data along today, I can tell you in the conditioned place preference model, if you block the histone acetyltransferase, you will also reduce that evidence of spontaneous pain, if I might call it that for the moment, that the animals seem to be displaying.

We did, however, move into a model of latent pain sensitization in these animals. Why would we do that? Because this latent sensitization or hyperalgesic priming, as it's sometimes referred, might reflect one of the types of processes reflective of persistent pain. This might be something that sets the animals up, perhaps the humans up, for persistent pain.

So in this particular model, we make our

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incision back here with or without the epigenetic agents, and we let the animals recover for a couple of weeks. And two weeks, they are back to their baselines when you look at their nociceptive thresholds.

Then we would inject a little bit of PGE2, which normally leads to a couple of hours of sensitization in the hind paws, and we ask what happens. Well, what happens is if you make an incision back here, even using a vehicle, the period of time the animals show sensitization after injecting the PGE2 is much prolonged, days to recover rather than hours to recover.

We did not, as we thought we might have, make it worse by virtue of giving the deacetylase inhibitor, that's SAHA, give the deaceytlate inhibitor at the same time that we made the incision, so the same level of enhanced sensitization.

However, if we had given the animals a histone acetyltransferase inhibitor, here we used anacardic acid, then we see that we were able to block some of that sensitization two weeks hence. So

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we feel that perhaps there's a reason to suppose there are epigenetic processes leading to that more persistent sensitization in the animals.

Moving on from simply looking at nociceptive and pain-related matters, we are more and more asked to determine what's happening with animals functionally not only because that might give greater basis for any conclusion we draw about what our drugs are doing, but also I'd point out, in the clinical world, restoration of function is a very important goal. And I think if you look at things like impact guidelines and what you should be looking at when you judge the efficacy of an analgesic, you'll see functional goals included as well.

So we decided since we're making hind paw incisions, our function would be gait. We did our gait analyses using a video-assisted system. This has a transparent moving treadmill. You take little videos, and you let the computer spit out lots and lots of parameters related to gait. If we do this in our incised animals, we find nine parameters. They're listed on the side here, which are made

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abnormal in the setting of incision.

This is two days later, sort of 1 to 3 days is where we see this. If curcumin, here we're using it as a probe compound to block histone acetyltransferase activity. If we include that drug at the time of incision and for the first two days afterwards, in fact, we normalize 8 of 9 of those parameters.

So perhaps we can even normalize function if we can block some of these changes. That would be our hope.

So I'm showing you some pharmacological data and some behavioral data such as they are, implying there could be some epigenetic changes we might be interested in, in understanding persistent pain after incision. But we haven't really talked about histone acetylation and evidence specifically for it.

So remember, histone acetylation is one of an array of things that can happen to histone proteins to change gene transcription. Acetylation occurs in all kinds of residues on these tails. This is the ninth lysine residue on the H3 histone subunit, and

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I'm going to call that H3K9 just for short. It is only one of many, but a particularly well studied residue that controls things like memory and drug addiction and has really quite a history at this point of being associated with plastic changes in the central nervous system.

So we chose to focus in our initial experiments on that particular derivative, H3K9, and we began then by simply staining spinal cord tissue, asking do we find more acetylated H3K9 in the dorsal horns of the spinal cords. And the answer is we do, and most of that -- and if we include a deacetylase inhibitor, one would make incisions, the level of acetylated histone is that much higher.

Most of that turns out to be in neuronal cells but not all of it. We haven't made careful study of exactly what the proportions are in this model of glial versus neural. It's way weighted towards the neuronal, but I'm not going to exclude non-neuronal cell types from being involved in these phenotypes.

We have now some evidence of acetylation, and

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as the previous speakers discussed, you might want to have a candidate gene or try to understand what it is that particular epigenetic change is altering. Ultimately, this should be a change in transcription level.

So what we did was we did a big series of array experiments, which were long and painful. And the outcome is when we made incisions, the animals included histone acetyltransferase inhibitors and deacetylase inhibitors and tried to probe which genes are likely epigenetically regulated in this model, we came up with a list of about a dozen or so that we were pretty convinced were altered epigenetically in the model.

We chose to use as our sort of probe set of genes this pair. This CXCR2-CXCL1, you'll recognize as a chemokine signaling receptor and ligand pair. Why did we do that? Well, one, we found the pair, and we found that kind of intriguing that both would be regulated under similar conditions. They're well established to play roles in nociceptive sensitization in other models, both centrally -- and

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that comes -- and these are recent papers that were published looking at neuropathic and inflammatory pain.

Even in the periphery -- and we'll get to some peripheral stuff as well -- where CXCR2-CXCL1 seem to be operative, what was very encouraging was others had shown these already to be epigenetically regulated genes. So that was just too good to pass up. And we proceeded by looking at CXCR2.

So we took our animals, made incisions, included the deacetylase inhibitor. And sure enough, we had one message for CXCR2, and that translated to increased protein levels as well. Where is that stuff? CXCR2 is primarily in neurons. That is where we expected to find it, and that's where, in fact, we did find it. And those particular neurons expressing CRCX2 also had, for the most part, elevated levels of acetylated H3K9. So we saw that sort of come together.

That would be the receptor side of the story. What about the ligand side of the story? Just to broaden this a little bit, CXCL1 is one of the

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endogenous ligands. CXCL2 is another. I recall that these things, these chemokines, are named based on the number of spacing residues between those, crosslinking the backbone of the chemokine structure. So we're dealing with the CXC chemokines here.

When we went ahead and made the incisions and looked at the spinal cord tissue, it was really only for CXCL1, which also goes by KC and about a half a dozen other names as well. But KC or CXCL1 is elevated, and it's pushed up just a little more, we found, not maybe as much more as would have made me say dramatically, but a little more if you include these deacetylase inhibitor.

No such change in our hands for the other endogenous ligand.

Now, we have some pharmacological data, and we've measured some message levels and things. But I haven't shown you yet that this has anything to do with histone acetylation. Does histone acetylation control the transcription of those genes?

To get at that question, we used what's called a ChIP assay. That's chromatin amino

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precipitation, and that is conducted as follows. You crosslink DNA to histone proteins. You fragment the DNA somehow. You can use sonication. You can use other means. You can use enzymes. You then precipitate based on the particular, in our case, histone derivative that you're interested in studying.

So we used an antibody against H3K9, acetylated H3K9, and then you separate your DNA, amplify it, or put it on a ChIP or sequence it, or do something. And you'll find out how histone that's been acetylated is near the promoter region of your gene.

So that's our basic ChIP analysis. And when we did that, we found that both for CXCR2 and CXCL1 or KC, we had this enhanced level of acetylated histone protein near the promoter regions of those two genes, which helped to complete our understanding.

By the way, if you do intrathecally inject a little CXCR2 antagonist, you can reverse mechanical, not thermal, sensitization in this particular model.

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So that worked out as well.

Now I'm going to change direction just a little bit. And here I'm going to go back to the observation that this chemokine signaling pathway, epigenetically regulated, is seen not only in central nervous system tissue, but in the periphery, it had been looked at as well. And not only has it been looked at in the periphery, but part of our laboratory's interest happens to be in how opioids might change chemokine signaling peripherally.

This was just happenstance, but we've had a long interest in why patients who have had chronic treatment with opioids seem to be so difficult to manage postoperatively. And by the same token, patients who have additional injuries who are chronically managed with opiates tend to be difficult as well.

So they have higher pain scores. They have much higher opioid requirements. They have more frequent side effects probably because you're pumping more opiate into them trying to control the pain postoperatively. And where it's been looked at,

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functional outcomes in these patients are also poor. It is a problem area for clinical medicine.

Once we can model some of this, we can certainly show greater sensitization, longer duration. And some people in the audience have been involved for years in doing just those experiments.

Again, coincidentally, we had several years ago looked at what opiates did to peripheral levels of cytokines and chemokines. One of those chemokines we had included in the panels happened to be CXCL1, which was known as KC at the time. And, in fact, in the morphine-treated animals after incision, there was more of it. We invoked that as an explanation for why the animals had more pain-related behaviors.

So we're going to return. It was a homecoming to that particular system. We used the model that's displayed here. And basically what that shows is if you make an incision in an animal here and measure mechanical sensitization -- why you find it -- and it gets better over the course of days. If you give an animal morphine for 4 or 5 days, it too becomes sensitive to noxious stimuli. If you stop

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giving them morphine, it recovers.
If you take that sensitized animal, though, and make an incision, the double whammy, then you have an animal that's sensitive for a prolonged period of time, maybe a week or more, under typical conditions, but very reproducibly all demonstrable in now several laboratories.
So what we did was we took the animals, made the incisions with or without morphine treatment, measured cytokine levels in the skin. And we did that first at the level of message, and sure enough, we found a lot more CXCL1. We expected that. It's an inflammatory lesion. It's not a surprise.
If morphine was given beforehand, there was a little bit more 1 and 3 days afterwards. No such change, no such real strong morphine effect for the receptor, I'll point out. It was only for the ligand here that we found the difference. And in the spinal cord under these conditions, we did not see it, which gets back to one of the questions that was asked earlier.
These epigenetic changes are very tissue

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specific. I want people to understand that as you find an epigenetically-regulated change on a gene in one tissue does not mean under similar circumstances you will see it in another. There's a lot of additional regulatory machinery that's at play there.
We did ask then if we have any reason to suppose there was an epigenetic effect here in the periphery controlling that CXCL1 expression. We looked first at the epidermis, and this is a stain for acetylated H3K9. What you'll see is that there's just a whole lot of it there, whether or not you make an incision in an animal and whether or not it's treated with morphine; so in the epidermis, and no evidence that our morphine or even our incision is changing the levels of H3K9 near that wound edge.
If you go down just a little further, though, and look at what's seen in the dermis, what we found was there was more evidence of this histone acetylation in incised animals and more yet if the animals had been treated with the opiate before the incision. If we co-labeled with CXCL1 and acetylated H3K9, this is not the loveliest of

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immunohistochemistry we've ever generated, but it did seem that we had co-labeling of CXCL1 and acetylated H3K9.
Maybe this is the most useful set of panels here, which shows that if you look at the number of cells co-expressing CXCL1 and acetylated H39, it goes up after incision. It goes up about three times as much if the animals had been treated with morphine.
Finally, we asked here on what cell types, then, is the CXCL1 being produced in these wounds. And it turns out almost all of it is being produced by neutrophils coming into the wounds. You'll see here the co-labeling. It is not incidentally just that morphine treatment means more neutrophils move in. So we both counted the neutrophils and did a peroxidase assay, which is another way to look at neutrophil abundance. And there's really no difference between the animals treated with morphine and not.
So it seems to be more the case that the neutrophils are just primed to make more chemokine as they arrive in the damaged areas.

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CXCL1 is algogenic in these animals. If you inject a little bit of CXCL1, it does in fact make the animals sensitive. And injection of the antagonist just into that wound area where we're measuring the ligand, it reverses the mechanical sensitization I would say modestly, not a large effect but some modest one. It, in fact, is only one of many mediators in those wounds, so perhaps we should not be terribly surprised.
Now, this is the third and, I promise you, the last twist I will try to take you through in this particular talk. We've talked about chemokines, and we've talked about central functions and peripheral functions. Those are molecules involved in pain mechanisms. Another place you might look at epigenetics is in how those processes control or govern, make better or make worse responses to our pain relieving drugs. So this is more of a pharmacoepigenetic issue here.
Two of the principal limiting processes for maintaining a stable effect when patients are given opioids are opiate hyperalgesia and tolerance. I

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learned last week, in fact, on the new Department of Veterans Affairs' consent form, all veterans will be asked to sign if they're going to be treated chronically with opiates. Listed as side effects, they need to understand, are opiate hyperalgesia and tolerance. And no, I didn't have anything to do with writing it, and I don't know if Frank did. But I was a little surprised, frankly.

But there they were. They might have something to do with it. I don't know if it belongs in a consent form or not, but we do study them in the laboratory.

Basically what we observe is, again, if you give animals morphine for several days, you can show that they sensitize. If you include some curcumin or try to block histone acetyltransferase, in fact, you don't see the hyperalgesia. If you include the deacetylase inhibitor, which should work the opposite way, you might even make that sensitization greater.

The epigenetic drugs by themselves aren't changing sensitization, by the way, in these animals. It has to be given along with the morphine.

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But maybe you don't believe that that's so much more sensitive than that, but if you look then at the recovery from sensitization after you stop giving the morphine, notice here that you have a fairly rapid recovery if the animals simply have morphine for four days. If they had morphine plus the SAHA, they're sensitive even a month later, and that's just when we got tired of measuring the stuff in the animals.

So these epigenetic processes are very long-lived, and I think that it is what is to be expected of them.

The story for tolerance was much the same. You give curcumin along with morphine, they don't get as tolerant. You give the SAHA and block histone deacetylation, you make the tolerance worse, and you also make it more persistent.

Finally, we had to move here then from the level of understanding pharmacology and behavior to what genes might be responsible. This time instead of using a genome-wide approach, we put together a panel of genes we knew to be epigenetically regulated

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or at least had been reported to be, that had been invoked to explain either tolerance or hyperalgesia.

What we did was we studied their expression in spinal cord tissue. We treated the animals for 4 days with morphine with or without the deacetylase inhibitor SAHA. And then we waited a week, which is really that period over which most animals recover if they've just had morphine. But we asked for which genes is there still an elevation if you block deacetylation. That was our clue that there might be something epigenetic going on.

It was really here for BDNF and prodynorphin that we had the greatest evidence that there was this persistent effect of the blockade of histone deacetylation, and maybe NRB2 as well. But we really pursued BDNF and prodynorphin.

We asked in the ChIP assay, again probing is this really epigenetic or not, was there a greater association of acetylated histone with the promoter region of the protein? There was for one of the several BDNF promoters and for the promoter of prodynorphin. And, in fact, the data are identical

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for BDNF and prodynorphin. There was more BDNF, both immediately after ceasing morphine administration and a week later if the animals had this deacetylase inhibitor. These are protein ELISA measurements, actually, in spinal cord tissue.

Both BDNF and prodynorphin are functional in supporting hyperalgesia and tolerance, so here we use a TrkB inhibitor, ANA-12, and can reverse hyperalgesia both the day after morphine is done and a week later in those animals. The data are similar for the use of a kappa opiate receptor antagonist, which may back at least part of the actions of prodynorphin.

One final data slide, and that is to say that I appreciate being here at the ACTTION conference and the interest in the audience in developing drugs to treat pain. Another way, though, to look at these epigenetic mechanisms is to understand why pain is persistent independent of your intention to ever develop a drug around the process.

So leaving aside drugs and getting back to one of our lab's core interests, it's what factors go

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together to make a pain problem a lot worse. And one set of factors that does prolong postoperative pain recovery, and is a clinical problem, is the use of an opiate long-term plus having an injury like an incision if you have an operation.

So here we're going to measure BDNF and prodynorphin. This is the day after we cease administering morphine. We find a little more message for BDNF and a little more message for prodynorphin. If we make incisions and look at those the day after, yes, there's a little more BDNF message, a little more prodynorphin, yes. And you'll measure the protein, and it's up, too.

But if we do both things, we have a sort of additive effect of doing both on the level of message. And, in fact, I think the key part here is that that additivity is seen in our ChIP assay. So if you put drug plus injury together and you have greater activation of that acetylation of the process that's controlling the expression of those genes, I think that is what we found most interesting.

So to end with a model, this is, of course, a

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very oversimplified cartoon, but it sort of is driving conceptually some of what we'd like to do experimentally. And what it represents here fundamentally is lots of processes coming together to converge on epigenetic mechanisms then ultimately controlling pain sensitization.

A few of the key players are with additional data that I've not shown you this morning, there's classes of nociceptive neurotransmitters. Neuropeptides are displayed here. Excitatory amino acids do the same thing in our neuro cultures. The presence of drugs can do this. Descending facilitating or inhibitory fibers might drive this as well.

But ultimately, we suppose those things might converse on some set of epigenetic mechanisms. Here I have drawn in the phosphorylation of CREB, which is one plausible mechanism, but it could be many others that then control the expression of a finite group of genes in a specific group of cells controlling nociceptive transmission.

So I'm going to end on that model slide, and

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then I guess invite any questions.

(Applause.)

DR. CARR: Hi. Dan Carr, Tufts University. I very much enjoyed your presentation.

Very often there are claims made for dietary supplements like the red wine, resveratrol and curcumin. Would you comment on the doses that were given understanding there are species difference --

DR. CLARK: Large doses of red wine.

(Laughter.)

DR. CARR: In your experiments, understanding it may not be generalizable across species, are the doses remotely akin to those that can be achieved with dietary intake of turmeric?

DR. CLARK: This is a good question because this is actually where some of these agents come from. I think the dietary supplement we've used here is curcumin. And it is very difficult to answer this specific question, but I'll tell you what the literature -- it's poorly absorbed.

It has been used in many clinical trials as a drug in an attempt to better control cancers. The

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presumed -- I don't think people really knew what the mechanism was, but one that was written about in the discussion sections is, in fact, the regulation of histone acetyltransferase.

They have been used in doses from milligrams per day to tens of grams per day with serum levels often documented to be below those, which probably interact with the particular enzyme systems that they would purport to control.

So I can't tell you that the doses we achieve in the animals are things that we can in a human. I think here, rather, we're just using it strictly as probe compounds, and I really don't advocate people go out and use a lot of curcumin around the time of an injury, even though there is one very interesting paper which shows people might recover a little faster; not a terribly good study but intriguing.

DR. MAIXNER: Laura.

DR. STONE: The double whammy of being on chronic opioids and then having surgery, that's the population of patients with chronic back pain that have tried all the drugs, are on chronic opioids, and

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then go out for surgery.
So in your opinion or do you have any data to suggest is it better to wean people off the opioids first with all of the stress that's associated with that and then have surgery, or just proceed with surgery as is?
DR. CLARK: Yes, so again, this question, it gets into the clinical realities of managing patients on these opioids. You have to hope -- let me just take this as a tack that I might have to change my mind on at some point.
If really this is activating epigenetic processes, you might not expect an acute detoxification or an acute change to alter the outcome. And it's because if what you've done is you've primed these guys to go and methylated their DNA or acetylated their histone proteins, it may be some time before that would be reversed. And so I think I would have some skepticism that an acute change would really get you to where you need to be.
The other thing is, you just try to get the drugs away from these guys before their operations.

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It's a difficult thing to do. So if it doesn't really show a benefit, I would say we have to put our thought more on not getting people in that situation to begin with.
DR. MAIXNER: We're into the break. I would like to ask, though, one quick question for David to address.
Curcumin also I think has profound effects on the NF-kappaB pathway. And it begs the question as to whether these effects are direct on the decarboxylation or methylation or whether they're upstream factors such as IkappaB, which are very important in this process and really maybe represent a target more so than deeper into the nucleus.
DR. CLARK: Yes, so especially for curcumin, there are probably multiple sites at which that drug is working. And you've named another prime site that certainly could be -- it's probably targeted by curcumin and is a legitimate one for analgesic development.
DR. MAIXNER: Let's come back at 11:00 if we can. I'm sorry. Let's come back -- Bob, can we come

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back at, say, 11:15? We're back at 11:15. Thank you.
(Whereupon, a recess was taken.)
DR. MAIXNER: Well, welcome back to round 2. It's my pleasure to introduce our next speaker, Chas Bountra from Oxford. Chas is a professor of translational medicine at Oxford and director and chief scientist at the Consortium for Structural Genomics.
He has a remarkable history, and I think one that's very germane to this workshop. Chas was a few years ago, about six years ago, vice president at GSK, head of biology, and I think comes to us with a lot of experience in the concept of translational medicine.
I think we will hear today of some of his work, which Stephen mentioned earlier this morning, a little bit about epigenetics and histones, I think, and various ways in which we can approach the question of epigenetics. Chas.
Presentation – Chas Bountra
DR. BOUNTRA: Well, ladies and gentlemen,

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thank you very much. Good morning, and thank you to the organizers for the invite.
I brought this title, Epigenetic Probes for Pain and also Neuropsychiatric Disorders. So my basic premise in this talk is that I believe the way we are currently doing drug discovery is incredibly inefficient. I'm going to highlight four problems. I'll share with you what we've been doing over the past few years to try and transform drug discovery, and I'll also share with you some of our plans going forward.
Most of what we've done to date has been in the cancer space or the inflammation space, but frankly, many of the lessons we've learned or things that we've doing are equally applicable to neuroscience and pain.
So my disclosures, payment, shareholding and research income.
So let me just set the scene. I think research and development, the way we're currently doing it, is frankly becoming unaffordable. And you've seen the Forbes analysis at the beginning of

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2012, where they estimated that AstraZeneca was spending about $12 billion a year for each product they launched. That is just unsustainable.

Frankly, many new medicines are also becoming unaffordable. So you're all aware the Vertex example, the cystic fibrosis drug, that they're charging, what, $300,000 a year. That's $800 a day. Who can afford that?

With an aging society, of course, we desperately, desperately need more new medicines, and also, diseases of modern living; I'm thinking of diabetes, obesity, et cetera. The thing that continually worries me is that in biomedical research, in both academia and in industry, there is massive duplication.

The tragedy is that most of our ideas, most of the targets, most of the assets that we work on when we take them into phase 2, they fail. This is a horrendous waste of money.

The problem, the first one, target selection is difficult. We've got hundreds of targets. We could sit here and come up with a whole list of

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targets. Actually picking which one to invest in is the problem. We're not good at it, even today in 2014.

The fact is complex diseases are all caused not by a single gene, but they're polygenic. They're caused by multiple gene defects. Complex diseases are immensely heterogeneous. We've heard today about some of these epigenetic environmental influences, but there are sex differences, age differences, chronicity, stress effects, drug exposures, early life experiences, injury, et cetera. The whole body, the nervous system and the immune system in particular, are incredibly plastic.

What we know in the clinic is that dirty molecules or combinations of existing drugs are usually more effective. So I think one of the challenges we've had in the past two decades is we've been seduced by single molecular targets.

So my hypothesis is that many of these epigenetic proteins that Steve touched on, I believe they may represent single early stage mediators of complex diseases. So by affecting one of these

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proteins, I believe we can actually target many of these polygenic diseases, if you like, and actually try and deliver something that's more optimally effective.

The second problem is that in academia, there's a lot of expertise and infrastructure and resources, but academia lacks a lot of tools. Academia lacks high quality, novel inhibitors for novel proteins. So to dissect biology, we need those tools.

Of course, PhRMA is brilliant is at medicinal chemistry. They've got the resources, and they've got the strength. And they're good at generating the tools, but most of the tools sit inside PhRMA.

I mentioned that there's massive duplication in biomedical research and there's too many targets to choose from. So what we've chosen to do is we've created a public/private partnership to generate novel tools, inhibitors, and make them freely available in an effort to crowd-source science, to use the whole of global academia.

So this is what we do. We only work on new

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human proteins. We work on proteins that most other people are not working on because we think this is where the new drugs are going to come from. And for those proteins, we generate what we call target prioritization tools. We generate the human protein. We generate assays for that protein. We generate small molecule inhibitors for that protein. We generate antibodies, and we generate the three-dimensional X-ray structure.

I should say that generating each of these inhibitors, these are incredibly high quality molecules, better than molecules that have often been generated at that stage within PhRMA. They're costing us in the region of two and a half to $3 million each to generate.

But the thing is, all of these reagents, we make them freely available. We give them away to anybody in academia, anybody in biotech, and anybody in PhRMA because we believe that's the best thing we can do to facilitate science, and therefore facilitate drug discovery.

As a consequence, we've come a hub for

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innovation. We only work on new proteins. We've also become a hub for PhRMA. We're now working closely with nine large pharmaceutical companies, GSK, Pfizer, Novartis, Lilly, AbbVie, Takeda, Boehringer Ingelheim, Janssen, and Bayer. And each of these companies has given us $8 million of funding. We have $72 million of private funding to do this work.

We've also become a hub for academia. You can imagine every academic who comes into my office wants to work with us because they know we've got all these new tools, new reagents. We've got no secrets. We share all of our know-how, all of our expertise. And frankly, that transparency creates a lot of trust, which is great for collaboration, it's great for science, and it's great for drug discovery.

So we're now collaborating with more than 250 of the best academic labs all over the world. This is a way of pooling resources, it's a way of sharing risk, and it's a way of crowdsourcing early discovery.

We work on families of proteins. These are

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our sort of therapeutic foci. The red is where I'm going to give you some examples in the next few minutes.

So kinases. In humans, there are 518 kinases. Just to show you the sort of throughput that we've got, this is time along here. The red is all the kinase structures that have ever been done in academia outside the SGC. The green is all the kinase structures done in industry. And this is all the kinase structures done by the group in Oxford in the past few years. And you can see, we've done as many kinase structures as the rest of academia put together.

It's worth saying there are many structural biology labs in Oxford that generate one structure every two or three years. We're generating in the order of five or six structures every month.

Another example, I talked about sort of making these reagents available. But the important thing is also to disseminate the data. Part of the problem we have at the moment is that these expensive clinical studies that are done inside PhRMA often are

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not published, or they're not published quickly enough, or they're not published in enough detail. We're not learning from those experiments.

This is just a simple example in our own area. So as you know, generating structures of human membrane proteins is phenomenally difficult. Four years ago in Oxford, nobody had ever generated a structure of a human membrane protein. In the past four years, we've done four of them.

This was one that we did in 2012. As soon as we did it in May 2012, we immediately told the whole world about it, how we did it. It's a zinc metalloproteinase involved in premature aging.

Because we disclosed all of this data, in the summer of that year, this allowed a lab in the U.S. to solve the yeast structure. In November that year, both papers were submitted, this one and ours. And April of last year, both were published. And I guarantee, there is no other lab on the planet that would have done this; as soon as we generated that data, sharing it with the world even before we'd started writing the manuscript.

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Last year, we did the structure of the first human ion channel ever done in the U.K. We deposited it in June 2013. Immediately, we told the whole world about it. I'm embarrassed to say we've yet to submit the manuscript, and it's because we're generating the functional data for that channel.

Now, I talked about inhibitors. So one of the epigenetic protein families that we're working on is called bromodomains here. Now, in humans, there are 61 members of these bromodomains. So five years ago, we decided we were going to generate inhibitors for these proteins and make them freely available. Five years ago, many people said forget it, you'll never do it, it's a protein-protein interaction. It's too difficult.

Well, in 2010, we generated this molecule. It exists as two enantiomers. This one actually switches off the protein, and this is inactive. And this compound, you can see it only works at this subset of bromodomains here, largely BRD4. It has little or no activity down here.

Now, when we generated this inhibitor, we

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thought, okay, now what are we going to do with it. And there was some literature that said that this protein, BRD4, if it exists with another protein effusion and not BRD fusion, then those patients develop a very rare cancer called NUT midline carcinoma. It's so rare many oncologists can't even diagnose it.

We worked with Jay Bradner at Harvard. He generated this data. He is the world expert in NUT midline carcinoma. He has access to patient-derived cells.

So what he did was a simple proliferation assay and put the inhibitor on. So this is the compound, and these are two patient-derived cells. And you can see the compound reduces proliferation. The compound reduces proliferation.

He also then, using annexin V and propidium iodide, showed that it increases apoptosis. So for example, this is in vehicle. Twenty-four hours after the compound, it's gone up, and 48 hours later, it's gone up even further. So apoptosis goes up. Proliferation goes down.

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Then he put it into a xenograph model. So he took these patient-derived cells, put them into a mouse. The tumor volume got bigger and bigger over days. This is the vehicle. This is the compound.

When we got this data, Jay wanted to take this molecule into patients because there is nothing out there that treats this site of cancer. Anybody diagnosed with NUT midline carcinoma, they're dead within three to six months.

Since then, we've given that molecule to more than 400 labs across the world. They've now shown that that molecule works in a whole range of other cancers. There was also a paper in 2012 that the compound could be a target for male contraception.

Last year, there was a paper in Cell that the compound reduces cardiac hypertrophy. And then GSK generated their own molecule and showed that it prevented and inhibited LPS-induced endotoxic shock, so septic shock. If you give the compound before or afterwards, it stops the animals dying. This is the vehicle treated. They're all dead in 40 hours, and the compound stops it dying.

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I deliberately share this data because it illustrates we do not know how to do target discovery. Here's a new target, new molecule. I've shown you data in cancer. I've shown you data as a male contraceptive. I've shown you data in cardiac hypertrophy. And here's the data in septic shock. And this is the challenge that industry has when they have these new targets, which disease area do they take it forward?

Well, since we generated that molecule, let's see what's happened. We've given that molecule to more than 400 labs all over the world and many companies. And, of course, I pay nothing for that. GSK couldn't afford to do it. Pfizer couldn't afford to do it.

We published that paper in Nature in December 2010. It's been cited more than 400 times since. As a consequence of that one paper, there's now been in three and a half years more than 200 papers on that target, many of them using that molecule. This is crowdsourcing science in a totally unprecedented way.

We've enabled proprietary efforts. Today,

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there are five companies with five molecules in the clinic, again, unprecedented. And Jay Bradner secured $15 million of VC funding to set up a biotech to take that target into the clinic. This has been the impact of one probe freely available to the whole of the biomedical community.

Let me give you another example, another family of proteins, and we've heard the speakers talk about it this morning, the demethylases. We worked with GSK to generate an inhibitor of this demethylase called JMJD3. You can see it's potent and it's selective over these other demethylases.

We took that compound and put it onto human macrophages, and it produced a dose-related reduction in the release of TNF from human macrophages. Now, anti-TNF, many of you're aware, are currently selling $27 billion a year for the treatment of RA and IBD. Here's a new mechanism for reducing TNF production.

We put the same compound onto osteoclasts and showed that it reduces bone resorption. We put it onto human breast cancer cells and showed that it increases apoptosis. Again, another new target, a

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new molecule, and I've shown you three different indications: inflammation, bone, cancer. I would argue there is nobody on the planet that can tell me where that target is going to end up.

We've now generated 24 of these inhibitors. I've just shown you details of two of them. We've generated 24. We intend to generate about 10 more per year in the coming years, and they're all freely available to anybody who wants to use them. These are the other epigenetic targets that we're working on, and we're trying to optimize these molecules. And I say again, each of these molecules is costing us working closely with industry in the order of 2 and a half to $3 million.

The third problem is target prioritization. Now, I'm afraid I have little confidence in data in cell lines. I think until some of the recent work that Frank's been doing on animal models, I largely believe animal models have not been predictive of what happens in the clinic. I think often the challenge we've got is that data generated in different labs is often different and conflicting.

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So what we now intend to do is we're going to set up a panel of primary human healthy and diseased cells and tissues. We're going to focus on inflammation initially, and then we're going to systematically profile our normal inhibitors in these cells.

So, for example, we can get T cells, B cells, macrophages from healthy individuals and patients, patients with MS, IBDs, RA, et cetera, et cetera. We will compare our inhibitors in those assays, and this will be a way of comparing healthy with disease, disease with disease, target with target, and cell type with cell type. I think this is going to be a better way to discover new targets for inflammatory diseases.

We've already started working with a company called Discovery. This is data they've generated for us for free. So they took a novel CREB inhibitor that David talked about, David mentioned in his early talk, CREBBP. This is a novel inhibitor of this bromodomain protein. These are 12 different human cells, different epithelial, fibroblast, smooth

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muscle. And basically what they've done is they've profiled this inhibitor at four different concentrations, as shown by these different colored lines. And they've come to a conclusion that the compound has antiproliferative immunomodulatory effects matrix remodeling, and it also affects angiogenesis.

This is just a quick way of trying to get a steer of where these targets are likely to be useful, and then we can probe the biology more deeply.

Here's another example, another inhibitor, and you can see by comparison, that seemed to have little effect. But there was again some suggestion of these sort of effects.

As I said, we're now building a human immune platform with all these different primary cells. We'll do medium throughput screening using high content readouts. It's going to be based in Oxford but also at the Karolinska. We've just got a grant from the Innovative Medicines Initiative to do this. And our intention is once we've set this up, to apply exactly the same to neurons derived from iPSCs from

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patients.

The fourth problem, there is massive attrition in phase 2. Most novel targets fail in patients. Most companies work on the same few targets in parallel and in secret. Frankly, the way we're doing drug discovery, we're wasting funds, we're wasting people's careers, and we're needlessly exposing patients to molecules destined for failure.

So what we intend to do, create a public/private partnership to evaluate novel inhibitors now in patients, sharing all the data and all the reagents freely. If that's the cliff, that's where at 9 times out of 10, we fail. Let's pool our resources, do the experiment once. Let's do it well. Let's share the data. Let's not do it 20 times in parallel and in secret, and then not publish the data.

This is what's happening in drug discovery. We come up with a target. We run a screen. We get a hit. We do lead optimization. We get a clinical molecule. We make sure it's safe in animals, safe in humans, and then efficacy in patients.

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This is the attrition at each of the stages. Now, we could argue about the figures, but this roughly my experience in GSK. We lose about half here, 10 percent here, a third here, a third here. But look at this.

Whenever we take a novel target -- and I'm talking about novel targets, not me toos and new formulations. We take a novel target into patients, the failure rate is 9 in 10. And this frankly is killing our industry. And of course, the tragedy is this process takes five, six, seven years.

Now, that's a horrendous waste of money for any one company, but when you think that 20 companies are doing it in parallel and in secret, now when you just add up how much money we're wasting, how many careers we're wasting, but importantly, how many patients we're needlessly exposing to molecules destined for failure.

A target that we all know about and love, TRPV1, this is just showing the patents that have been taken out on that target in the past 15 years or so color coded by company. It's not a complete list.

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It finishes at M. There's a lot more companies down here.

Now, I can tell you my own story on this one. I was working on this target in 1997. And then in '99, 2000, Dave Julius and John Davies published their knockout papers, and the whole industry jumped on that target for pain.

In 2003 when I was in GSK, we came up with a very potent selective molecule. We took it into the clinic, and basically, there was no analgesia, but there was an increase in body temperature. Now, we didn't publish it, I'm embarrassed to say.

In 2006, there was still 60 companies working on that target. And in 2010, there was still academics, biotech, and PhRMA working on that target. And then AstraZeneca published their clinical data, no efficacy, increase in body temperature. Just imagine in 13 years how much money we wasted on that target and how many patients we exposed.

Now, this is color coded, the same target TRPV1. Now the patents are color coded by therapeutic area. Now, you can't see the list because I can hardly see it from here, but there's allergy, Alzheimer's, asthma, COPD, headache, hearing loss. I mean, the list just goes on, and it's not even a complete list. The red is pain, and all the others are different disease areas. Maybe this just says we don't know how to do target discovery.

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Now, I'm fortunate to be working with Tom McCarthy, who's sitting at the back, who started this company called Spinifex. And Andrew Rice is going to give, no doubt, a brilliant presentation tomorrow on some of our recent clinical data. But I wanted to share two slides without stealing Andrew's thunder.

This is a clinical study that we completed with this compound here in postherpetic neuralgia patients dosed for four weeks. This is a placebo, and this is the active compound. And you can see clearly there's a significant difference down here.

Now, let me share with you the target. We know angiotensin works at two receptors, AT1 and AT2. If you block AT1, you get antihypertensives like losartan. Tom's compound is a blocker of AT2.

Now, I know when we first got this data, there was many, many people in the pain area thinking where the hell did this target come from. I can also tell you if I was still inside GSK, there was no way I would have been allowed to take an AT2 antagonist into the clinic for pain because everybody thinks, oh, it's cardiovascular, blood pressure. It's nothing to do with neuroscience and pain. It's worth bearing that in mind.

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So what are we now going to do? We're now going to take some new targets all the way through to what we call proof of clinical mechanism, so phase 2A, experimental medicine study.

In the process, we will generate these reagents, shown here in blue, and we'll make them freely available to anybody who wants them. We're going to generate these publications shown here in red. Those reagents in blue and the publications in red, they will facilitate more collaboration, more leverage funds, and more of these proof of clinical mechanism studies.

So I may decide to take Target X into, let's say, schizophrenia. But if Frank Porreca says to me,

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Chas, I think Target X is going to work in osteoarthritis, we'll say, Frank, here's the molecule. You go away and get your own funds and do that clinical study. At least it saves Frank from generating that molecule.

The patient groups we've talked to, they've said that they will help us recruit patients, and they'll minimize the payment. So potentially, the studies could be cheaper and faster.

We've talked to many of our friends and the regulators. They've said because this is a knowledge creation endeavor, this isn't about me trying to make money. This isn't about GSK trying to sell a drug, et cetera. This is a knowledge creation endeavor. They will help us design new clinical studies, help validate new biomarkers, and help pave the path to new targets, a totally unprecedented way of working with the regulators.

Where have we got to? We've started the project in cancer with Cancer Research U.K., the world's biggest cancer charity. And on the 5th of February this year, and we're sorting this out,

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Takeda has agreed to fund a new target for Alzheimer's. Now, let me just repeat that, a new target for Alzheimer's.

They want us to pick the target, which is what we're doing now. They will then fund the high throughput screen, the lead optimization, the toxicology, the phase 1 and the phase 2A study completely in the open. We're working with the Canadian government, the CIHR, Canadian Institute for Health Research. They want us to do this in neuropsychiatry, and they're willing to fund $30 million into this effort.

So this is what we've been doing. We've been working on new targets. We've been generating open high quality tools. We've been crowdsourcing science. The green is what we've been doing.

What we've now started to do is we want to profile these tools in human disease cells and start generating open clinical candidates. And then ultimately, we want to do open phase 2A studies. This is what we intend to.

I think this should be the precompetitive

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boundary. Once we've worked out that 1 in 10 target that's likely to do something, that industry can generate proprietary assets. At least they know they're working on a clinically validated target, a de-risk target, not another TRPV1 or another NK1.

So summary. Target validation occurs in patients. We have to get to phase 2A as quickly as possible. Pioneer drug discovery, it's too high risk, too expensive, and it takes too long. There's too much duplicative activity on targets destined for failure. We have to pool public and private resources, access the best academics, work with patient groups and regulators to de-risk pioneer targets precompetitively. Industry can then generate proprietary molecules for such targets.

I believe I think it is worth taking some of our novel epigenetic probes and profiling them in pain relevant cellular assays, whatever they may be. Some of our molecules have got good PK in animals, and it may be worth evaluating some of those in some of the assays that people like Frank are developing. And I think it's worth considering building a

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public/private partnership to take a novel target all the way into phase 2A.

Ladies and gentlemen, we're doing this in the interest of patients. Thank you very much.

(Applause.)

DR. MAIXNER: Questions?

DR. BASBAUM: Just going to play a little devil's advocate. I won't quote the individual, but it was somebody who we all know. And I remember having lunch with this individual who told me that he was incredibly successful in industry. Now, I happen to know that he worked very high up at a company, and when he was there, never got a compound into the clinic that was successful. But he was convinced that he was incredibly successful. Why? Because he had brought 20 compounds into phase 3. And what he was basically saying, my interpretation, is the pipeline is what drove his market.

So success in that case meant that the stock was doing incredibly well. So I wonder whether the endpoint desire of PhRMA is not necessarily aligned with the endpoint desire that we really want. That's

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a little bit -- it's an unpleasant perspective on the industry, but are there -- somebody wants to make money.

DR. BOUNTRA: Allan, of course, you're always right. And the challenge we've got in this game is that most of the targets, the new targets, that we work on, we take them into phase 2, and 9 times out of 10, they fail. And then the 1 in 10 that we think works in phase 2, we take them into phase 3, and 6 times out of 10, they fail. They don't replicate in phase 3. And that's, of course, where the company spends an absolute fortune.

Then, of course, there have been many examples where we've taken stuff to the market and we've ended up pulling it because of side effects, et cetera.

This is just such a difficult game. I believe that industry has got some phenomenal strengths. They've got strengths in areas that require scale and infrastructure, so high throughput screening, lead optimization, the tox, the regulated, the really big clinical studies.

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But in academia, there are many things that are easier to do. I didn't say we are better. I said it's easier to do. In academia, it's easier to work with clinicians. It's easier to access patient material. It's easier to work with patient groups. It's easier to access patient databases.

So what we're trying to do is to bring together the strengths of both, but we're trying to underpin it with public funding. We're trying to bring in the regulators. We're trying to bring in some of the patient organizations, et cetera, et cetera, because frankly, this is the only way we're going to do it.

I shared an example at Frank's meeting last week in the Alzheimer's space, and I'll share it again. In Alzheimer's, there's one target we've been working on for 31 years, amyloid. The idea is this protein accumulates in the brain. As a consequence, neurons die. You get dementia, cognitive decline, et cetera.

Thirty-one years, we've been working on that target. If you added how much money we've spent in

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academia, in biotech, and in PhRMA, the estimate is more than $30 billion. After 31 years and $30 billion, we still don't know the answer. Everybody is saying which form of amyloid, where in the brain do you need to lower it, how much do you need to lower it by, and if you do lower it, is it going to have any effect, and when do you lower it?

We can't carry on like this. Alzheimer's in one disease -- FYI, in the past decade, there's been 12 failed phase 3 clinical studies in Alzheimer's. And many of those studies cost several hundred million dollars. One of the Lilly studies cost $750 million. And this is one disease, if we don't come up with a treatment in the next 20 years, it's going to financially cripple many societies.

DR. BACKONJA: Misha Backonja from Salt Lake City. You really make an excellent case for a way to look at this problem in new ways. But the onus is really on phase 1 and phase 2 trials. Spending the past couple years in that arena, I realize that that phase is really way beyond the times. There's so much data that's not collected. It's not paid

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attention to.

So really to follow your model, the whole idea of phase 1 and phase 2 has to be reconsidered and redesigned and revised because, as I said, for this to be a source of information and to help facilitate translation, the data really needs to be captured rather than kind of shunted aside, because it's all down to this kind of mad rush to cross that as fast as you can, really missing the opportunities.

DR. BOUNTRA: And I totally agree. Many of my sort of clinical academic friends, they're sitting on new ways of stratifying patients or new biomarkers, but they need to test them. And they need a molecule to test them, and they're willing to do this. All they care about is just testing their hypotheses. They're not worried about making money, et cetera. So I think it's a beautiful intersection, I agree.

Thank you, Bill. Thank you very much.

(Applause.)

DR. MAIXNER: Thank you, Chas.

Our next speaker is Dr. Jordana Bell from

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Kings College, and she will speak to us today on DNA methylation studies in twins.

She is a senior lecturer in the Department of Twin Studies. Maybe it's totally unique to Kings. I don't know if there are other departments across the world, but an amazing opportunity to dig deeply into genetic factors by having a whole unit that focuses on twin studies.

So, Jordana, we're very interested to hear what you have to say.

Presentation – Jordana Bell

DR. BELL: Great. I'd like to thank the organizers for inviting me to speak here.

So I'm coming to this from a slightly different angle. My focus is very much DNA methylation and other epigenetic processes in twins, and one of the studies that we did was to apply these methods to pain.

So first, disclosure. The bulk of the work that I'm going to present today has recently been published in this study. And on the far side are the funding bodies that contributed to this, so Pfizer in

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part funded some of this work. In particular, Pfizer paid for methylation sequencing in a subset of the individuals that went into the final paper.

So I'm interested in looking at DNA methylation variation in human populations and looking at this from two perspectives. On the one hand side, we have drivers of methylation variability itself, and on the other side, we have potential consequences of that variation to complex phenotypes.

So first, what potentially causes variation in methylation? So if we consider the DNA methylation landscape across the genome, we know that we can identify very different subsets of regions.

So one subset of regions, which are so far a relatively small subset of regions, we know that genes or changes in DNA sequences strongly associates with changes in DNA methylation profiles. So these particular regions show very heritable patterns of DNA methylation. And particular variants usually very close to the methylation site itself, these genetic variants strongly drive methylation variability. So this is one set of regions.

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Another set of regions, we know that environmental exposure drives variability in methylation. And this is currently a very much hot topic in research in many groups internationally, and the type of factors that people are looking at include diet, smoking, alcohol, chemical pollutants, and many others.

In my opinion, the strongest evidence that there is to date is for tobacco smoking. So I think that 20 regions genome-wide have been identified where methylation profiles in current smokers are very different from those in nonsmokers. And interestingly, ex-smokers show an attenuation, so they fall somewhere in between. And some studies show that it depends on when exactly they quit smoking.

Another interesting link to this is that some of these changes you also see in newborns from mothers who smoke during pregnancy. So that's a second set of regions where environmental exposure leads to methylation variability.

Then there's a third set of regions that

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change presumably stochastically over time, and research is ongoing in trying to exactly characterize these changes.

Of course, these are the interesting regions. There's also a whole other set of regions that don't change over time, but we predominantly focus on the variable regions.

The other aspect is how does methylation link to complex phenotypes. So one simplistic hypothesis here would be to say that methylation regulates gene expression. So presumably especially in promoter regions, changes in methylation would drive changes in gene expression, which would lead to a complex phenotype, but this is a little too simplistic.

So many of the changes that have been identified in disease fall not just in promoter regions but in enhancers and intergenic regions, and gene body regions, which presumably also link to gene expression regulation. But it's also very much possible that a complex disease would change the cellular environment, which would then drive changes in methylation. So in that respect, methylation

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would be a marker of disease rather than a cause of disease.

So I'm talking about twins. How do twins fit into this? So one way would be to think of using twin studies to understand more about the causes of methylation variability, so applying the classical twin study. So here methylation would be the phenotype, and you would compare methylation profiles in dizygotic or dizygotic twins who on average share 50 percent of their genetic variants to monozygotic twins, who are genetically identical, to establish what proportion of methylation variation is attributed to genetic factors versus environment.

I'm not really going to talk about this today. What I'm going to talk about is discordant genetically identical twins.

So in this aspect, again, you can use twins who are discordant for environment exposures such as smoking to identify methylation markers of environment exposure. But the focus of my talk will be on disease discordant monozygotic twins. So one potential mechanism that we're seeking to identify

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here is environmental exposure driving changes in methylation, which then translates to changes in our complex disease.

So discordant monozygotic twins, you would think are very rare because monozygotic twins themselves are relatively rare events. So twinning, about one in 250 individuals has an identical twin. But actually, if you look at some rates, it turns out that concordance rates for some typical common diseases are not as high as you might expect.

So here's some estimates from published work as well as our own cohort. In fact, discordance for some of these common traits ranges between 40 to 80 percent, so there's quite a lot of variability. And you do identify discordant monozygotic twins.

So one could say that disease discordant monozygotic twins is an ideal study design for identifying non-genetic risk factors in disease because compared to unrelated individuals, they are controlled for the same germ line genetic variants. Somatic variants are a different story.

They have the same or very similar in utero

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environment, and they're controlled for age, sex and cohort effects. And it's the environment that really varies, so similar early life but different adult life environment.

So to date, people have used this design to identify environmental risk factors in disease, for example, alcohol and bone mineral density. And currently, many studies are using this design to identify molecular mechanisms and disease such as somatic DNA mutations and epigenetic changes.

So I'm going to talk about the epigenetic changes in a discordant twin design, but before I get to that, just a few slides on doing an epigenome-wide association study.

So many groups are currently performing epigenome-wide association studies to identify DNA methylation changes and disease. Typically most would use a case control design using unrelated individuals. There are also a bunch of groups using the discordant MZ pair design. And I guess in addition to the potential confounders that I mentioned before, one potential benefit of comparing

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these different types of design is that in the unrelated case control design, you might be able to identify genetic changes that associate with the phenotype that are mediated through DNA methylation variation, which would be of interest to dissecting the molecular mechanism of disease, while with the discordant twin design as discussed, presumably you're looking for environmentally driven changes.

But both really cannot tell you -- they cannot establish whether this is a cause or a consequence completely. So ideally, what you really want is to have some longitudinal samples where you can look at DNA methylation over time prior to disease onset and post disease onset.

Here's some of the issues that one needs to keep in mind when conducting an EWAS study. So clearly, EWAS study design and power and sample size, so most of the DNA methylation changes link to a complex disease that have been identified to date are relatively modest. So clearly, you need large samples.

Are you in the right cell and tissue for the

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phenotype of interest? Time, in my opinion, as I've already discussed, is really the key thing to consider here. And this relates to the question is this a cause or a consequence of disease; of course, DNA methylation assay, and as always, it's a question of coverage versus sensitivity.

Once you do identify something, I think it's important to follow up using functional genomic data, in particular expression profiles, and also to be able to validate and replicate the finding.

So going on to these specific examples, so we did an epigenome-wide association scan for pain. What we started off with, this was a pilot study. We started off with 25 monozygotic twin pairs where one twin was classified as pain sensitive, and the other one was pain insensitive. In each one of these 50 individuals, we characterized their DNA methylation profiles using MeDIP sequencing, and I'll describe that in a minute.

So the approach was to take these 50 twins, quantify their methylation across the genome, and then compare at each region of the genome the

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profiles across the 25 MZ twin pairs to identify differentially methylated regions that showed an association with pain, or from now on, I'll refer to these as pain DMRs.

So just a few slides on the methods. The twins come from the TwinsUK Cohort, which is based at the Department of Twin Research at Kings College. It was established in '92 by Tim Spector. And currently we have about 12,000 volunteer adult twins in the cohort. And approximately 6,000 of these have genotype data as well as multiple omics.

The phenotype -- and I think this was very well covered by previous speaker -- is on pain, so clearly, members of multiple systems are likely to play a significant role in pain sensitivity. In our sample, we looked at heat pain sensitivity using the HPST.

So briefly, a probe was attached to the forearm of each individual, and the temperature of the probe increased. And at the point at which the subject felt that the pain was unbearable, they pressed the button, and the experiment stopped. So

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that temperature is the phenotype.

That particular test has been shown to be heritable, and candidate genes exist. And the lower plot, you can see the temperature profiles in our twins. So our twins were discordant by at least 2 degrees Centigrade.

The methylation data was obtained by MeDIP sequencing. So here what you do is you would take the DNA sonicated, add an antibody to the 5-methyl C, extract the methylated chunks of the genome, sequence them, and quantify them into a methylation read profile across a genomic region. And then you would compare this across the two twins.

So briefly, we took this approach, and at the end of the day using a number of normalization techniques for strand bias and so on, we ended up with approximately 5 million overlapping 1kb regions across the genome. So at each one of these regions, we looked at the DNA methylation profiles across the 50 twins.

So here's a very large summary plot of what we saw, and what it's meant to show you is three

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levels of information. So first, we looked at levels of DNA methylation at a very crude estimate, looking at 1 megabase bins, and we saw that these were very similar with the exception of central meric [ph] regions, which is what we expected to see.

Second in red, we looked at the similarity between twins versus unrelated individuals, and we saw some variability in this across the genome. And third, we looked at allele-specific methylation, so cases where only one allele was methylated but the other one was not. So we were only able to assess this at only 100 SNPs across the genome.

So another way to look at these results is shown here. So overall in terms of levels of DNA methylation, we saw what we expected to see. So promoters and CpG islands tended to show a reduced level of DNA methylation relative to the rest of the genome.

Second, monozygotic twins are more similar in DNA methylation profiles compared to unrelated pairs of individuals. And if you consider this allele-specific methylation, then the monozygotic

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twins are highly concordant for ASM skew or which allele is methylated.

third, this relates to the assays of DNA methylation. So we used MeDIP sequencing, which has very good coverage, but it's not very sensitive because you can only go down to 200 base pairs. So you cannot get the single base pair prediction of methylation. So when we compare our data to a different array which is single base pair array but has relatively poor coverage, then we see good correlation across the two.

So then moving on to comparing methylation to pain, when we compare methylation to pain across the entire data set of individuals, here are the results. So this plot shows the Q-Q plot of the observed versus expected data. In black are the observed results, and the shaded area in gray is what we expect to see under the null hypothesis that pain does not associate with methylation. And this was done by permutation.

So relatively modest enrichment of signal basically. And overall, there was one genome-wide

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significant region. And this region did not fall in a gene.

So as I said, once we did this, we know that the majority or a large proportion of the genome is not variable in terms of DNA methylation patterns. So what we did then is to only look at the proportion of the genome that falls in interesting functional genomic categories. And specifically, we looked at CpG islands. We looked at the regions right at the borders of CpG islands, which are called CpG island shores that others have shown to be functionally interesting, promoters and other regions. And what we saw was a weak enrichment when we look particularly in CpG island shores.

So then we went on, and we looked at what hits are we getting specifically, just looking in the top 100 ranked hits across all different analyses. And this is what we get.

So genome-wide, looking at 5 million tests, when we examined the top 100 hits, we saw some interesting genes in that list. And importantly, TRPA1 was ranked top 44th. When we then look at CpG

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island shores, TRPA1 is now ranked top fourth, and we also see V1 and V3 in that list of top 100 genes. This is only 50,000 tests genome-wide. And then when we restrict it to CpG islands, we see some more interesting genes.

If you look at gene ontology analysis of these sets of genes, then you do come up with terms that are relevant to pain.

So at this point, we stopped this pilot study, and we decided that the results we were getting were interesting. And we decided to spend some more money and extend the study to a larger study design.

So here's the final study design. So what I've told you so far is the discovery stage in monozygotic twins. We then went on and profiled another 50 unrelated individuals where the HPST scores were just representative of the cohort.

We did the same type of analysis that I showed you so far. We then meta-analyzed the findings, took the top hits from the meta-analysis, and went on to validate them.

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So the validation included two analyses which basically validated the methylation signal we were getting using a different methylation assay based on bisulfite conversions, so either bisulfite sequencing or this Illumina 450k array that I mentioned. And we also did a longitudinal follow-up where we took 33 of the original twins, and we recalled them back in clinic three years later, and we looked at their HPST and methylation profiles.

So from the meta-analysis across the 100 combined individuals, what do we get? We were very excited to see that the top region genome-wide was in TRPA1. And in addition to TRPA1, there were altogether nine regions that surpassed statistical significance at a permutation based, false discovery rate of 5 percent.

These nine regions actually fall in eight unique regions. ST6GalNAc3 appears twice. It's a contiguous DMR. So we thought that TRPA1 and potentially some other genes in this list where quite interesting. We focused on TRPA1.

I think many of you know TRPA1 much better

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than I do, but it's an ion channel, and it has been reported to be involved in the detection of pain for cold pain and chemical irritants.

Here is the structure of the gene in humans. So this is the promoter. There's a CpG island overlapping the promoter, and the pain DMR that we detect is right at the border of the CpG island.

What we see in our data, here's the original finding, is that as methylation increases, you become sensitive to pain. So the temperature decreases or you're detecting pain at a lower temperature. And this finding in the MeDIP seq data is validated in the bisulfite sequencing of this region.

So then as a functional follow-up, we ideally would like to look at methylation in neurons as well as expression in neurons, but we did not have access to such data. So what we did next was to look at gene expression profile that we could get access to as well as HPST. So we had gene expression estimates from 340 twins from skin, and this is what we used to follow up.

What we see in skin is, as expected, a

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slightly increased gene expression with pain sensitivity, which would suggest that there is a regulatory DNA methylation region in the CpG island shore next to the TRPA1 promoter where increased methylation suppresses gene expression. And this is linked to heat pain sensitivity.

Going back to the meta-analysis results, so here are the top 9 findings. We then wanted to see, because we used a different types of EWAS study design, to try and understand what are the driving causes of DNA methylation variability at these regions. So we went back to this idea of using different types of designs to try and differentiate between potential genetically-driven effects that are mediated by methylation and potential environmentally-driven effects. And, of course, these could also be markers of pain rather than a cause of pain.

So with this in mind, here are the top 9 results. And for each one of these, we did a comparison between genetic variability and methylation. And of the top 9, two regions, so the

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olfactory receptor and the ST6GalNAc3 DMR showed strong evidence that there is a genetic variant associated with a methylation change. And all of them showed very strong negative correlation in the inter-twin analysis comparing the sensitive twin to the insensitive twin.

The other follow-up was to do a longitudinal analysis. So as I said before, we recalled 33 of these twins back in clinic, and we looked at the change in the phenotype and methylation. So here in gray, you can see the change in phenotype from the original HPST score to the new one. So with a few exceptions of the extremes, the majority of the sample did not show large changes in the phenotype.

The heat map shows the change in the DNA methylation profiles, and this is coded as follows: So red is a gain in methylation over three years, and in blue is loss in methylation over three years. And these are the top 100 regions from the genome-wide meta-analysis.

So one thing that is kind of obvious is that there is a subset of genomic regions that are very

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variable over time, so they gain and lose methylation. And then there is another two subsets of regions that are relatively stable over time, and a small subset of regions shows a slight loss in methylation over time.

Where do the nine DMRs fall in this? So nearly all of them -- I mean, it depends what you call variability and what is not variability, but most of them show a very stable pattern over time. So we conclude that the top 9 results are relatively stable over time.

Another issue is, of course, are we in the right cell and tissue, and to a certain level, this depends on what does right mean. So are you talking about a marker, or are you talking about trying to understand the mechanism of detecting pain and being sensitive to pain?

So with respect to the latter question, we are looking at whole blood, so there are several issues with this. One is that blood is a heterogeneous collection of cells, and this could skew the DNA methylation profiles that we're seeing.

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So what we did is for the top 9 regions, we corrected the results because in a subset of individuals, we had facts data from lymphocytes, neutrophils, monocytes and eosinophils. And we saw that generally the results were very similar within one region, on chromosome 4, the top 6 region showed an associated with lymphocyte count.

The other issue was okay, well, blood is clearly perhaps not the best tissue for pain sensitivity, and so we looked at data across different tissues. And we had access to a study of two female donors for whom we had blood and methylation profiles from seven brain regions, and we compared demethylation profiles across these different regions. And we saw that five of the top pain DMRs showed very similar patterns. But in general, that data was a little bit difficult to interpret because there's quite a lot of variability. For example, cerebellum and brain was as different as blood and brain.

So in conclusion, that study used a discordant monozygotic twin design to assess the

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effect of methylation on pain. We identified some differentially methylated regions that associated with pain, and these validated using a different technique. We combined the twin design with an unrelated design to try and understand more about the mechanisms driving the methylation variation and the epigenetic associations.

I think in general, epigenetics can complement and extend genetic studies to help us understand molecular mechanisms in disease. And this was a pilot study of a larger project, the EpiTwin Project in which we're profiling methylation of 5,000 twins in blood using predominantly methylation sequencing as well as array data.

Here are some of the traits that we're looking at the moment. In red are the ones that we've completed, and for each of these, we have profiled methylation predominantly focusing on disease in discordant monozygotic twins. We're performing epigenome-wide association and exploring the differentially methylated regions in disease.

Thank you.

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(Applause.)

DR. MAIXNER: Questions?

DR. PORRECA: A very, very interesting study. I was thinking about the identifications of the differences in methylation in the different genes that you were finding and your link of those to pain. And I guess this is -- I mean, what if the twins have other changes going on that are still linked to that particular gene, such as TRPA1, for example?

That is not necessarily -- that is identified by the pain sensitivity, but it's actually not linked to pain. I guess I'm not being very clear on that question. But how do you actually link it to that pain sensitivity I guess is what I'm trying to ask.

DR. BELL: So I guess another way of saying that is you're saying is there a confounder that is -- I mean, we tried to --

(Laughter.)

DR. BELL: We tried to control for potential confounders as much as we could. And so age, BMI, these are all female twins. Smoking status and so on, we explored all of these. We also explored cell

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composition because we thought that might influence the effects. And as much as we could, none of these factors confound the finding. But of course, there are many other confounders for which we simply don't have data, especially environmental exposures is one. And characterizing the twins very well is very difficult to do that.

I mean, for TRPA1, it was quite interesting that we not only see it in the twin sample but also in an unrelated sample that was completely independent. So that gives me confidence to say there's likely something there. Of course, it could be confounded, but until we have better environmental data, I can't really answer that question.

DR. BOUNTRA: Jordana, could I ask Frank's question in a slightly different way? Do you believe that TRPA1 is more likely now to be a therapeutic target?

DR. BELL: That's a difficult question. I believe that there's a methylation change that associates with a measure of pain. So I believe it could be a marker of pain. I think our study on its

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own cannot at all say whether it's a potentially causing pain or a consequence of pain. I think a follow-up needs to be done with the proper experiment in a model organism presumably to address causality.

DR. BASBAUM: This is again a follow-up to Frank's attempts.

(Laughter.)

DR. BASBAUM: So A1 has been implicated in a variety of respiratory conditions, asthma, for example. Do you know anything about the status of the twins with respect to asthma?

DR. BELL: We have phenotype data on that. I haven't looked in the sample, but we could look at TRPA1 on asthma. We have data on those. I haven't looked at this.

DR. BASBAUM: Thanks.

DR. MAIXNER: Thank you very much.

(Applause.)

DR. BASBAUM: Try to be lighthearted around lunchtime. So I get to introduce Bill. And I didn't think I needed to do any reading to know about him because I've known Bill for so long. But I did

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learn -- I mean, I know he got his PhD at University of Iowa in pharmacology and his dental degree there, and he went to NIH.

This the part I had to write down. He is the Mary Lily Kenan Flagler Bingham Distinguished Professor -- that is very impressive -- at the University of North Carolina. And he is also director of the neurosensory disorders group in the Department of Endodontics.

A few years ago, he turned to genetics and has done some spectacular work specifically concerning pain, and we're going to hear about that today.

Presentation – William Maixner

DR. MAIXNER: Thank you, Allan.

I want to thank the organizers for the opportunity to present some of our actually historical and some ongoing work that we've been doing with many collaborators across the country.

Actually, disclosure, I should disclose I'm a cofounder and equity shareholder in a small biotech company called Algynomics, which focuses on

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diagnostics and therapeutics.

So today what I'd like to do is really cover two general areas, a heuristic model, which many in this room have seen, but each time I look at it, I see something different. It is a mosaic, a model that is a mosaic, which can become a kaleidoscope as one works through it. And then I'd like to show a single example of a translational approach that our problem is using to identify putative targets in the pain area, and based on that identification, to go through a series of translational steps, which we feel are important in validating and potentially bringing a drug to phase 2.

We call it the translational clock, which was in the title, and we'll discuss and I'll present our approach to the translational clock, which is essentially a reverse translation going from human genetic association studies, identification of potential pathways, identifying key elements of those pathways that are potentially druggable, and then to show proof of concept or proof of principle using that approach, with the desire of really identifying

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both novel targets and also trying to speed the development and identification and validation of target.

So first, let's start with the conceptual model. Many have seen this before. This is a model that was developed several years ago, published back in 2006 by my colleague Dr. Luda Diatchenko, and many of us in the field, myself, Roger Fillingim, several were involved in putting this together.

It's really not a quantum leap. This is really an attempt to begin to integrate several features that we think are important in -- in fact, not what we think, the field thinks is important in contributing to the onset and maintenance of a variety of complex persistent pain conditions. And many of these features, we've already spoken about this morning.

So there's much literature, much research that shows that both psychological factors and factors involved in pain transmission, pain modulation, interact in a way that produces temporally dependent, temporally dynamic changes in

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the signs and symptoms that a patient expresses in the clinic.

So if we look at these black arrows underlying the ovals of psychological distress and pain amplification, there's several intermediate phenotypes or endophenotypes, which can be assessed in a given patient and in a population of patients that expresses signs and symptoms that are collected in the clinic.

These intermediate phenotypes are temporally dependent, and they depend largely on environmental exposure, the types of exposures that the individual experiences during their lifetime. And several of those exposures, at least the nature of those exposures, are shown on the Y axis as various variables, which are involved in inducing methylation, histone changes, so physical trauma, abuse, infection, psychological life stressors, and chemical events such as exposure to cigarettes.

We in OPPERA just recently published that the odds of being a chronic pain case versus a control non-pain case is about 2.4 if you have a history of

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smoking at least 100 cigarettes in your lifetime. So this goes well with some of the previous statements made that exposure to certain chemicals can produce long-term changes in the organism that not only is detrimental in the short term but produces long-term changes that put them at risk for developing a variety of conditions, including chronic pain.

On the X axis is merely a schematic of several genes, multiple genes. As noted this morning, I think few of us believe that there is a pain gene. There are many genes that contribute to complex and common chronic pain conditions. And polymorphisms in each of several genes contribute differentially to produce this temporally dependent, very dynamic changes in signs and symptoms and susceptibility to a variety of chronic pain conditions.

So we see that environmental exposures through epigenetic mechanisms that we've spoken about today can change the expression patterns of multiple genes, each which have potentially small effects on the intermediate phenotypes and which are temporally

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changing like the city lights that Laura spoke about earlier.

One important feature to remember that we haven't really gone in detail is that chronic pain patients, patients with fibromyalgia, TMD, vulvodynia, lower back pain, as well as individuals who are going to experience postoperative pain, they're not a homogenous population.

There's great heterogeneity in the population, and one of the tricks that we have, as Chas mentioned, is to somehow identify stratification procedures that permit us to begin to differentiate individuals into subgroups based on phenotypes, these intermediate phenotypes, and then based on those phenotypic clusters, the molecular profiles that underlie those clusters.

Unfortunately, I won't have time to show you some of the approaches that we've been taking in some of our large cohort studies, but this is a very important trend in the field and now has begun to respect the pleiotropic manifestations that these individuals express with and try to capture those

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manifestations by cluster analysis in particular; and then to look at the specific molecular profiles, looking for specific targets that may underlie these specific clusters.

So I'd like to give an example of how we use the translational research clock, which we recently published in Nature Rheumatology. And again, this is a reverse translational process where we start with the genetic association studies, confirm functionality using cellular systems, then move to animal models to show that the target or the putative target has biological relevance. And then if we have an agent in hand, a proof of concept trial leading to some validity of the target.

I will say that we've evolved this translational clock several times. The example I'm going to show relates to catecholamine-o-methyltransferase and the identification of a beta 2, beta 3 pathway, and a drug that's been developed in this area. We have identified the EGFR pathways, a very important pathway in many of our cohorts, and have a paper under review in Cell now that I think

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will help demonstrate that using this reverse methodology, the EGFR pathway, and various inhibitors of that pathway may prove to be very potent analgesics in both the chronic pain and acute pain.

We also have identified new splice variants in the mu opioid receptor using this approach. And then finally, the angiotensin 2 pathway, we observed back in about 2006 and are still working on some additional data sets related to angiotensin 2.

So there are many other examples, but those are four. And I'll present in some detail the one that is most mature in this translational experience.

So starting with the genetic association studies, Luda and group identified in 2005 genetic variants in the gene that codes for catecholamine-o-methyltransferase as an important genetic variable that's associated with human pain sensitivity and the risk of developing a condition known as TMD.

Since this study, there have been literally a couple of hundred studies now that have validated COMT as an important enzyme in the pain system and that it is polymorphisms in COMT, especially those

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polymorphisms which lead to a reduced expression of COMT. And potentially high catecholamine burdens in the organism lead to increased pain sensitivity and greater likelihood of developing a variety of chronic pain conditions.

So this is an example of one gene that has pleiotropic effects across several of the domains I showed in the heuristic model and may contribute to a variety of pain states.

Now, downstream from COMT are obviously, adrenergic receptors, which are sensitive to catecholamines. And we've published that polymorphisms in the beta 2 adrenergic receptor are associated with both psychological profiles, psychological distress, alternations in blood pressure regulation, and increased risk of TMD as well.

We have similar examples for the alpha-1A and beta 3 adrenergic receptor as well. So starting with COMT as, if you will, a hub enzyme which is controlling catecholamines that influence a variety of processes, which I'll review in a moment, we've

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been able to identify a putative target, potentially the beta 2 adrenergic receptor or the beta adrenergic pathway, which may be important in modifying and modulating pain and risk for a variety of conditions.

I'll just note that in the genetic studies that were conducted by Luda and group, we identified three SNP combinations, three haplotypes, that were associated with differential pain sensitivity in the human population. One haplotype we called low pain sensitive because it was associated with reduced pain sensitivity, where we phenotyped across five different measures, thermal, mechanical, ischemic measures. And using a combined normalized Z score associating the haplotype with specific measures of pain sensitivity, we identified a low pain sensitive, an average pain sensitive, and a high pain sensitive grouping of haplotypes, which explained about 80 percent of the population that was examined in this study.

So we had an initial association study pointing towards the COMT pathway and potentially beta 2 adrenergic pathway, but then we wanted to ask

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the question as to whether there's some functional relationship between these polymorphisms, these haplotypes, and a biological pathway that was functional in nature and not merely an association.

So we published then shortly thereafter the findings that alteration in transcript expression associated with these three haplotypes led to different levels of protein expression. So in panel A is basically the COMT activity based on whether cells were transfected with the LPS, APS, or HPS haplotype. And you can see cells transfected with the HPS haplotype showed very little enzymatic activity.

In panel B, which is partially covered, if you look at the transcript levels generated by these three cell lines that were transfected, you see that there was surprisingly no change in transcript level. And then if you look at transcript survival time in panel G, what you see is actually the HPS transcript, which again codes for the lowest amount of protein, had the greatest survival time in the group assay, a paradox, low protein levels, transcript levels the

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same and survival time greatest for the transcript that's actually producing the least amount of protein.

We were able to go on and show, using a variety of informatic tools and site-directed mutagenesis procedures, that in fact what is happening is that individuals who harbor the HPS haplotype code for a transcript which has much greater free energy for uncoiling so that translation into protein is greatly impacted by the amount of free energy that's required at the transcript level to induce into produce translation.

In contrast, those harboring the LPS haplotype, the amount of free energy for uncoiling and translation is much less, so it represents a relatively novel and an important mechanism by which we can influence translation into protein that doesn't involve promoter site.

Whether they're epigenetic phenomenon that are involved in transcript production outside of promoter site, I think is an important question that may also influence some of these patterns that

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develop.

So in 2006, we demonstrated that we, in fact, have a functional consequence of these polymorphisms, and then that then begged the question as to whether we could show some type of an effect in the whole organism. In various types of in vivo models are we able to show that when we suppress COMT activity, are we able to create a human phenotype, which mimics the human condition?

Andy Nackley, who at that time was a postdoctoral fellow with Luda and myself, conducted a series of studies where she showed that using different COMT inhibitors in rodents, that she could, in fact, induce a human phenotype associated with increased thermal pain sensitivity, mechanical pain sensitivity, changes in bowel function, changes in GI function beyond bowel function, and changes in locomotor activities suggestive of a very complex phenotype that was being produced by this inhibition of COMT activity.

When we tried to examine what types of downstream effectors may be involved with this,

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looking at alpha, beta, dopaminergic antagonists, we were able to demonstrate that the use of relatively selective beta 2 and beta 3 receptor antagonists were able to restore or rescue that phenotype in the rat.

So this just shows data on the upper left panel on mechanical threshold following the administration of a COMT inhibitor, in orange, the mechanical allodynia that develops. And this can be rescued -- the behavior can be rescued with a beta 2 selective and a relatively selective beta 3 antagonist given in combination. If we give them individually, we restore about 50 percent of the phenotype. And we see this for both mechanical threshold and mechanical hyperalgesia.

The lower panel shows the effects on thermal pain. We see a thermal hyperalgesia that is expressed with the COMT inhibitor, and again, rescued with the combined combination of a beta 2, beta 3.

Coincident with these behaviors, we also see elevation in circulating cytokines, IL-6, TNF-alpha and IL-1 beta, and those can be restored with beta 2, beta 3 antagonist.

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So we have in a short period of time, in two years, identified a putative target, something that would block beta 2, beta 3 and that would, in fact, potentially be useful target to assess in a clinical trial.

So in 2010, we conducted a small clinical trial on 40 individuals, and I'll show in just a minute. And we showed that the nonselective beta antagonist, propranolol, which blocks beta 1, beta 2 and beta 3 somewhat weakly, is able to reverse and to ameliorate the signs and symptoms of TMD, a very common chronic musculoskeletal pain condition.

In this study, 40 individuals were recruited with chronic TMD. A two-period, double blind, placebo controlled randomized crossover design was used. And a very low dose of propranolol was used for seven days, wash out, and then seven days with either propranolol or placebo. So there was randomization by treatment arm as well.

This data just shows the pain relieving effects of propranolol versus placebo. In this study, we used what we called the pain relief index,

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which was essentially the average pain at a given epoch in time multiplied by the percentage of time during the waking day that the individual experiences pain.

So this is a way of integrating the severity of pain over time. So it's an average pain multiplied by percentage of time during the waking day that the individual experiences pain. And we find this to be a very sensitive index for pain assessment and pain relief because it respects the temporal characteristics of change in pain.

On the far left independent of genotype, you see the pain relief index, which represents about a 50 percent reduction relative to their clinical pain at baseline. And if we stratify these individuals based on genotype, a COMT genotype, individuals who had zero copies of the low pain sensitive haplotype -- remember, this haplotype is coding for a lot of enzyme, a lot of COMT. So we're really chewing up catecholamines in these individuals, and so they have very little, we think, catecholamine burden.

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These individuals who have zero copies of LPS have a lot of catecholamine burden because they have very low levels of COMT. They're expressing high catecholamines because they're not able to metabolize it as well. And because of that, we believe propranolol is more effective in this subpopulation.

Individuals who have two copies of LPS are the population that can really chew up catecholamines and are in essence free of the catecholamine burden, and we believe should be less sensitive to the effects of propranolol. And about two-thirds of the population carry either zero or one copy of the LPS haplotype.

So this really caused us a lot of thought as we looked at these studies, which occurred in a relatively short period of time, as to whether beta adrenergic receptors could play a role in pain or pain processing and represent potentially new drug target. And there's considerable evidence in the literature that beta adrenergic pathways play a role in pain, involved especially the beta 2 receptor, is associated with hyperalgesic responses.

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John Levine and coworkers and many others have shown that the application of beta 2 receptor agonists, both centrally and peripherally, can induce hyperalgesic states involved in human models of arthritis. High dose propranolol has been shown to be effective in treating rheumatoid arthritis. A number of inflammatory cytokines and proteins like NGF are regulated by beta 2 receptors.

We know that many ROS products released in the periphery and the CNS that are able to produce pro-pain and pro-inflammatory states can be diminished with beta adrenergic blockade.

We know that beta 2 receptors activate microglia when agonists are present. Beta 2 receptors stimulate classic cascades involved in pain pathways. And EGFR, which we are showing to be an important pathway in pain, is also regulated by beta adrenergic receptors. And finally, there's a considerable work that beta adrenergic receptors contribute, as David has shown, to opioid-induced hyperalgesia, tolerance, and seem to be also involved in opioid sparing in the postoperative environment.

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So there are a number of attractive features about the beta adrenergic receptor and agonists that would suggest that it's worth pursuing the identification of new chemical entities. And I can say that our program over a two-year period spent considerable time trying to identify a new chemical entity that would be superior to propranolol as a model therapy, as an analgesic, and potentially as an agent that is able to produce opioid sparing.

Our search resulted in a kissing cousin of propranolol called bupranolol, and bupranolol has a number of very unique features. If you look at its ability to block beta 1, beta 2, beta 3 receptors and HEK cells that are overexpressing a specific receptor, you see in panel A the dose response to the agonist isoproterenol.

Using the EC50 for each of these, we then were able to show that the r-enantiomer of bupranolol is able to produce a new dose-dependent suppression of beta 1 activities. It also has very nice beta 2 receptor activities, antagonist activity, and is able to block beta 3 receptors as well. And it has a

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number of pharmacodynamic properties that appear to be superior to propranolol.

Though I don't show it, propranolol actually is a fairly effective agonist of the beta 3 receptors at low to moderate doses, and the r-enantiomer of bupranolol fails to show that agonist behavior.

If we look at some of the pharmacodynamic properties of these agents, S bupranolol and R bupranolol versus propranolol, we find that the r-enantiomer of bupranolol is able to produce nice dose-dependent reductions in the formalin tests. Both the early phase and late phase, in inflammatory models, caragenin model, the r-enantiomer of bupranolol is able to rescue the inflammatory state induced by caragenin.

This just shows the relative potency of the r-enantiomer, s-enantiomer of bupranolol versus propranolol. And R appears to have a nice profile relative to propranolol. Neuropathic pain, the same story, that the r-enantiomer seems quite able to reverse a neuropathic pain state as well.

If we look at sedation using rotarod ataxia,

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you see that both enantiomers of bupranolol are relatively free of ataxia at 60 milligrams while propranolol is really knocking them off the rod. And if you look at the therapeutic index, you see that both the R and S show superiority to propranolol for therapeutic index as related to lethality in mice.

These behavioral data were collected by Loren Martin and Jeff Mogil at McGill.

So finally, what I'd like to do is just make a few comments about future direction and the translational research clock. I think this is one example whereby one can reverse engineer, if you will, from genetic variables back to biological plausibility and identify either new targets that can be druggable by existing agents that can be pulled off the shelf, repurposed, or the creation through synthesis of new chemical moieties.

I think this is one of several future directions whereby target identification and I think facilitation to proof of concept in a phase 2A is expedited in a substantial manner. So I think this is one of many ways to the future, and I think the

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application and the collection of very large cohorts and stratification procedures will help in the identification of large subpopulations in the pain field that will benefit from this type of approach in identifying putative targets that can rapidly be validated in animal models and then moving forward clinically.

Finally, I'd like to thank my many, many colleagues who've contributed over the last several years to both conceptual framework in the conduct of these studies and to really the thousands of patients who have contributed to our cohort studies over the years, who've made some of these initial thoughts possible. And I thank you for your attention.

(Applause.)

DR. CLARK: Thank you very much, Bill. I've admired your work for many years.

You mentioned here and key on variants of the catechol methyltransferase gene, and then the other half of that is the receptors for the product. And I wondered if you had looked therefore at protective or influences that would tend to indicate exacerbation

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if you combined your analysis of variants of the beta receptor genes with those of the COMT to see if there are instances where there are particularly bad or particularly favorable outcomes.

DR. MAIXNER: Right.

DR. CLARK: And my second question is actually did you see any -- are you anticipating any problems with bronchospasm and pulmonary problems if you go too far in antagonizing beta 2?

DR. MAIXNER: The latter question first, it could very well be that side effects would be the same as propranolol with respiratory capacity given the cellular data that beta 2 antagonistic properties are about equally potent between propranolol and both the r- and s-enantiomer of bupranolol.

So this could become a factor for selection. Current day thoughts on low dose beta blockers and asthma are changing. Obviously, exercise-evoked asthma is a problem, but the issue of non-exercise-evoked asthma, it appears that beta blockers are being used commonly now. And Cochrane Reviews are supporting the use in asthma without exercise-induced

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components.
Your first question related to basically looking at gene-gene interactions, truly epigenetic relationships. The issue is numbers, having sufficient number of individuals. Anytime that you add an interaction, power becomes a real problem for the analysis.
We have under review a 400-patient clinical trial under review looking at propranolol for a variety of pain conditions but focused primarily on TMD. And in that analysis, we should have sufficient power to begin to look at interactions.
Chas?
DR. BOUNTRA: Bill, your compound seems to be as potent as propranolol beta 1, beta 2 and beta 3 as an antagonist. So why do you think the ataxia profile of the therapeutic index is better?
DR. MAIXNER: I think that propranolol has some very unique properties that are not shared by bupranolol. And a couple of those properties is that actually propranolol in our hands is appearing as a mixed agonist/antagonist while bupranolol is

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appearing to be more of a pure antagonist.
So the issues of potentially effects on cardiac rhythm may be important. There are some unique beta 1 variants that have been identified, and we think that propranolol may be an agonist of that variant, which is involved in arrhythmias.
Finally, propranolol has pretty profound effects on calcium channels, and we do not see literature. We have not looked at this, but it's very likely, we think, that the effects on calcium channels may be a problem for propranolol, which is not shared by bupranolol.
DR. BOUNTRA: And can I ask, do you think big PhRMA would progress this target?
DR. MAIXNER: I don't know. I think a lot of things that for big PhRMA -- impair this. I think that it is definitely ripe for a clinical phase 2 or at least a phase 1 where a proof of concept can be generated.
I think the issue of intellectual property is a big thing for big PhRMA. If they're going to invest money, they want to make sure of two things.

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They want to make sure that there's intellectual property that can be held. The second is big PhRMA likes to see something that's already been in man and to progress it. I think it has a profile, and I think it has potentially a market, not only as monotherapy for chronic pain but potentially as an opioid sparing agent in the postoperative area.
So I think that it's unknown. I think there are a lot of attractive features, and there are some detractions from a big PhRMA perspective. Smaller PhRMA may have interest in this and be willing to go after a 5, 10 or something like that.
DR. BASBAUM: [Inaudible – off microphone.]
(Whereupon, at 12:57 p.m., a luncheon recess was taken.)

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A F T E R N O O N S E S S I O N
(2:02 p.m.)
Q&A and Panel Discussion
DR. BASBAUM: So in addition to the speakers, we have Sharon Hertz, and maybe we should begin by allowing Sharon to introduce herself and tell us.
I'm Sharon Hertz. I'm deputy director for the division of anesthesia, analgesia and addiction products here at FDA.
Do you guys need anything more than that? No? Okay.
DR. BASBAUM: You need to know that everything you're going to say is going to be on tape.
(Laughter.)
DR. BASBAUM: So thanks, everyone, for coming back.
In order to get the ball rolling, I asked everyone on the panel to prepare a question or think of a question for someone else on the panel, and then hopefully, we can evolve from there. So it should be freewheeling. Don't be shy, and let's see where this

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takes us.
We'll begin -- you want to start, Mac? All right.
DR. MCMAHON: There are a few questions I didn't get to ask earlier, and if I can start, one was for Chas.
So your model is I think very refreshing, but I still wondered how you get to nominate or how the -- does the outside world have any chance to affect your nomination procedure, or is that really dealt with by the money that you raise for the whole process?
DR. BOUNTRA: Which process are you thinking, Stephen? In terms of the generating the probes --
DR. MCMAHON: Yes.
DR. BOUNTRA: -- or in terms of taking targets into the clinic?
DR. MCMAHON: Well, both because the concepts, if I understand it, is sort of similar, that you're pursuing one avenue of research, but by making everything public, lots of other people could be incidental bystander gainers from it. So all of

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them really, yes.
DR. BOUNTRA: So in terms of generating the probes, we've made a strategic decision that what we want to do is to generate as many inhibitors for as many epigenetic proteins as quickly as possible. So we've decided to focus on families. We've deliberately decided not to do the HDACs because everybody else is doing the HDACs.
So we're trying to break into new families and show that they're tractable and druggable and so on and so forth, and that's why we picked bromodomains and demethylases. We're working on the HATs. They're proving to be incredibly difficult. We're making a lot of progress with the methyltransferases, et cetera.
In terms of selecting targets to take into the clinic, we will do that with our academic friends but also our friends inside big PhRMA because at the end of the day, if we do show something works in the clinic in phase 2A, we want the PhRMA guys to take it all the way. So those guys are involved.
We're just about to -- and I didn't talk

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about this, this morning, but we're putting in -- as we're chatting this morning over breakfast -- we're trying to get some money out of the Wellcome Trust, and I'm pretty hopeful we'll do it. And what we're going to do is to pick out sort of with a full set of disease experts and ask them to nominate their highest priority targets that have come out of genetic studies or clinical studies. And we will take those targets, and we will do what we're good at, generating the human protein, assay inhibitors, et cetera, et cetera.
Again, we'll make all of those freely available because one of the problems at the moment is in the past 15, 20 years, we've done all these big GWAS studies. We've identified lots of hits, but most academics can't do anything with them because they have no tools. So we're going to try and create those tools to try and exploit that biology. So three bits to your answer.
DR. BASBAUM: Now you get to ask the question unless anybody else wants to comment on that.
DR. BOUNTRA: Okay. I think the thing that

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worries me is -- and I suppose when asking Jordana Frank Porreca's question, is TRPA1 a therapeutic target, that's what worries me day and night, is how do we pick targets that are so fundamental to the disease process that if we modulate them, we will get a therapeutic that will be efficacious and safe. And that's what we're struggling with.
I think coming up with lots of targets is dead easy. And I think of the study that Jordana's done with yourself, Steve, taking monozygotic twins and seeing differences, it's superb. I can't think of a better experiment, but even then, I'm not convinced that TRPA1 is a therapeutic target.
It just goes back to the discussions Frank and I were having last week. I'm sure substance PNK1 is associated with the pain pathway. Lots of cytokines are involved. Amines are involved. Peptides are involved. But at which point do we intervene at to get a therapeutic? And that's what we're struggling with. So I don't know how to answer that.
I thought the talk just before lunch from

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yourself on the beta 1, beta 2, beta 3, I thought that was superb. But I worry there that I don't think PhRMA will take that forward because they will be worried about the effects in asthmatics or COPD patients, and the industry has become so risk averse.

So my question, Allan, is how do we pick that key target? And I don't know.

DR. MCMAHON: I'll just come back on that a second. I mean, one of the points of doing an unbiased screen is that you're unbiased about what you want to see. But nonetheless, when you see a whole lot of hits that don't mean anything to you, you think, well, that's a bit disappointing. Where do I go from here?

So seeing some old friends in that list is, of course, reassuring. It makes you feel that the process is at least appropriate, can pick out things that we know are involved at some level in some of the processes we're interested in.

But I agree with you, that the tough bit is all of these targets that don't mean anything to you, how much time and energy are you going to invest in

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those because it's almost inevitably going to be for an academic at least several years' work to really make an progress against each of them.

DR. PORRECA: Well, just to speak to your point, Steve, so isn't this -- once you see these potential targets that you don't recognize and you don't know what they mean, isn't this where the crowdsourcing concept really would have its impact, where people may be looking at this list with completely divergent concepts of how and why they may be interested in that particular target?

I would think that it can't be an individual effort. It really has to be something that's broad, that it calls upon the creativity of the whole community.

DR. MCMAHON: But it's quite difficult to publish the list as a fishing list, actually. There should be better opportunities to do that, but it's difficult to just say, here's a list of the data, let other people go and mine it. You nearly always have to make some kind of story up around the list.

DR. BASBAUM: Bill?

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DR. MAIXNER: I think one of the actually most difficult aspect of this from my perspective is trying to find the signal, the signal amongst the noise where a given variant or a given pathway may only contribute a small effect to the overall phenotype. And I think there are two areas that need to be developed that will help identify signals.

One is more detailed and substantial phenotyping of populations, populations of interest where the phenotypes are tapping into the pathophysiological processes that contribute to the condition of interest.

So I think the ability to detail in a quantitative manner in several of the domains that I've shown and then taking those data and developing the strata, the subpopulations where you take a very heterogeneous population of, say, fibromyalgia patients, and then through various procedures like cluster analysis, many others, you develop subpopulations, which are more homogenous with respect to their underlying pathophysiology. They're giving these complex presentations.

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So I think one of the first things that needs to be done is population-based studies where the population is well phenotyped and then they are stratified based on those phenotypic portraits and then contrasting those different subpopulations for molecular profiles.

I think that when we start to reduce the heterogeneity, we'll find that a given variant has a much stronger effect size and that we're now finding pathways, not single genes but multiple genes that are contributing to fundamental pathways that are controlling the expressions of the phenotypes in these subpopulations.

So I think one way to increase signal production is to decrease the noise, and they have several populations where that's decreased.

Then lastly, I think comment to this would be the need for the development of bioinformatics tools. Our pathway analyses are fraught with problems, statistical problems, conceptual problems. They're gleaning some information for us. But being able to take large arrays of data, big data, and reduce it

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into pathways using bioinformatics tools is sorely needed and I think further increases the signal that you can see in these strata that are developed.

DR. BASBAUM: Steve, I do want the audience to ask, and they always have priority.

DR. EDVINSSON: Lars Edvinsson from Sweden. Concerning the TRPV aspect, I wonder if it's not a little bit overstated. You need to go to -- have some kind of a migrant model to look on it. And when this was done, it turned out that the amount of CGRP that was released from the trigeminal system was rather small. So its inhibition was not like during a real migrant attack, and that could be a reason why when they tried blockers against that mechanism, it had not a significant effect.

So it's important to have a good model that represents the disease. So that's very important, I am sure.

The other thing we learned from the CGRP story is primates have a different kind of CGRP receptor. So with the CGRP blockers, the initial ones, they were equal sensitive like CGRP A237, but

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the new ones, they were much more potent on primates compared to rodents, for example. So that's another kind of interesting aspect.

That was my small comments here. So I agree with you that it's important to look on patients or primates at least.

Your nice data, Laura, on the disks and the ingrowth of the PGP nerves, very nice. Do you know what kind of fibers they are?

DR. STONE: That's a great question. We don't know. We know that many of them are CGRP positive, but we're right now doing the studies to find out also if there's sympathetic fibers in there or if there's IB4 positive or P2X3 expressing and so forth. But it's a great question.

DR. BASBAUM: I think there was a question, it was kind of directed at Jordana, and it got shifted somewhat sideways when we were talking about TRPA1. And I wonder if I could elaborate perhaps because you were asking -- you said is it a plausible pain therapeutic target.

The question I'm curious about, and I don't

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know. It's not a rhetorical question. I did ask about the asthma. The approach that you took was to do the epigenetic-wide analysis, and then came up with A1. If you go the other route and say in twin studies, are there differences in the incidence of pain associated with particular diseases -- arthritis, headache, migraine, et cetera, et cetera -- and then look for differences in the cohort that is already distinguished by a clinical entity -- is that being done in the pain domain?

The question is, of course, would you come up with A1 under those circumstances as well?

DR. BELL: So I think in the twin sample that I presented, the Discovery sample, we were very careful to document what other traits and whether they were not on pain medication, for example.

So going back to asthma and other related traits, there would be some cases, but those would be representative of the levels that you see in the population.

I mean, related to the comment that is it a target, I can say it's a marker from our results.

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How you go forward, then I think you need to address it with experimental --

DR. BASBAUM: I'll probe a little more deeply, and maybe it's unfair because I'm coming from a pain world. A1 is in the animal literature implicated, as you mentioned, and some people think it's involved in cold pain sensitivity and maybe in injury associated, and certainly in irritant receptor.

The one thing I don't think there's any evidence for is a heat sensor. And I was curious why you decided to use heat as your QTL or your measure -- oh, this is going to blame --

DR. MCMAHON: No, actually, we did have a bit of a disagreement about phenotyping the cohort. I think as Jordana said, this was part of a bigger effort to phenotype 2 and a half, 3,000 individuals. And I was very keen. We did a more exhaustive phenotyping, which would have allowed us to look at secondary hyperalgesia, sensitization.

But actually, we just didn't have the resources to spend that long phenotyping them. So

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the choice of heat pain was entirely pragmatic, and I agree, arguably, not necessarily the best for a quick test, either. You can argue about that.

Once you've got that phenotype, though, are you surprised with TRPA1? Well, as it happens, I would have thought in other contexts, TRPA1 is an excellent target, actually. And I'm sure many drug companies are pursuing it.

In this one to say, well, does it make sense for heat pain? It doesn't superficially, but I notice again in the latest edition of Nature Neuroscience, there's an article that talks about small variants. So many non-mammalian species, TRPA1, is a heat sensor, actually, snakes. Small variants in TRPA1 confer heat sensitivity on TRPA1. So you're not a million miles away.

Then secondly, there's, I think, quite an interesting story now that TRPA1 and TRPV1 form heterodimers, quadromers. And so if that's true, the properties of those mixed receptors are really unknown. I mean, they're likely to be a hybrid, and of course, TRPV1, everyone agrees, is a good heat

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sensor.

So I think asking what TRPA1 might do in the context of I'm assuming sensory neurons -- it doesn't have to be there. It could be in the keratinocytes, which have low levels as well. Asking whether or not it might be playing a role as a heat sensor in a real cell in situ rather than in some hydraulical system I think is a different question.

So I know that the knockout data doesn't support a role for heat pain sensitivity, but I think there's a bit of wiggle room about it where you might say it's associated with, or a modified form of TRPA1 may actually form a different kind of interaction with TRPV1.

So there's lots -- I agree. It's this issue of cause and effect. We don't have -- you can't tape that in these populations, but it's not inconceivable that it's true.

DR. BASBAUM: Sharon?

DR. HERTZ: Can we get a follow-up on that or -- because I have a different --

DR. BASBAUM: New questions are good, too.

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DR. HERTZ: So I think that this morning has been very exciting for me. I'm not in your caliber of sophistication when it comes to this area, and it was really informative.

Some of the questions that occurred to me as I was listening to the presentations regarding the future and pain therapeutics was a lot of the mechanisms that seem to be epigenetic that are influencing the phenotypes are nonspecific to this condition and, in fact, may be very adaptive to other epigenetic phenomena that are occurring.

So what promise or what opportunity do you think there may be ultimately for identification of ways of intervening or influencing the system that can limit some of the effects to where we're interested? It's a question that is in every -- it spans well beyond this particular approach to therapeutics, but I think in this one, it could be perhaps even more challenging.

Has any of the work done so far suggested some possible ways of targeting this system of mechanisms that are responsible for the development

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of chronic pain states, the hyperalgesias, all the problems that we encounter?

DR. MCMAHON: I'll just make a comment. I'm not sure in the pain field whether -- I mean, there are kind of several things going on. Some people are looking for processes, which are pretty much exclusively and highly selectively in operation in nociceptors or in pain signaling part of the nervous system, and they're trying to target those. And I think you could argue that some of the sodium channels are expressed peripherally in which mutations leading to a loss of function do result in analgesia. They are great examples of a very selective target that's unlikely to have side effects.

But I would point out that none of those so far have been successful in the clinic, whereas I think there is a surprising amount of success, or claimed success, around very nonspecific things, kinase inhibitors. There are some classes of compounds you'd think would be hopeless to treat anything, and yet that's not my understanding.

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Maybe you can comment, Chas, as to the successes that industry has seen with those nonspecific targets.

DR. BOUNTRA: I agree. I mean, I think, well, first of all, we can get hopefully better therapeutics by specifically targeting specific targets. That makes sense. Instead of having nonselective molecules like the HDACs, if you can get real potent selective ligands, that could be one way forward.

I think the second thing is -- and touching on Steve's comment -- if we think of things that do work in pain, opioids, NSAIDs, et cetera, the mu opioid receptor is everywhere. It's in the GI tract. COX-2 is up regulated in many systems if they're stimulated, et cetera.

So I know we've again been seduced by things like SNSs and the AV1.879, et cetera, very target specific proteins, but I don't think from a drug discovery perspective we always need that. And I know it's about risk/benefit.

I also think that sort of -- I often think in

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terms of neurobiology. If you've got neurons and you expose them to some sort of nerve injury or tissue damage or ischemia or whatever, the response of the neurons is very similar. They'll sort of start firing crazily. Sodium channels will go up, et cetera, et cetera.

That's probably why things that, say, for example, dampen neuronal hyper-reactivity, they may work in certain pain states. They may work in epilepsy. They may be neuro protective in sort of stroke conditions or whatever.

So I think there are mechanisms that translate across disease areas, if you like, especially in neuroscience.

DR. BASBAUM: Dave, I think you might have a comment on this.

DR. CLARK: So I might. So my comments are taking this from a slightly different angle. I think there are two opportunities. One is when you give the drug. So not all pain is acute. Most of these problems we're talking about are rather chronic.

But what I spoke about this morning was pain

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that begins at the time of surgery, which is a very predictable thing. There if what we're trying to do is to use the epigenetic agents to prevent plasticity, which is ultimately supporting these longer-term or relatively persistent states, we might have an opportunity to use something that would be suboptimal for a huge population for a long term in the acute setting for several days, as we tend do in hospitals and around the time of surgery. So there we might profitably pursue something not suitable for the longer term.

The second is I think targeting is a very important part of things, and I know we're going to have some talks about that later. So the other thing we can do is use some of these agents that might not be welcome to do their thing in every cell in your body. But if delivery can be to those cells, which are more involved through a virus, through any kind of targeting strategy, that might help us use the overall strategy more effectively.

DR. BASBAUM: I wonder if I could follow up with a bit of a corollary on that comment, and it's

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just something that has -- and it bears a little bit on Chas' perspective, the whole point of targeting.

I happen to personally believe that there's an enormous opportunity for spinal delivery of drugs that already exist, and one of the best examples are obviously opioids. But we could go through a whole list. If Tony Yaksh were here, he could probably give us a list of 20 different compounds that when administered to the spinal cord have a much better therapeutic window than when given systemically. And a lot of the failure is due to systemic adverse side effects, not because the drug doesn't work.

Yet now, we're faced with the problem of money. Why is big PhRMA not interested in a spinally-delivered, good molecule? Well, because the market's not big enough. And I'm curious how that would affect your philosophy when I still believe that the rainbow, the pot of gold at the end of the rainbow, is a big driver of whether or not PhRMA is going to get interested in a compound, because I personally believe that the spinal cord is a terrific target, and you can't get people interested in it.

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DR. BOUNTRA: I suppose, Allan -- I mean, I honestly feel that the current PhRMA model is broken. I think many people accept it. It's going to be hard to change, and we can have long discussions about all the things that are wrong with it.

I think with the model that we were proposing this morning -- if, say, for example, we generated a molecule and you went off and showed that it worked in a very small subset of patients, but it worked beautifully, and big PhRMA was not interested in taking it all the way to the market, I guarantee that we can get money from either the public funders or the charities or philanthropy to make that happen.

If that's not the case, then I'll give up today. But I honestly believe that that's a possibility.

DR. BASBAUM: I'll remember the promise. I think that's great.\

(Laughter.)

DR. BASBAUM: Anybody else want to comment on that?

DR. MCMAHON: Just on the issue of whether

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epidural or spinal deliveries are feasible. I mean, it is striking that the devices companies like Medtronics, they now are selling $2 billion a year of implantable devices. So I think a lot of the prejudice against putting anything in these dangerous spaces is just that. It's prejudice.

So I can imagine a world where people would be more open minded to these unusual routes of delivery. Don't know what the regulators would feel, presumably want to be reassured. But as I say, at least we have quite a bit data set now coming from the devices people about the incidence of adverse effects.

DR. BASBAUM: Somebody, unless somebody in the audience would like to ask a question? Laura, it's your turn.

DR. STONE: First, I just want to comment on that because with spinal delivery, it's not just the drugs that are already in clinical use that you can get a better therapeutic window. We know if you give them spinally, that a lot of very effective targets are not being developed because we can't get drugs

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into the CNS. So you get around that problem, and the world just opens up to all kinds of different targets and types of molecules that we couldn't use otherwise we have to give them systemically.

So my question is for Bill. And that is, so you had a lot of really strong evidence that beta blockers are going to be useful analgesics. And you also have massive data sets from your cohorts, where I'm assuming that you've asked them what drugs they're on, right? And a lot of people are on beta blockers.

So I'm curious if you've gone back to the data and if your data would have predicted, just by looking at who's on what drugs, this analgesic effect.

DR. MAIXNER: Yes, thank you, Laura. We have access to a few cohorts, some that we've collected, so the OPPERA cohort, which now is about 4,000 individuals, aged 18 to 34. And now we have OPPERA II, which is going to go up to 60. And we have followed OPPERA I longitudinally for about six years being very deeply phenotyped in the domains I

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showed at baseline and then following some for conversion to caseness.

During that process, we have collected data on medication usage. In fact, at three-month intervals we have on a portion of the cohort information related to medications. We have baseline treatments if they're a pain patient. We also at these quarterly updates have information related to treatments that they're involved with.

We have not yet looked at data of drug or treatment by baseline characteristics, but we'll do so in this.

So there are other data sets available, as talking to Chas at the lunch break. The cancer registries in particular have a lot of information related to obviously, survival but also opioid consumption, medication use. And there's evidence, at least for the beta adrenergic system, that individuals on beta blockers have longer survival times. They have reduced opioid consumption compared to those not on beta blockers.

So I think there's some convenience cohorts

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out there. I think the data just needs to be mined.
DR. CLARK: Bill, I wonder if I could ask a follow-up on the beta question, and that is, as you know, beta receptors in a sense more or less directly regulate nociceptive pathways. That's one.
But then your data, and those of many other groups, points to conditions like anxiety and things supported by adrenergic signaling as really very important co-factors in related phenotypes. How do you think they might be working in your samples?
DR. MAIXNER: Right. I think this gets to many of the questions that have been asked of the panel. What are the characteristics of an analgesic in actually an acute or a chronic pain population because it's more than nociceptor transmission that we're trying to modify. It is really a gestalt of bio, psycho, social events that occur in response to injury, at least bio/psycho events that occur in response to injury and maybe even sociological events.
But trying to find an agent by reducing to the nociceptor, in my opinion, in many cases is not

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going to resolve the mosaic, the mosaic that oftentimes consists of anxiety, somatic awareness, which is a big factor we find to be predictive of onset of these conditions and maintenance of these conditions.
So I think that nonselective agents, maybe those which would be so-called off target from the nociceptors, have great potential. And beta blockers, there are beta receptors throughout the central and certain regions of the peripheral nervous system. The amygdala, areas of the limbic system, are highly concentrated with beta receptors, beta 2 in particular.
So I think one can make an argument by reading literature that many receptors, including beta receptors, are strategically located in pathways involved in pain transmission, nociceptive transmission, but also in areas of the brain involved in affect and, mood which are components that influence both acute and chronic pain perception.
So I think rather than going -- reducing to potentially a molecule that is selective for

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nociception, that we need to go back to the old amitriptyline model where we're getting more broad base, so-called off target effects, but in areas that are associated with the larger phenotype that we're trying to treat.
DR. BELL: So just to follow up on that, related to the COMT results, I think COMT has been also implicated in other related conditions in similar vein, extending the previous question.
Have you looked at pleiotropic effects or following up on the gene interaction question, and how much are you planning to --
DR. MAIXNER: So we have looked at pleiotropic effects that are associated with polymorphisms in COMT, not only looking at pain sensitivity across a variety of modalities -- so heat, ischemic, pressure pain, pinprick pain -- we find that almost independent of sensory modality, there are relationships between COMT polymorphisms and sensitivity to these stimuli in the direction that I showed today.
There's strong evidence in psychiatry that

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COMT is involved in a variety of affective disorders, including anxiety. There are fundamental pathways in prefrontal cortex that are COMT dependent. The amygdala limbic system, also COMT plays an extremely important role.
So I think it is an example, one example of a gene that actually has rather profound pleiotropic effects. And so I think we've sort of stumbled on this by knowledge and pathway to date.
I'll say that maybe it's just a bias of an early discovery in the pain genetics field, but if you look at the field now with respect to the number of replicated findings by gene, you find that COMT is by far, amongst all candidate genes, the most replicated for both experimental pain as well as for chronic pain conditions.
So either it is just being investigated more substantially because of existing evidence, or it's beginning to hold weight as a pleiotropic gene across several pain conditions.
DR. BASBAUM: I'm going to ask an elephant in the room question. HDAC inhibitors, HAT inhibitors,

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they're dirty, and Steve, you certainly highlighted that fact.

How concerned should we be about their use in preclinical studies, and what do we need to do to have a little more confidence that, in fact, the effects being observed are due to what we think they're supposed to be doing?

DR. MCMAHON: Well, I mean, they're what we've got so it's not a bad starting place. But I think you can definitely do better. And you can write to Chas and say, will you make a XYZ block.

As I said, we've got a methyltransferase inhibitor from Chas that works in vivo. So there will be better tools coming along.

Of course, you can always go the transgenic route, and I suspect that that ultimately will be cleaner. Obviously, you know the problems there. It's time consuming. You're stuck with a mouse, but ultimately, I think it's likely to throw more light. And the power of the manipulability of targets in the mouse is fantastic now, so you can turn it on and off pretty much wherever you want whenever you want if

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you've got the money to invest in making the tool.

DR. BASBAUM: Chas?

DR. BOUNTRA: Can I put another elephant in the room or whatever your phrase was, Allan?

I think -- and Frank and I have had this conversation a few times. What do we as a community need to do to persuade big PhRMA to start reinvesting in the pain area? Because we know many companies have literally pulled out of pain completely, and they've pulled out because they've done loads of clinical studies, and they've all failed and they've lost a fortune, and so on and so forth.

Big PhRMA, at the moment, what they're trying to do is reduce their risk. And here we're talking about novel medicines that come from novel targets, novel delivery devices, novel biomarkers, novel stratification methodologies. Novel, novel, novel, novel means more risk, more risk, more risk, more risk, et cetera.

I'm just thinking, say, if Andrew Witty, who's the CEO of GSK was sitting here, would he start reinvesting in the pain area? And I think until we

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provide a clear path of this is how to develop an analgesic, these are the assays, this is how we're going to minimize risk, they're not going to reinvest. But as a society, we desperately need new medicines, so somehow I think we need to start helping PhRMA to do this.

My concern is if I think from a drug discovery perspective, we have this global pot of money for medical research. And that pot of money is not getting any bigger. Governments can't afford it. Charities can't afford it. PhRMA can't afford it.

So yet we want more medicines out with that pot, and the way to get more medicines out is to make that pot more efficient. And so what I was advocating this morning was reducing all this duplication because there is massive duplication. And I think we have to start somehow, we have to stop competing with each other and working together to try and help industry deliver new medicines for patients.

DR. BASBAUM: Please?

DR. KHALSA: Partap Khalsa from NIH. I wanted to see if I could add another elephant to this

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growing herd that we're building. And I was particularly struck by some of Laura's data on exercise and how consistent it is when you look at a whole series of physical activity, movement-based interventions, that particularly for most chronic conditions, chronic clinical conditions, they tend to work close to, if not equal to, if not better than most of the pharmaceutical medicines that we have. And in some cases, opioid use, I think there's a growing bed of evidence that suggests long-term opioid use is actually detrimental to these conditions.

So the elephant in this case is, given that you've got these wide range of physical activity-based interventions that seem to be at least modestly effective if not moderately effective overall, isn't that a source of sort of looking particularly from an epigenetics approach to what's common amongst all these different interventions? And can't that then somehow -- sort of the inverse approach here of sort of looking at a specific protein and trying to figure out then what you can

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use it for and what target -- but sort of saying, well, what seems to work and can we than somehow look at the epigenetic pathways to help then identify, well, what could we make that would be druggable that might work in these cases?

It may not be receptor specific, but it might have this sort of dirty effect that seems to be nonetheless beneficial.

DR. BASBAUM: Laura?

DR. STONE: So one of the things, if we think about epigenetics and target identification as not looking for a way to target the epigenetic mechanisms but using these kinds of screens that we've talked about this morning to identify pathways that are involved in pain -- so we might get 4,000 genes that are disregulated in the dorsal root ganglion, but they might only belong to five or six pathways that have to do with glutamate-related plasticity or nerve inflammation or something like that.

So the first point that I want to make is that we should be using these big data sets to do this sort of pathway analysis and then go after

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things that are within those pathways where there's a lot of convergence.

The second thing is if you think of the global landscape, something like exercise has shifted that global landscape. So if we could figure out what's different in the disease state versus somebody who's having therapeutic benefit from exercise, do pathway analysis there, we're going to find out what pathways are the positive therapeutic ones. Maybe we go after them pharmacologically as well.

DR. CLARK: So I'll add that it's not an epigenetic project, but we are taking a similar approach in a model of complex regional pain syndrome, which for years was studied using small molecules and antibodies, and did experiments very similar actually to the ones that were described here.

Exercise is a brilliant treatment for these animals. I think I can cure any rat of anything. I don't know about any human of anything, but the rats and mice do seem to get much better with exercise. And we're undertaking the effort of looking for

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overlapping pathways but on a much more focused group of molecules which will be easier for us to approach.

I think we're having some success in looking at things like inflammatory mediators generated by dorsal horn glial cells as one of those pathways, which might not be a surprise, I suppose. But I think that it will be a good idea to incorporate these many different modalities to get at that core group of targets that we'd hoped to find.

DR. MCMAHON: So as a neuroscientist, I don't feel I have to kind of apologize for being interested in the basic biology, and I'm happy to do that. But I'm 100 percent with you that to not utilize easy things that could offer a fair degree, maybe a lot of protection or improvement, is crazy. But that really is a political and a regulatory issue in many ways.

So here, if I understand it, there's the attempt by New York to regulate the size of soda that could be sold was overturned. It's unconstitutional in some way. So I think if we don't take advantage of the ordinary interventions, if we don't educate people, if we don't regulate, we've only got

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ourselves to blame. The solution is not going to be just to take a pill, to develop a pill for everything. But the pills will help, can help, are life changing in some circumstances, so I would agree that some holistic approach is necessary.

I mean, but definitely, I think we could do a lot by lobbying to get better regulation, better enforcement of the opportunities we already have.

DR. MAIXNER: Just one comment on the question about non-pharmacological agents. I think it's really interesting that pharmacological agents that we use today, the majority, if not all, don't create new biological events, new processes. They merely work with endogenous processes and have many off target effects in general.

In contrast, some of the behavioral interventions tap into those very same endogenous processes and are largely free of side effect. So I think it would be extremely interesting to really dissect out in substantial detail those behavioral therapies and interventions that we know to work and to understand them well because I think that will

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help reveal therapeutic targets that could be combined with some of the behavioral interventions.

So I think there's a real window there that's not being captured that could inform us for better on target effects and better targets for development.

DR. BOUNTRA: Could I just respond to that? So maybe this comment's better saved for tomorrow afternoon, but I sometimes think as a community we need to be a lot slicker and faster. So I think of the anti-CGRP approach for migraine. I know Boehringer Ingelheim had some early clinical efficacy data at the start of this century, so now 14 years later, we still actually don't have a molecule on the market.

If I think of the NGF approach, I think some of the efficacy we've seen in patients -- and David's here. He can tell us what we're saying wrong. But it's absolutely remarkable. And from an efficacy perspective, that's one of the biggest successes.

Now, I know the preclinical literature on NGF blockade or TrkA antagonist was -- Frank, I'm looking at you because you know it all -- 15, 20 years ago at

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least? And we still haven't translated that into patient benefit, et cetera.

The sodium channel blockers, I was working with John Wood in 1990 on a DRG-specific sodium channel, and 24 years later, we still don't have a drug on the market.

So somehow even with these really high priority targets, I don't know how we can rally round and make it happen.

DR. BASBAUM: I don't even know what to say. I really think that's two ships passing in the night, the fact that there isn't a good NEV1.8 blocker because some of the animal data predicted is not because -- and everyone went after it. I'm not quite sure what you're saying that they went about it the wrong way? It's a very difficult drug to target selectively. That's one of the first problems. We know that.

The anti-CGRP story is a complex one. That was small molecule when it started, and now the -- as you know, Merck had a small molecule that unfortunately had an off target failure in liver, and

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now its antibodies to CGRP.

These things take time. It's very different from the cancer world where tox is less of a concern, unfortunately, but it is when you're trying to kill a cell. If you have some normal cells get killed, you might tolerate it a little bit more.

So I think there's a little bit of throwing babies out with bathwater here to dump on the way that the science and the development has occurred. I think you're sort of looking at the cup really half full.

DR. BOUNTRA: No, no, no, not at all, Allan. All I'm trying to say is we have these clinical hints, and we're desperate for new analgesics. So how do we make it go faster? And I'm just trying to think, are there lessons we can learn?

So that was it. I mean, so with CGRP we knew at the start of this century, albeit not with an optimal molecule, that there was potential for clinical efficacy. All I'm saying is 14 years later, that's not translated into patient benefit.

The sodium channel blocker, I know absolutely

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all the issues around tractability and selectivity and so on and so forth. And maybe we don't even need a selective molecule, but we've been working on it for 24 years. That's a long time.

The NGF, Dave Shelton's doing a brilliant job, I'm sure, but I worry about will PhRMA take it all the way because they might be afraid of being sued for osteonecrosis.

DR. BASBAUM: That's a good place to end.

(Laughter.)

DR. BASBAUM: Misha, I think you were going to give us a little more lighthearted approach, please.

DR. BACKONJA: I don't know how lighthearted it's going to be, but at least put some light on the perspective. As a clinician scientist going to meetings and seeing so many targets and mechanisms, one of the things that was always leading me with like which one matters -- and what really was primary impression of these series of presentations -- is that finally, we're talking about really what's relevant in the clinical world is the fact that

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there's a time course to everything that's happening, and that now there are possibly or potentially ways to start prioritizing these mechanisms.

So that was a probably high point of my realizing what's going on here, but I'm still not convinced which of these approaches is the way to go. And I don't know if anybody dares to step out and tell us that.

DR. MCMAHON: We've obviously got no idea, either. That's why we're doing these.

(Laughter.)

DR. BASBAUM: I was going to turn it to the audience, and Roy, let me ask you a question, if I may.

So Roy Freeman is a neurologist, who you have done -- and others in the audience who have done comparable studies, Misha included, clinical trials. So now we've gone through the preclinical phase. It's gone through phase 1. And now someone approaches you and say, we want to take this to the patient, PHN trial.

To what extent do you sort of look at all the

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previous work to decide whether do I really want to take the time to do this work? You must have a lot more confidence in the compound than Chas has that you're taking it in and it may fail.

I'm using -- I'm sorry, Chas, but it's easy.

Roy, any thoughts?

DR. FREEMAN: I lost you with the question. As soon as you brought Chas in, I --

(Laughter.)

DR. BASBAUM: No, the question is somebody says, Roy, we'd like you to take this compound into the clinic and test it in X. And you are probably making some decision as to what the target should be. I don't mean the site, but the patient population. And you're building that decision on the preclinical, what they've told you, the phase 1 data

Do you ever sort of take the perspective that I'm probably wasting my time because it's probably going to fail?

DR. FREEMAN: Well, I go along with the series of points -- the continuum of points that Chas showed on his slide of which things fail. You've got

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to be a realist over here and say that things are going to fail at every point along the way and then just -- even if we get to phase 3 and they work, there are probably going to be toxic side effects. I think that's the nature of the business.

If the question was -- which I thought it was going to be -- what do we want preclinically, what kind of preclinical package will give us some clue, I have the same nihilistic attitude there as well. I think we want some clue that it is going to work preclinically, that there is some preclinical evidence. We want some clue from the models.

But I think we can't put too much weight on it, and I think tomorrow afternoon's session gives us some sense as to how many molecules have actually moved from the what we think of bench to bedside route. And you can count them on much less than one hand, unfortunately.

DR. STONE: If I could comment on that? There's a couple of things that we can do in the preclinical community, and one of them is if we're going to model chronic pain, then we should model

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chronic pain and not inject CFA and test the animal 24 hours later and call it arthritis. That's not chronic pain. You have to wait for a long time. Just like in people, chronic pain takes a long time to develop and is very intractable once it develops.

So why are we not waiting three months after nerve injury or joint inflammation to test our drugs? Because the system is going to be completely different three months later than it is a week later.

The second comment is that in the preclinical world -- and I think we're all guilty of this -- we'll take some animals, and we'll inject a drug once. We'll test the animal an hour later, and then we'll make conclusions about its efficacy. You would never do a clinical trial like that, right? Why are we not giving the drug for a month in the animals?

In the exercise study that I showed, if we had stopped looking after a week, we never would have seen what I believe are disease modifying effects of running exercise because they weren't there after a week. It took a couple months. So that's one

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suggestion.
DR. BASBAUM: Frank?
DR. PORRECA: Well, I just had to comment a little bit on Roy's point about the preclinical studies and just remind everybody that much of what we know about nociceptive pathways and modulatory pathways have come from the preclinical science. So saying we shouldn't put too much weight on this, I think is not right. That's the first thing.
The second thing, the point was made about spinal administration. And I just want to circle back to that for a moment and just remind everybody that the correspondence between preclinical evaluation of reflex responses with spinal administration and efficacy in humans with spinal administration is essentially 100 percent.
So the point is that the biology translates -- and I think we've got a talk that's coming tomorrow by Toto Olivera that I think is going to remind us about this, translation of biology across species, the mechanisms translate.
Now, making a drug is a different question,

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it's a hard question, for systemic administration. But I don't think you can question the fundamental relevance of biology, and that's something that we shouldn't forget.
DR. FARRAR: Yes, John Farrar, University of Pennsylvania again. Frank, I agree with you 100 percent, that the biology has been spectacular in terms of dealing with earlier models.
I do have to say that I also couldn't agree more that when we get to thinking about clinical effectiveness, whether we think we're about clinical efficacy, we do need to study chronic pain models. And there are some chronic pain models in animals that certainly could serve as potential useful treatments. Yes, we probably need to study them longer.
But I wanted to actually get back to the point that you raised with Roy, which is what about moving this forward into people. And one of the things that I think that has come out of the IMPACT ACTTION process has been an understanding of the way in which we should move forward with proof of concept

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trials.
That's obviously affected by a push from industry to get done faster and to try and combine the dose finding and proof of concept in the same. But if you take a step back, one of the things that's true from everything I've heard today is that the population that's likely to be benefitted by any one particular entity that we've looked at today is going to be relatively small.
Identifying that group a priori in a large population of patients with back pain is nigh short of impossible at least currently. One way around that is to do and to start out with proof of concept trials that are either randomized withdrawal or enhanced in some other way.
Randomized withdrawal trials, there's a long history of using them. They're not perfect. They certainly don't serve as the only trials that you can do to get approval. But it gives you the opportunity to use your drug, to identify the patients that are likely to benefit from that drug, and then to see whether if you take it away and give it back to them

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or just take it away in a randomized way, whether you can demonstrate that they actually saw a difference.
It gives us the ability within a large population to identify at least one subpopulation using the drug to identify one that benefits. If you can't prove it there, then it's time to give up.
But it seems to me that we waste -- in the past, we certainly have wasted a fair amount of time going from sort of small randomized trials parallel groups to large randomized trials to show that it doesn't show anything. And so I wondered what the panel might think about that and what others in the audience might think.
DR. BASBAUM: If the panels wants to comment, and then I think we will take a break because we're going to continue after. We have a whole day tomorrow.
DR. MAIXNER: I'd like to comment. And if there is any breathing room for one last question, I'd like to pose a question to the audience, too. We have a few minutes.
DR. BASBAUM: Minute and then the question.

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DR. MAIXNER: The comment to John's thought is I think that this is maybe one of the main reasons why there's failure from phase 2 to phase 3, that if you look at the way that most clinical trials are designed in phase 2, it's to select individuals who have only that specific condition.

For example, many of the fibromyalgia trials, individuals were recruited, selected, who only have fibromyalgia. Well, that's a rare bird. Most chronic pain conditions, 15 percent or less only have that select condition. The vast majority have other comorbid conditions.

So I think that we have a selection bias that is working against us. And so I think I advocate for case definitions and inclusion of individuals who are more common to the clinic. And I think that that will promote success, and I think we'll then entice PhRMA further, not all the way, but further into the process when there's success in larger portions of the population rather than the 30/30/30 rule. And that will continue as long as we limit the nature of the population that we try a randomized control trial

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on.

The question I'd like to pose to the group relates to the theme of the workshop today, epigenetics. We as a group do very well at characterizing pain populations, genotyping them, the methodology is great. What I think we're lacking here are good instruments that assess the environmental exposures that individuals have.

So I'm curious from the audience perspective, what instruments should IMPACT or ACTTION be thinking about that can capture the key environmental exposures that will lead to stratification capabilities, that may help us inform us as to why an individual may or may not respond to a drug. So we have good ways of phenotyping, molecular profiling, but I think we really have a long way to for assessing environmental exposures.

So what instruments should be used?

DR. BASBAUM: Sounds like a good topic for a next meeting because no one has the answer yet, and I think people are looking for a cup of coffee. So we'll sit on that one.

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Thank you all. Thanks to the panel and everyone. We'll be back in an hour.

(Whereupon, a recess was taken.)

DR. PORRECA: All right. We're going to get started on the afternoon session, and I think we had a very lively and interesting discussion this morning. And I'm very pleased to move on to the topic of imaging in translation.

I think that one of the things that emerged from the morning discussion is the concept of de-risking targets and investment and how we can increase confidence. And I think that one of the things that didn't come up that is a barrier is exactly how we measure pain.

So we all know the complexity of pain and the subjective characteristics of pain in individual patients. And so when we think about investing in development of pain therapeutics, as opposed to investing in some other area of biology where there is an objective outcome measure, one can make the argument that it's a safer bet to go with a disease that would have some sort of an objective readout.

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That's not the case in pain at least at the moment. I think that things are moving forward into gaining better insight into how we actually evaluate pain, and imaging has played a tremendous part in this.

So it's a pleasure to introduce Catherine Bushnell, who's going to give the first talk entitled From Mice to Men, the Role of Brain Imaging in Translational Pain Research, which I think is exactly the topic.

Catherine is the senior director of the NCCAM here in Washington and was the director of the McGill pain center in Montreal. Thanks, Catherine.

Presentation – Catherine Bushnell

DR. BUSHNELL: Okay. Thank you, Frank.

But I'm afraid if you want somebody to say that imaging can tell that somebody's in pain, you should have invited Irene and not me.

(Laughter.)

DR. BUSHNELL: But I will try to talk about how we can use brain imaging as a tool and possible translational research.

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So I have nothing to disclose, but I'm obliged now as a government employee to tell you that these are my personal views and not that of the NIH, or the Public Health Service, or the Department of Health and Human Services.

So I just want to start off with some of the reasons why one might want to conduct parallel brain imaging studies in animals and in rodents as preclinical models. And one thing is we have these clinical observations and the ability to kind of back translate into our animal models and to then look at possible mechanisms of this that imaging can make the link between the clinical observations and being able to go back to rodent models and see, using a parallel technique as a whole brain imaging to see if there's similarities at that level.

Also, as we're developing mini genetically modified animals and people are looking a lot in the spinal cord at what's happening in these animals to be able to get an overview, a whole brain overview of possible changes in the pain system or modulatory systems so you know where in the brain to be possibly

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targeting more detailed analyses. So this gives you kind of a whole brain overview that can be useful. And together these things maybe can lead to better translation between preclinical models.

So I'll go through and give examples of different types of imaging that's involved in human studies and how it's starting to be applied to rodent studies, and then come back to whether or not we can actually use these things successfully.

So just an overview, we all know that there are many parts of the brain that are now involved in pain processing in the human brain. And imaging shows us that -- it's an example of just heat pain standard imaging of a phasic heat pain stimulus in healthy people, and seeing that in fact there are multiple parts of the brain that are activated by the pain stimuli. And these involve some meta-sensory areas such as primary and secondary somatosensory cortex and also areas in the limbic system and pre-limbic system, including the anterior cingulate cortex and the insular cortex.

So if you go into the rodents, we actually

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can see that both these sensory and affective areas can be activated with an acute pain stimulus in rodent models similar to what we see in the human models.

I'll show you -- this is an example that Scott Thompson in our lab did. Throughout this whole talk, I'll be bringing up the issue, and I'll talk more about it at the end, about anesthesia and stress and other things that when you try to image a rat or a mouse can become quite an interesting issue that may not apply to the human studies.

So when we do human imaging studies, first of all, we make sure they're not claustrophobic and that they are comfortable in the scanner. At NIH, we have a mock scanner that we put them in first to condition them. But then you take a rat or a mouse and you either anesthetize them or you tie them down. And he's not going in there voluntarily and does sign a consent form, and he may be having a very different experience.

One issue when you're doing whole brain imaging is you're imaging everything that that little

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guy is thinking and feeling, and so you're not just imaging the stimulus you're putting on the skin, you're imaging everything that's going on. And that complicates the issue and something we have to be aware of.

But one way to get around that is to do what we've done in our lab that has pros and cons, but is to do -- this is PET imaging, where we use a radioactive tracer to look at metabolic activity as a surrogate for neuronal activity.

The temporal parameters are such that the uptake period is before -- you can have an uptake period where the animal is just in a box, and you can expose them to whatever you want to expose them to. And then you can anesthetize them and put them in the PET scanner, and you can see the residual from the activity, the uptake that occurred during this pre-scanning period. So the animal's not stressed. He's just sitting in a normal environment.

So you can do this where -- what we did is did a formalin injection into the rat's hind paw, had him sit there and then during this uptake

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period -- of course, rats don't just sit there when they get formalin. They shake their paw, and they lick their paw and do various things.

So what you can -- and when you scan, then you're going to be scanning not only the pain that they may be feeling but also these behavioral things that they're doing to compensate for the pain. But you can measure all this. You can videotape it, and you can record all of this. And then you can look at what's happening in the brain during this uptake period.

We saw found activations in the primary somatosensory cortex, the hind limb area where we had injected the formalin, and in the cingulate cortex, CG1, but also in the face area. But then if we looked at the videotapes of the animal licking and shaking, we found that the licking behavior correlated perfectly with the activity that we saw in the face area of the cortex, suggesting that this was being driven by the licking, biting behavior, whereas the activity we saw in the hind limb area was unrelated to the shaking -- the hind limb motor

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behavior, suggesting that this was not being caused by the movement. And from that, we assumed it was being caused by the afferent input, the, quote, "pain."

Then the cingulate cortex that I'm showing you here in the graphs also did not correlate with any behavior, suggesting again that this was more related to the afferent side.

So you're seeing these things. So this is a case where the animal is in a non-stressed, non-anesthetized condition, but the downside of doing it this way is you do have to factor out the other things that you know are going on.

So I'm going to go through -- there's a number of imaging techniques that are used for studying pain in humans. One is anatomical imaging that we have the BOLD technique, the pain evoked responses, the most commonly used functional brain imaging technique. I'll explain all these as we're going along.

One is resting state imaging, which is something I'll explain to you and how it's going to

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be altered by pain. And then more recently people instead of using BOLD, they're using this arterial spin labeling, which gives you an absolute measure of blood flow as opposed to a relative measure.

Then you can actually -- you can compare data between scans, and you can look at a more chronic condition. You don't have to have very quick on and off as you have to do with using a BOLD technique that requires kind of a change in signal that actually has a slow drift. And so you can't look at slowly occurring events. You have to look at very rapidly occurring events; and then PET imaging to be able to look at various radial ligands and looking at changes in neurotransmitters or the chemicals.

Anatomical imaging. Over the last decade or so, there have been many, many studies. I think it was Vania Apkarian in 2004 was the first study out there showing that chronic pain patients have changes in their brain anatomy, and many studies find small -- there's slight differences from study to study. But overall, we find that people who have chronic pain have decreases in the amount of gray

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matter, particularly in areas that are involved in pain processing and pain modulation.

Just as a couple examples, this is from Dave Seminowicz with Laura Stone, where they looked at chronic pain patients and compared them with matched healthy controls. And these blue areas on these brains show areas where there's significantly thinner cortices in the pain patients than the matched control subjects. So these are age, sex, lifestyle, socioeconomically matched people.

So you see that particularly a lot of these frontal areas, dorsal, prefrontal cortex, as Laura pointed out earlier, another medial -- a lot of this prefrontal areas, in fact, are thinner in the pain patients than in the healthy controls.

We've done this with fibromyalgia patients. And again, you find this -- here the red shows area where there's significantly less gray matter in the fibromyalgia patients compared to controls. And again, these prefrontal areas dominate in the areas where you find thinner cortices.

So you can take this general finding that we

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have in many, many studies now in pain patients, and you can back translate into your rodent model. One issue that's going on with the pain patients is that you don't know what's cause and what's effect. It may be -- we talk about -- lots of people say with back pain, lots of people hurt their backs, and most people get better, and they don't go on to have chronic pain, whereas other people do go on to have chronic pain.

Well, if you go back to this, it could be that after an injury, the people that just have really crummy dorsal lateral prefrontal cortices are more inclined to have their pain become chronic than people that don't. So we don't know if these brains were already different and that's a factor that makes them susceptible to having chronic pain or if the pain itself is leading to their having reduced gray matter.

Now, with these cross-sectional studies, you can factor in how long they've had their pain. And in the studies that do that, if you use that as a covariate, we generally find that the longer the

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people have had their pain, the less gray matter they have, suggesting that, in fact, it is causative, but it's not really definitive.

But in the animal models, you can actually take genetically identical animals, and you can expose them to a pain stimulus. And you can look across time. And this is what we did again with Dave Seminowicz in our lab, where we took rats and we did an SNI model. And half of them had sham surgery, and half of them had the SNI injury. And then we looked over time for up to 24 weeks after the surgery. And we did every week -- well, not every week, but every few weeks, we put them in a little MRI scanner, and we did an anatomical scan of their brain.

We found that, in fact, this prefrontal cortex of the red glob there, which is prefrontal cortex, in the animals that were injured ended up ultimately being thinner than in the animals that just had the sham surgery. But you see this is not significantly different. These were actually young animals that were growing, so their brains were growing. And you see the sham animals were growing

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more rapidly than the SNI animals. But we didn't see a significant difference until 20 weeks after surgery.

So this goes back to this issue about doing these experiments where you do an injury, and then you look what's happened in the next day or week or two weeks or three weeks, and it's still not going to be sufficient to see these kind of changes. So you get a chronic pain patient who comes in and you do -- you put them in a drug trial, and their brains have been changed while you do these drug trials in animals where they're nice and healthy.

So I think this is an important thing. It also allows you -- in this study -- we haven't had great success with our tissue, and we're doing some more studies now -- that you can then after you -- people say what do these changes mean physiologically in humans?

So you see these using anatomical MRI that your cortices are less thick. And is this neuronal death? Is this changes in dendritic arborization and synaptic connectivity? Is this related to

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non-neuronal cells? Is it related to water? Who knows?

So this has been a big issue that's not really resolved, and I think this can be resolved. The data are starting to suggest that this is not really related to frank neuronal death but maybe more related to changes in dendritic arborization and synaptic connectivity. But the data, there's just not much out there yet on this.

But this kind of model allows you then to take tissue and go back and do other analyses in the animals that then hopefully will inform you about the human models. And again, it allows you to control their environment.

We were talking a lot about epigenetics and environmental factors. And then you can take these animals, and you can make them smoke for those 20 weeks or you can do all sorts of things that people -- make them drink Coke, liter Coke bottles for 20 weeks. But you can do all the things that we do to undermine our health.

So I think this provides a very nice model

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and an easy way to be able to start to understand the changes you're seeing -- the anatomical brain changes that you're saying with humans and human pain patients. And this is easy in the sense that you don't have to worry about anesthetics because you're not doing functional but just anatomical.

Now we get into the functional brain imaging. So there's this BOLD technique, which is used for a lot of human studies. As I said, it requires -- it's a relative signal that requires a fast on and off with stimuli, so it works best with acute types of stimuli.

There have been some studies with rats using strong electrical stimuli under anesthesia, and you can get something similar. But Becerra and Borsook have been kind of leading the way in trying to do awake imaging in rats, where they have several days of conditioning, where they put them in a little restraint thing, and they condition them for several days, and then put them in a scanner.

Towards the end of the talk, I'll show you what I think are some of the problems with this. But

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this is a study they published recently where they did heat stimuli, parallel stimuli in humans and rats, and they looked specifically at nucleus accumbens and the anterior cingulate, and showed that they got analogous responses to the pain onset and pain offset in these regions in their rats and in their humans. And I think using a parallel technique, just showing that, in fact, you can get very parallel data with using these methodologies.

Now, the mouse is very interesting to many, many people. Now, we're getting away from rats, and then if you want to start getting into genetic manipulations, you want to be imaging the mouse. So obviously, their brains are a lot smaller, so your resolution is not as good.

But now the imaging techniques are getting better. At NIH, we're getting a cryoprobe, which is supposed to really enhance it even more. So coming out of these labs now when you have 11 Tesla scanners and you have cryoprobes and things, the resolution is getting better.

This is an example of a study that just came

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out recently using -- it's electrical stimulation, so again, it's a very strong stimulation that we know in rat studies seem to survive anesthesia. And that's what this is, but you can see -- but then they are actually looking at the effects of a new adrenergic acting anesthetic or whatever it is. I'm sure it's not a classic anesthetic, but showing that, in fact, that even with higher and higher -- these three graphs are showing higher and higher levels of the anesthetic and showing that this electrical stimulation survives the anesthetic.

But it's saying that actually, electrical stimulation can survive lots of anesthetics; but just showing activation and sensory motor cortex. It's showing that it follows the temporal parameters quite well. So people are starting to image more and more in mice.

So there's a whole other type of imaging that's kind of come into the pain world, and it's called resting state imaging. And this is something that was first identified by Fox and showing that when you have humans and you're doing tasks in the

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scanner, there's a period of time between the task where you're resting. And there are brain circuitry.

If you look at how different parts of the brain are activated in time and do temporal correlations between various brain areas -- so you take areas A and B and look how each of them is increasing and decreasing activity across time and seeing which ones in fact have a strong correlation -- you find that there is a network that involves primarily the posterior cingulate/parietal cortex and this big frontal area, so the stuff in blue and green, that are activated more when a person is not doing a task than when he is. And it turns off when a person is doing a task. And that's why it's negatively correlated with a task, and it's called the resting state network.

Then there are others that no matter what task you're doing, other parts of the brain are active whenever you are doing something, engaged in something. And so people have now identified a bunch of networks in the brain that are just coherently across time activated or co-activate.

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So this default-mode network, the one that's activated when you're at rest, has been studied now in humans and in monkeys and in rats, and basically has been identified in all three of these species. And so it seems to translate across species, and it's important because it can be -- chronic pain itself can alter this default-mode network, And this is something -- I'm giving you an example from our lab from Marta Ceko's work with fibromyalgia patients.

What she did is she put a seed here in this posterior cingulate, in this part here. And then she looked at connectivity with other parts of the brain and found in the healthy people and in fibromyalgia patients, and found that this connectivity with this anterior part is disrupted in the fibromyalgia patients. They're shown in red.

This is actually independent of the pain that they're experiencing while lying in the scanner because, obviously, if a pain patient's in the scanner -- like you take a back pain patient and you make them lie in a scanner for an hour, they could be experiencing substantial pain from their back. And

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so if you're looking at something that's supposed to be active when a person's at rest and they're not at rest because they're cursing you about the fact that you're making them stay in a scanner with their back hurt, then they're not resting.

But even if you take into account possible pain that's going on by the patient while they're in the scanner, you have a disruption in this default-mode network. We don't know the meaning of that, but we know that somehow pain is altering the way the brain is functioning.

Again, David Borsook and Becerra have identified a number of resting state networks in the awake rat. And I can't exactly explain this whole graph, but it's just showing -- because it's not from me. But it's just showing the areas that are co-activated together during a resting state with the idea that awake rats can also have this resting state activity.

But more interestingly to me is the work that David Seminowicz did in Baltimore recently, where he basically looked at how a nerve injury can alter some

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of this connectivity in the brain. And he found that -- I'm just giving you one example from his paper. There were a couple of other networks that were disrupted. But the relationship between the somatosensory thalamus and the somatosensory cortex was disrupted in these animals that had a nerve injury.

So after surgery, you see that as time goes by, it becomes more disrupted. If you look at the difference between the normalized to the shams, that the -- this red and blue is showing connectivity between the two sides of the thalamus, and the green and black are showing connectivity to the somatosensory cortex.

So you have increased -- in the nerve injured animals, you have increased intrathalamic connectivity and decreased thalamocortical connectivity.

So again, I'm not sure of the meaning of this, but it just shows that, in fact, in the animals models, as in the human models, you can find alterations. And this is not anatomical

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connectivity. This is functional connectivity, just meaning synchrony and activation in these areas that's disrupted.

Now, again, system analysis, it's interesting this group that's in Europe that's been doing the mouse is showing that functional connectivity -- and again, they're using their three levels of anesthetic. And interestingly here, whereas the anesthetic did not alter, did not have an effect on electrical stimulation, produced functional activity, it does not have an effect on -- this highest dose of anesthesia has an effect on these intrinsic functional connectivity within the brain, as you can see by this less red.

So it's something again to be aware of with anesthetics, that this could be affecting this type of activation where it survived -- the electrical stimulation can survive. And this just graphically shows the inter-hemispheric connectivity in the resting mouse brain, and again, disrupted with this highest level of anesthetic. But the lower levels, it does not seem to be disrupted and looks pretty

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good that you can give them some anesthetics and still show this inter-hemispheric connectivity.

So these are possible. In the mouse, they haven't taken it to the level of looking at chronic pain and how it's disruptive, but it looks like it's a viable model.

So now, arterial spin labeling. If you want to look at a chronic condition, you can't really use the BOLD technique. So you can look at things like disruption of resting state. But if you want to actually look at what's activated in the brain related to your pain, you either need to do something like we did with deoxyglucose, or you need to do what they call arterial spin labeling, which allows you to be able to get a real absolute measure of blood flow.

Using that -- again, this is from David Borsook's lab where -- and there's been several -- my student Marco Loggia's done some really nice work with arterial spin labeling. But people are starting to use this for pain stimuli, and it seems to work pretty well.

The pattern you're seeing here with the heat

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pain stimulus looks pretty much the same as you'd see using the BOLD technique with activations and the insula and the anterior cingulate and the thalamus and hippocampus. And it looks pretty similar to what you see using the BOLD technique, suggesting that, in fact, this is a pretty good measure.

This again has been taken to the rat with arterial spin labeling. We're starting to do this with the mouse, so next year, hopefully, I can tell you that it worked. Keep my fingers crossed. But with the rat, you can see nice temporal correspondence with the electrical stimulation. Using arterial spin labeling looks pretty much the same. The same people have done this with BOLD, and it looks pretty much the same as the BOLD.

So the nice thing about it, with these short stimuli, you don't need it. But if you want to use a longer stimulus, then you -- so if you're going to have a more chronic pain type of a measure, then you want this is a technique that could work. So it seems like it's working pretty well.

Then finally, imaging

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neurotransmitters -- and this is something that -- in our lab we started doing this with -- so there have been a number of studies in chronic pain patients -- or with acute pain and also with chronic pain patients -- that you can get alterations in the availability of either opiates or dopamine in the brain related to chronic pain.

So if you take an acute pain stimulus and you give it to a healthy person, what happens is every part of the -- and then you do this competitive binding of -- this is diprenorphine for opiates. If an area -- if the pain itself leads to the release of the opiate in that area, then the receptors are bound, and then you give this exogenous ligand, and it can't bind. So you look at reduction in binding potential.

So in order to -- and there's now studies showing that chronic pain patients have alterations in the way the brain reacts to a pain stimulus itself, suggesting either that the receptors are already -- they're either down regulated, or they're occupied. Again, we don't know the mechanisms. So

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if we can recreate this type of thing in a rodent model, then we can go back and we can look at mechanistically what was happening.

But if you look, this is just showing the baseline distribution of opiates in the brain as identified using PET. And you see here that there is high opiate receptor binding in many parts of the pain system; so thalamus and anterior cingulate and the amygdale. So many of the parts that -- the insular cortex that we identify as being part of the pain system, you have this high opiate receptor binding.

So in our lab, we've started off -- as I just said -- and this is just an example -- chronic pain can alter this binding, and there's a number of different studies. This is just one that I just pulled out with central post-stroke pain and showing that these are -- the regions in yellow are regions where the binding is altered by chronic pain.

So in our lab with Scott, we've now started doing this in rats. And so the only thing we've done now is identified, taking it a step a time, as the

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baseline levels of opiate binding throughout the brain. And now we're seeing it's a very, very nice correspondence without human studies, which is nice.

So on top is a binding that we see in our awake rats, and below is the example I showed you before of the human binding with diprenorphine. And we see that it's very, very similar across the brain area. So now we're going on to the competitive binding with an acute stimulus and then with chronic pain to see if, in fact, we can alter this.

Then it shows you the regions where it can be altered, where then you can go back and do histological studies to see what's happening, to see if, in fact, it's due to down regulation of the receptors or whatever. But this gives you a whole brain overview that then allows you to access these things.

So quickly here, the main pitfalls in making these translations is that, first of all, anesthesia can suppress nociceptive responses. And this is what we did with Scott, where I showed you before that with this formalin, when the animals were awake, with

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formalin, you get a nice response in S1 hind limb. We then imaged animals that had SNI injury for several months, and we found that even though they looked perfectly normal, we had an increase in the activity in S1 cortex in these animals, suggesting that there was something going on in their brains related to their chronic pain.

This was without an acute stimulus being applied. But when we did the same study with isoflurane, we saw nothing. The baseline went down, and there was nothing. There was no difference between the SNI and the shams.

So I think that that anesthesia can have a big effect when you have a more subtle manipulation. Electric shock, okay, but not with something like looking at possible ongoing activity in a chronic pain model.

I'm not trying to bash David Borsook, but I just want to show you their very first awake rat BOLD heat pain study, they had 72 activations, significant activations in the brain, whereas with human studies, you see 5 or 6 or 10.

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These guys, they conditioned them. They habituated them for several days. They were measuring heart rate, and they found that the heat pain stimulus didn't alter their heart rate. But their heart rate was quite high at baseline, and we've now gone back and taken animals and exposed them to the same restraint.

I don't have the data now because I couldn't track down my postdoc, who seemed to have disappeared on me yesterday -- but measuring cortisol levels. And these guys, their cortisol is through the roof after the three days of conditioning.

So I think we need to do a lot more of measuring what's going on because there seems to be a lot more going on in these rats' brains than just what you'd expect from a little heat pain stimulus. So these are just things that we all have to try to deal with one way or another.

So it seems the functional anatomical brain imaging techniques developed in humans are now being successfully applied to rats and mice. And these techniques, I think, can allow us to examine

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mechanisms of clinical observations, provide whole brain analysis of altered circuitry in genetically modified animals, and lead to better translation between preclinical and clinical models. But we must remain aware of the effects of anesthesia and experimental stress in interpreting the rodent imaging data.

These are some of my main colleagues in the studies that I talked about from our lab. Thank you.

(Applause.)

DR. PORRECA: Since we only have two speakers in this session, we thought we'd go ahead and have both presentations given, and then spend the rest of the time with the open question and answer.

So it's my pleasure to introduce our next speaker, Dr. Yves De Koninck, who is in Laval running an extremely innovative and creative pain research center. And Yves is going to speak about the potential that's associated with the emerging application of optogenetics to understanding pain mechanisms.

So you see Yves' title here, so thanks, Yves.

Presentation – Yves De Koninck

DR. DE KONINCK: Thanks, Frank.

As Frank said, I guess my job here is to try to give you a feel for why I think that optogenetics or optical approaches are going to be hopefully very useful to bridge in what's been missing and what's we've been discussing.

Catherine gave a great talk to really show how trying to use the same modality in humans and animals can be quite instrumental.

Another place where I think we need to bridge is between cellular activity and behavior. So pain is a complex behavior. Frank is always bashing this on us and saying we have to be assessing pain as a more complex behavior than just a reflex. But then we are interested in targets that are working at the cellular level, enzymatic level, biophysical level, and we want to try to bridge between them.

I think this is where optical approaches and optogenetics, in the broad sense, I think is going to be quite instrumental as allowing us to do cell biology, but in vivo, in context-sensitive

situations, context-relevant situation.

I guess as disclosure, I just wanted to mention that I am actually -- we have a license agreement with Hoffman-La Roche on something that has absolutely nothing to do with today's talk, on our compounds, on chloride transport modulators. But I thought I'd mention it because along with that, we actually have a collaborative grant with them as well.

Again, that's big PhRMA coming to us because they are interested in these cellular imaging approaches in vivo to push further their target discovery and validation approaches. So it really is another example of where my speech today is maybe relevant to drug discovery.

So ultimately we want to figure out how this whole thing works. The bridging part that's perhaps missing in what Catherine's talk was before is that the imaging approaches that she's talked about are great in trying to get different regions of the brain working away, trying to connect them. At some point, as I said, you need to try to go down to single cells

and try to decipher how the microcircuits work, how the information is encoded there, and how cellular processes and even molecular processes occur in these small networks within the functioning brain. And that's where, again, we think that optical approaches are a really good translational tool for that.

What I like to always stress here is that I take optogenetics in its broad sense where it's exploiting genetically encoded proteins that respond to light so that you can exploit them to both observe and control. And the two things are very, very, very interdependent and equally important, I think.

There's a lot of hype. Optogenetics, we can certainly give the credit to Karl Deisseroth for coining the term, and Karl really certainly pushes a lot of the control aspect. And that's where there's a lot of hype for that and a lot of hopes and rightfully so. But I think sensing is equally important. And hopefully, I'll be showing you some examples of that; so both actuators and sensors to be able to control the system but also observe down to single cells, down to cellular process, inside cells,

in the in vivo context.

So in my mind, the first optogenetic protein -- optogenetics goes back to green fluorescent protein -- any protein that is photoresponsive. And then you exploit that. And there's been obviously enormous work, especially from Roger Chan's lab, to turn these photoresponsive proteins into incredible sensors.

So the context is we want to figure out -- we still today want to figure out today how somatosensory input is encoded. And taking this slide from a review by Steve Prescott, who's bringing up again the concept that we have to think of the system in terms of combinatorics with a combinatorics view.

Even Allan had a review in Neuron recently talking back about the gate control theory, and reflecting 50 years later, we're still debating how the signals are processed in the spinal cord. And certainly, there are labeled lines, but everyone, I think, would agree that there are interactions between them and complex interactions. And we need

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to figure out these interactions are.
We'd like to think about them -- I mean, the problem poses itself already at the spinal level, but it recurs at every point in the relay, at the spinal cord level. We can transpose the same thing to higher brain structures, all the way to the cortical areas.
So there's complex processing, multiple cells interacting. We need to decode that. Optical approach or optogenetic approaches offer some advantage over classical electrophysiology approaches in that they can bridge the genetics. We can harness the genetics to do that, to monitor the different players in these networks and also control the different players in these networks. And hopefully, as the technology progress, we can complexify this so that we can monitor multiple, multiple players together and control them as well.
So the optogenetics toolbox keeps growing very fast. Perhaps it's frustrating for some of you because the hopes and the promises are great, and you're saying, well, how come it's not really

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translating. There's still quite a lot of debugging that is occurring in the field. It's moving very fast, and the number of Nature, whatnot, papers on this is creeping up because of the expectations are very high. But I think one thing to keep in mind is that a lot of these -- there are still a number of hurdles in building these tools.
But certainly, you have a bunch of tools, and it will allow you to control things, to be able to identify cells, monitor them and whatnot; also, using these genetically encoded tools to use tracing techniques and mixing these tracing techniques with optogenetic tools and so on, a number of tools to try to decipher the network.
So one example, let's start with the beginning, just how the signal is encoded from the periphery to the central nervous system. We have these classes of -- this massive class of sensory fibers. We'd like to be able to dissect out what each of these are doing. Again, I was talking about hurdles.
It takes a while before we could really get

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these tools right to work even in something simple as a sensory system. In the last year to two, there's been some good breakthroughs.
Here's one example that I'm illustrating where we can actually use these certain viruses. This was pioneered by a few groups, including David Anderson's group, and finding that when you inject certain viruses in the newborn, in the periphery, you can actually selectively transduce sensory fibers, and just sensory fibers. It's quite nice.
So you can then play with whatever, your favorite mouse, here, an Na(v)1.8-Cre for the proof of concept here, and have a virus that will encode channelrhodopsin, which when activated by light will excite the cell so that you can probably use light to selectively activate sensory neurons.
So here's this Na(v)1.8 mouse, so you can see just a subset of the cells are transduced here. You can even see the channelrhodopsin. The reason why you see is that the protein channelrhodopsin is coupled to YFP, so you can actually see it in the nervous system. This is the periphery. The sensory

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neurons are expressing it.
This strategy was necessary to get enough -- just simple bug, just get enough channelrhodopsin expressed in the afferents so that you could envision being able to activate the afferents with light through the skin.
So here's an example. Here's one mouse out of these four that has it. I hope you saw which one that was. The other ones don't really bother. So basically, you're able to just with light -- see, that little mouse is reacting to light, so we're able to activate those sensory fibers with light.
So why is that particularly interesting? This is again just proof of concept. It's just using the Na(v)1.8-Cre mouse. But one place where it's already bridging is to bridge between this and this; the idea that now you can have an excised preparation and apply the same stimulus, the same parameters and whatnot, the same class of afferents selectively, and study in the cellular process, for example, your synaptic plasticity, an example of long-term potentiation in the spinal cord that you trigger by

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activating these afferents.
Then you can do the same in the behaving animal to try to produce sensitization through the skin. And here is an example. When you're doing withdrawal reflex to mechanical stimulation before and after, you've conditioned the animal with stimulating the nociceptors at a high frequency. And you can see that you can produce that sensitization.
So, of course, the promise is then you can start activating selectively each of your favorite afferent and look for the responses that is evoked there. There's an example. And, of course, by doing this presumably you'll be producing a cleaner response as you see here with this light as opposed to electrical stimulation where you have a more complex response here.
So the idea would be to try to dissect out how activation of each of these afferents both in vitro and in vivo will be happening.
Now, challenge again, high expectation but challenges again, the genetic targeting and the genetic identification of each of our favorite cell

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types still remains a challenge. I think -- and here I'm illustrating one of the strategies that Quifu Ma here is highlighting, and he's been pioneering with Martin Goulding at UCSD, trying to do combinations.
I think it's going to -- I'm a strong believer that if we want to start targeting our favorite cells, we'll have to start doing combinations of expression of different factors -- here's one strategy using these two gene activators, if you want -- but also combining genetic targeting with different promoters and tracing techniques and whatnot. We'll have to be able to play with all of these things to really narrow down on the selective subsets of cells that we're interested in.
So the beauty of this, of course, is that if you have just the cell that you're interested in express the light sensitive channels or light sensitive whatever you're interested in, it's nice because then you can just do bulk light excitation. Here it is illustrated with just a fiber optic stuck in the head of the animal. And then you will

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hopefully be able to just target, activate, just the cells that you're interested in.
The same logic is true for sensing. By targeting sensors, so protein sensors that will be sensoring activity, enzymatic activity, neuronal activity or whatnot, neuronal morphology and whatnot, you can really follow that in vivo.
So to illustrate that, just a few examples. Again, challenges, we need to develop tools to be able to follow these small networks. Here's an example of fancy microscopes that we're designing in our group to try to be able to follow the networks of cells in sensory ganglia and in dorsal horn.
One of the simple issues that you face when you develop these things, just to illustrate the hurdles that are there, is that spinal cord tissue moves big time. Here's an example of stabilization process, which you saw systems stabilizing when you turn it on, based on a feedback system that actually senses the surface of the tissue and feeds back on the objective.
So what we're actually seeing there is a

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stable image. The tissue is not stable. The image is stable because the microscope follows in real time.
So then the promise of this, of course, these imaging approaches is that to record from a number of cells at the same time and see how they can encode the information.
So let's step back again to our sensory fibers, sort of maybe plead for the opposite of what I said before. I said that with the genetic targeting, one nice thing is that we'll be able to dissect out how each component works. But it's also interesting to try to monitor how the whole ensemble is dancing and dancing around.
So here again using our viral strategy to transduce selectively sensory neurons, here we're putting in the neurons A sensor to GCaMP6, calcium sensing protein, which will be a proxy of activity of the cells. Again, the field is moving now very fast thanks to this game changer GCaMP6. Before that, a lot of iterations before that, a lot of hurdles, a lot of limitations, and then especially, translation

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in using them as tools for in vivo.
So again, as I told you, the beauty of this AAV9 is that if you inject that in the periphery, you really infect -- transduce -- the vast majority of the sensory fibers just in the DRG, like you see this. You see that in the dorsal horn. You see just the sensory fibers entering, but there's no dorsal horn neuron that were transduced by that. And you can really label the vast majority of the DRG neurons here just going through the ganglia here to see all cells that are labeled with GCaMP.
So that you can just then monitor an ensemble of neuron responding to a sensory input. Here's an example of the cells that are just responding to a noxious heat to the foot, and we're recording in the DRG.
The point I want to make here with this slide, just to provoke your thoughts, is this is just a early mapping, crude mapping, of the array of responses that you have in the DRG to your stimuli. So you have cells -- you're getting cold stimuli, warm stimuli, heat stimuli. You have a few cells

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that are responding just to heat, a few that are responding just to cold, some to warm, but you have a whole array of cells in the gray zone here that are responding, to some degree, to all of these modalities.
So that in the end when you give a stimulus -- so you have cells. We know we have cells that are tuned optimally to a stimulus. But when you give a stimulus in real life, stimulus is always acting in a number of different cells. Each cell has a spectrum of activation, so that you'll be activating an array of cells. And, in fact, I would push even further that in many stimuli, never occur in isolation. I can't pinch you without touching you before. So the fact that these two stimuli are occurring often concurrently probably means something to our neuro system.
So the ultimate, I think, experiment will be to decode or to figure out how all of this information is encoded, something as early as the DRG, and then play it back in the system and see how the system is going to behave. And what I'm

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illustrating here in the DRG, you can transport each level in the CNS, spinal cord, brain and so on. Each of these places, information is going to be encoded, translated, sent further down to be encoded further.
So actually to link to what Catherine was saying before, this is great. You saw the heavy microscope. I have to anesthetize the animal in that case. Anesthesia could be an issue for sensory neurons, primary sensory neurons, perhaps not too much, but then as you go further down the brain, down the neuraxis, it's going to become more critical. So obviously, you want to develop approaches to try to follow this in vivo. Again, a lot of development in the works.
Here's an example of a spinal window that you can use that we've been developing and others have been developing to try to follow things at the spinal level. Again, spinal just being more challenging than the rest of the higher up in the brain. You can see that these little mice move happily with this little window.
There were inflammation issues. This has

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been a lot debugged and worked out. Here I reference work by Franck Debarbieux, who's done a lot of great work at re-optimizing the spinal windows to minimize any inflammatory responses.
What I want to illustrate here is that -- well, so this window perhaps is a bit invasive. Another approach that is being explored a lot in the field and developed is to try to develop these small micro-endoscopes to try to have the most minimal intrusion into the brain for the spinal cord. This turns out to be great because, in fact, even in little mice, you can insert these micro-endoscopes in between vertebrae without having to break any of the bones. And you can do the imaging across the dura, so we can do relatively noninvasive imaging.
So what I showed you before is a lot of imaging using GCaMPs, calcium imaging as a proxy of neuronal activity. There are a lot of other cells in CNS. Here's just an example of another cell that we like or hate, whatever, the little microglial cells. They don't have the same type of electrical activity that neurons have, but they are very active. You

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need to be able to follow how these cells are behaving in tissue.

So here's an example of where again imaging, in situ, through the optogenetic approach so that you can just see those cells can be quite instrumental. Here's a mouse that expresses GFP inside microglial cells. You see the little cells are just scanning. That's what these cells do. That's one of their behaviors that's critical, is that they move around scanning the environment, and they respond to injury.

So what you can do is you can come in with a laser and just make a little hole in the middle to see how these cells will react to that hole, and you see that they do come in and actually are circumscribing the area to protect that damaged area. This is done through the dura without going into the tissue.

Now, the reason I'm showing this again is to say that the physical behavior of these cells characterizes them, and you can actually quantify that. Just to illustrate my point here, for example, when you do a nerve injury and you're measuring in

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the spinal cord, not the area that you've damaged, you can see that the cells decrease a lot their mobility. They become activated and whatnot, fat. They just don't move. But when you actually do a lesion, they actually respond faster. So something has happened that is priming these cells, some signaling mechanisms are ongoing there, and you can actually see that in the dynamic behavior of the cells.

Just again to illustrate that, seeing things in action through these approaches can reveal things that you wouldn't be able to see in static approaches, for example.

Now, you want to go higher up in the brain. That's the area that Frank only cares about now. And certainly, ultimately, we want to go and see how is information encoded higher up there. Of course, there are several techniques to monitor cortical activities, and Catherine has shown great stuff that you can do with imaging.

What optical approaches offer perhaps over other approaches is very fast time scale; again,

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trying to figure out how the brain encodes information needs fast time scale monitoring.

Here's an example from work from Tim Murphy's group who's actually using voltage sensitive dyes. This is a dye in this case, though it's not optogenetics. But it really shows that you can really follow across levels cortical activity, and then you can -- just doing the same type of correlative activity that the MRI community is doing now to look at functional connectivity between different areas. You can do this at a smaller spatial scale but much faster temporal scale to try to figure out how the connectivity is organized there.

You could argue that this is surface recording in the cortex. That's one of the problems with light is you record very much at the surface. Tissue is very scattering. Brain tissue is very scattering, so you can't really go deep into the tissue to figure out how things are working there. So you could argue, well, maybe electrical approaches or EEGs have been telling us quite a bit on the

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cortical surface activity. And if we want to be able to really decipher, especially in the pain system, how things are happening, you want to be able to go deeper into the tissue.

So that's where, again, these little micro-endoscope approach that I told you about are quite instrumental, something that's been pioneered by Mark Schnitzer at Stanford. Here you have one of his little favorite mini, mini microscope, small enough that a little mouse can walk with it on the head, and you can insert the little micro-endoscope in the head.

The objective here is to monitor cellular activity in the network. Here you have it inserted in the hippocampus. This is actually a mouse moving around and looking at place cells in the hippocampus. And you can see how this mouse responds, how the network responds to a specific context and whatnot. The objective ultimately is to again decode how all of this information is happening.

Sometimes what you want, though -- or some of the cells that you're interested in are very rare and

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sparsely distributed. Some of us are obsessed with some little cell types that are very rare. This is an example of cells that we're interested in, the little cholinergic neurons in the dorsal horn of the spinal cord. I just used that as an example. You have tons of cholinergic neurons in the ventral horn, but in the dorsal horn is actually forming a very, very small subpopulation of cells.

So you need to be able to actually go in, in context again, and follow how each of these cells are behaving inside the intact animal. And even though some of them may be scarcely distributed, they sometimes have very important impact on overall network activity, like, for example, these cholinergic interneurons.

So one way to get at that that we developed is a little microprobe that's basically making a glass micro-electrode that we've been using for ages to record electrical activity, but making it through a fiber optic. So it's actually using an all glass strategy, fiber optic that has an optical core. So fiber optic is just glass inside glass with an

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interface to guide the light, but then we also add a little hollow tube in the perform.

So then you can have your glass probe that you can pull down. You have the hollow core that you can fill with an electrolyte, and then have electrical contact to the tip as well as optical contact in the tip. So the idea is then to go down and be able to use both electrical activity to study your favorite cell, but also of an optical channel to see what's happening there.

So use light again to probe beyond just electrical activity, for example, in the cell. So the beauty of that is if you can inject the light and collect the light through the same fibers, so you can go down to your bottom, if you want.

So the idea is you just go down. You scan down, and as you're getting close to the cell, the light will go up and down so you can identify your cell. This is an example where we've actually used this in a mouse that had green and red cells. This is in the striatum, the D1 and D2 cells.

You can see that when you get close to a

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green cell, you see the cell as you're passing by, or a red cell, you see it as you're passing by. We get the same proportion of cells with either just looking at the tissue or sampling them with our probes. So that's making us confident that we can sample the different types of cells.

So you can envisage then going in vivo, studying your favorite cell and how it responds to different interventions. Think of drug intervention, for example. Understanding how the cell that you're interested in, the sub-cell that you're interested in, will respond to drug application, but in an intact brain. And ultimately, you'd want to do that in a behaving animal. We'll get to that.

Of course, if you have a probe like that, you can try to play with it and do optogenetics. Well, optogenetics -- see, I'm using the same bias -- optical activation.

This is a transgenic mouse where you have channelrhodopsin distributed in different areas. You can go down next to cells that you're interested in, and then you can flash light. And you can see that

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your cell can respond to your light. See the little thing here; this is action potentials of the cells as you're shining light on it.

Now, this is superficial. You can ask what are the things that you can do. There are a number of things that you could do now that you can manipulate cells with light at the single cell level deep into the tissue.

But I want to come back to what is actually more dear to me, is monitoring, using this to monitor activity. This is an example taken from the thalamus. You go down to thalamic cells that respond to whiskers. You can move the whiskers around. These thalamic cells now have calcium indicators in them. So you can see that when you move the whisker, you get an action potential, and you get a calcium response.

You see it better here. The cell is bursting. You see nice calcium response, single traces, very nice signal to noise ratio.

So this is telling us that now we can use light to actually monitor in vivo deep in the brain

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individual cell activity or individual signaling events. Here this is just an ionic mechanism. You could imagine an enzymatic mechanism, whatnot.

Now, just to close, this is going full circle to what perhaps what's dear to Frank at least. Frank would be telling you, well, if you want to assess pain, you have to assess what goes on here in the brain, and there's a whole bunch of nuclei there that are important, reward pathways, whatnot.

So what Frank is using as a strategy is saying, well, I'm going to be assessing this using behavioral assays, and they're going to be my endpoints or my proxies of how these pathways are acting. And they are reflecting, if you want, the state of the animal, closer proxy to actual pain than these reflex mechanisms that we've been studying.

So my sort of dream project through this is to try to add, go further in this direction in saying, well, okay, you can study this in behavior. But it would be nice if we had ways of actually monitoring in these different areas activity as the animal is just walking around and doing things.

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One of the ways that you can do that is to pick up on that little microprobe that I told you about and go into the different little brain areas that you're interested in. And the reason why I think our approach may be a little more translatable towards that is because the idea is you want to have an animal that has a few needles in its head that are just monitoring activity.

So you could say, well, people have been doing this with electrophysiology over the years. Well, sure, electrophysiology will -- you can do chronic electrophysiology in animals. It won't give you easily information on the molecular identities of the cells that you're interested in. And, of course, it won't allow you to control these cells.

Again, what the optogenetic approach is offering to us is a way of targeting the different cells that you're interested in and multiplexing the information. So here is actually exploiting, for example, the recent developments on the GCaMPs, on the calcium sensitive fluorescent proteins. And Robert Campbell in Canada has made green, blue, red

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variants of that. So now you can have multiple signals that you could target to different compartments.

So the difference here, I showed you before Mark Schnitzer's micro-endoscope who can record, image the network. You can do imaging. It's fairly invasive, though, because you have to image a fairly large area, bulky thing. This little microelectrode that I told you is not an imaging device. With fibers, you don't image. It's a point measurement. So you actually don't have spatial information. But specific spatial if you want information, you can try to regain it by using colors.

So here's an example from Robert's collaboration with Nagai's group. Here they've actually targeted the different colors to different cellular compartments, so nucleus, endoplasmic reticulum, cytoplasm. And you can see that the cells are responding. So each of these are signaling calcium, but each in their own respective compartments.

If you forget the spatial information that

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you have there in the image and you just look at it temporally, you see that calcium oscillations are following different kinetics in each of these compartments.

So if I'm interested in calcium in the endoplasmic reticulum, I just need to target something there and just study what happens in the endoplasmic reticulum. I don't necessarily need to actually see the endoplasmic reticulum.

So the same concept you can transpose in the brain area, the your favorite brain nucleus that you care about, that has five, whatever, three through two, seven cell types that you can target with different colors.

So then you can envisage that you can bring your probe down, and the probe can be able to be specific to one color, for example. So only the cells that are blue, for example, are going to be firing away. Then you can try to scale this back, try to sample a larger area and do multicolor sampling or even further sampling.

So the idea is to envisage having a small

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probe in one area and sample activity without spatial information in the sense of imaging but monitoring activity from X, Y and Z cell type. And that, I think, is going to be step forward from, for example, one of the criticisms that we often have with conventional imaging approaches with MRI or functional MRI is that you don't necessarily know which cells are actually firing during this or that behavior.

All right. So this is just my usual advertisement for those who are interested or have students. We run the summer school. What I told you about today is actually happening in a center that I founded, which is actually a whole platform that's an interface between physicists and biologists. It's all aimed at developing new techniques.

So there's a huge work force that are working on trying to improve the hardware to do the kinds of things that I'm telling you, and we run a summer school every year on many of these advanced techniques. Et voila.

(Applause.)

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Q&A – Bushnell and Koninck

DR. BUSHNELL: Some people are now beginning to combine these things, so you stimulate specific cells with optogenetics, and then you can do whole brain imaging about where they're going, which is cool.

DR. DE KONINCK: Sure, absolutely. And, in fact, I think the future is going to -- the concept right now, light is very amenable to this. But I think there are other modalities that will emerge again with the same principle of being able to control very selectively.

DR. BASBAUM: I'm just going to let you change direction for a second, and I'm surprised actually, Yves, that there was no mention of DREADD technology.

DR. DE KONINCK: Yes, okay. Good point, Allan, and I guess it's just because optogenetics was the title, and I just tried to focus on there.

Again, as we were just saying, other modalities, and one of them is chemicals, so chemical specificity. The optogenetics field is sort of often

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the most talked about out there because the promise there is to have very precise temporal control of the cells.

So the idea, the ultimate experiment in a sense is to have the -- we see these networks of neurons that are firing away. If you could just basically tape this and play it back, it would be really great to see whether this or that pattern of activity in these different cells is sufficient to explain this or that behavior.

There the temporal precision is quite important, and the optical approaches are great. But for certain, other interventions, which you would want is to replicate like, say, for example, continuous activation of a pathway. And in that case, the chemical approaches might be more appropriate than having to inject light continuously.

I assume everyone understood what Allan said when he said "DREADD." So he's just talking about essentially the idea is to use a non-endogenous receptor that's coupled to an effect or mechanism that will activate or inactivate cells. And if this

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receptor is responding to an agonist that is not endogenous, that the animal never sees, then you can use that agonist to selectively activate the cells that expressed, that you've made express that receptor.

DR. MCMAHON: So the optical approach obviously is dramatic and fantastic, but there must be some snags. And you didn't tell us about any of the disadvantages.

DR. DE KONINCK: Yes.

DR. MCMAHON: So in the spirit of cooperation, things like the sensitivity of different cells or sustainability of current with continuous light, I mean, are there significant problems?

DR. DE KONINCK: Yes, and, in fact, old timers like you and I, Steve, who've been doing electrophysiology for years, are seeing that the whole field of optogenetics is just rediscovering electrophysiology. And they're rediscovering a number of artifacts of electrophysiology. And there are definitely a number of artifacts.

One thing that you mentioned is the fact

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that, for example, I guess in your field with sensory fibers that are transducers, they are transducing a physical stimulus that has a certain physical flavor. With light, when you are activating the afferent with light, you're not necessarily replicating that. So there's something that may not be physiological there.

Another bigger aspect that's -- well, again, I was mentioning that there's a lot of debugging in the field. Channelrhodopsin actually turns out to have been the tool that won the battle because it's all genetically encoded single channel. You don't have to add extraneous agents to make it light responsive.

But channelrhodopsin turns out to be one of the worst of all. Its conductance is awful. And the properties of its conductance are really awful. So the unit conductance is very small, so you need tons of it. That's one of the problems.

That's why, for example, when Mark Zylka tried to make his first light activated MrgD mouse -- so he had this subclass of afferents, and he

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just said I'll just make it transgenic and be able to activate these subclass of afferents -- it worked fine, and the DRG worked fine in the spinal cord. But through the skin didn't work. Basically the idea was that there was not enough channelrhodopsin.

So you need a lot of protein. If you need a lot of protein, you're a little worried. That could be a problem for the cells.

A lot of work out there, tremendous amount of work in improving the conductance, kinetics, properties of this ion channels. So that's improving over time.

One thing that is emerging, for example, in people that are studying sense synaptic physiology is that we used to do it nicely with stimulating electrodes, and we'd get responses. What you do there is you generate an action potential, let's say in the axon, and it's translating down to the terminals. Some people are trying to use optical approaches to stimulate directly the terminals.

But when you do that, again, you're doing it in a very different way than an invading action

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potential. So people see papers emerging now that are showing that, well, the plasticity properties are not necessarily exactly the same when you do it like that. So there's a number of things that people have to be worried about, the usual with every new technique that's coming out.

DR. PORRECA: Yves, you mentioned a couple of things that I found very striking. The difference between the activation of the afferents with light as opposed to the electrical stimulus, where you get the --

DR. DE KONINCK: No, to natural stimulus I meant. Oh, well, okay. Go ahead.

DR. PORRECA: You were pointing that you could be very, very selective in which fiber class you activate, and that that's not the normal way in which the nervous system sees the afferent input. So you're deconstructing the signal into its component parts, but how does --

DR. DE KONINCK: But eventually you want to reconstruct it, yes.

DR. PORRECA: Yes, exactly.

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DR. DE KONINCK: Absolutely. So I think the two approaches are very complementary, and that's why I wanted to show them like that; that it will be interesting to dissect things out, but we have to understand again that these things are very artificial.

That's why eventually we want to bring everything back in vivo. It's the same with all of our approaches, our reduction to these approaches. We like to isolate components, but you need to actually be able to look at them back in the functional context.

DR. PORRECA: And to understand what the meaning is to the brain if the brain has never seen that isolated signal.

DR. DE KONINCK: Absolutely.

DR. PORRECA: So the other question is that -- since we were talking --

DR. DE KONINCK: But that may be what pain is, you know, input from some afferents when others are not coming in.

DR. PORRECA: Well, maybe. So related to

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this, I think is the question of since the aversiveness of pain is highly dependent on stimulus intensity, how do you control the stimulus intensity in a way that you can modulate in a graded fashion, for example? How do you really get a finer control of --

DR. DE KONINCK: Well, I guess it depends -- can see it at the single cell level. So if the cell is going to be responding to one spike or several spikes or a burst of spiking, whatnot, to a given stimulus, or as an ensemble, whether 1 or 2 or 12 cells are going to be firing to your stimulus.

I think you can replicate that, to a certain extent, with light. It has its limitation, but you can certainly control duration, frequencies with light. Again, that's where the temporal aspect -- the fact that you can control very fast cells has promises, where you try to replicate the same type of strains that you see in an afferent. But again, threshold may be an issue.

DR. BASBAUM: You can all leave if you want. We're just going to keep asking questions.

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(Laughter.)

DR. BASBAUM: I have a kind of general question for everybody, and I wanted to ask it before. We talk about -- and there was an interesting discussion about TRPV1, and just now we talked about using heat or light to activate. You showed subpopulation of dorsal root ganglion cells. And, Catherine, you will often use heat as a stimulus certainly in humans.

I've always thought of heat, in engaging TRPV1 afferents, for example, as really a surrogate for some stimulus that produces pain in humans. Patients don't -- they might come in with nerve injury and complain about burning pain, but they rarely come in complaining that, well, gee, I have arthritis and my hand is hot. No, they're sensitized. It hurts when they move. There's allodynia, even though the TRPV1 afferents might be engaged under those conditions.

So I see heat as really a surrogate that we're using to study something that is happening clinically.

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How do you translate -- can you translate in the animal, by imaging other patients or even in an mouse model in the brain, a heat stimulus to the injury stimulus? Is the heat stimulus too specific to really understand what's going on? In other words, in the injury condition, does that population ever really get stimulated independently? It's kind of the question that I think Frank was getting at.

Do you see where I'm going? I don't believe patients come in and say, "Oh, my TRPV1 afferents really got activated badly last night."

DR. BUSHNELL: That might be one reason why taking this to the clinic doesn't work very well.

DR. DE KONINCK: But I think that's why I was showing that slide at the DRG, where you're getting a natural stimulus, and the stimulus itself is actually a very heterogeneous stimulus. It takes time before you reach noxious heat. You're activating a whole bunch of afferents before. There are a wide array of afferents that are responding to that stimulus.

Again, I think being able to do monitoring of multiple cells at the same time, and that's one of

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the promises of the optical approaches over electrophysiology. In fact, we've done enormous amount of work in the sensory neurons characterizing individual cells, their dynamic response to stimulus and so on because with electrophysiology, we could isolate each of them.

I think, indeed, we have to go back into the more complex thing. And the same that I showed at the DRG level is true at each level. And that's why I think Catherine is saying we need to basically use these approaches to see what matrix of activation occurs in the brain to that stimulus.

DR. BUSHNELL: I mean, being able to selectively monitor certain cell types and then taking it out to the chronic pain condition and seeing what's happening to the very stimuli, that we know what happens acutely. But knowing specifically the different cell types, I mean -- which is the beauty of that, being able to --

DR. DE KONINCK: Yes, and Vanya is not there to see it, so I'll see it. And of course, we can't just do this in control animals. We have to do this

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in models of chronic pain.
DR. PORRECA: Right, and so that was where I was just going to pull up and ask Catherine if you could comment on whether or not the imaging in animals has progressed to the point where we can start to look at the effects of drugs in chronic pain situations, or even if not in chronic pain situations but at least in sensitized states.
Can you see differential effects of drugs with the animal imaging, as I think has been done in a couple of examples of human imaging?
DR. BUSHNELL: Yes, I mean, I think imaging chronic pain -- so we have one study where we show cortical activation in SNI rats that are just walking around looking normal, and whether this is evoked or spontaneous, but at least you're showing differential cortical activation.
Although we saw that in the sensory areas, we didn't see it in the cingulate cortex in the more affective areas. And I don't know if that's because they really don't have the same affect as with acute pain. But I think by being able to do a whole brain

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imaging where you can specifically look at chronic pain activation -- but this requires either -- this development of the arterial spin labeling will allow us to be able to do that much better with the whole BOLD technique that is not amenable to looking at chronic pain.
So either using PET imaging for metabolic activity, which is more complicated, or using arterial spin labeling with MRI will allow us to be able to look at activations related to chronic pain, which would be a lot easier to --
DR. PORRECA: I was thinking of the study that was done by Iannetti and Irene Tracey with the gabapentin, showing activation and deactivation only in sensitized states in human subjects, and whether or not we could capture that kind of activity in a preclinical setting to differentiate mechanisms in areas of actions of new drugs.
DR. DE KONINCK: And when you say chronic, you mean how much time after the injury, to come back to Laura's point, and Catherine? Are we talking --
DR. BUSHNELL Well, I was just saying that

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when we looked at what happened to these anatomical changes in the brain that we see consistently in pain patients, it took 20 weeks in a rat for that to be significant. And it was interesting that -- I didn't show this, but we were doing these anxiety tests and things, other tests, and the animals looked perfectly normal until about that same time period where they started getting prefrontal changes. They started developing anxiety-like behavior then, and they didn't have it for the first 20 weeks after injury.
So I think that really these rats are not looking like chronic pain patients until way out there, and we have to start going --
DR. BOUNTRA: Thanks, Frank.
I mean, Catherine, I appreciate all the advances that you're making in brain imaging. And, Yves, what you're doing on the optogenetics is just mind blowing. But of course, we can only do that in animals. We can't do it in humans.
I can see with the optogenetics, we can ask questions like sort of what cell type's involved or maybe what's the pathway, and we can come up with

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hypotheses, et cetera. But I'm just thinking in the clinical situation, what we really want to know is where's the pathology. Is it the afferent nerves, the cord going up or down, in the brain, where in the brain, et cetera? Which treatment is most likely to work?
Now, I don't know the literature well enough, but in terms of sort of non-invasive electrical measurements in humans, how are those advancing? Because I think those could be very powerful in terms of really trying to understand what is causing the pain in a particular individual and which treatment is most likely to work.
DR. BUSHNELL: When you say non-invasive electrical stimulation, what are you referring to?
DR. BOUNTRA: Yes, ideally in the human, of course, we want something that's non-invasive. But I just -- Yves, you're the technical genius here. I mean, I just don't know what advances are being made there.
DR. BUSHNELL: Of being able to look at the single unit level in the humans you're saying or --

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DR. BOUNTRA: No, not necessarily. We don't need that. I mean, I think in the human -- at the moment I sense a lot of neurologists, when patients come in, they just try a whole battery of drugs one by one. They'll try one anticonvulsant, and then they'll try another one. And they'll try tricyclic antidepressants. They have no idea in many cases where the pathology is. It's a bit hit and miss. They don't know which patients are going to respond.

Is there some way we could improve that?

DR. BUSHNELL: I just think the whole issue of using, say, human brain imaging as a surrogate has problems in that if the patient is getting pain relief but you're not seeing it on a brain image, well, which is right? Is it the brain image, or vice versa?

I think that it's too crude to be able to use it predictively. And again, these drugs are ones that take time to develop. And so you still have to do a long time frame. So not asking the patient and trying to use these as a surrogate is not going to get us very far. It's just not. It's too crude.

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I mean, there may be some instances where somebody is not able to report their pain and you can use it in that case as a crude surrogate, that's fine. But I think if you have patient reports, I'm personally -- this is my personal opinion and not all people who do brain imaging agree with me. But I think that it's not getting you a lot farther than patient reports.

DR. DE KONINCK: What I'd like to add to this, though, is one of the promises, and one I often talk about -- I focus here on optogenetics because that was the topic. But a lot of the technologies that we push in our center are optical approaches that allow us to do measurements in tissue that are beyond just optogenetic measurement.

One of the promises of optical approaches is molecular specificity, the fact that you can measure things more directly, neurovascular response, neurometabolic response, detect directly metabolic proxies, even chemicals directly with light.

I'd like to make a link here to one of the things that Steve mentioned earlier, that in the

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instrumentation field, new things are advancing fast. And there are a lot of things that we stick in people, and certainly, spinal stimulators are a great example of that.

What I think is missing a lot in this whole field is the other part of the loop, the ability to actually measure what happens in the tissue, to tailor your intervention. And I think I'm one that believes that fiber optic technologies, which is quite translatable to humans for implants like microelectrodes are and whatnot in different areas, are going to be very enabling in that, using them to actually measure the impact of our intervention or even doing early measurements to guide intervention.

DR. BOUNTRA: I'm sorry. I think that's exactly what I was trying to get at, Yves. I was just thinking if we had a room full of clinicians here, what are the questions they want to know, or a group full of people trying to do drug discovery, what are the key questions?

The questions are where is the pathology, which treatment is most likely to work in this

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individual, how long is it going to take, is this compound getting across the blood-brain barrier, is this compound acting peripherally or centrally, those sorts of things --

DR. DE KONINCK: And coincidence of events, so the temporal aspect is quite important. Like in the epilepsy field, people are trying to detect the early signs of a seizure coming in to intervene. I think the same type of logic has to be used in the field of pain or migraine or whatnot.

DR. BOUNTRA: Yes, I agree.

DR. PORRECA: Any other questions from the audience?

(No response.)

Adjournment

DR. PORRECA: Well, okay. Well, thank you, everybody. I think it was a very outstanding session, amazing work. Thank you very much.

(Applause.)

(Whereupon, at 5:06 p.m., the workshop was adjourned.)

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